

(RESEARCH ARTICLE)



SALIVARY BIOMARKER PROFILING AND EVALUATION OF A *GLYCYRRHIZA GLABRA* EXTRACT IN ORAL POTENTIALLY MALIGNANT DISORDERS AND ORAL SQUAMOUS CELL CARCINOMA

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GSC Biological and Pharmaceutical Sciences, 2026, 36(03), 242–251

Publication history: Received on 17 August 2026; revised on 27 September 2026; accepted on 29 September 2026

Article DOI: <https://doi.org/10.30574/gscbps.2026.36.3.0336>

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ABSTRACT

Background: Oral potentially malignant disorders (OPMD) can serve as precursors to the development of oral squamous cell carcinoma (OSCC), which shows the need for new non-invasive markers ideal for other treatment options. The present study assessed salivary profiles of oxidative stress, inflammatory condition, and miRNAs among healthy controls, with OPMD and OSCC individuals, along with studying the effects of extract from medicinal plants on apoptosis and cytotoxicity in OSCC.

Methods: Saliva samples without stimulation were gathered from healthy people, OPMD patients, and OSCC patients. Salivary IL-6 and IL-8 concentrations, malondialdehyde (MDA), and reduced glutathione (GSH) were measured, and miR-21 and miR-31 expression quantification was performed using qRT-PCR. The cytotoxic activity of the ethanolic *Glycyrrhiza glabra* extract was performed by using the MTT method, and apoptosis was measured through Annexin V/PI flow cytometry. Correlation and receiver operating characteristic (ROC) testing were performed to understand biomarker interactions.

Results: Salivary levels of IL-6, IL-8, and MDA showed an upward trend from the control group to OPMD and OSCC, while levels of GSH were reduced. Additionally, the levels of miR-21 and miR-31 also followed a similar trend. According to the biological activity of the plant, it was found to be toxic toward the OSCC cell line in a dose-dependent way. The calculated IC₅₀ value was 210.6 ± 18.7 µg/mL. The analysis of Annexin V/PI showed not only the decrease in the number of viable cells after treatment but also the increase in early and late apoptosis effects. Strong correlation was detected for a number of inflammatory, oxidative, and miRNA parameters. The results of ROC analysis showed that AUC values ranged from 0.86 to 0.94, with miR-21 presenting the best result of 0.94.

Conclusion: The observed changes in salivary inflammatory, oxidative stress, and miRNA profiles indicate that they can be useful for distinguishing healthy, OPMD, and OSCC groups. Moreover, the *Glycyrrhiza glabra* extract showed cytotoxicity and effect on apoptosis in OSCC cells allowing new therapies in anticancer.

Keywords: Saliva; *Glycyrrhiza glabra*, Oral Lesions; Oral Cancer; IL-6; IL-8; Mir-21

1 INTRODUCTION

Oral squamous cell carcinoma (OSCC) is regarded as a significant oral cancer, while potentially malignant disorders of the oral cavity (OPMDs) refer to clinical diseases that may develop before the appearance of cancer (Bouaoud *et al.*, 2022); hence, the significance of the early identification of biomolecular changes that occur before cancer development cannot be underestimated.

In light of these developments, saliva has been shown to be a non-invasive biological specimen that can be simply collected and can contain biomarkers of cancer-related inflammation and molecular shifts (Kaczor-Urbanowicz *et al.*, 2017). A recent review also suggested that saliva may help differentiate healthy individuals from patients diagnosed with OPMD and OSCC, but more standard and larger follow-up trials must be conducted (Bastías *et al.*, 2024; López-Galindo & Sousa-Roques, 2026).

The connection between inflammation and oxidative stress in the context of oral cancer is well established. Two types of interleukins, IL-6 and IL-8, are the two important biomarkers found in saliva connected with the inflammatory response, and malondialdehyde (MDA) indicates oxidative stress level determined by lipid peroxidation, while reduced glutathione (GSH) acts as an essential element of the antioxidant defense. Recent systematic reviews and meta-analyses proved that the level of IL-6, IL-8, MDA, and GSH is altered in oral cancer patients, thus proving the necessity of the analysis of those biomarkers together, rather than relying on single markers only (Huang *et al.*, 2024; Zhou *et al.*, 2023).

