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Original Research Article

Modulatory effect of *Glycyrrhiza glabra* on the cytokine secretion by stromal cells derived from oral submucous fibrosis and oral squamous cell carcinoma tissue

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Abstract

Objective: Research on inflammation pathways that influence cancer cells, infiltrating leukocytes, and surrounding stromal cells promoting tumour growth has gained significant attention. This study explains how mesenchymal stem cells from oral submucous fibrosis and oral squamous cell carcinoma tissue have their cytokine secretion influenced by ethanolic extract of Glycyrrhiza glabra root, highlighting their roles in tumour development and progression.

Methods: Mesenchymal cells from oral submucous fibrosis and oral cancer tissues were identified, characterized, and cultivated. We used the MTT assay to assess cell viability. Cytokine evaluation was performed in mesenchymal cells from oral submucous fibrosis and oral squamous cell carcinoma tissues.

Results: Oral squamous cell carcinoma mesenchymal stem cells showed higher levels of CXCL-10 (Chemokine (C-X-C motif) ligand 10). CCL-2, IL-6, and TGF-beta were analyzed in relation to oral submucous fibrosis mesenchymal stromal cells through cytokine profiling. Glycyrrhiza glabra lowered inflammation and tumor-promoting factors CXCL-10, CCL-2, and TGF-beta in OSCC-mesenchymal stem cells. The result also caused a reduction in IL-4 and IL-1β levels in oral submucous fibrosis-mesenchymal stromal cells.

Conclusion: Our research indicates that mesenchymal stem cells and stromal cells could be important targets for treating oral submucous fibrosis and oral squamous cell carcinoma. These cells can reduce inflammation in the tumour microenvironment, slow tumour growth, and stop malignant transformation by influencing key cytokines.

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Introduction

Cancer development is influenced by the regulation of cellular processes and signaling pathways in the microenvironment. The tumor microenvironment role in cancer promotion is well recognized. The microenvironment role in oral potentially malignant disorders (OPMD) related cancer is unclear. Studying the molecular and cellular mechanisms of OPMD development and progression will enhance early detection, diagnosis, and treatment strategies. Globocan 2022 reported 389,485 new cases and 188,230 deaths from lip and oral cavity cancer. 10 per 100,000 cases in countries with low human development indices, like India have been recorded (Bray et al. 2024). Cancer metastasis requires tumor microenvironment mesenchymal stem cells (MSCs). MSCs are present in the tumor microenvironment due to their inflammatory affinity, but their significance is unclear. Research has used MSCs or their secretome in cancer treatment, resulting in varied outcomes. MSC activation mechanisms and their phenotypes could clarify their diverse functions; however, these areas are still mostly unexamined.

Researchers are studying the immunomodulating soluble components of MSCs, as shown by previous works (Bernardo et al. 2011,Aggarwal and Pittenger 2005,Ortiz et al. 2007, Nasef et al. 2007, Djouad et al. 2007,Sato et al. 2007,Lee et al. 2009,Bai et al. 2012,Kim and Hematti 2009,Liu et al. 2014). Mesenchymal stem cells affect immune cell responses via various factors, including Transforming growth factor beta (TGF-β), Hepatocyte growth factor (HGF), prostaglandin E2, Interleukin-10 (IL-10), IL-1 receptor antagonist, IL-6, human leukocyte antigen-G, leukocyte inhibitory factor, indoleamine-2,3-dioxygenase, nitric oxide, galectins-1 and -9, and Tumor Necrosis Factor (TNF-α)-stimulated gene. MSCs can effectively influence immune responses, which is advantageous in situations where uncontrolled growth of T cells, dendritic cells, macrophages, and natural killer cells may lead to an overproduction of cytokines. Different cell types produce cytokines, showing varied responses in normal and pathological situations (Lan et al. 2021). Cytokines are made by immune cells, tumor microenvironment cells, and nearly all nucleated cells (Raeburn et al. 2002). Studies show that cytokines play a key role in the formation, progression, invasion, and chemoresistance of Head and Neck Squamous Cell Carcinoma (HNSCC), especially in the oral mucosa. Cytokines have various roles in head and neck squamous cell carcinoma, often interacting in complex ways. This has shifted scientific attention from tumor cells to their communicators (Georgescu et al. 2020,Kartikasari et al. 2021)**.**

Advanced head and neck cancer is usually treated with surgery, radiation, and chemotherapy. This approach targets metastatic disease and localized cancers. Surgical interventions or high-dose radiation therapies can lead to serious complications and morbidity. Improving outcomes and reducing morbidity requires careful patient selection and customized treatment approaches (Posner 2010)**.** Platinum-based chemotherapy can reduce kidney, ear, and bone marrow functions depending on the dosage given (Yip et al. 2006,Cohen et al. 2004,Chandana and Conley 2009.Adelstein et al. 2010). Despite progress in research and treatment, clinical outcomes and survival rates for HNSCC have not improved for decades (Argiris et al. 2004; EE et al. 1993). Alternative treatments for head and neck cancer are being sought due to the limitations and side effects of conventional therapies.

Ayurveda aims to preserve health and prevent illness, using Rasayana remedies for quick patient recovery. Contemporary Rasayana therapy includes antioxidants, immunomodulators, adaptogens, anabolics, nutraceuticals, and anti-aging agents. The Ayurvedic text Charak Samhita recommends Rasayanas like *Withania*

somnifera, *Guduchi*, *Glycyrrhiza glabra*, and *Brahmi* for treating various ailments. Ayurveda uses Yastimadhu, known scientifically as *G. glabra* (GG) or Licorice, to treat dental and oral cavity issues. Licorice extract paste may reduce the duration and size of aphthous ulcers, ease discomfort, and speed up healing, common symptoms of oral submucous fibrosis (OSMF) (Jeffrey A Burgess et al 2008) **.**

This research studied mesenchymal cells isolated from oral squamous cell carcinoma (OSCC) and OSMF tissues, focusing on their biological role in the immune response. We evaluated isolated primary mesenchymal cells to assess their stemness, categorizing them as OSCC-mesenchymal stem cells (OSSC-MSCs) and OSMF-mesenchymal stromal cells (OSMF-MStrCs). The treatment with *G. glabra* extract altered the cytokine production by these cells. Findings indicate that *G. glabra* may protect against OPMD and OSCC by regulating cytokine release from OSSC-MSCs and OSMF-MStrCs.

Materials and Methods

Glycyrrhiza glabra ethanol extract

The G. glabra extract of root in the form of a dry powder (Ethanolic Extract) was utilized in this study. This was procured from Pharmanza Company PVT [Batch No: GGEP/RAD/031].

Formulation of stock solution of the extract

To create a stock solution with a concentration of 10 mg/mL, a 10 mg ethanolic extract of GG was diluted in 1 mL of Dimethyl Sulfoxide (DMSO) and subsequently stored at -80°C. A stock solution with a concentration of 10 mg/ml was employed to prepare a 1 mg/mL extract in complete medium. The solution underwent filtration through aseptic 0.22 µm filters and was thereafter employed to create dilutions.

