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The effects of basil (*Ocimum basilicum*) leaf extract on Ca9-22 oral cancer cells

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Abstract: Basil (*Ocimum basilicum*), as a prominent member of the *Lamiaceae* family, is known to possess anti-inflammatory, anti-oxidant, and anti-cancer properties. This study investigated the inhibitory effect of basil extract on oral cancer cells. Basil leaves were dried and extracted with ethanol. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used to assess the cytotoxic effect of basil extract (12.5, 25, 50, 100, 200, 500, and 1000 μg/mL) on Ca9-22, a human gingival squamous carcinoma cell line, after 24, 48, and 72 h. Gene expression of cell cycle regulators (cyclin D1, cyclin-dependent kinase 4 (CDK4), p21, p53) and inflammatory markers (cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β and IL-6) was analyzed using real-time polymerase chain reaction (RT-PCR). Additionally, these markers were measured in culture supernatants via enzyme-linked immunosorbent assay (ELISA). The MTT assay revealed a concentration-dependent reduction in cell viability, with IC50 values of 350 μg/mL for Ca9-22 cells. RT-PCR analysis revealed that treated cells exhibited downregulation of cyclin D1 and CDK4, along with upregulation of p21 and p53, compared to control Ca9-22 cells, which were only exposed to nutrient medium. These changes were observed at both mRNA and protein levels. Inflammatory genes (COX-2, iNOS, TNF-α, IL-1β, IL-6) were significantly decreased at both mRNA and protein levels. Basil extract exerts cytotoxic effects on oral cancer cells by inhibiting cell cycle progression and inflammatory mediators. These findings point to the potential use of *O. basilicum* extract as a therapeutic agent against oral cancer.

Keywords: oral cancer, anti-inflammatory, gene expression, basil, *Ocimum basilicum*

INTRODUCTION

Oral cancer, along with pharyngeal cancer, significantly adds to the global cancer burden, representing approximately 4% of all cancer cases and ranking as the sixth most prevalent type of cancer worldwide [1,2]. The most common type of oral cancer is squamous cell carcinoma, with tobacco and alcohol recognized as the primary risk factors [3,4]. Despite advancements in treatment, the outlook for advanced oral cancer remains poor. While early-stage disease can potentially be cured through surgical or radiation-based interventions, the overall 5-year survival rate for oral cancer remains around 50% [5]. New preventive and therapeutic approaches are urgently required to combat this deadly disease. Inflammation is a central process in cancer progression. It triggers the production of reactive oxygen/nitrogen species that damage DNA, the release of growth factors and cytokines that promote cell growth and survival, and facilitates angiogenesis and metastasis [6]. Chronic inflammatory conditions like gingivitis and periodontitis are closely linked to oral cancer development [7]. Persistent inflammation in the oral cavity creates a conducive environment for cancer growth by activating cell proliferation signals, inhibiting apoptosis, and compromising immune surveillance mechanisms [8].

In recent years, the integration of medicinal plants with standard cancer treatments has increased due to their anti-cancer properties [9,10]. The National Cancer Institute (NCI) has conducted extensive screening, evaluating around 35,000 plant samples and 114,000 extracts to identify potential anti-cancer effects [11]. According to the sources provided, basil, also referred to as Saint Joseph's wort or *Ocimum basilicum*, is a prominent member of the Lamiaceae family, commonly known as the mint family [12,13]. Research on the effects of basil leaf extract on Ca9-22 oral cancer cells is limited. However, studies on related species

suggest potential therapeutic benefits. Luke et al. (2021) reported that *Ocimum sanctum*, a close relative of basil, exhibited anti-cancerous activity on oral cancer cell lines [14]. Similarly, Shivpuje [15] and Naghul [16] found significant cytotoxic effects of *O. sanctum* on oral cancer cell lines. Naghul observed an increase in p53 expression, a critical regulator of apoptosis [16]. Hanachi [17] demonstrated that *O. basilicum*, another species closely related to *O. sanctum*, had cytotoxic effects on cancer cell lines. These studies suggest that basil leaf extract from *O. sanctum* and *O. basilicum* may hold promise as a treatment for oral cancer, including Ca9-22 cells. Nevertheless, additional research is necessary to substantiate these findings. The present study aimed to investigate the impact of ethanolic basil leaf extract on the viability, growth, and cell cycle progression of Ca9-22 cells (a human gingival squamous carcinoma cell line). The research focused on evaluating key indicators of inflammation and proliferation to uncover the molecular pathways affected by *O. basilicum*.

