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Original Article

Bioinformatics and Experimental Validation of *Safranal* Modulating the Epithelial-Mesenchymal Transition in Oral Cancer by Regulating Wnt/β-Catenin and Transforming Growth Factor-β/Smad Signalling

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ABSTRACT

Objectives: Oral Squamous Cell Carcinoma (OSCC), indicating a health challenge due to recurrence and metastasis. Epithelial-Mesenchymal Transition (EMT), regulated key signaling pathways like Smad /TGF-β and Wnt/ β-catenin plays an important role in cancer progression. *Safranal*, derived from *Crocus sativus*, possess notable antioxidant and anti-inflammatory activities. To investigate the *Safranal* inhibition of EMT via Smad /TGF-β and Wnt/ β-catenin pathways. **Materials and Methods:** Antioxidant and Anti-inflammatory activities of *Safranal* were evaluated using DPPH and protein denaturation assays. KB oral cancer cell line was used to assess the cytotoxicity via MTT assay. The interactions of TGF-β, SMAD, β-catenin and Wnt genes were analysed by docking. Network pharmacology analysis was applied to analyze the pathway involved in EMT regulation. **Results:** Antioxidant and anti-inflammatory effects with significant reduction in OSCC cell viability and apoptosis related morphological changes of *Safranal* in a dose dependent manner. Moderate binding affinities of *Safranal* with EMT-related targets including β-catenin, Wnt (-5.4 kcal/mol), SMAD (-5.3 kcal/mol) and TGF-β (-5.1 kcal/mol) highlighting its role in signaling pathways. **Conclusion:** *Safranal* modulates EMT signalling in OSCC through anti-inflammatory and antioxidant properties. These results suggest that it may be used as a therapeutic agent to lower metastasis and enhance therapy approaches.

Keywords: Cancer, Health, Molecular docking, Network pharmacology, *Safranal*.

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INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) comprising of 90% head and neck malignancies globally. Because of lifestyle factors like drinking alcohol, smoking tobacco and chewing betel quid, it is more common especially in Southeast Asia (Warnakulasuriya, 2009). The prognosis of OSCC is poor, with a survival rate below 50%, due to delayed diagnosis, therapeutic resistance and recurrence rate (Shield *et al*., 2017). Chronic inflammation, DNA damage and Epithelial Mesenchymal Transition (EMT) are the contributors to OSCC initiation and progression (Gupta and Johnson, 2014). Dysregulation of the Smad /Transforming Growth Factor-β and Wnt/ β-catenin pathways was a key factor in EMT, where TGF-β pathway exhibits a carcinogenic role in early stages of tumour suppression and progression of tumour. Activation of Smad-dependent signaling induces Snail, Twist and Slug leading to E-cadherin repression and enhancement of mesenchymal phenotype (Zhang *et al*., 2020). When the Wnt/β-catenin pathway was abnormally activated, cytoplasm gets stabilized and the β-catenin nucleus is translocated, which promotes the transcription of genes linked to invasion, proliferation and dispersion which is responsible for the aggressive behaviour of OSCC (Xu *et al*., 2009).

Natural compounds with potential therapeutic targets have been gaining attention due to multi-targeted actions and safety profiles. *Safranal*, a monoterpene derived from *Crocus sativus* (saffron), was known for its antioxidants, anti-inflammatory and anticancer properties (Clevers and Nusse *et al*, 2012). It has also demonstrated the cytotoxicity against the cancer cell lines,

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highlighting its therapeutic potential with less toxicity (Amin and Hosseinzadeh, 2009). Emerging evidence suggests that *Safranal* exerts anticancer effects by inducing apoptosis, oxidative stress modulation and key signaling pathway interference such as PI3K/AKT (Aung *et al*., 2009). In oral cancer models, *Safranal* has shown to inhibit EMT by restoring the epithelial characteristics and suppressing mesenchymal markers such as Vimentin and Cadherin. Colorectal and lung cancers indicated that *Safranal* enhances chemosensitivity by inducing cell cycle arrest and impairs DNA repair (Samarghandian *et al*., 2009; Lim *et al*., 2021). *In vivo* studies support low systemic toxicity and pharmacological benefits (Ziaee *et al*., 2014).