Collection of tissue samples

Tissue samples from primary OSCC and OSMF cases were procured from Dr. D. Y. Patil Medical College and Hospital and Research Centre, accompanied by documented patient consent. The tissues were efficiently processed after being transported in a 50 ml/15 ml tube filled with complete cell culture media DMEM (Dulbecco's Modified Eagle's Medium, Gibco)

Isolation and cultivation of OSMF and OSCC derived mesenchymal cells

The methodologies for OSMF and OSCC derived cells were executed as outlined in prior documentation (Ladke et al. 2023). Following a period of 15 to 19 days, an adherent monolayer was established, showcasing diverse morphologies that included elongated, slender cells. Notably, in some cases, the OSMF-derived cells predominantly displayed a fibroblast-like appearance.

Identification and characterization of cells originating from OSMF and OSCC tissues through the utilization of surface markers using flowcytometry

Mesenchymal cells derived from OSMF and OSCC were isolated and characterized through the application of specific surface markers (Dominici et al. 2006) like human CD105, CD73, CD24, HLA-DR, CD90, and CD34 (Biosciences, California, USA).

Viability assay

The assessment of cellular viability was conducted through the MTT conversion test (Mosmann 1983). OSMF and OSCC-MSCs were introduced onto a 96-well plate at a concentration of 5 x 10^3 cells/mL. Various concentrations of *G. glabra*, specifically 50, 100, 200, 300, 400, and 500 µg/ml, were employed in the study. Following this, 20 µl of 5 mg/ml MTT was added to each well and allowed to incubate for a further 4 hr. The formazan crystals in each well were solubilized through the addition of 100 µl of DMSO. The absorbance at 570 nm was

measured using a Multi-SkanGo Thermo Fisher Scientific ELISA plate reader. The target concentration (IC_{50}) was determined through the application of a standard formula derived from a Microsoft Excel spreadsheet (KUMBHAR et al. 2025, (Kumbhar et al. 2024).

Assessment of cytokine, chemokine, and growth factor concentrations

The concentrations of cytokines, chemokines, and growth factors present in cell supernatants were measured utilizing the LEGEND plexTM HU Essential Immune Response Panel (13-plex) kit, in accordance with the manufacturer's instructions. The assessment involved the concentrations of 13 distinct cytokines (IL-4, IL-2, CXCL10 (IP-10), IL-1β, TNF-α, CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN-γ, IL-12p70, CXCL8 (IL-8), and TGF-1).

Statistical analysis

The experiments were conducted in duplicates and triplicate accordingly, with results expressed as the mean ± standard deviation (SD). Data analysis was performed utilizing GraphPad Prism 8. “Two-way ANOVA” was conducted, followed by Tukey post-hoc tests which was determined and recommended by the software at ****p<0.0001, ***p<0.001, and **p<0.01 to assess the statistical significance among the groups in comparison to the control.

Results

The morphology of mesenchymal cells derived from oral submucous fibrosis and oral squamous cell carcinoma (OSMF-MStrCs and OSCC-MSCs)

The OSMF-MStrCs showed a fibroblast-like morphology, characterized by a broad, irregular shape and minimal branching (Figure 1 A). OSCC-MSCs showed a slender, elongated shape without branching (Figure 1B). Both tissues showed changes in their cellular structure. Haematoxylin and eosin staining was conducted on fixed OSMF-MStrCs and OSCC-MSCs. The OSCC-MSCs showed compact spindle-shaped cells with significant nuclear and cellular pleomorphism (Figure 1C). The OSMF-MStrCs showed a wide, branched cellular structure with cytoplasmic vacuolization (Figure 1D).

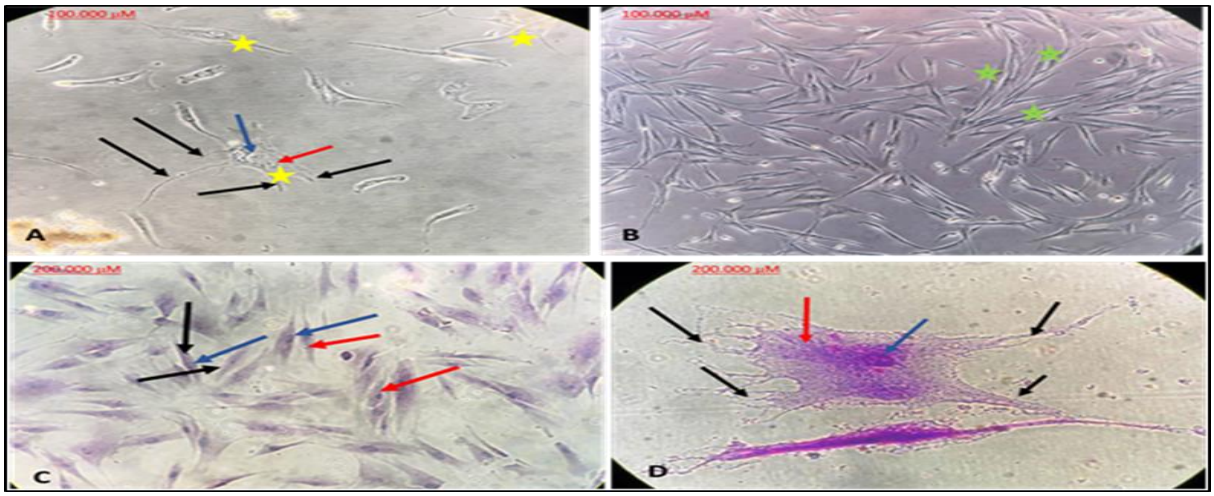

Figure 1. The morphology of OSMF-MStrCs and OSMF-MSCs. A) More dispersed, wide fibroblast-like cells [yellow star], Cytoplasmic branching indicated by black arrows, a small nucleus highlighted by a blue arrow, and abundant, vacuolated cytoplasm marked with a red arrow. Magnification at 200 X. Scale bar of 100µM.B) Elongated, slender, and compact cellular structures [green stars], Magnification at 200 X. Scale bar of 100µM. C) Haematoxylin and Eosin staining of elongated slender OSCC-MSCs cells [Black Arrows]. A small, round nucleus is indicated by the blue arrows. Consistent, reduced quantity of cytoplasm [Red arrows], Magnification at 400 X. Scale bar of 200µM. D) Extensive, dendritic fibroblast-like cells. Cytoplasmic branching indicated by black arrows, a small nucleus highlighted by a blue arrow, and abundant, vacuolated cytoplasm marked with a red arrow, Magnification at 400 X. Scale bar of 200µM.

Identification and characterization of OSMF and OSCC-derived mesenchymal

OSCC-MSCs displayed positivity for CD105, CD73, and CD90 (Figure 2A-C), while showing negativity for HLA-DR, CD34, and CD45 (Figure 2D-F). OSMF-MStrCs demonstrated an absence of positivity for stem cell markers (Figure 3A-F). OSSC-derived cells demonstrated a significantly greater enrichment of positive CD90 [96.88%], CD105 [79.35%] and CD73 [96.59%] cells, suggesting that OSCC-derived cells are indeed Mesenchymal Stem Cells, hence are termed as OSCC- Mesenchymal Stem Cells (OSCC-MSCs) in contrast to OSMF-derived cells which are simply mesenchymal stromal cells rather than stem cells hence are termed as OSMF-Mesenchymal Stromal Cells (OSMF-MStrCs) (Figure 4).