MATERIALS AND METHODS

Ethics statement

The research study was conducted exclusively *in vitro* using a cell line, with no human subjects or animals involved.

Cell line and cell culture

The Ca9-22 human oral squamous cell carcinoma (OSCC) cell line, sourced from AcceGen, USA, was grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and 0.03% glutamine at 37°C in a humidified environment with 5% CO_2.

Ethanol extract preparation of basil (*Ocimum basilicum* L.) leaves

Fresh *O. basilicum* leaves were locally cultivated. The collected basil leaves were macerated with 96% ethanol as the solvent and dried for 2 days at a controlled temperature of 30-35°C to eliminate excess moisture. The leaves were finely powdered using a blender and sifted to ensure consistent particle size. The dried and powdered basil leaves were then immersed in 1 L of 96% ethanol in an Erlenmeyer flask and allowed to soak overnight, enabling the active compounds to dissolve in the solvent. This soaking process was done three times to maximize the extraction of bioactive compounds. Following the final soaking, the ethanol was removed using a vacuum evaporator at 90°C, the boiling point of ethanol. As the ethanol solution stopped dripping into the flask, a dense, concentrated extract of the basil leaves was obtained. This extract was stored at 4°C until future use [18]. The dosages of the basil leaf extract were determined based on [19]. Increasing concentrations of basil extract (12.5, 25, 50, 100, 200, 500, and 1000 µg/mL) were used.

Cell viability assay

To assess the impact of basil extract on the proliferation of cultured Ca9-22 cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out. Initially, a standardized number of cells (1×10^5) were maintained at 37°C under 5% CO_2 humidified conditions for 24 h in DMEM containing 10% fetal bovine serum (FBS), 0.03% L-glutamine, and 1% penicillin-streptomycin. Once the cells formed a monolayer, the growth medium was removed, and the cells were exposed to the different concentrations of basil extract (12.5, 25, 50, 100, 200, 500, and 1000 µg/mL) for 24, 48, and 72 h. The medium was then removed, and the cells were incubated in the MTT solution for 4 h. The medium was aspirated, and the formazan crystals were dissolved in 100 µL of solubilization reagent. The plates were incubated overnight at 37°C in a 5% CO_2 humidified environment. Finally, the absorbance was measured at 545 nm using an enzyme-linked immunosorbent assay (ELISA) reader (MultiscanGo, Thermo Scientific). The concentration that inhibited 50% of cell growth (half maximal inhibitory concentration (IC50)) was determined. Two assays with six replicates per dilution were analyzed in 96-well culture plates.

RNA extraction and real-time PCR

Total RNA from Ca9-22 cells was extracted using TRIzol, following the manufacturer's guidelines (Invitrogen, Massachusetts, United States). The extracted RNA was reverse-transcribed into complementary DNA (cDNA) using the Takara PrimeScript RT Master Mix (Takara Bio Inc., Japan), and the resulting cDNA was used for qRT-PCR analysis. The SYBR Green (Invitrogen, Massachusetts, United States) Premix was utilized. A real-time PCR system (7500 system, Applied Biosystems, Carlsbad, California, USA) was used to measure gene expression. β-actin was the internal control and the expression levels of genes involved in the cell cycle (cyclin D1, cyclin-dependent kinase 4 (CDK4), p21, p53) and inflammation (cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6) markers were evaluated. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method, allowing relative gene expression calculation by comparing the Ct values of the target genes with those of a reference gene. The results are presented as fold changes, indicating the extent of up- or downregulation of the target genes relative to the control sample to which only nutrient medium was added.