On the other hand, microRNAs represent another molecular layer that can manifest changes in carcinogenesis. Among the relevant miRNAs in oral squamous cell carcinoma were miR-21 and miR-31, which have attracted considerable attention, as research showed differences in their expression in the saliva of oral cancer patients. In this respect, one recent systematic review analyzed a total of 42 studies showing that several salivary miRNAs, including miR-21 and miR-31, possess some potential in diagnostics; nevertheless, the authors noted that further confirmation in larger groups would be beneficial (Hashmi *et al.*, 2026; Kumari *et al.*, 2021). In this light, inflammatory and oxidative stress markers could be combined with miRNAs in order to get a more informative assessment of their discriminative capabilities.

Besides biomarker evaluation, assessing the anticancer properties of plant extracts has become an important area of development in the search for auxiliary therapeutics (Al-Burki *et al.*, 2026). The current study examined the anti-cancer potential of an alcoholic *Glycyrrhiza glabra* extract on OSCC cells utilizing the MTT method, a common assay to determine cell viability (Mosmann, 1983). The cell death mechanism was then determined using Annexin V/PI staining and flow cytometry, a valuable technique for distinguishing viable from early and late apoptotic and necrotic cells (Koopman *et al.*, 1994).

Moreover, qRT-PCR determination of the expression of miR-21 and miR-31 was performed using the 2- $\Delta\Delta C_t$ approach (Livak & Schmittgen, 2001). Therefore, the current study will seek to assess the changes that occur in salivary inflammatory cytokines (IL-6 and IL-8), markers of oxidative stress (MDA and GSH), and the levels of miR-21 and miR-31 in healthy, OPMD, and OSCC patients, while analyzing their correlations and ability to discriminate; this will be done in conjunction with the examination of the cytotoxicity and apoptosis of the *Glycyrrhiza glabra* extract on OSCC.

2 MATERIALS AND METHODS

2.1 Collection of saliva sample

The study involved three groups of participants: healthy people, patients suffering from Oral Potentially Malignant Disorders (OPMDs), and patients suffering from Oral Squamous Cell Carcinoma (OSCC). The saliva collection was done in the right way, so the patient did not eat, drink or smoke before the collection.

Saliva was obtained in sterile and RNase-free tubes and taken to a laboratory quickly where it was stored at low temperatures before analysis. Participants' clinical and demographic information was kept using specific criteria for each category.

2.2 Assessment of Salivary Inflammatory Markers

Levels of IL-6 and IL-8 in saliva were assessed with specific validated ELISA kits for each marker according to the guide provided by its maker. After preparing the specimen and adding the standards and samples to the wells, the incubation,

washing and detection stages were performed according to the prescribed method in the instructions for the diagnostic kit.

Absorbance was assessed with a plate reader at the wavelength specified in the instructions and the value of each marker was understood via its calibration plot. Results are expressed in pg/mL.

2.3 Assessment of Oxidative Stress Markers

The oxidative status of saliva samples was evaluated by measuring malondialdehyde (MDA)—an indicator of lipid peroxidation- and reduced glutathione (GSH)- a key cellular antioxidant.

MDA was measured using an appropriate assay kit based on the reaction products of MDA with a specific reagent, while GSH was measured using a kit in accordance with the manufacturer's instructions. Absorbance was recorded using a microplate reader or a suitable spectrophotometer, and the concentrations of both markers were calculated using calibration curves. Results were expressed relative to protein content or sample volume, depending on the specific assay protocol.