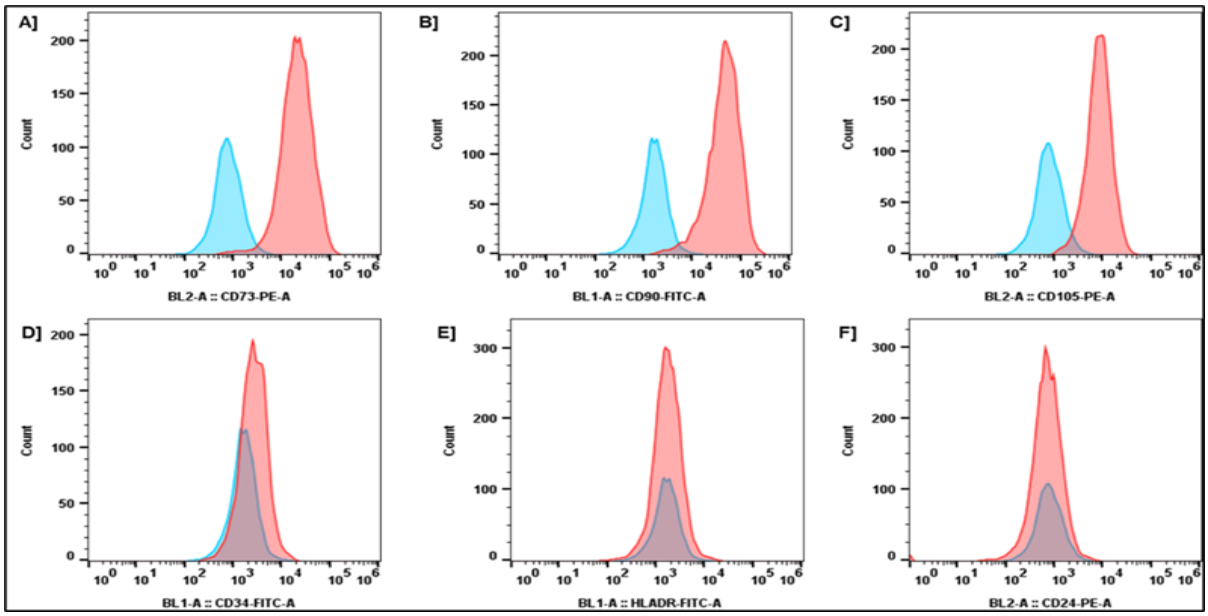

Figure 2. Flowcytometry analysis of stem cell surface markers in OSCC- derived cells. The representative flow cytometry histogram illustrates the cell surface marker molecules (depicted in Red) alongside their corresponding unstained control (shown in Blue) for A] CD73 B] CD90 C] CD105 D] CD34 E] HLADR and F] CD24.

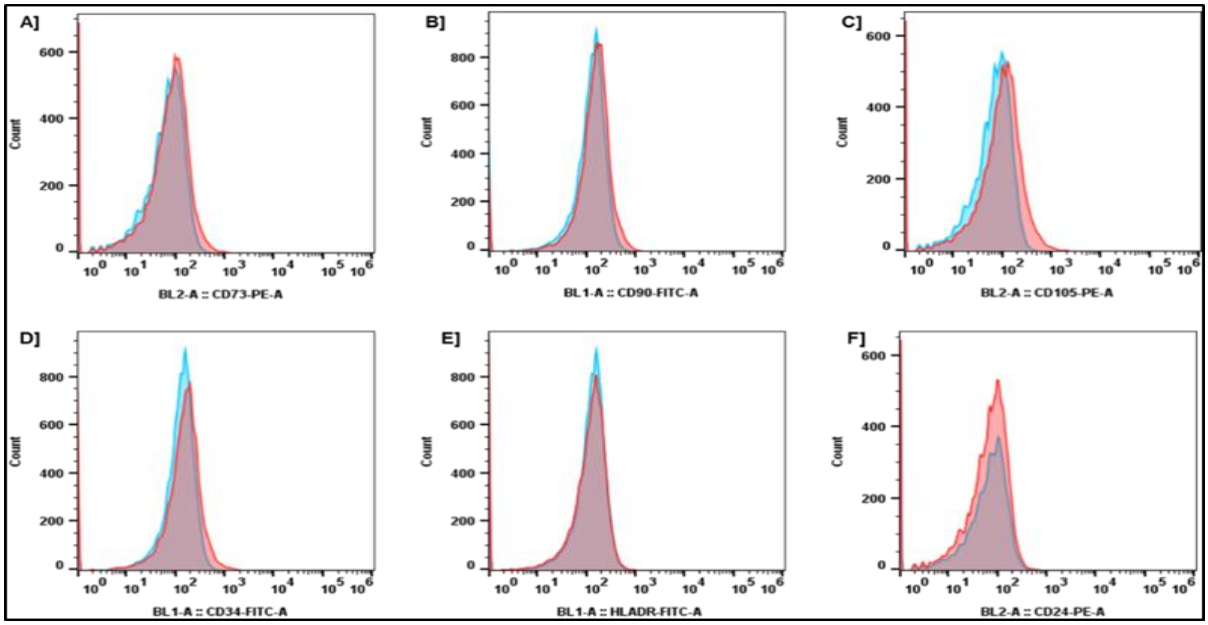

Figure 3. Flowcytometry analysis of stem cell surface markers in OSMF-derived cells. The representative flow cytometry histogram illustrates the cell surface marker molecules (depicted in Red) alongside their corresponding unstained control (shown in Blue) for A] CD73 B] CD90 C] CD105 D] CD34 E] HLADR and F] CD24.