Enzyme-linked immunosorbent assay

To determine the cell cycle status and the level of inflammation markers in the culture supernatant, ELISA kits for human cyclin D1 (RayBiotech, USA), CDK4 (MyBioSource, USA), p21 (MyBioSource, USA), p53 (R&D system, USA), COX-2 (RayBiotech, USA), iNOS (Abcam, USA), TNF-α (R&D system, USA), IL-1β (R&D system, USA), and IL-6 (R&D system, USA) were used. Subsequently, marker levels were quantified following the manufacturers' instructions. For the stimulation of cytokine releases, samples were stimulated with lipopolysaccharide (LPS; 1000 ng/mL; *Escherichia coli* O111:B4).

Statistical analysis

Each experiment was repeated a minimum of three times. The results are presented as the mean±standard deviation (SD). Statistical significance was assessed using the Student's t-test or the Mann-Whitney U test. $P<0.05$ was considered statistically significant.

RESULTS

Cytotoxic effects of basil extract on Ca9-22 cells

The current study measured the survival rates of Ca9-22 cells using the MTT cell viability assay at different concentrations of basil extract. The results revealed significant effects of the concentration of basil extract and treatment duration ($P<0.001$). Cells treated with basil extract for 24 h exhibited the highest viability, which gradually decreased after 48 and 72 h of treatment. There was a concentration-dependent decrease in cell viability across all treatment durations. Additionally, the analysis identified a significant interaction between the time and concentration of basil extract, suggesting that the extract's effects on cell viability varied depending on both factors. Based on these findings, the study concluded that a 48-h treatment duration was most suitable for further investigations.

The results revealed a marked decrease in cell viability following basil extract treatment, particularly noticeable when Ca9-22 cells were exposed to concentrations of extract exceeding 12.5 μg/mL. A significant negative correlation between the basil extract dosage and Ca9-22 cell viability was observed, with higher extract doses resulting in a notable decline in cell viability ($P<0.001$). The IC50 values were 2143 μg/mL, 350 μg/mL, and 10^7 μg/mL after 24 h, 48 h, and 72 h of treatment, respectively. A concentration of 350 μg/mL inhibited half of the cells at 48 h, prompting its use for further investigations (Fig. 1).

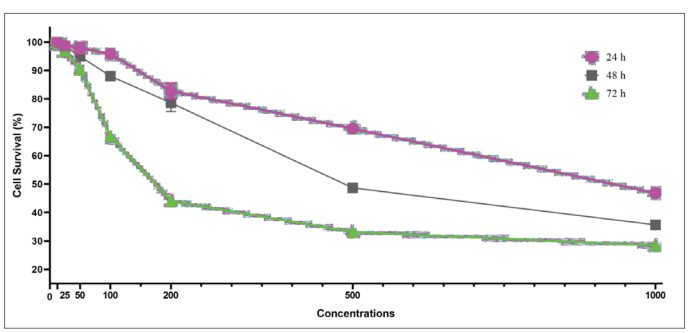


Fig. 1. The effect of different concentrations and times of treatment with the basil extract on cell viability. Effects of basil extract concentrations and time on Ca9-22 cell viability. MTT analysis was performed to define cell viability percentages of cells exposed to different concentrations of EA at 24, 48, and 72 h.