Despite these findings, the molecular mechanism of *Safranal* in the EMT-associated pathway in OSCC was poorly understood. Research has primarily concentrated on its cytotoxic and apoptotic effects, with limited investigation into its role in modulating signaling networks such as the Smad /Transforming Growth Factor-β and Wnt/ β-catenin pathways. This study aimed to provide mechanistic insights to support *Safranal* as a safe and effective anti-oxidant and anti-inflammatory agent in oral cancer management.

MATERIALS AND METHODS

Preparation of plant extract

The study was done between November 2025 and February 2026 after obtaining approval from Scientific committee Board of Saveetha Dental College, Chennai, Tamilnadu, India (SRB/SDC/UG-2068/26/GPATH/134). *Crocus sativus* was obtained from botanical garden in Chennai, Tamil Nadu. It was authenticated and identified by Sri Vidya Mandir Arts and Science College (Autonomous), Katteri, Krishnagiri District, Tamil Nadu, India, under certificate number of SVMC/BOT/274/2025-26. 10 g of plant powder were combined with 70% ethanol, followed by filtration and concentration under 40°C pressure to get a crude extract. The extract was stored at 4°C in amber containers.

Antioxidant activity

DPPH radical scavenging activity

DPPH free radical scavenging assay was performed according to the method described by Vallinayaki *et al*. (2024) to assess the antioxidant activity of *Safranal* (Vallinayaki *et al*., 2024). Methanolic solution was prepared by dissolving 4.3 mg of 1,1-diphenyl-2-picrylhydrazyl in 3.3 mL of methanol to create a fresh DPPH solution, which was then protected from light with aluminum foil. After mixing 150 µL of DPPH solution with 3 mL of methanol, absorbance at 517 nm was measured.

For test samples, extracts at different concentrations and ascorbic acid (standard reference) were prepared. Each sample of 50 µL was adjusted to the final volume of 3 mL using methanol, followed by adding 150 µL of DPPH solution. After 15 min of dark incubation, the absorbance was recorded at 517 nm using a Shimadzu UV-1601, Japan with methanol serving as blank solution. The percentage of DPPH radical scavenging activity was determined by the below formula:

$$\% \text{ scavenging} = (\text{control absorbance - sample absorbance/control absorbance}) \text{ X } 100$$

Anti-inflammatory activity

Protein denaturation inhibition assay

Safranal's potential was evaluated using a technique outlined by Nandita *et al*. (2019) based on its capacity to prevent protein denaturation (Nandita *et al*., 2024). The reaction mixture, with the total volume of 5 mL, consisted of 0.2 mL of 1% bovine serum albumin, phosphate buffered saline (pH 6.4) of 4.78 mL, test extract of 0.02 mL. To cause denaturation of the protein, the mixture was heated to 70°C for 5 min after being incubated at 37°C for 15 min.

UV-visible spectrophotometer (Optima SP-3000, Tokyo, Japan) was used to measure turbidity at 660 nm and cooled to room temperature. The control was a phosphate buffer solution. The percentage of inhibition was measured by.

$$\% \text{ Inhibition} = \frac{\text{Control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

Cell culture

The National Centre of Cell Science provided the KB cells, a human oral cancer cell line. Dulbecco's Modified Eagles Medium (DMEM) supplemented 1% antibiotic solution (HiMedia, India) comprising of streptomycin and penicillin was used to sustain the cells and 10% Fetal Bovine Serum (FBS) followed by culture with 5% CO_2 at 37°C.

Cell viability assay

Safranal's cytocompatibility was examined using the MTT colorimetric test on KB cells. 1*10^4 cells are seeded into each well of 96-well plates, and the cells are allowed to adhere overnight. Dulbecco Modified Eagles Medium (DMEM) with Dimethyl Sulfoxide (DMSO) were used to dissolve the compounds; the final DMSO concentration was kept between 10 and 100 µM. After incubation with *Safranal* for 24 and 48 hr, the culture media was withdrawn, and the cells were extracted. After adding 10 µL of MTT solution to the well, plates were incubated for 2 hr to allow formazan crystals to form. After dissolving the crystals in DMSO, the absorbance was measured at 590 nm (Echhpal *et al*., 2024).