2.4 miRNA Extraction and Analysis

After acquiring samples of saliva, total RNA containing miRNA was collected using a suitable kit according to the provided protocol by the manufacturer. The quality and quantity of RNA were measured by the use of a spectrophotometer, thus making it possible to use high-quality RNA for reverse transcription.

miRNA relative expression was studied based on qRT-PCR, hereby calculation of relative changes was conducted with the help of the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001) with following the rules stated in MIQE guidelines (Bustin et al., 2009).

2.5 Preparation of the plant extract

Plant parts from *Glycyrrhiza glabra* were gathered from the Al-Shabaka region near the Saudi Border (approx. 135 km from Najaf governorate, Iraq). Air-dried at (25-28 °C), the parts were ground into powder. Extraction of plant powder was completed by maceration using 70% ethanol, the solvent being added in 1:10 (w/v) ratio and soaked for 72 hours with agitating it from time to time. After filtering, the extract was evaporated using rotary evaporator, not raising the temperature above 40 °C and later on stored at 4 °C until needed.

2.6 Assessment of plant extract's cytotoxicity using MTT method

The cytotoxicity of the plant extract was assessed with the help of OSCC cells cultured under optimal cell culture conditions. The cells were cultured in 96-well plates in varying concentrations of the plant extract for a specified duration.

After treatment, the MTT reagent was added to the cells, which were then incubated to allow the formation of formazan products. The medium was carefully discarded, and the formed crystals were dissolved in an appropriate solution, following which absorbance was measured at 570 nm on a microplate reader. The cell viability was calculated with respect to the untreated group, and if applicable, the IC_{50} value was determined as well (Mosmann, 1983).

2.7 Evaluating the apoptosis using Annexin V/PI

To evaluate the function of the toxicity, in OSCC cells, the apoptosis was examined using the Annexin V/PI staining. The cells were treated with the extract at an effective concentration based on the MTT assay data, with an untreated control included.

Post-treatment, the cells were collected, washed, and stained with Annexin V and Propidium Iodide (PI) as per the instructions provided with the kit. Flow cytometry was used to analyze the cells and categorize them as viable, early apoptotic, late apoptotic, or dead based on the fluorescence patterns.

2.8 Statistical Analysis

Statistical analysis of the data was performed using relevant statistical software. Quantitative variables were presented as mean $\pm$ standard deviation or in accordance with their distribution. The comparison of biomarker levels and miRNA expressions between the healthy, OPMD and OSCC groups was performed using appropriate statistical tests depending on the number of groups and their distribution.

Correlation analysis was performed to determine the relationship of biomarkers, miRNA and significance variables. ROC and AUC were used to evaluate the performance of biomarkers. The differences were considered statistically significant at $p < 0.05$.

3 RESULTS

3.1 Clinical and demographic characteristics of the involved study groups

The clinical and demographic characteristics of the groups involved in the study are given in Table (1). The average age of participation across the three groups is 42.4 ± 8.2 years in the normal group, 46.8 ± 9.3 years in the OPMD group, and 54.7 ± 10.2 years for the OSCC group. The percentage of male participants is 53.3%, 60.0%, and 60.0%, respectively, whereas that for females is 46.7%, 40.0%, and 40.0%, respectively. The age ranges were as follows: 28-56 years for the normal group, 30-62 years for the OPMD group, and 34-70 years for the OSCC group. In terms of smoking, the percentage of smokers was observed to be 26.7%, 43.3%, and 63.3% for the groups studied respectively. Non-smokers comprised 73.3%, 56.7%, and 36.7% in the three respective groups.

Table 1: Clinical and Demographic Characteristics of the Study Groups

Variable	Healthy (n=30)	OPMD (n=30)	OSCC (n=30)
Age (years)	42.4 ± 8.2	46.8 ± 9.3	54.7 ± 10.2
Male, n (%)	16 (53.3)	18 (60.0)	18 (60.0)
Female, n (%)	14 (46.7)	12 (40.0)	12 (40.0)
Age range (years)	28–56	30–62	34–70
Smokers, n (%)	8 (26.7)	13 (43.3)	19 (63.3)
Non-smokers, n (%)	22 (73.3)	17 (56.7)	11 (36.7)

Values are presented as mean $\pm$ SD or n (%).