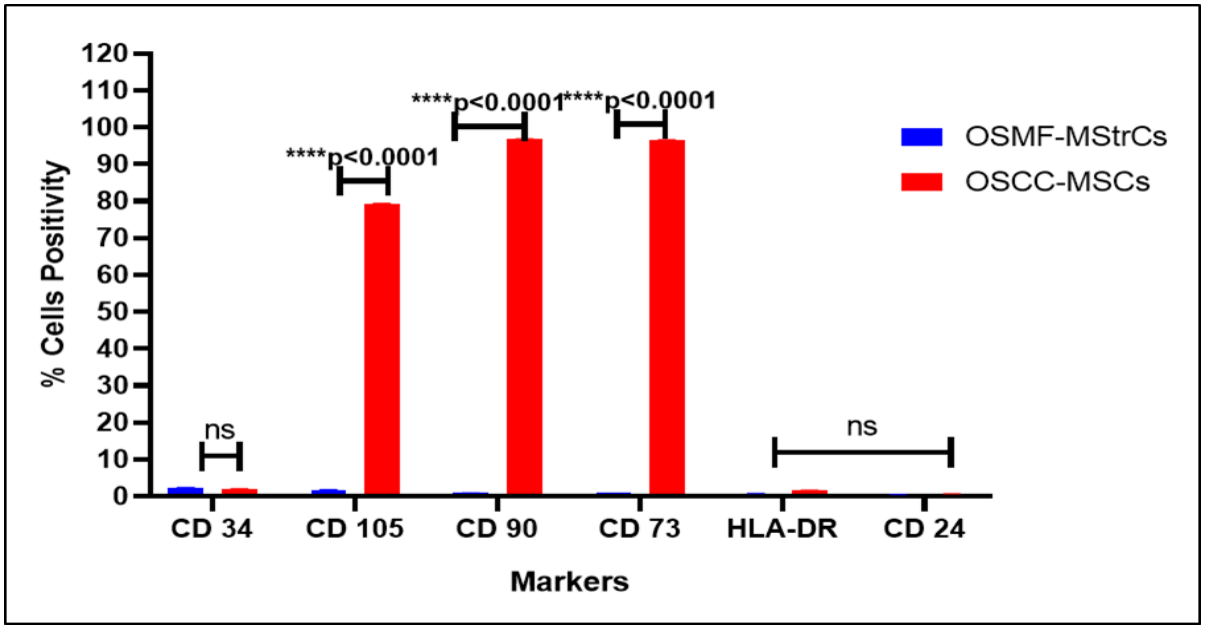

Figure 4. Evaluation and characterization of OSMF and OSCC derived mesenchymal cells for stemness markers. The data are presented as mean ± SD for n = 3, with analysis conducted using "Two-way ANOVA" followed by "Tukey" at significance levels of ***p<0.0001; **p<0.01; and *p<0.05. analyzing the differences between OSMF-MStrCs and OSCC-MSCs concerning their stemness.

Assessment of the viability activity of *G. glabra* on OSMF-MStrCs and OSCC-MSCs

Concentrations of GG ranging from 50 to 500 µg/ml were employed for the experimental procedure. With the help of formula in Microsoft excel the IC_{50} value was calculated. This concentration that is IC50 value 238 µg/ml was used for further cytokines analysis (Figure 5).

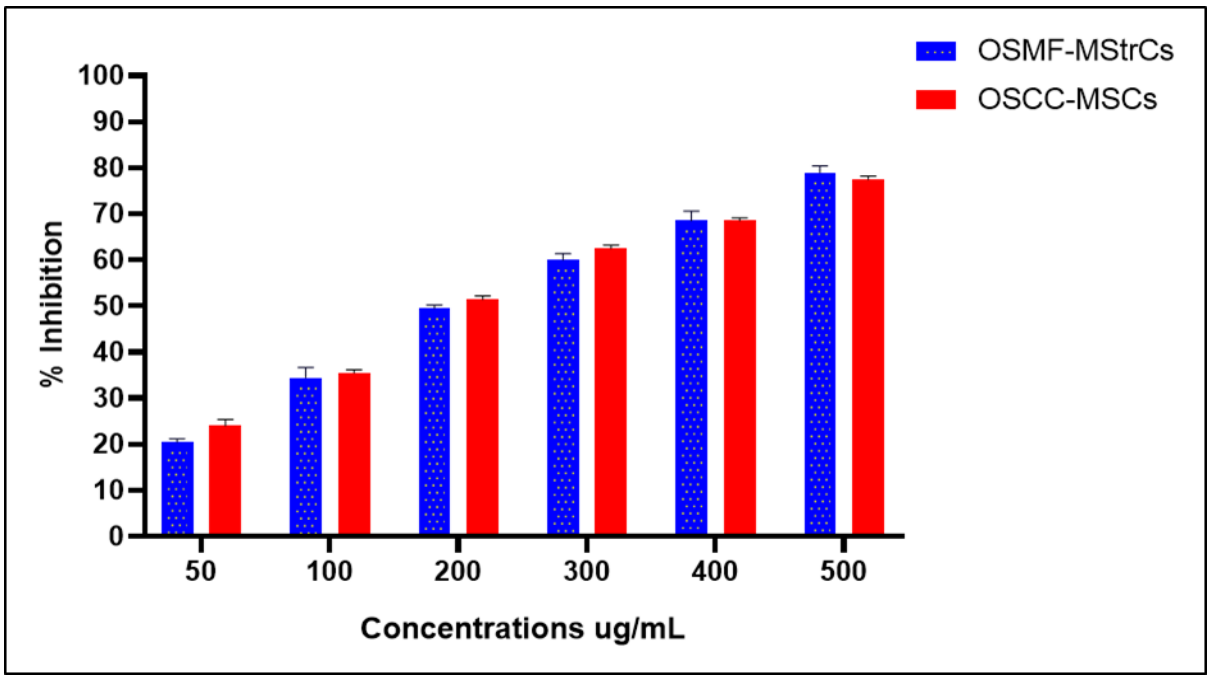

Figure 5. Cytotoxicity activity of *Glycyrrhiza glabra* (50 to 500 µg/ml) on OSMF-MStrCs and OSCC-MSCs. Bar graph represent the % inhibition effect of *G. glabra* on OSMF-MStrCs and OSCC-MSCs for 24 hr. Data is presented as mean± SD where n = 2 of independent experiments.

Comparative examination of cytokine concentrations between OSMF-MStrCs and OSCC-MSCs cell secretome

OSMF-MStrCs and OSCC-MSCs underwent treatment with GG at a concentration of 238 µg/ml for a duration of 48 hr, during which 13 cytokines were assessed in accordance with the kit's specifications. Our analysis uncovered increased levels of cytokines CCL2, TNF-α, CXCL-10, TGFβ, IL-6, and IL-17 in OSCC-MSC when compared with OSMF-MStrCs. Conversely, OSCC-MSC cells demonstrated diminished concentrations of IL-1β and IL-4 when compared with OSMF-MStrC cells. The absence of cytokines IL-2, CXCL-8, IL-12, IFN-γ, and IL-10 was distinctly observed in both cell lines, as illustrated in Table 1 and Figure 6. The various biologically based explanations account for their absence or diminished levels. This may exemplify the immunologically inert or suppressed tumor environment characteristic of OSCC, wherein anti-tumor cytokines are limited, while IL-8 and IL-6 prevail, and immunosuppressive factors may exert their effects either systemically or at minimal tissue concentrations. This warrants a more thorough investigation through comprehensive assays involving a greater number of samples.

The influence of *G. glabra* treatment on cytokine secretion within OSMF-MStrCs

We observed an increase in the concentrations of cytokines TNF-α, TGFβ, IL-6, and IL-17 when compared to the untreated group. Conversely, there was a notable reduction in the concentrations of the cytokines IL-1β, CXCL-10, and IL-4. (Table 2, Figure 7).