Bioinformatic analysis

Database collection

The molecular targets were identified through bioinformatics approach. GeneCards and comparative toxicogenomics databases were used to obtain protein information and corresponding

genes. The genes associated with oral cancer and those linked to exposure by *Safranal* were collected using these datasets.

To determine the therapeutic interactions of *Safranal*, the targets that overlap between genes linked to oral cancer were selected. The purpose of identifying these targets was to assess the possibility for therapeutic intervention. FunRich Software (Version 3.1.3) was used to display these targets, allowing Venn diagrams to be created to show the similar genes. This method has made it easier to identify important molecular entities and gain understanding of the biological processes underlying the pathophysiology of oral cancer (Davis *et al*., 2023).

Network pharmacology analysis

A Drug-Protein Interaction (DPI) network has been employed to investigate the interactions between *Safranal* and its molecular targets. The STITCH database (http://stitch.embl.de/), an online tool for predicting chemical and protein interactions, was utilized to examine Differentially Expressed Genes (DEGs). Network creation was done using Cytoscape (http://www.cytoscape.org; version 3.5.1). The network was enhanced by altering edge thickness depending on confidence score interaction, which corresponds to the degree of association between nodes. The CytoHubba plugin was used to find the regulatory elements, allowing hub gene identification and key cluster interaction (Shannon *et al*., 2003).

Functional enrichment and pathway analysis

Annotation and analysis of enrichment pathways were carried out using the significance of DEGs. The genes were categorized into Gene Ontology categories (GO), such as Cellular Components (CC), Molecular Functions (MF), and Biological Processes (BP), using the ENRICHR database. Furthermore, the enrichment pathway analysis employed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome databases to identify the signaling pathways associated with the target genes pathways related to TGF-β/Smad and Wnt/β-catenin signaling in EMT and cancer progression. This integrative method offered a thorough comprehension of the molecular pathways impacted by *Safranal* (Natarajan *et al*., 2024).

Molecular Docking Analysis

The purpose of the investigation was to assess the binding relationship between *Safranal* and the target proteins associated with apoptosis. The PyRx virtual screening tool was used to stimulate docking. The crystal structures are provided by the Protein Data Bank (PDB). To ensure coverage of the active site, docking simulations were carried out using a grid box measuring 90 x 90 x 90 with a grid spacing of 0.45. For docking calculations, the Lamarckian Genetic Algorithm (LGA) was used, with 100 independent runs for each ligand-protein combination to guarantee the best binding conformation. The binding affinity scores and interaction patterns were used to examine the data. To analyze docked complexes and evaluate ligand-protein interactions and binding, BIOVIA Discovery Studio was utilized (Jejurikar and Rohane., 2021).

Statistical analysis

All experimental data were expressed as Mean±Standard Deviation (SD) from these independent experiments. GraphPad prism was used for statistical evaluation. Differences among experimental groups were assessed by one way ANOVA. Student t test for pairwise comparison. A p-value of less than 0.05 was considered statistically significant. Significance was presented as $p<0.05$, $p<0.01$, $p<0.001$.

RESULTS

Antioxidant assay- DPPH radical scavenging activity

Safranal showed increase in free radical scavenging activity from 100-500 µg/mL concentration. The Percentage of inhibition gradually increased from lower concentrations to higher concentrations, indicating its progressive antioxidant potential. However, when compared to ascorbic acid as standard, *Safranal* showed moderate antioxidant activity, with consistently lower percentage of inhibition values at all concentrations. At 500 µg/mL, the maximum concentration, *Safranal* reached approximately ~70% inhibition, whereas the standard exceeded ~80%, demonstrating a comparatively stronger radical scavenging capacity. Overall, these findings suggest that *Safranal* possesses significant but comparatively lower antioxidant potential, which may contribute to its protective role against oxidative stress-mediated signaling pathways, including those involved in EMT progression such as the Wnt/β-catenin and TGF-β/Smad pathways (Figure 1).

Anti-inflammatory activity

Safranal demonstrated a concentration-dependent inhibition of protein denaturation, indicating its anti-inflammatory potential. The percentage inhibition increased steadily from ~30% at 100 µg/mL to ~60% at 500 µg/mL. In comparison to the standard (diclofenac), *Safranal* exhibited moderate inhibitory activity, with the standard showing higher inhibition (~90% at 500 µg/mL). In spite of this, the steady rise in *Safranal* activity indicates that it can stabilize proteins and stop denaturation in stressful situations. The statistical significance of the observed variations across concentrations was confirmed by the existence of statistical groupings. These findings suggest that *Safranal* has significant anti-inflammatory qualities, which are essential for assessing oral cancer since inflammation was a major factor in EMT. *Safranal* may help to suppress EMT by lowering inflammatory reactions and protein denaturation through downstream pathways including TGF-β signaling (Figure 2).