3.2 Biomarkers in Saliva and Expressions of miRNA

The findings in relation to the salivary biomarkers showed a distinct difference in the study groups. The average concentration of IL-6 increased from 4.82 ± 1.21 pg/mL in the healthy group to 8.76 ± 2.14 pg/mL in OPMD and 14.63 ± 3.52 pg/mL in OSCC. Similarly, the concentration of IL-8 saw an increase from 18.45 ± 4.16 pg/mL to 31.72 ± 7.35 pg/mL and 52.86 ± 11.24 pg/mL, accordingly for the three groups.

The amount of MDA also increased continuously, from a level of 1.84 ± 0.39 nmol/mL in the healthy group, to 3.12 ± 0.67 nmol/mL in the OPMD group and 5.27 ± 1.08 nmol/mL in the OSCC group; on the contrary, the GSH method showed an opposite tendency with respect to the reduced quantities of GSH that decreased from 8.72 ± 1.34 μ mol/L in the healthy group to 6.31 ± 1.12 μ mol/L in the OPMD group and 4.18 ± 0.96 μ mol/L in the OSCC group.

The analysis conducted on miRNA expression demonstrated a gradual increase of miR-21 and miR-31 in the groups that were studied. miR-21 was estimated at 1.00 ± 0.18 for the healthy group, 1.86 ± 0.34 for the OPMD group, and 3.24 ± 0.61 for the OSCC group, while miR-31 was 1.00 ± 0.16 for the healthy group, 1.64 ± 0.29 for the OPMD group, and 2.87 ± 0.52 for the OSCC group. The changes were also presented in the figure (2).

Table 2: Salivary Biomarkers and miRNA Expression among Study Groups

Biomarker	Healthy (n=30)	OPMD (n=30)	OSCC (n=30)
IL-6 (pg/mL)	4.82 ± 1.21	8.76 ± 2.14	14.63 ± 3.52
IL-8 (pg/mL)	18.45 ± 4.16	31.72 ± 7.35	52.86 ± 11.24
MDA (nmol/mL)	1.84 ± 0.39	3.12 ± 0.67	5.27 ± 1.08
GSH (μ mol/L)	8.72 ± 1.34	6.31 ± 1.12	4.18 ± 0.96
miR-21 (fold change)	1.00 ± 0.18	1.86 ± 0.34	3.24 ± 0.61
miR-31 (fold change)	1.00 ± 0.16	1.64 ± 0.29	2.87 ± 0.52