Table 1. Summary of cytokine levels secreted by OSCC-MSCs and OSMF-MStrCs

	Analyte	OSCC-MSC Mean ± SD	OSMF-SC Mean ±SD	p Value
Higher levels	CCL2	196.2± 7.09	0±0	*0.016
	TNF-α	28.05± 1.89	22.3± 2.45	0.31
	CXCL-10	1692±35.21	26.91± 1.95	*0.01
	TGFβ	13.2±1.77	6.05±1.75	**0.002
	IL-6	1662±165.8	24.27±4.25	*0.046
	IL-17	75.92±1.08	64.51±6.64	0.28
Lower levels	IL-1β	18.12± 2.65	33.58± 4.71	0.059
	IL-4	1.5±0.9	7.8±0.7	*0.014
Not Detected (ND)	IL-2	ND	ND	-
	CXCL-8	ND	ND	-
	IL-12	ND	ND	-
	IFN-γ	ND	ND	-
	IL-10	ND	ND	-

The comparison of concentration of cytokines in OSMF-MStrCs and OSCC-MSCs assessed by *two*-way ANOVA with Tukey's post hoc analysis at *p<0.05, **p<0.01 and ***p<0.001 significant.

Table 2. Cytokine levels secreted by OSMF-MStrCs before and After 48-hour treatment with GG IC_{50} [238 µg/ml] concentration.

	Analyte	Untreated Mean ±SD	GG IC_{50} Mean ±SD	p Value
Higher levels	TNF-α	22.31±2.45	33.40± 1.88	0.17
	TGFβ	6.08±2.51	10.90±0.70	0.16
	IL-6	24.27±4.25	29.28±2.83	0.12
	IL-17	64.52±6.64	77.46±1.08	0.18
Lower levels	IL-1β	33.59±4.71	19.06±3.98	*0.022
	CXCL-10	26.92±1.95	22.99±2.27	*0.036
	IL-4	7.8±0.7	5.05±0.21	0.08
Not Detected (ND)	CCL2	ND	ND	-
	IL-2	ND	ND	-
	CXCL-8	ND	ND	-
	IL-12	ND	ND	-
	IFN-γ	ND	ND	-
	IL-10	ND	ND	-

The comparison of concentration of cytokines in OSMF-MStrCs before and After 48-hour treatment with GG assessed by *two*-way ANOVA with Tukey's post hoc analysis. Data are presented as mean ± standard deviation (SD). Statistical significance was denoted with p-values as *p<0.05 (significant), **p<0.01 (highly significant), and ***p<0.001 (very highly significant).

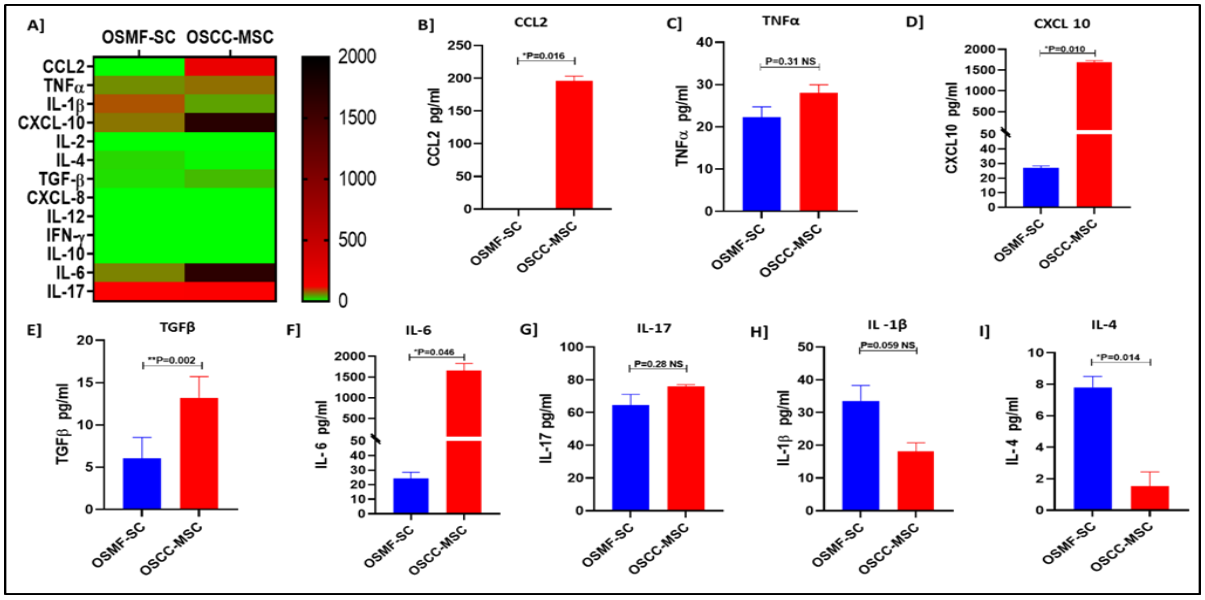

Figure 6. Comparative Analysis of cytokine levels between OSCC-MSCs and OSMF-MStrCs. A] Heat map representing cytokine concentrations in the OSCC-MSC and OSMF-SC cell lines. Each row of the heat map represents cytokines secreted and each column represents OSMF-MStrCs and OSCC-MSCs. The color scale corresponds to the relative expression of the cytokine for the minimum (0 pg/ml) and maximum (2000 pg/ml) of all values. The bar graphs represent the comparison of concentration of cytokines in OSMF-MStrCs and OSCC-MSCs assessed by two-way ANOVA with Tukey's post hoc analysis. B) CCL2, C) TNF-α, D) IL-1β E) CXCL-10, F) IL-4, G) TGFβ, H) IL-6 and I) IL-17. Data are presented as mean ± standard deviation (SD). Statistical significance was denoted with p-values as *p<0.05 (significant), **p<0.01 (highly significant), and ***p<0.001 (very highly significant).