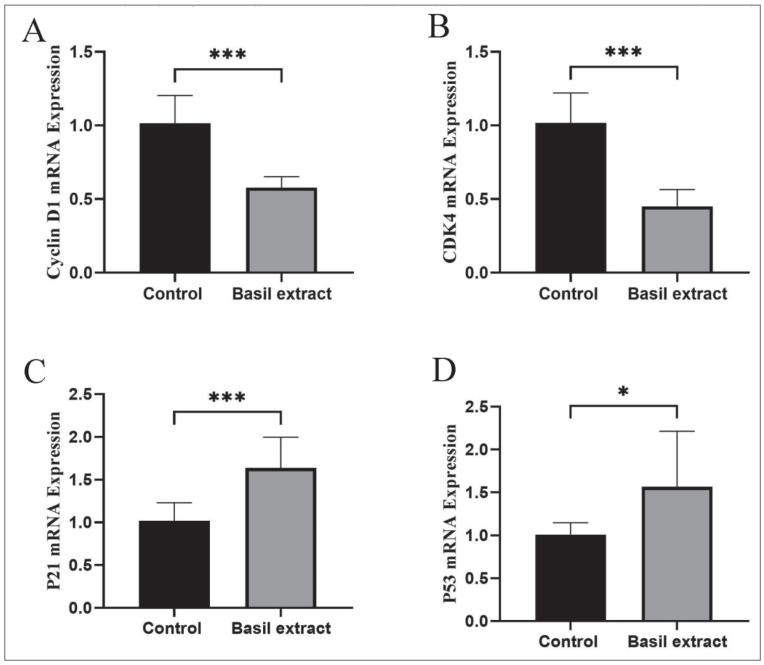
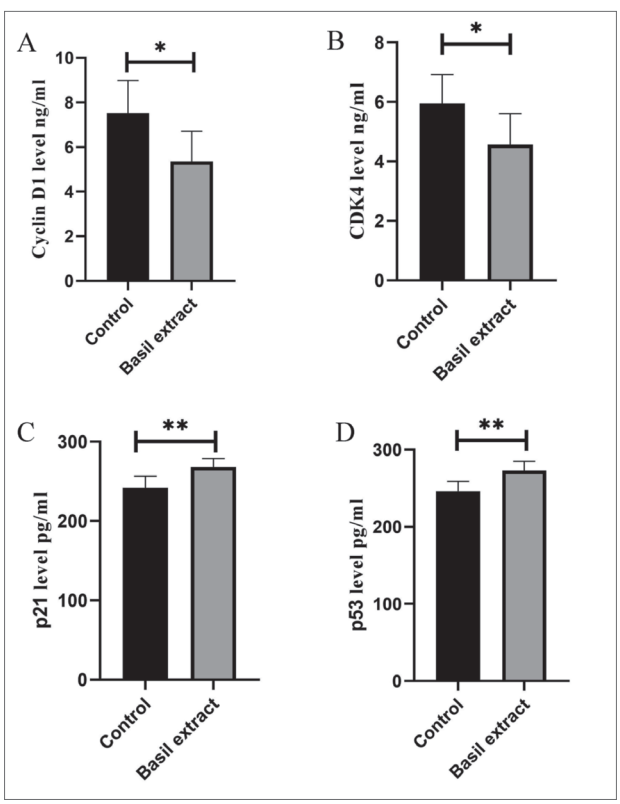


Fig. 2. Basil extract induces cell cycle arrest in Ca9-22 cells, as evidenced by RT-PCR analysis. The results of the RT-PCR analysis demonstrated a notable alteration in gene expression patterns in basil extract-treated cells compared to controls. Basil extract significantly decreased the expression of Cyclin D1 (**A**) and CDK4 (**B**) and, conversely, increased the expression of p21 (**C**) and p53 (**D**). The results are expressed as the mean ± standard deviation (SD). Statistical significance is indicated as *** for P<0.001, and * P<0.05. CDK4: Cyclin-dependent kinase 4.

Basil extract induces cell cycle arrest in Ca9-22 cells.

The results of the RT-PCR analysis demonstrated a notable alteration in gene expression patterns in basil extract-treated cells compared to controls. Specifically, there was a significant decrease in the expression levels of cyclin D1 and CDK4, the key regulators of the cell cycle, indicating a disruption in cell cycle progression. Conversely, the expression of p21 and p53 was markedly increased in treated cells, suggesting the activation of cell cycle arrest and apoptotic pathways (Fig. 2). These findings suggest that basil extract treatment induces molecular changes consistent with cell cycle arrest and apoptosis, contributing to the observed decrease in cell viability.

Utilizing the ELISA technique in supernatants of cultures, we corroborated the findings obtained through RT-PCR analysis, reaffirming the observed molecular alterations in response to basil extract treatment. Specifically, the ELISA results validated the decreased level of Cyclin D1 and CDK4, along with the concurrent increased level of p21 and p53 in the supernatant of treated cells compared to controls (Fig. 3). This confirmation provides additional support for the assumption that basil extract treatment induces cell cycle arrest and apoptosis pathways in Ca9-22 cells, contributing to the observed decrease in cell viability.



