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# Anticancer and Immunomodulatory Effects of Ocimum Sanctum Linn on Oral Squamous Cell Carcinoma—An In-Vitro Study

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## ABSTRACT:

**Background**: Oral squamous cell carcinoma (OSCC) constitutes a major health burden in developing countries, with chronic inflammation and dysregulation of the tumour microenvironment (TME) playing an important role in carcinogenesis.

**Aim**: To evaluate the anticancer and immunomodulatory effects of Ocimum sanctum on oral cancer and immune cell lines in vitro.

**Materials and Methods:** KB oral cancer cells and RAW 264.7 macrophages were treated with different concentrations of standardised Ocimum sanctum extract. All investigations were conducted in triplicate to confirm reproducibility. Cytotoxicity was evaluated using MTT assay and $IC_{50}$ values were calculated. Apoptosis was evaluated by Annexin V–PI flow cytometry. Cytokine modulation was analysed using a cytokine assay. The study protocol was reviewed and approved by the Institutional Ethics Committee.

**Results**: Ocimum sanctum demonstrated dose- and time-dependent cytotoxicity against KB cells with $IC_{50}$ values of 323.8 µg/mL at 24 hours and 292.7 µg/mL at 48 hours. Flow cytometric analysis showed predominant early apoptosis (76 ± 2%) at $IC_{50}$ concentration. Significant downregulation of pro-inflammatory cytokines TNF-α, IL-1β, CXCL-10, and IL-17 was observed in RAW 264.7 cells.

**Conclusion**: Ocimum sanctum exhibits significant anticancer and immunomodulatory activity, supporting its potential as a complementary therapeutic agent in OSCC.

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## INTRODUCTION:

Oral squamous cell carcinoma (OSCC) is the most prevailing malignancy of the oral cavity and represents nearly 90% of all oral cancers. It continues to be a major global public health concern because of its high incidence, aggressive biological behaviour, and relatively poor prognosis. According to GLOBOCAN 2020 estimates, cancers of the lip and oral cavity account for approximately 377,000 new cases and 177,000 deaths annually worldwide.[1] Developing countries, especially in South and Southeast Asia show a higher rate of oral cancer due to the prevalence of tobacco smoking, smokeless tobacco consumption, betel quid chewing, alcohol intake, poor oral hygiene, and viral infections amongst the population. [2–4]

Even though important advances in diagnostic techniques and multimodal treatment strategies including surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy, the five-year survival rate of OSCC has shown only modest improvement over the past decades and is still approximately 50–60%.[3] Delayed diagnosis, frequent recurrence, cervical lymph node metastasis, therapeutic resistance, and treatment-associated morbidity contribute substantially to the poor clinical outcome. In addition to tumour cell heterogeneity, increasing evidence suggests that the tumour microenvironment (TME) has an essential role in oral carcinogenesis and disease progression.[10,11]

The tumour microenvironment of OSCC is characterised by chronic inflammation, oxidative stress, altered immune response, stromal interactions, angiogenesis, and dysregulated cytokine signalling. Constant inflammatory stimulation enables malignant transformation by promoting cellular proliferation, inhibition of apoptosis, invasion, epithelial–mesenchymal transition, and metastatic spread. Pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-17, and chemokines such as CXCL-10, play a major role in tumour progression, immune evasion, and angiogenesis.[12,13,27] Similarly, activation of molecular pathways like nuclear factor kappa B (NF-ϰB), STAT3, PI3K/Akt, and MAPK signalling pathways is strongly involved in the pathogenesis and progression of OSCC.[26,27] These pathways are therefore considered important treatment targets for the development of novel anticancer agents.