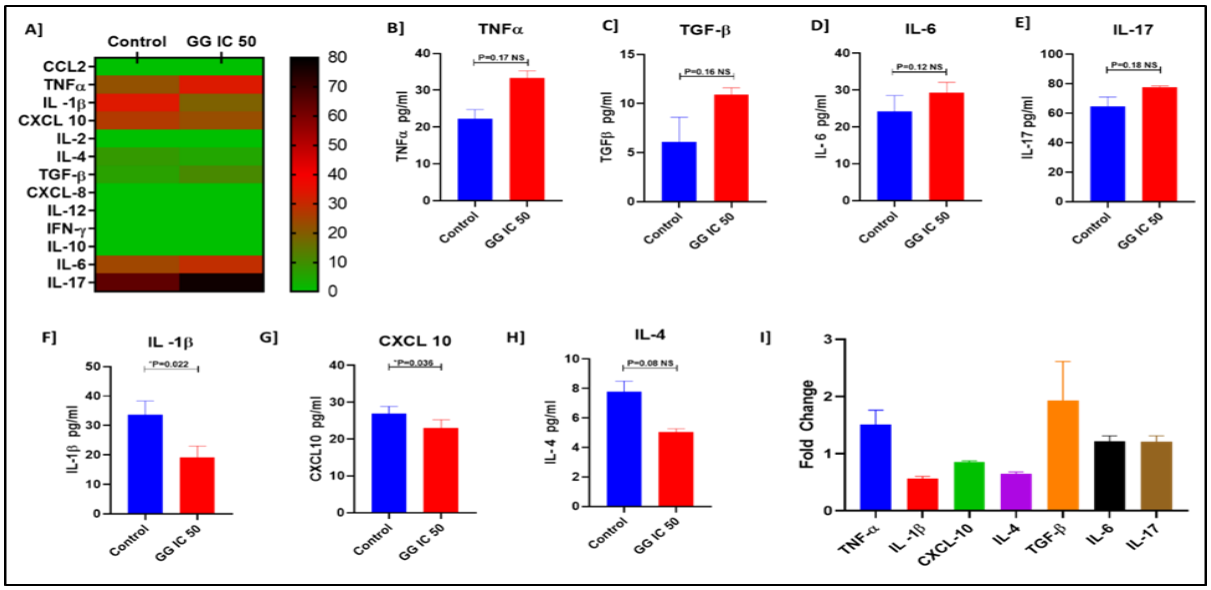

Figure 7. Effect of G. glabra IC50 treatment on Cytokine Secretion by OSMF-MStrCs. A] Heat map representing cytokine concentrations secreted by OSMF-MStrCs before and after of 48-hr treatment with GG IC_{50}. Each row of the heat map represents cytokines secreted and each column represents OSMF-MStrCs Control and after of 48-hr treatment with GG IC_{50}. The color scale corresponds to the relative expression of the cytokine for the minimum (0 pg/ml) and maximum (80 pg/ml) of all values. The bar graphs represent the comparison of concentration of cytokines in OSMF-MStrCs before and after of 48-hr treatment with *G. glabra* IC_{50} B) TNF-α C) TGFβ, D) IL-6, E) IL-17, F) IL-1β, G) CXCL-10, H) IL-4 and I) The bar graph shows the fold changes in levels of cytokines secreted by 48-hour treatment with GG IC_{50} as compared to control OSMF-MStrCs assessed by two-way ANOVA with Tukey's post hoc analysis. Data are presented as mean ± standard deviation (SD). Statistical significance was denoted with p-values as *p<0.05 (significant), **p<0.01 (highly significant), and ***p<0.001 (very highly significant).

Influence of GG Treatment on cytokine release in OSCC-MSC cell line

Our findings revealed a significant increase in the levels of cytokines IL-6 and IL-1β when compared to the untreated group. Conversely, there was a notable decrease in the concentrations of the cytokines CCL2, CXCL-10, and TGFβ. Nevertheless, the levels of TNF-α, IL-4, and IL-17 were comparable to those observed in the untreated group after 48 hr of GG (238 µg/ml) administration (Refer to Table 3 and Figure 8).

Table 3. Cytokine levels secreted by OSCC-MSCs before and after 48-hour treatment with *G. glabra (GG)* IC_{50} (238 µg/ml) concentration.

	Analyte	Control Mean ±SD	GG IC_{50} Mean ±SD	p Value
Higher levels	IL-6	1662±165.8	5802±123.3	*0.03
	IL-1β	18.12± 2.65	20.94±1.32	0.2
Lower levels	CCL2	196.2±7.0	-6.35±0.88	*0.013
	CXCL-10	1692±35.21	112.7±1.95	**0.009
	TGFβ	13.20±2.5	6.78±1.5	0.06
No change	TNF-Aα	28.05± 1.8	28.05±1.8	>0.99
	IL-4	1.53±0.9	1.40±1.0	0.94
	IL-17	75.92±1.08	72.09± 0.00	0.12
Not Detected (ND)	IL-2	ND	ND	-
	CXCL-8	ND	ND	-
	IL-12	ND	ND	-
	IFN-γ	ND	ND	-
	IL-10	ND	ND	-

*p<0.05, **p<0.01 and *** p <0.001 significant.

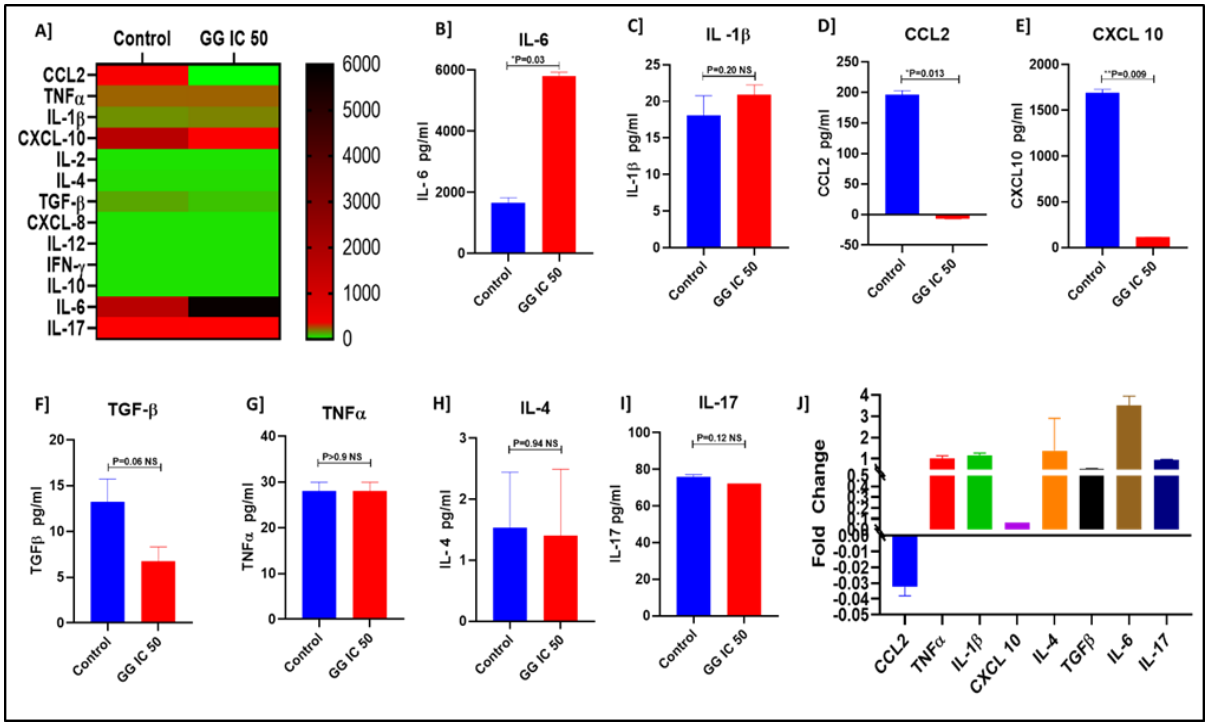

Figure 8. Effect of G. glabra treatment on cytokine secretion by OSCC-MSCs. A-J) Heat maps representing cytokine concentrations secreted by OSCC-MSCs before and after of 48-hr treatment with GG IC_{50}. Each row of the heat map represents cytokines secreted and each column represents OSCC-MSC Control and after of 48-hr treatment with GG IC_{50}. The color scale corresponds to the relative expression of the cytokine for the minimum (0 pg/ml) and maximum (6000 pg/ml) of all values. The bar graphs represent the comparison of concentration of cytokines secreted by OSCC-MSCs before and after of 48-hr treatment with GG IC_{50} B) IL-6, C) IL-1β, D) CCL-2 E) CXCL-10, F) TGFβ, G) TNF-α H) IL-4 and I) IL-17. J) The bar graph shows the fold changes in levels of cytokines secreted by 48-hr treatment with GG as compared to control OSCC-MSCs assessed by two-way ANOVA with Tukey's post hoc analysis. Data are presented as mean ± standard deviation (SD). Statistical significance was denoted with p-values as *p<0.05 (significant), **p<0.01 (highly significant), and ***p<0.001 (very highly significant).