Suppression of inflammatory markers by basil extract

The impact of basil extract on the mRNA expression levels of pivotal inflammatory markers, including COX-2, iNOS, TNF-α, IL-1β, and IL-6, was examined. The

Fig. 3. Basil extract induces cell cycle arrest in Ca9-22 Cells (ELISA analysis). Basil extract significantly decreased the levels of Cyclin D1 (**A**) and CDK4 (**B**) and, conversely, increased the level of p21 (**C**) and p53 (**D**) in the supernatant of Ca9-22 cell cultures. The results are expressed as the mean±standard deviation (SD). Statistical significance is indicated as *** for P<0.001 and ** for P<0.01. CDK4: Cyclin-dependent kinase 4; ELISA: enzyme-linked immunosorbent assay.

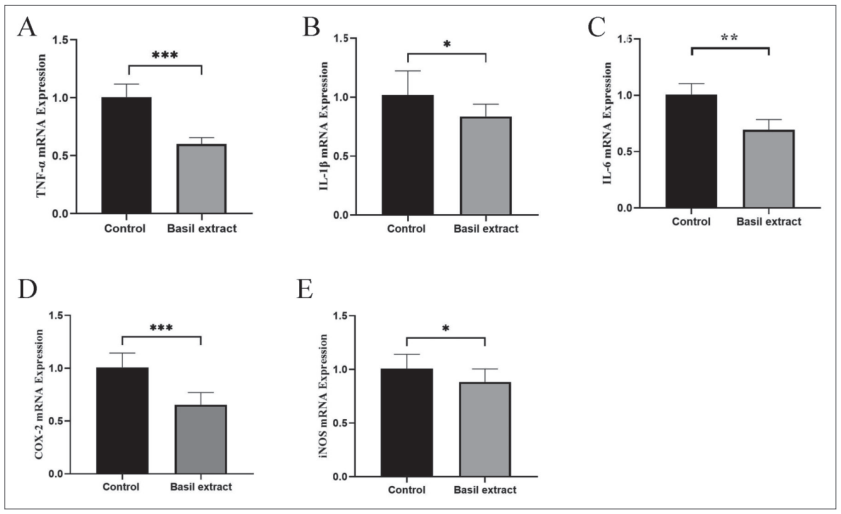


Fig. 4. Basil extract suppresses inflammatory marker expression in Ca9-22 cells (RT-PCR analysis). The results of the RT-PCR analysis unveiled a significant suppression of inflammatory mediators, including TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), COX-2 (**D**), and iNOS (**E**). The results are expressed as the mean±standard deviation (SD). Statistical significance is indicated as *** for p < 0.001, ** for P<0.01, and * for P<0.05. COX-2: cyclooxygenase-2, TNF-α: tumor necrosis factor-alpha, iNOS: inducible nitric oxide synthase, IL: interleukin.