Safranal's effect on viability of OSCC cells

Safranal's cytotoxic effect on cancer cells was assessed using the MTT assay, and morphological changes were demonstrated using phase-contrast microscopy to support the findings. The MTT assay reveals a dose-dependent decrease in cell viability after *Safranal* treatment at concentrations between 0 and 100 µM. The nearly 100% vitality of control cells (0 µM) indicates that the cells are healthy and multiplying rapidly. With increasing concentrations of *Safranal*, cell viability progressively declines approximately 90% at 20 µM, 80% at 40 µM, 70% at 60 µM, 60% at 80 µM, and further down to around 50% at 100 µM. *Safranal's* cytotoxic properties, become more noticeable at greater dosages. The morphological alterations linked to *Safranal* concentrations in the relevant phase-contrast images. The control group has a consistent distribution of long, spindle-shaped cells with intact membranes in a dense monolayer. The cells show obvious symptoms of stress at 50 µM, such as membrane blebbing, cell rounding, and partial separation from the surface, which are indicative of early stages of apoptosis. At 100 µM, showing substantial cell shrinkage, decreased cell density, and cellular debris indicating cytotoxicity. *Safranal* dramatically lowers cell viability and alters cellular architecture in a dose-dependent manner, as shown by the MTT assay. These findings showed that *Safranal* has the potential to be an effective anti-cancer agent with further research into its mechanisms for possible therapies (Figure 3).

Molecular Docking analysis

Safranal has modest binding affinities with proteins implicated in fibrosis, redox balance, and Wnt signaling, according to the molecular docking data. WNT and β-catenin showed the highest binding affinity (-5.4 kcal/mol), closely followed by TGF-β (-5.1 kcal/mol) and SMAD (-5.3 kcal/mol). These interactions imply that *Safranal* may have regulatory effects on the Wnt/β-catenin and TGF-β/SMAD pathways, as well as support antioxidant defence through WNT modulation. Overall, the results imply *Safranal's* potential role in modulating pathways associated with fibrosis, oxidative stress and tissue repair (Figure 4 and Table 1).

Network pharmacology analysis

The image illustrates a network pharmacology analysis of *Safranal*, revealing its potential molecular targets involved in EMT, a process in cancer progression and metastasis. *Safranal* (yellow triangle), connects to 70 green nodes representing genes associated with EMT regulation, including mesenchymal markers (like VIM, FN1 and ZEB1/2), epithelial markers (like CDH1, OCLN) and EMT-transcription factors (e.g., SNAI1/2, TWIST1/2). Gene Interactions suggests that *Safranal* has a wide range of effects on several EMT signaling including modulation of migration, invasion, and stemness. TGFB1, SMAD, WNT1A, and CTNNB1 are identified as important hub genes in the network by topological analysis based on centrality measurements. These genes are crucial components of the TGF-β/SMAD and WNT/β-catenin signaling pathways, which are both important promoters of EMT and tumour growth. Both decrease in epithelial characteristics and increase mesenchymal phenotypes are regulated by TGFB1 SMAD (yellow) while Cadherin-mediated

Table 1: Binding affinity values of *Safranal* with selected target proteins involved in signaling pathways.

Proteins	Binding affinity
Safranal	
SMAD	-5.3
TGF-β	-5.1
NNT	-5.4
β- CATENIN	-5.4