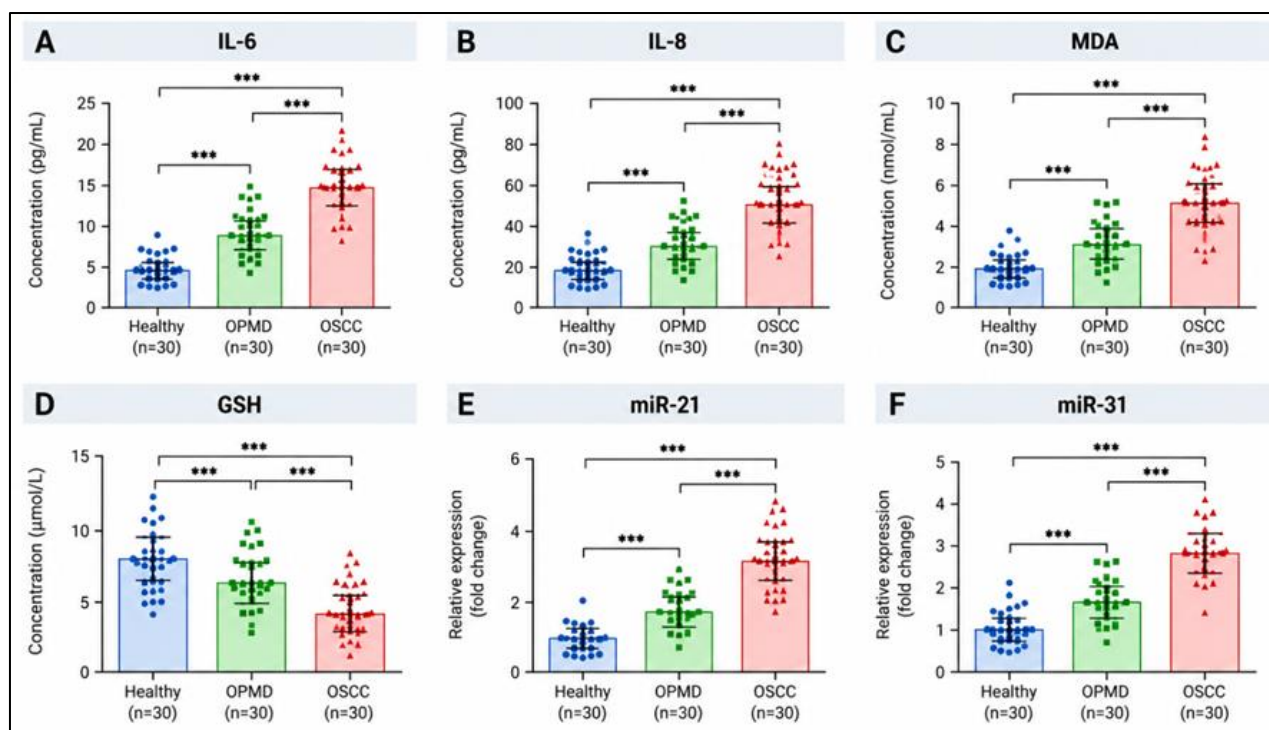


Figure 1: Salivary biomarkers and miRNA expression among the study groups. (A) IL-6, (B) IL-8, (C) MDA, (D) GSH, (E) miR-21, and (F) miR-31 levels in healthy controls, OPMD, and OSCC groups

3.3 Cytotoxicity of the plant extract against OSCC cells

The plant extract caused a concentration-dependent effect on the viability of OSCC cells. In the control group, the viability was measured at $100.0 \pm 4.1\%$, which then reduced to $91.7 \pm 3.8\%$, $82.5 \pm 4.2\%$, $68.8 \pm 3.7\%$, $51.4 \pm 3.3\%$, $32.7 \pm 2.9\%$, and $18.5 \pm 2.2\%$ with the increasing concentrations of 25, 50, 100, 200, 400, and 800 $\mu\text{g/mL}$, respectively, resulting in an IC_{50} of $210.6 \pm 18.7 \mu\text{g/mL}$ which is summarized in Table (3) and depicted in Figure (2).

Table 3: Cytotoxic Effect of the Ethanolic *Glycyrrhiza glabra* Extract on OSCC Cells

Extract concentration ($\mu\text{g/mL}$)	Cell viability (%)
0 (Control)	100.0 ± 4.1
25	91.7 ± 3.8
50	82.5 ± 4.2
100	68.8 ± 3.7
200	51.4 ± 3.3
400	32.7 ± 2.9
800	18.5 ± 2.2