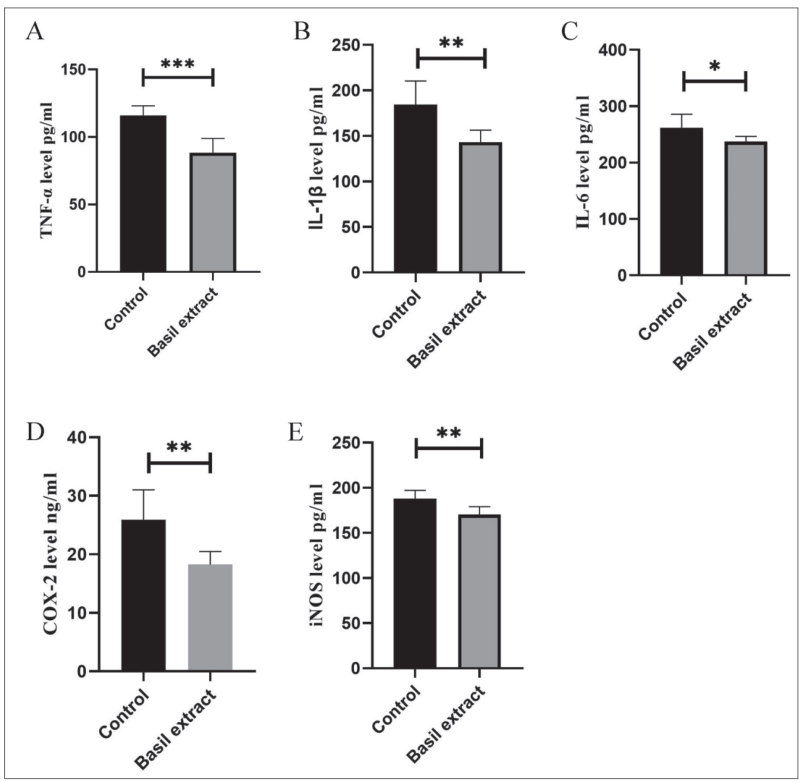


Fig. 5. Basil extract suppresses inflammatory markers in Ca9-22 cell culture supernatants (ELISA analysis). Basil extracts significantly reduced the protein levels of key inflammatory mediators, including TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), COX-2 (**D**), and iNOS (**E**). The results are expressed as the mean ± standard deviation (SD). Statistical significance is indicated as *** for p < 0.001, ** for P<0.01, and * P<0.05. COX-2: cyclooxygenase-2, TNF-α: tumor necrosis factor-alpha, iNOS: inducible nitric oxide synthase, IL: interleukin, ELISA: enzyme-linked immunosorbent assay.

results revealed significant suppression of inflammatory mediators, indicating the potent anti-inflammatory properties of basil extract (Fig. 4). Utilizing the ELISA technique, we substantiated our findings regarding the modulation of inflammatory markers by the basil extract. A significant reduction in the protein levels of key inflammatory mediators, including COX-2, iNOS, TNF-α, IL-1β, and IL-6 (Fig. 5), was revealed. The downregulation of COX-2 and iNOS mRNA expression suggests a reduction in the production of pro-inflammatory prostaglandin and nitric oxide production, respectively, which are key factors in inflammatory processes. Moreover, the decreased expression of TNF-α, IL-1β, and IL-6 mRNA signifies a dampened inflammatory response, as these cytokines play pivotal roles in orchestrating various aspects of inflammation.

DISCUSSION

The findings of this study present evidence that the ethanolic extract of basil leaves exerts potent cytotoxic effects on oral cancer cells by inhibiting the main regulators of cell proliferation and inflammation. These results are consistent with research highlighting basil's anticancer properties [20]. The affected molecular pathways suggest that basil extract holds promise as a novel therapeutic option against oral cancer, warranting further investigation.

Research has consistently demonstrated the anti-proliferative and anti-inflammatory activities of basil leaf extract against oral cancer cells. Elansary [21] found that basil leaf extract exhibited considerable antioxidant and anti-proliferative activities, with the highest activity observed in

the *O. basilicum* (Lamiaceae) cultivar. Manosroi et al. [22] further supported these findings, reporting that sweet basil oil showed the highest anti-proliferative activity on oral cancer cells. Additionally, Perna et al. [20] and Asl et al. [23] both highlighted the potential of basil leaf extract in inhibiting cancer cell growth and inducing apoptosis, with Perna et al. noting its ability to slow down tumor growth and progression. These studies collectively underscore the promising role of basil leaf extract in the treatment of oral cancer.

Basil leaf extract has been shown to inhibit the formation of DNA adducts of the procarcinogen 1'-hydroxyestragole, suggesting a potential protective effect against carcinogenesis [24]. It also exhibits important anti-cancer activities, including cell death, viability inhibition, cytotoxicity, apoptosis induction, and cell cycle arrest [20]. Furthermore, extracts of *Ocimum sanctum* (holy basil) leaves have been found to decrease tumorigenicity and metastasis of aggressive human pancreatic cancer cells, indicating a potential role in cancer therapy [25]. Similarly, extracts derived from the roots of *Scutellaria baicalensis* have been shown to inhibit the growth of human oral squamous cell carcinoma cells, suggesting potential anti-cancer activity [26]. These studies collectively indicate that basil leaf extract may have potential anti-cancer effects on Ca9-22 oral cancer cells.