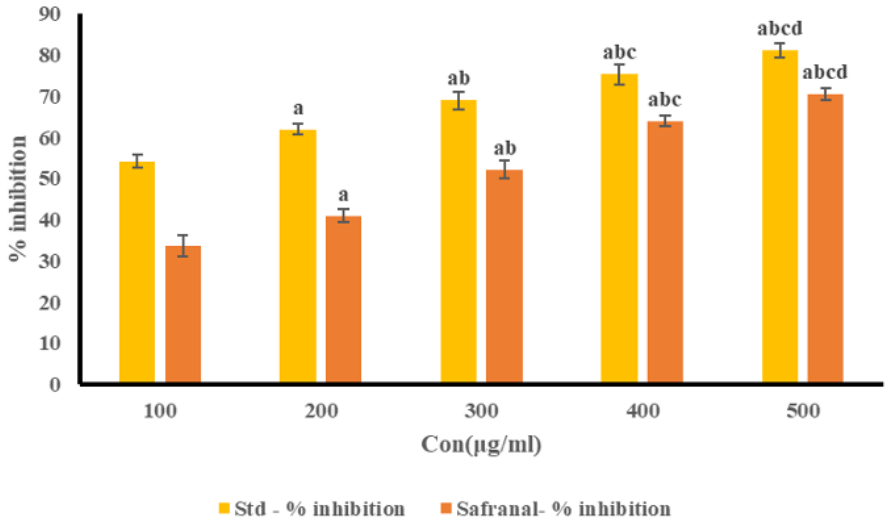


Figure 1: DPPH radical scavenging assay.

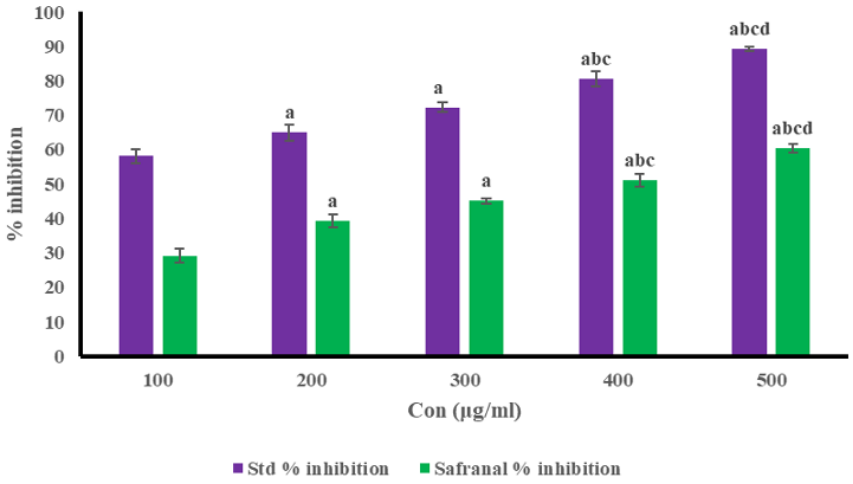


Figure 2: Protein denaturation.