IC_{50} : $210.6 \pm 18.7 \mu\text{g/mL}$

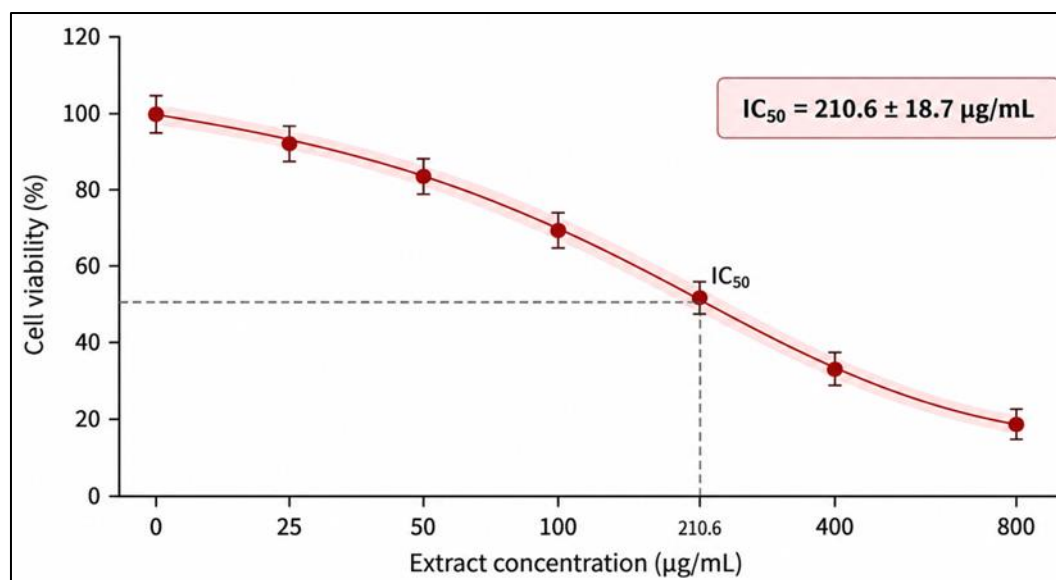


Figure 2: Cytotoxic of the ethanolic extract on OSCC cells

3.4 Influence of the plant extract on OSCC cell apoptosis

The results of the Annexin V/PI tests indicate that there was a variation in the amount of cells present in the groups without the plant extract and the groups that received the plant extract. The viable cell percentage was found to be $88.8 \pm 2.9\%$ in the group without the extract whereas the viable cell percentage fell after the treatment with the extract to $48.7 \pm 3.8\%$. The percentage of early apoptotic cells increased from $4.9 \pm 1.3\%$ to $17.9 \pm 2.3\%$, while the percentage of late apoptotic cells increased from $3.2 \pm 0.8\%$ to $27.7 \pm 3.2\%$. The necrotic cell percentage increased from $3.5 \pm 1.1\%$ to $5.9 \pm 1.4\%$. The overall distribution of the populations of different types of cells is shown in Figure (3).

Table 4: Effect of the Ethanolic Extract on Apoptosis in OSCC Cells

Treatment	Viable cells (%)	Early apoptosis (%)	Late apoptosis (%)	Necrosis (%)
Control	88.8 ± 2.9	4.9 ± 1.3	3.2 ± 0.8	3.5 ± 1.1
Plant extract	48.7 ± 3.8	17.9 ± 2.3	27.7 ± 3.2	5.9 ± 1.4