The MTT assay results provided evidence of a concentration-dependent decrease in the viability of Ca9-22 cells following treatment with increasing concentrations of basil extract. Additionally, time-dependent effects showed a decline in cell viability with longer treatment durations. These findings suggest that basil extract inhibits the proliferation of oral cancer cells in a manner that is both dose- and time-dependent, consistent with studies examining its effects on other types of cancer cells [27-30]. Future investigations into the effects of basil extract on non-cancerous oral keratinocytes will shed light on its selectivity and safety profile.

RT-PCR and ELISA analyses revealed significant alterations in key cell cycle regulators and inflammatory pathways. Specifically, the downregulation of cyclin D1 and CDK4 expression, coupled with the simultaneous upregulation of p21 and p53 expression following basil treatment, indicates the induction of cell cycle arrest, particularly in the G1/S phase. Cyclin D1 and CDK4 typically promote cell cycle progression through multiple mechanisms [31-33]. These observed modulations align with the decrease in cell viability, indicating that cell cycle inhibition significantly contributes to basil extract's anti-proliferative effects on oral cancer cells.

The suppression of COX-2, iNOS, TNF-α, IL-1β, and IL-6 expression further validates basil extract's anti-inflammatory potential [34,35]. Elevated levels of these mediators are believed to promote the proliferation of malignant cells by fostering local inflammation, inhibiting apoptosis, and facilitating angiogenesis and metastasis [36]. Therefore, their downregulation may offer clinical benefits through multiple mechanisms. Intriguingly, certain spices with known anti-inflammatory properties, such as turmeric, have shown synergistic effects with conventional therapies against various cancers, including oral cancer, suggesting potential adjunctive applications [37]. Based on these preliminary mechanistic insights, future studies exploring basil extract in combination with immunotherapy or chemoradiation regimens against oral cancer models are warranted.

A significant constraint of the present study is its reliance on a single oral cancer cell line; this calls for verification across diverse molecular subtypes to broaden the applicability of conclusions about the effects of basil extract. Broadening the scope of research to encompass validation in patient-derived xenografts and genetically engineered mouse models of oral cancer, which more accurately mimic tumor heterogeneity and the immune microenvironment, would strengthen preclinical evidence prior to clinical translation. Furthermore, investigating mechanisms underlying cell death induction, such as mitochondrial pathways, and assessing impacts on tumor-supporting stromal and immune cells, could provide a more comprehensive understanding of basil extract's anti-cancer properties. Additionally, evaluating its effects on cancer stem-like cell self-renewal and tumor-initiating potential may further substantiate its potential as an intervention targeting tumor propagation and recurrence.

From a translational standpoint, comprehensive toxicity profiling in animal models is imperative to determine the safety of systemic administration of basil

extract before advancing to clinical trials. However, parameters such as the maximum tolerated dose, reproductive and developmental effects, and potential drug interactions must still be meticulously evaluated because of interspecies variability. Also, to provide further insights, it would be beneficial to compare the effectiveness of the basil extract with standard agents commonly used for oral cancer treatment, such as cisplatin or 5-fluorouracil.

CONCLUSIONS

In conclusion, this study highlights the suppressive impact of basil leaf ethanolic extract on the survival and cell cycle progression of oral cancer cells *in vitro*, likely through the reduction of inflammatory mediators. Further investigations aimed at characterizing the bioactive constituents, confirming activity in more clinically relevant models, thoroughly evaluating toxicity profiles, and devising practical delivery approaches are essential steps toward translating these promising findings into clinical practice. Given the anti-proliferative and anti-inflammatory properties highlighted by this study, basil extract merits ongoing research efforts as a potential avenue for combating this lethal disease, for which no herbal preventive or curative options currently exist.

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Author contributions: ML; writing, original draft preparation, methodology, investigation, Conceptualization. YW; data curation, formal analysis, writing – review and editing. YB; supervision, writing – review and editing, project administration. All authors have read and agreed to the published version of the manuscript.

Conflict of interest disclosure: The authors declare that there are no conflicts of interest.

Data availability: Data underlying the reported findings have been provided as a raw dataset available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Li%20et%20al_Raw%20Dataset.docx

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