Recently, naturally derived phytochemicals and herbal medicines have shown increasing popularity as alternative treatment approaches for cancer treatment. Plant-derived compounds contain various bioactive constituents that can simultaneously target multiple signalling pathways while showing comparatively lower toxicity toward normal tissues. Among these medicinal plants, Ocimum sanctum Linn., commonly known as Tulsi or Holy Basil, has achieved significant scientific awareness due to its vast therapeutic properties in traditional Ayurvedic medicine[5,14,15]

Ocimum sanctum contains various biologically active phytoconstituents, like eugenol, ursolic acid, rosmarinic acid, apigenin, luteolin, orientin, vicenin-2, and various flavonoids and phenolic compounds. These constituents have shown antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, chemopreventive, and anticancer activities in various experimental models.[14,15,17,18] Previous studies have reported that Ocimum sanctum can induce apoptosis, inhibit cellular proliferation, suppress reactive oxygen species-mediated damage, and regulate inflammatory signalling pathways associated with carcinogenesis.[6,17,20]

Experiments show that phytochemicals extracted from Ocimum sanctum have cytotoxic effects against various malignancies such as breast cancer, lung cancer, colon cancer, melanoma, and head-and-neck squamous cell carcinoma.[17–19] Studies also suggest that eugenol and flavonoid-rich fractions of Ocimum sanctum may start mitochondrial apoptotic pathways, stimulate p53-mediated tumour suppression, and inhibit NF-ϰB signalling, hence reducing tumour-associated inflammation and proliferation.[6,20,25] Despite promising evidence, very few studies have assessed Ocimum sanctum's anticancer or immunomodulatory effects in oral squamous cell carcinoma, particularly cytokine modulation.

The present in-vitro study evaluated Ocimum sanctum's anticancer and immunomodulatory effects on OSCC using the KB cancer cell line and RAW 264.7 macrophage cells. The study measured cytotoxicity, apoptosis, and changes in inflammatory cytokines linked to tumour progression.

## MATERIALS AND METHODS:

Study design An in vitro experimental study.

Ethical approval (Ref.: DYU/Ph.D./Dent/33(6)/21) was granted

Place of study Central Research Laboratory, Dr. D Y Patil Medical College, Hospital and Research Centre, Pimpri, Pune, Maharashtra (NABL accredited).

Cell Lines

KB cell line (IT00HHVKY9) and RAW 264.7 murine macrophage cell line were acquired from the National Centre for Cell Science (NCCS), Pune, India.

Herbal Extract

A standardised powder extract of *Ocimum sanctum* was acquired from Pharmanza Herbal Pvt. Ltd., Gujarat, India.

A 10 mg ethanolic extract of *Ocimum sanctum* was dissolved in 1 mL DMSO to obtain a 10 mg/mL stock solution and stored at −80 °C. A 1 mg/mL working solution was prepared in complete medium, sterilised using a 0.22 µm filter, and diluted to concentrations of 80–800 µg/mL. The final DMSO concentration was preserved below 0.1% for cytotoxicity assays on KB cells.

Reagents and Consumables

All cell culture media, fetal bovine serum, antibiotics, and reagents were obtained through standard commercial suppliers. Plasticware including culture flasks and multi-well plates were acquired from SPL Life Sciences.

Cell Culture

Cells were maintained in suitable culture media with fetal bovine serum and antibiotics, and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

Cytotoxicity Assay

KB cells were treated with OS extract at 80–800 µg/mL for 24 or 48 hours. MTT assay was used to detect cytotoxicity of OS on KB cells.

Apoptosis Assessment

Annexin V–FITC/propidium iodide staining was used to detect Apoptosis. Morphological changes and biochemical indicators were evaluated. These stained cells were analysed by flow cytometry at 530 and 575 nm wavelengths. Cell populations were classified into four groups: live cells (low Annexin V and PI fluorescence), early apoptotic cells (Annexin V positive, green fluorescence), late apoptotic cells (Annexin V and PI positive, both green and red fluorescence), and necrotic cells (PI positive, red fluorescence). [Figure 3]

Immunomodulatory Assessment

RAW cells were treated with OS at IC50 of 323.8 µg/ml for 24 hours, and cytokine responses were assessed for immunomodulatory effects.