Discussion

Current treatments for head and neck cancer show poor clinical outcomes and high toxicity, leading researchers to explore gentler alternatives. Evidence suggests that complementary and alternative medicine may help in managing cancer. Numerous ethnobotanical studies have examined plant use. Rasayana in Ayurveda represents a specialized domain dedicated to the principles of rejuvenation, longevity, and vitality. It encompasses the use of particular herbs, dietary practices, and ethical conduct (Achara Rasayana) aimed at nourishing bodily tissues (Dhatus), enhancing immunity (Ojas), postponing the aging process, and improving both mental and physical well-being. This practice fundamentally seeks to ensure the optimal flow (Ayana) of the body's essence (Rasa). Rasayana substances can neutralize free radicals, regulate immune responses, and fight cancerous growths. These traits make them strong candidates for cancer treatment (P S Rekha, G Kuttan et al. 2001**,** Mukherjee et al. 2011**,** Baliga et al. 2013**,** Ladke et al. 2022). We based our investigations on the principles of Rasayana therapy. Ayurvedic Rasayana therapy restores normal physiological functions and immune responses at the cellular level of each tissue, or Dhatu (according to Ayurveda these are seven essential tissues or structural components that construct, maintain, and uphold the functions of the body). Rasayana was chosen to improve immune response regulation and slow disease progression. Mesenchymal stem cells and tumor cells interact in the tumor microenvironment, showing both pro- and anti-tumor effects (Mishra et al. 2008,Spaeth et al. 2009). Many MSCs show migratory behavior and often cluster at tumor sites during tumor development (Hu et al. 2013).

Mesenchymal stem cells do not exert a direct influence on cancer cells; however, neighboring cells within a common environment with mesenchymal stem cells may play a role in immunomodulation. The present investigation identified mesenchymal cells-derived OSCC and OSMF tissues as OSCC-MSCs and OSMF-MStrCs and assessed their immune response along with the influence of GG on cytokine secretion.

A scoping review conducted by Wu et al. (Wu et al. 2024) examined Ayurvedic Rasayanas adaptogens in oncology. They reported that the mechanisms of action (MOA) identified in the literature across all 15 Rasayanas were categorized as follows, from most to least frequently mentioned: apoptotic (n = 298, 29.2%), anti-proliferative/anti-growth (n = 249, 24.5%), antioxidant (n = 167, 16.4%), anti-metastatic/anti-invasive (n = 68, 6.8%), anti-inflammatory (n = 47, 4.6%), DNA/RNA degeneration (n = 41, 4.0%), anti-angiogenic (n = 36, 3.5%), cell cycle arrest (n = 32, 3.1%), anticarcinogenic/anti-metastatic (n = 26, 2.6%), and immunomodulatory (n = 24, 2.4%). Their study indicates that there has been a limited number of investigations into the immunomodulatory aspect. The existing research has examined cytokines and their implications in murine studies, (Raveendran Nair et al. 2004,Kaur et al. 2017); however, there remains a notable gap in the literature regarding the investigation of MSCs derived from OSCC and the influence of Rasayana on these cells.

Previous research showed that MSCs from breast cancer can alter the secretion profile, which is significantly dysregulated. Breast tumor cells exhibit a more aggressive phenotype in both *in vitro* and *in vivo* settings due to the activation of epithelial-mesenchymal transition (EMT) (Plava et al. 2020). The present study examined changes in cytokine secretion caused by GG.

Li and colleagues (Li et al. 2012) demonstrated that IL-1beta may enhance the development and invasion of colon tumors in HCT-116 cells by facilitating the self-renewal of cancer stem cells and the epithelial-mesenchymal transition process,

with Zeb1 as a crucial factor in this mechanism. Our findings aligned with these results.

Very few studies have examined the progression of malignancy and metastasis by affecting immune cells and their secretions (Plava et al. 2020**,**Li et al. 2019). Present research showed that OSCC-MSCs expressed and secreted CXCL10, CCL-2, and TGF-beta, contributing to OSCC metastasis. GG administration led to a significant reduction in CXCL10, CCL-2, and TGF-beta, showing its effectiveness against tumors.

Krampera et al (Krampera et al. 2006) noted that IFN-γ and TNF-α induced BM-MSCs to produce CXCL10, significantly affecting T cells. This study found that OSCC-MSCs produce CXCL10 in similar conditions, promoting tumor growth. GG therapy significantly reduced production of CXCL10, suggesting potential anti-tumor effects through immunomodulation.

Interleukin-6 (IL-6) is produced during inflammation and immune responses and is recognized as a factor in tumorigenesis within the tumor microenvironment (Tanaka et al. 2018**,**Karakasheva et al. 2018). HNSCC treatment resistance links to IL-6 overexpression. The link between the IL-6 pathway and chemoresistance is not directly established (O'Keefe et al. 2020)**.** A study by Babiuch, Kuśnierz-Cabala and colleagues (Babiuch et al. 2020) involved measuring IL-1, IL-6, IL-8, and TNF-α in tissue and saliva samples from patients with OSCC and OPMDs, using Immunohistochemistry (IHC) and ELISA techniques. Immunohistochemistry showed that OSCCs and OPMDs with dysplasia have higher levels of IL-8 and TNF-α expression than normal oral mucosa. Patients with OSCC showed significantly higher saliva levels of IL-6, IL-8, and TNF-α compared to OPMDs without dysplasia. GG significantly reduced IL-8 levels in the current investigation. OSMF-MSCs showed elevated levels of TNF-α and IL-6 due to GG. This finding needs to be explored further.

Research showed that blocking IL-4, common in the tumor microenvironment, improves treatment responses (Ito et al. 2017**,**Kim et al. 2016). IL-10 levels are high in HNSCC and linked to tumor growth via the JAK-STAT signaling pathway, affecting IL-6 expression (Bornstein et al. 2016). This study found that OSCC-MSCs showed increased IL-6 secretion, suggesting potential pro-tumor activity. Reduced IL-4 secretion by OSMF-MSCs suggests a protective role against the progression from OSMF to OSCC malignancy. TGF-beta activates the PI3K-AKT-mTOR pathways, enhancing cancer cell survival, proliferation, migration, invasion, angiogenesis, and metastasis. Except for IL-6 and IL-17 secretion, their findings match our study's results.