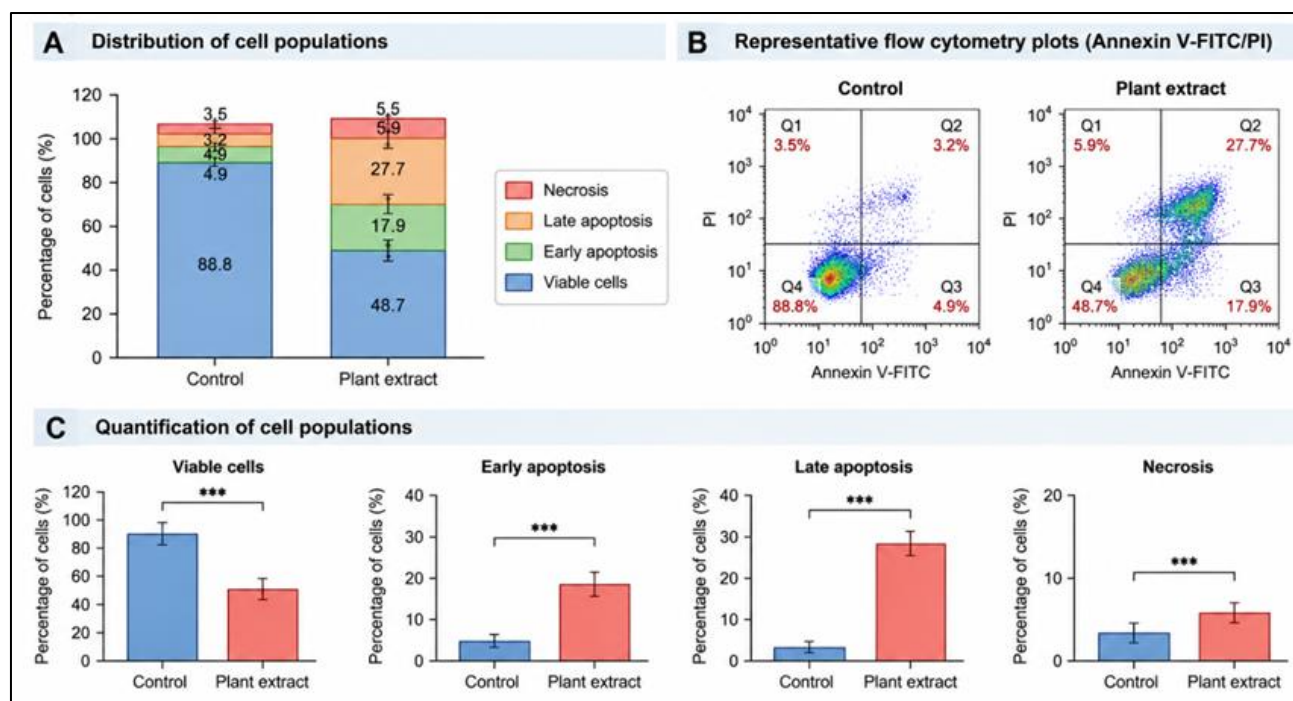


Figure 3: Effect of the ethanolic extract on apoptosis OSCC cells

3.5 Interrelation and Diagnostic Efficiency of Salivary Biomarkers

A high positive correlation is noted between IL-6 and miR-21 ($r = 0.62$, $p < 0.001$), IL-8 and miR-31 ($r = 0.58$, $p < 0.001$), MDA and miR-21 ($r = 0.65$, $p < 0.001$), as well as MDA and miR-31 ($r = 0.59$, $p < 0.001$), on the contrary, GSH exhibited a high negative correlation with miR-21 ($r = -0.53$, $p < 0.001$) (Table 5A).

The relation and the diagnostic efficiency of the biomarkers are presented diagrammatically in Figures 4A and 4B.

In the ROC analysis that examined OSCC against healthy controls, the AUC ranged from 0.86 to 0.94. The AUCs for IL-6, IL-8, MDA, GSH, miR-21, and miR-31 were as follows: 0.88, 0.92, 0.89, 0.86, 0.94, and 0.91. For sensitivity, values varied from 80.0% to 90.0% and related specificity values from 80.0% to 86.7%, with the results summarized in table B5.

Table 5: Diagnostic Performance and Correlations of Selected Salivary Biomarkers

A. Correlation analysis				
Biomarker pair		Pearson's r	p-value	Interpretation
IL-6 vs miR-21		0.62	<0.001	Positive correlation
IL-8 vs miR-31		0.58	<0.001	Positive correlation
MDA vs miR-21		0.65	<0.001	Positive correlation
GSH vs miR-21		−0.53	<0.001	Negative correlation
MDA vs miR-31		0.59	<0.001	Positive correlation
B. ROC analysis: OSCC versus Healthy				
Biomarker	AUC	95% CI	Sensitivity (%)	Specificity (%)
IL-6	0.88	0.80–0.95	83.3	80.0
IL-8	0.92	0.86–0.97	86.7	86.7
MDA	0.89	0.82–0.95	83.3	83.3
GSH	0.86	0.78–0.93	80.0	80.0
miR-21	0.94	0.89–0.98	90.0	86.7