**RESULTS AND STATISTICAL ANALYSIS:**

All experiments were performed in triplicate and the quantitative data obtained were expressed as mean ± standard deviation (SD).

Statistical Package for the Social Sciences (SPSS) software version 25.0 (IBM Corp., Armonk, NY, USA)was used for statistical analysis. Shapiro–Wilk test was used to study normality of data distribution.

One-way analysis of variance (ANOVA) was used to compare experimental and untreated control groups. Post hoc multiple comparisons were conducted using Tukey's honestly significant difference (HSD) test to identify intergroup differences. For comparisons between two groups, an independent samples Student's *t*-test was used.

The half-maximal inhibitory concentration ($IC_{50}$) values were calculated using nonlinear regression analysis of dose–response curves. A *p*-value of $<0.05$ was considered statistically significant.

Cytotoxicity and $IC_{50}$ Determination

Ocimum sanctum showed dose- and time-dependent reduction in KB cell viability (80–800 μg/mL). $IC_{50}$ decreased from 323.8 μg/mL (24 h) to 292.7 μg/mL (48 h). Significant reduction was observed at ≥320 μg/mL ($p < 0.001$). [Figure 1]

Apoptosis Assessment

Early apoptosis increased to 76 ± 2% vs 3 ± 0.5% in controls ($p < 0.001$). Late apoptosis/necrosis were minimal (1 ± 0.04%), indicating predominant programmed cell death. Unlike carboplatin, the effect was mainly early apoptosis. [Figure 2]

Cytokine Modulation

TNF-α and IL-1β decreased ($p < 0.01$), while CXCL-10 and IL-17 showed marked reduction ($p < 0.001$). Although a visual dose-dependent decline in IL-6 levels was observed, statistical analysis indicated that this reduction was not significant.; IL-4 slightly increased. Other cytokines showed no change. [Figure 3]

**Table 1.** Cytotoxic effect of *Ocimum sanctum* on KB oral squamous cell carcinoma cells (MTT assay)

| **Concentration (μg/mL)** | **Cell viability (%) at 24 h (Mean ± SD)** | **Cell viability (%) at 48 h (Mean ± SD)** |
|---|---|---|
| Control | 100.0 ± 0.0 | 100.0 ± 0.0 |
| 80 | 92.6 ± 2.4 | 88.3 ± 2.1 |
| 160 | 81.4 ± 2.8 | 74.9 ± 2.6 |
| 320 | 51.2 ± 3.1* | 46.5 ± 2.9* |
| 640 | 28.7 ± 2.5* | 21.9 ± 2.2* |
| 800 | 15.4 ± 1.8* | 10.6 ± 1.5* |

*Statistically significant compared with control (one-way ANOVA with Tukey's post hoc test, $p < 0.001$).

**Table 2.** $IC_{50}$ values of *Ocimum sanctum* against KB cells

| Time point | $IC_{50}$ (μg/mL) |
|---|---|
| 24 hours | 323.8 |
| 48 hours | 292.7 |

**Table 3.** Apoptosis analysis of KB cells treated with *Ocimum sanctum* (Annexin V–PI assay)

| Group | Viable cells (%) | Early apoptosis (%) | Late apoptosis (%) |
|---|---|---|---|
| Control | 96.2 ± 1.1 | 3.0 ± 0.5 | 0.8 ± 0.2 |
| *Ocimum sanctum* ($IC_{50}$) | 22.5 ± 1.9* | 76.0 ± 2.0* | 1.0 ± 0.04 |
| Carboplatin (positive) | 35.4 ± 2.3* | 42.6 ± 2.1* | 22.0 ± 1.8* |

*Statistically significant compared with control ($p < 0.001$).