CCL2 is a powerful chemoattractant for macrophage recruitment and a key initiator of the inflammatory response. Our study presented that CCL2 can recruit more host cells in the tumor microenvironment and affect their differentiation alongside other cytokines. CCL2 negatively impacts tumor prognosis by promoting the accumulation of immunosuppressive cell subtypes. CXCL10 promoted cell proliferation, migration, and epithelial-mesenchymal transition in MCF-7 and MDA-MB-231 breast cancer cell lines (Kim et al. 2021)**.** This study found that CXCL-10, CCL-2, IL-6, and TGF-beta were expressed at higher levels in OSCC-MSCs than in OSMF-MStCs. These genes may significantly contribute to the pathogenesis of OSCC from OSMF. GG reduced CXCL-10, CCL-2, and TGF-beta levels in OSCC-MSCs, linked to pro-inflammatory and pro-tumor activities. GG can enhance pro-tumor activity through immunosuppression and the formation of cancer-associated fibroblasts, as shown by increased IL-6 levels. GG reduced IL-1beta, CXCL-10, and IL-4 levels in OSMF- MStCs, which have anti-tumor effects and affect fibroblasts and immune cells to decrease collagen production and fibrosis. These cytokines may be important cancer

biomarkers, diagnostic tools, and therapeutic targets.

In summary, the present study demonstrated that OSMF-MStCs and OSMF-MStCs secrete important pro and anti-tumor cytokines which are present in different levels in these two cell types. After treating these cells with GG at IC_{50} it was observed that certain protumor cytokines were reduced by GG whereas few anti-tumor cytokines were elevated. But, Il-6 was elevated by GG which is tumor promoting and this finding needs to evaluated in detail. Il-17 was increased by OSMF-MStCs whereas it was reduced by OSMF-MStCs. This finding indicates that certain cytokines like IL-17 are having dual function depending on the duration of tumor in which they are present requiring further analysis. The summary of the effect of GG on OSMF-MStCs and OSMF-MStCs is given in Figure 9.

The intricate interplay among cytokines and their regulatory pathways is underscored by the findings of the current study which revealed an elevation of IL-6 induced by GG in both cell types. Additionally, IL-17 exhibited an increase in OSMF-MStrCs while showing a decrease in OSCC-MSCs upon treatment with GG. Further comprehensive investigation is essential to substantiate these findings.

Our investigation revealed that the stromal cells found in OSCC are Mesenchymal Stem Cells, which could influence the tumor microenvironment. The interplay between cancer cells and stromal cells within the tumor microenvironment profoundly influences the secretion of cytokines by tumors. *G. glabra* influenced the secretion of cytokines by these cells, presenting a promising avenue for targeted therapy in the management of OSMF and OSCC. Therefore, *G. glabra* can be utilized as a complementary treatment as a Rasayana for the management of OSMF and OSCC, particularly regarding its immunomodulatory effects within the tumor microenvironment. It is imperative that these findings undergo validation, accompanied by a thorough evaluation of *G. glabra*'s clinical safety and efficacy.

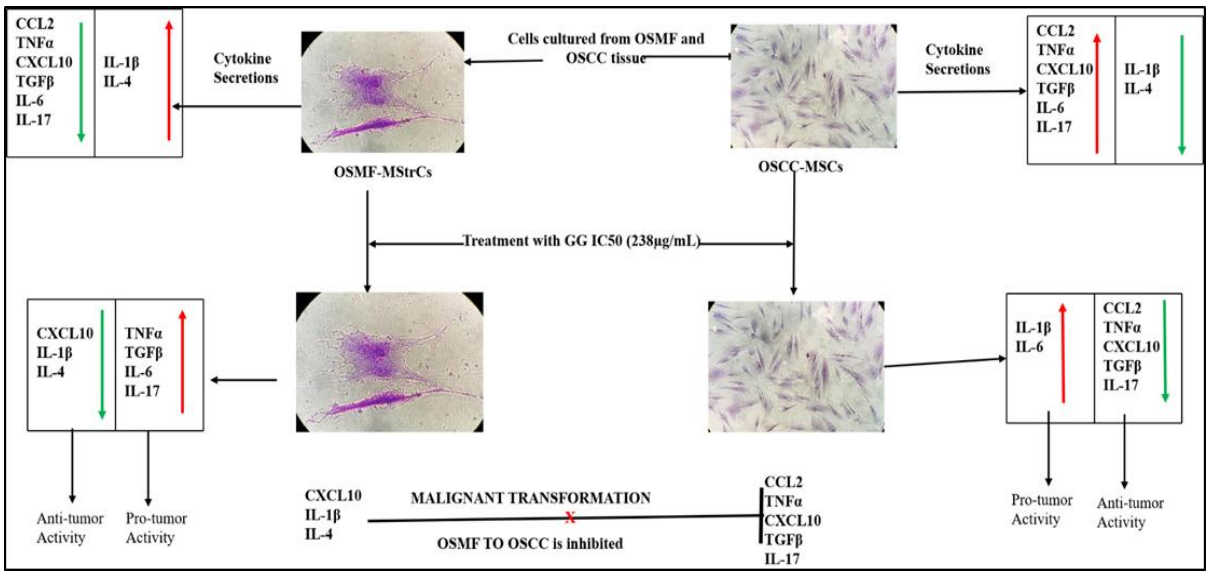

Figure 9. Effect of *G. glabra* on secretion of cytokines secreted by oral squamous cell carcinoma -Mesenchymal Stem cells (OSCC-MSCs) and oral submucous fibrosis-Mesenchymal Stromal cells (OSMF-MStCs). Important pro and anti-tumor cytokines are secreted which are present in different levels in these two cell types. After treating these cells with GG at IC_{50} it was observed that certain protumor cytokines were reduced by GG whereas few anti-tumor cytokines were elevated. But, Il-6 was elevated by GG which is tumor promoting and Il-17 was increased by OSMF-MStCs whereas it was reduced by OSMF-MStCs. Inhibitory effect of GG on CXCL10, IL-1β, and IL-4 will prevent OSMF to OSCC transformation.

Conflicts of interest

There are no conflicts of interest

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Ethical Considerations

The ethical and scientific committee approval for the study was obtained from Dr. D. Y. Patil Vidyapeeth, Pimpri, Pune, India with approval number DYPDCH/EC/404//2019.

Authors' Contributions

VL: conceived the study and performed formal analysis, investigation, validation, and helped write the original daft. GK: provided methodology, data curation, software and formal analysis, and writing the original draft. PS: helped with data curation, formal analysis, software, and methodology. KJ: conceived the study and performed formal analysis, investigation, validation, and helped write the original daft. All authors approved the submitted manuscript.

Abbreviations

GG: *Glycyrrhiza glabra*. HNSCC: Head and Neck Squamous cell Carcinoma. IHC: Immunohistochemistry.
MSC: Mesenchymal stem cells. MStrC: Mesenchymal stromal cells. OSCC: Oral squamous cell carcinoma. OSMF: Oral Submucous Fibrosis. TME: tumor microenvironment

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