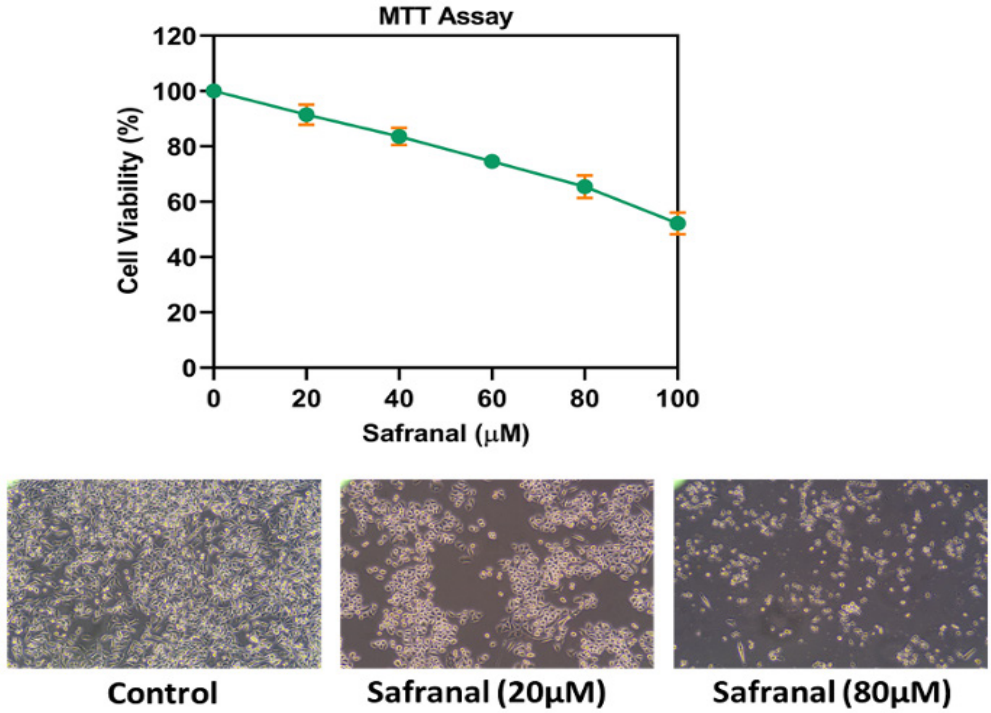


Figure 3: Safranal Suppresses Epithelial Mesenchymal Transition (EMT) in oral cancer by targeting the TGF-B/Smad and Wnt/ß-Catenin Pathways: A bioinformatics and experimental perspective.

adhesion and WNT signaling are connected by CTNNB1 (blue), which encodes β-catenin. *Safranal* may have therapeutic promise in preventing cancer invasion and metastasis by interfering with EMT-associated signaling, as suggested by the prevalence of these hub genes (Figure 5).

DISCUSSION

OSCC remains an aggressive malignancy with a tendency of invasion and metastasis, mediated largely by EMT. EMT is a dynamic biological process regulated by multiple cascades of signaling, mainly Transforming Growth Factor (TGF-β)/Smad and Wnt/β-catenin pathways, which are involved in progression of tumour and metastasis (Ibi *et al.*, 2024, Yim and Laronde, 2024). The present study has provided integrated experimental evidence demonstrating *Safranal* might act as a potent inhibitor of EMT in OSCC targeting key signaling pathways.

The anti-inflammatory and antioxidant activity observed in this study are consistent with the established role of oxidative stress and chronic inflammation in EMT promotion. *Safranal* exhibited free radical scavenging and protein denaturation inhibition, although its activity was moderately less than the standard

compounds. Previous studies have shown that *Safranal* regulated the oxidative stress through activation of antioxidant pathways via Nrf2 and inflammatory mediators, thereby limiting the tumour promoting microenvironments (Niu *et al.*, 2022, Esmaealzadeh *et al.*, 2023). Oxidative stress was known to activate EMT-related factors, whereas *Safranal*, through its antioxidant properties, may counteract the stress and thereby inhibit EMT progression.

The cytotoxic effects of *Safranal* observed in the KB cell line supports its anticancer potential. Reduction in cell viability along with features of cell shrinkage and detachment, indicates apoptosis induction. These findings are in agreement with transcriptomic and proteomic findings demonstrating that *Safranal* causes cell cycle arrest and apoptosis in a variety of cancer cell lines (Ali *et al.*, 2025; Zarrineh *et al.*, 2022). Unlike conventional chemotherapeutic agents, which exerts systemic toxicity, *Safranal* appears to exert a selective cytotoxicity, highlighting its potential as a safer alternative.

The key strength of this study lies by integrating the molecular docking and network pharmacology analysis to elucidate the *Safranal's* action. *Safranal* action binding affinities were shown by the docking data. The docking data showed that *Safranal* had a strong affinity for EMT-related proteins. Including β-catenin, SMAD and TGF-β. Recent studies have supported the findings indicating that natural compounds exert anticancer effects through the multiple interactions rather than single inhibition pathway (Fang *et al.*, 2023).

Network pharmacology analysis have further identified TGFB1, SMAD and CTNNB1 as a central hub of genes reinforcing the importance of Wnt/β-catenin and TGF-β/Smad signaling in progression of OSCC. TGF-β activation promotes EMT through Smad- dependent transcription of key regulators like Snail and Twist, leading to epithelial repression and acquisition of mesenchymal characteristics [26]. Similarly, Wnt/β-catenin signaling facilitates the translocation of β-catenin, promoting the gene transcription associated with proliferation, invasion and metastasis (Yu *et al.*, 2020). The present findings suggest that *Safranal* might disrupt the signaling cascade, thereby inhibiting EMT and tumour aggressiveness.

Furthermore, recent studies on phytochemicals, like celastrol and fisetin have shown targeting Wnt/β-catenin and TGF-β/Smad pathways which inhibits EMT and OSCC progression effectively (Li *et al.*, 2024; Chen *et al.*, 2025). This highlights the therapeutic strategy of natural compounds which act as multi-target modulators in EMT signaling networks.