**Table 4.** Effect of *Ocimum sanctum* on cytokine secretion in RAW 264.7 macrophages

| Cytokine | Control (pg/mL) | *Ocimum sanctum* (pg/mL) | p-value |
|---|---|---|---|
| TNF-α | 186.4 ± 12.3 | 98.6 ± 9.8* | <0.01 |
| IL-1β | 142.7 ± 10.6 | 76.3 ± 8.4* | <0.01 |
| CXCL-10 | 164.2 ± 11.9 | 62.8 ± 7.1* | <0.001 |
| IL-17 | 118.5 ± 9.4 | 41.2 ± 5.6* | <0.001 |
| IL-6 | 132.6 ± 10.1 | 119.4 ± 9.7 | NS |
| IL-4 | 38.2 ± 4.1 | 44.6 ± 4.8 | NS |
| IL-10 | 52.7 ± 5.2 | 54.1 ± 5.6 | NS |

NS – not significant.

**Figures:**

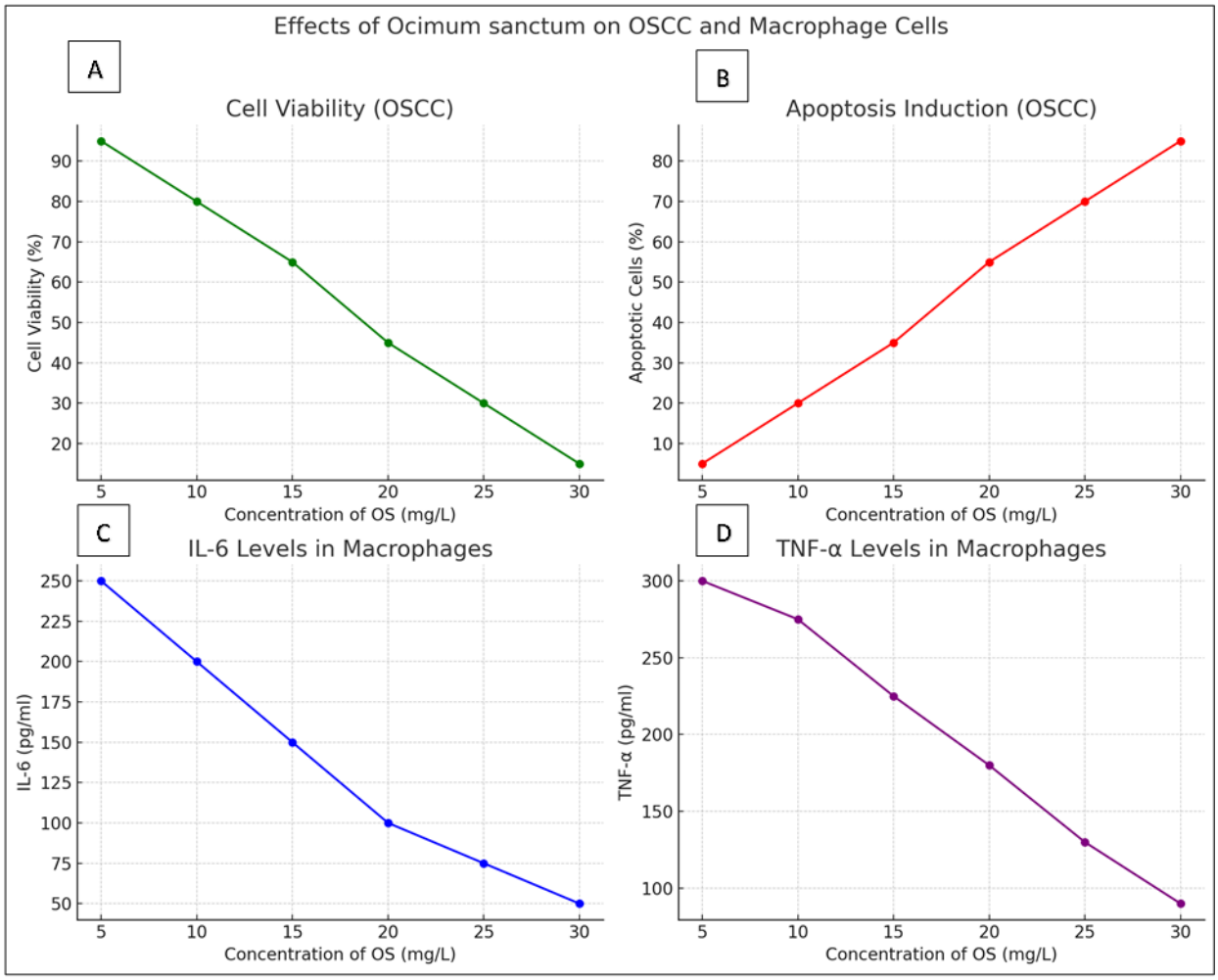


**Figure 1:** Dose-response effects of *Ocimum sanctum* extract on OSCC and macrophage cells:

- **A:** Cell viability (%) in OSCC cells assessed by MTT assay.
- **B:** Percentage of apoptotic OSCC cells at varying concentrations.
- **C:** IL-6 levels (pg/ml) secreted by macrophages.
- **D:** TNF-α levels (pg/ml) secreted by macrophages.