From a clinical standpoint, focusing on EMT improves OSCC patient's lifespan by preventing metastasis. Because of its anti-inflammatory, anti-oxidant, and anti-cancer characteristics, *Safranal* acts as a potential adjuvant therapy. Its low toxicity and place of origin increase its potential as a long-term chemotherapeutic agent.

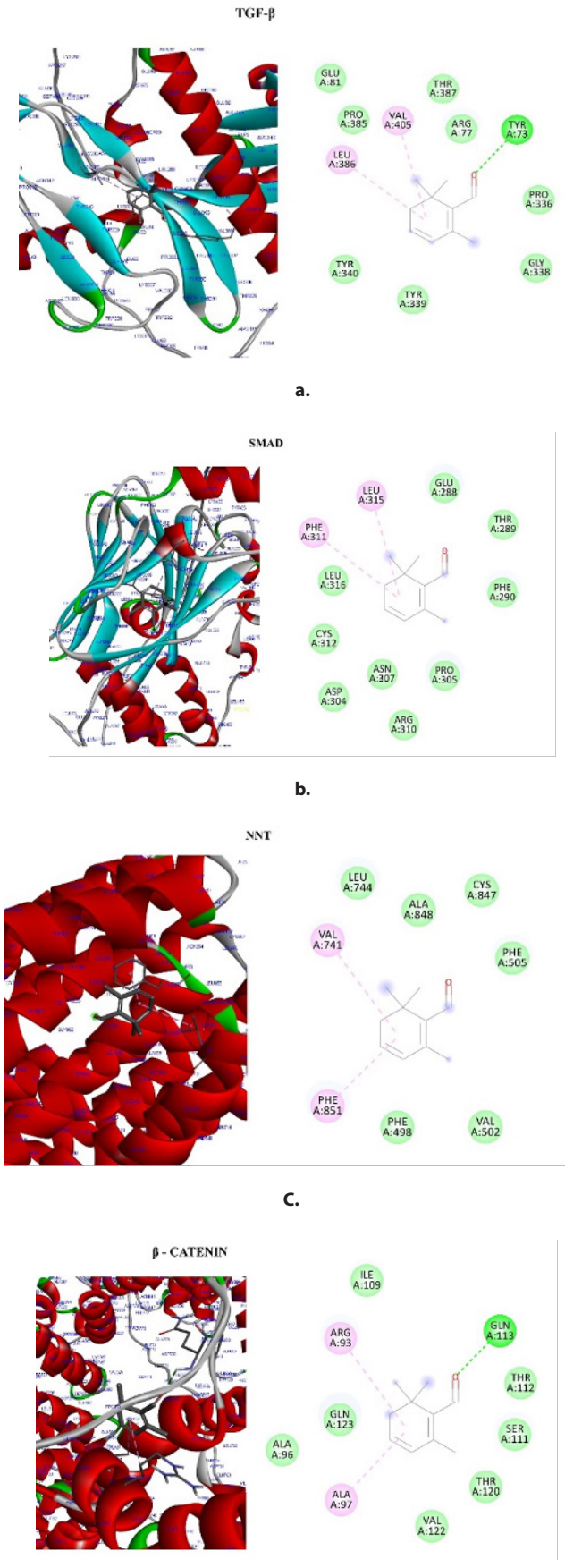


Figure 4: Binding interactions of safranal with target proteins in the Wnt/β-catenin and TGF-β/SMAD pathways.

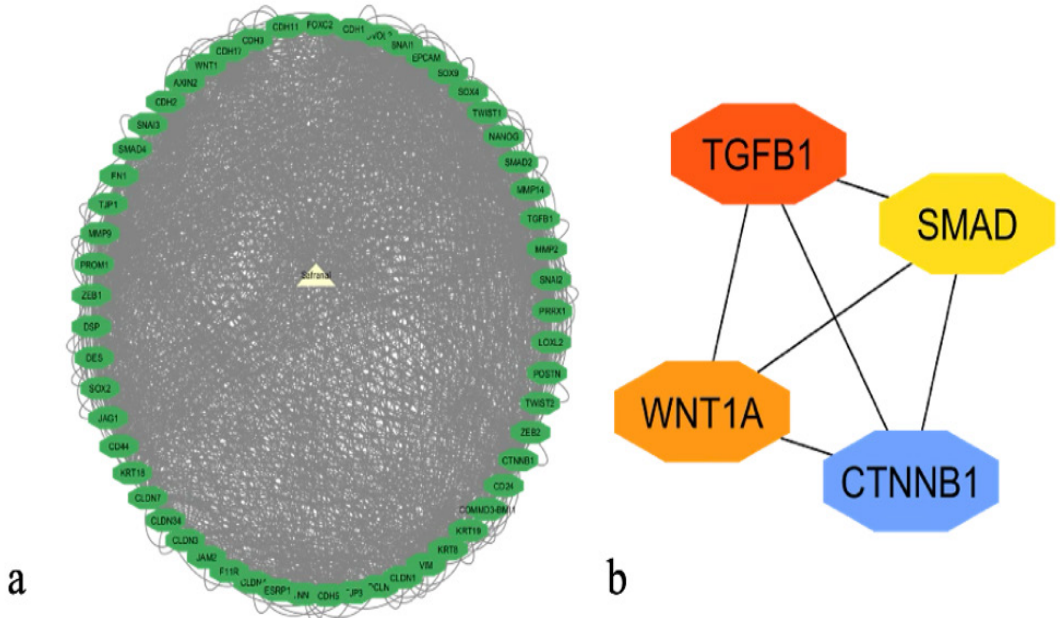


Figure 5: Safranal Suppresses Epithelial Mesenchymal Transition (EMT) in oral cancer by targeting the TGF-B/Smad and Wnt/ß-Catenin Pathways: A bioinformatics and experimental perspective.

Further, Binding affinities in docking studies require validation through molecular dynamics and gene expression studies. *In vivo* models and clinical trials should be the main focus of future research to validate *Safranal's* effectiveness.

CONCLUSION

The present study demonstrated that *Safranal* exhibits antioxidant, anti-inflammatory and cytotoxic effects and inhibits EMT in OSCC by modulating key signaling pathways like Wnt/β-catenin and TGF-β/Smad. These findings, supported by recent literature, highlights the potential of *Safranal* as a multi targeted agent for controlling progression of tumour and metastasis in Oral cancer.

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Concept and design: Priyadharshini R, Selvaraj Jayaraman, Bhavya Rohatgi.

ABBREVIATIONS

EMT: Epithelial-Mesenchymal Transition; **TGF-β:** Transforming Growth Factor-Beta; **SMAD:** SMAD Family of Signal Transducers; **Wnt:** Wingless-related Integration Site; **β-catenin:** Beta-Catenin; **OSCC:** Oral Squamous Cell Carcinoma; **KB:** Kohlenberg-Berg carcinoma cell line; **PI3K:** Phosphoinositide 3-Kinase; **AKT:** Protein Kinase B; **PI3K/AKT:** Phosphoinositide 3-Kinase/Protein Kinase B Signaling Pathway; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl Sulfoxide; **FBS:** Fetal Bovine Serum; **LGA:** Lamarckian Genetic Algorithm; **PDB:** Protein Data Bank; **SD:** Standard Deviation; **VIM:** Vimentin; **FN1:** Fibronectin 1; **ZEB1:** Zinc Finger E-Box Binding Homeobox 1; **ZEB2:** Zinc Finger E-Box Binding Homeobox 2; **ZEB1/2:** Zinc Finger E-Box Binding Homeobox 1 and 2; **CDH1:** Cadherin 1; **OCLN:** Occludin; **WNT1A:** Wnt Family Member 1A; **CTNNB1:** Catenin Beta 1; **Nrf2:** Nuclear Factor Erythroid 2-Related Factor 2.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Acquisition, analysis, or interpretation of data: **Priyadharshini R**, **Selvaraj Jayaraman**, **Bhavya Rohatgi.** Drafting of the manuscript: **Priyadharshini R, Selvaraj Jayaraman**, **Bhavya Rohatgi**. Critical review of the manuscript for important intellectual content: **Priyadharshini R, Selvaraj Jayaraman, Bhavya Rohatgi**. Supervision: **Priyadharshini R, Selvaraj Jayaraman.**

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