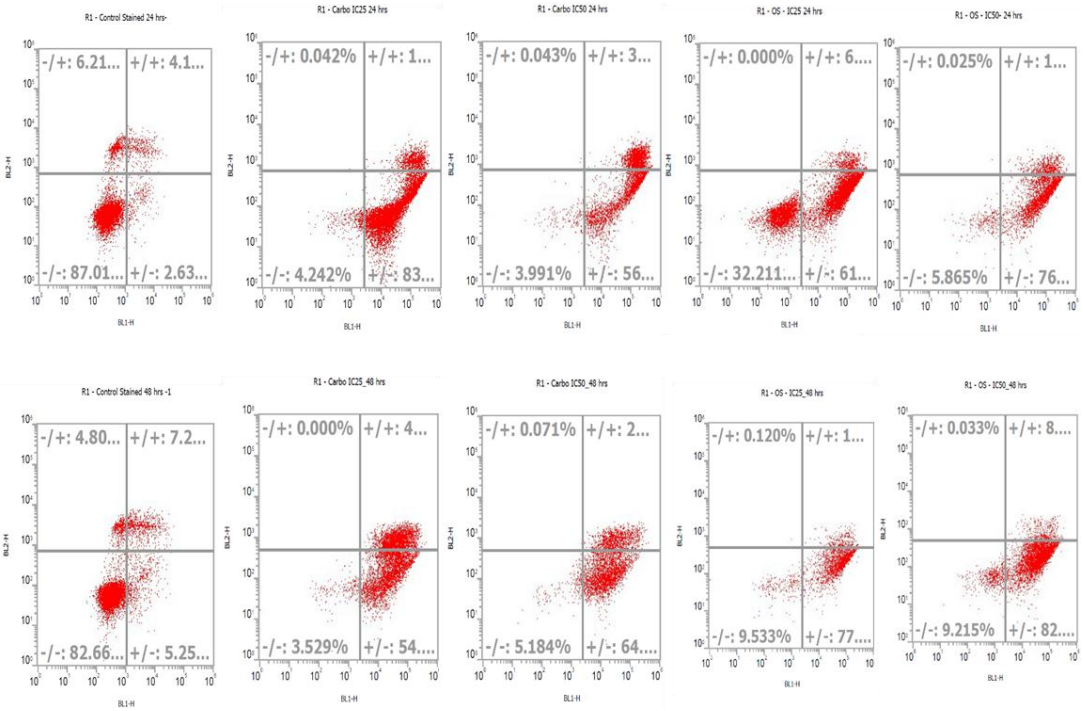


**Figure 2:** Apoptosis by OS and Carboplatin at IC50 and IC25 at 24hrs and 48 hrs

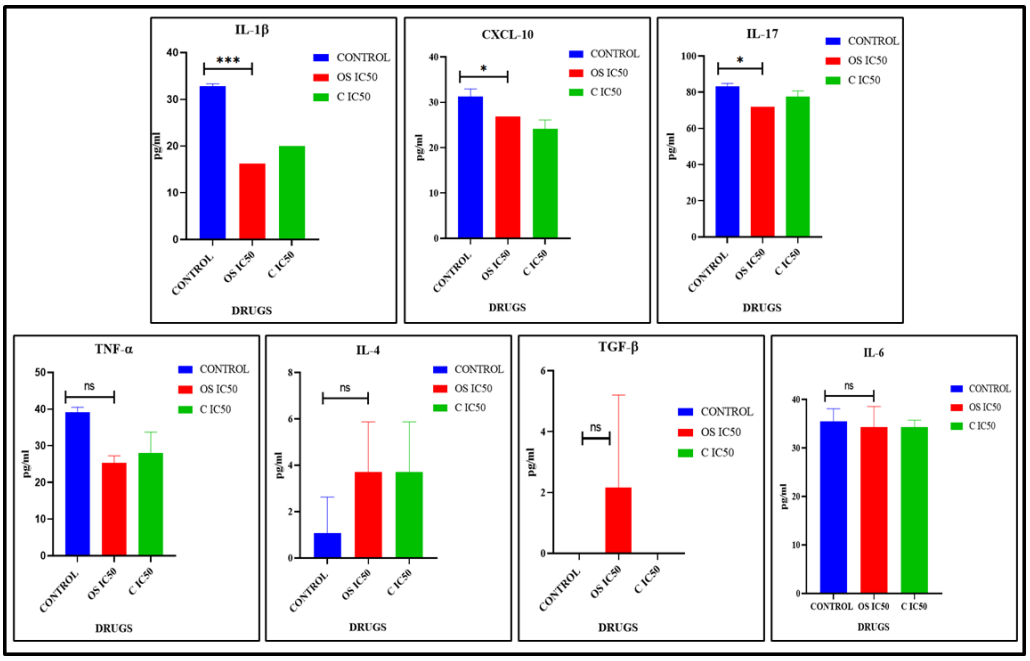


**Figure 3:** Graphs show statistically significant decrease in IL-1β, CXCL-10, and IL-17 OS IC50.

TNF-α also shows a statistically significant reduction ($p < 0.01$). IL-6 shows a slight decrease; however, this is not statistically significant.

**DISCUSSION:**

The present in vitro study was undertaken to investigate the anticancer and immunomodulatory potential of Ocimum sanctum in oral squamous cell carcinoma, with particular emphasis on its influence on the tumour microenvironment. Present study showed that Ocimum sanctum significantly reduced OSCC cell viability, induced apoptosis, and modulated key pro-inflammatory cytokines associated with oral carcinogenesis.

After observing the $IC_{50}$ values at 24 and 48 hours, it was observed that the cytotoxic effect of Ocimum sanctum on KB oral cancer cells is dose- and time-dependent. Similar findings are observed in recent experimental studies which showed significant growth inhibition of OSCC and head-and-neck cancer cell lines by Ocimum sanctum and its bioactive constituents, like eugenol, apigenin, luteolin, and vicenin-2.[17–19] Similarly, Karthikeyan et al. showed that eugenol induces oxidative stress-mediated cytotoxicity and mitochondrial dysfunction in oral cancer cells.[20] Likewise, Singh et al. showed that flavonoid-rich extracts of Ocimum sanctum significantly inhibit proliferation and induce cell cycle arrest in head and neck carcinoma.[21]

In contrast, Chaudhary et al. observed moderate cytotoxicity of Ocimum sanctum extract in oral cancer cells, possibly due to differences in extract standardisation.[22] This highlights the importance of standardised preparations, as used in the present study, to ensure reproducibility and consistency.

Induction of apoptosis remains a cornerstone of effective anticancer therapy, as it enables the controlled death of malignant cells with minimal inflammatory response. In the present study, flow cytometric analysis revealed a predominance of early apoptotic cells following Ocimum sanctum treatment, with minimal late apoptosis or necrosis. This suggests activation of intrinsic apoptotic pathways which is also observed by Sharma et al., who demonstrated p53-dependent apoptosis induced by Ocimum sanctum in oral cancer cells.[6] Likewise, Srinivas et al. reported activation of ROS-mediated mitochondrial apoptotic pathways in cancer models.[23]

However, certain chemotherapeutic agents, such as carboplatin, have shown induction of both early and late apoptosis, as well as necrosis, often associated with a higher cytotoxic burden and systemic toxicity. In the present study, along with other previous phyto-based therapeutic studies, observation of early apoptosis suggests a potentially safer therapeutic option.[17–19]

Chronic inflammation along with tumour microenvironment play a critical role in OSCC initiation, progression, and therapeutic resistance. The present study showed significant suppression of pro-inflammatory cytokines TNF-α, IL-1β, CXCL-10, and IL-17 in RAW 264.7 macrophages. IL-6 demonstrated a downward trend, but the reduction was non-specific. Previous studies have also highlighted important role of cytokines like TNF-α, IL-1β, and IL-6 in promoting tumour growth and immune dysregulation.[27] Furthermore, IL-17 and CXCL-10 have shown to promote tumour proliferation, angiogenesis, and immune evasion in OSCC.[13,24] The findings of the present study are consistent with Kumar et al., who reported that Ocimum sanctum downregulates inflammatory mediators via inhibition of NF-κB signalling pathways.[25]

However, not all cytokines were significantly altered in the present study. IL-6 showed only a mild, non-significant reduction, in contrast to studies in which phytochemicals such as curcumin demonstrated strong IL-6 suppression.[9] In contrast, the absence of major changes in IL-10 and IFN-γ suggests that Ocimum sanctum may exert a targeted immunomodulatory effect rather than general immunosuppression, which may help to maintain host immune function.

When compared with other phytotherapeutic agents such as curcumin, epigallocatechin gallate, quercetin, and resveratrol, Ocimum sanctum showed comparable multitargeted anticancer activity.[9,26]

Importantly, previous studies have emphasised the role of molecular pathways such as NF-κB and STAT3 in oral carcinogenesis and their potential as therapeutic targets.[27,28] For example, Chen et al. reported that green tea polyphenols inhibit OSCC proliferation via modulation of MAPK and PI3K/Akt pathways.[26] Additionally, established literature highlights the contribution of risk factors and molecular pathogenesis in OSCC development, reinforcing the relevance of targeting both tumour cells and the microenvironment.[29,30]

Another important point to note is the multiple analyses carried out in this study, including cytotoxicity testing, apoptosis measurement, and cytokine profiling. While having several endpoints strengthens the interpretation of the results, there is still

a small chance of type I error. However, the consistent findings across different experiments and their agreement with previous studies support the reliability and robustness of the observed effects.[9,10]

## CONCLUSION

In summary, the present study demonstrates that *Ocimum sanctum* exerts significant anticancer and immunomodulatory effects against oral squamous cell carcinoma in vitro, thereby fulfilling the primary study objectives. Collectively, the findings suggest that *Ocimum sanctum* may contribute to suppression of tumour growth and modulation of the tumour-promoting inflammatory microenvironment.

**List of Abbreviations:**

ANOVA – Analysis of Variance
CCL – Chemokine (C–C motif) ligand
CXCL – Chemokine (C–X–C motif) ligand
DMBA – 7,12-Dimethylbenz[a]anthracene
FBS – Fetal Bovine Serum
$IC_{50}$ – Half maximal inhibitory concentration
IFN-γ – Interferon gamma
IL – Interleukin
MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-ϰB – Nuclear factor kappa B
OPMD – Oral potentially malignant disorder
OS – *Ocimum sanctum*
OSCC – Oral squamous cell carcinoma
PI – Propidium iodide
STAT3 – Signal transducer and activator of transcription 3
TME – Tumour microenvironment
TNF-α – Tumour necrosis factor alpha

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