miR-31	0.91	0.85–0.96	86.7	83.3
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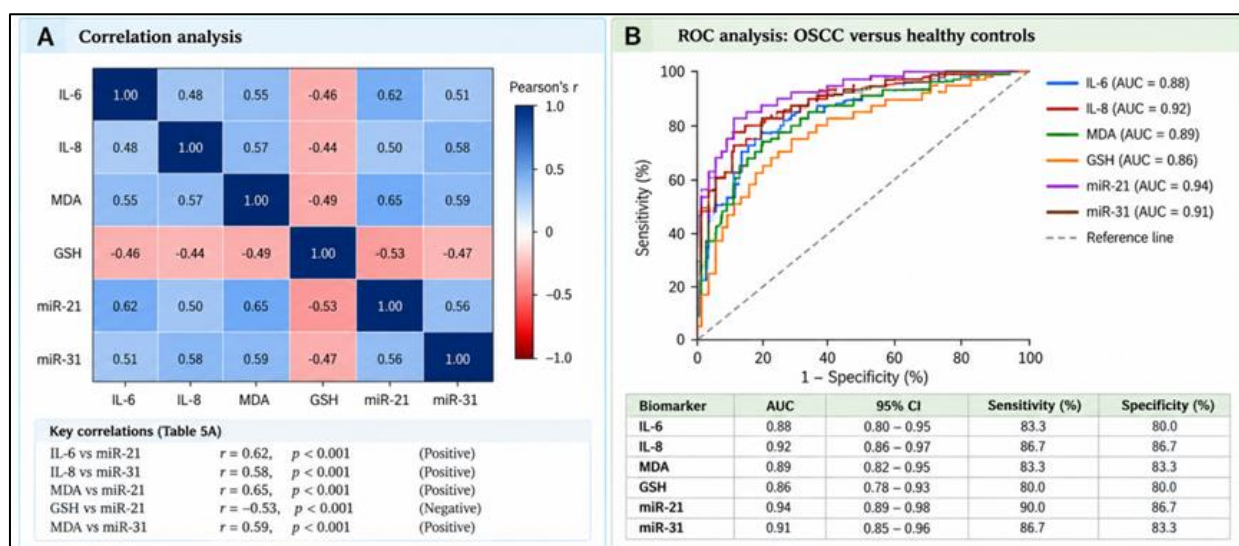


Figure 4: Correlation and diagnostic performance of salivary biomarkers

4 DISCUSSION

The current investigation recognized synchronized changes in inflammation, oxidation, and molecules from healthy stage through OPMD until OSCC. The systematic increase in salivary concentration of IL-6 and IL-8 is in agreement with studies showing the link between inflammation and cancer of the mouth, whereas the increase in MDA and drop in GSH is indicative of disturbed state of redox equilibrium. Other systematic reviews have demonstrated alteration of salivary inflammatory and oxidative markers in OSCC as well (Benito-Ramal *et al.*, 2023; Huang *et al.*, 2024; Zhou *et al.*, 2023). The findings validate the theory of interrelated inflammatory and oxidative changes associated with OPMD and OSCC, as opposed to a simple molecular modification.

Interestingly, the expression of both miR-21 and miR-31 gives further meaning to these profile results. Identification of salivary miRNAs as new markers has been growing over time, and recent systematic reviews have acknowledged miR-21 and miR-31 as potential markers of oral cancers. The dynamics of alteration discovered in this investigation coupled with previously demonstrated correlations between miRNAs and inflammatory and oxidative stress markers allow concluding on some biological interrelations within the disease context.

Nevertheless, this association does not imply causality, and longitudinal studies need to be conducted to verify whether these biomarkers are able to predict the malignant progression of OPMD lesions in individual patients (Osan *et al.*, 2025; Hashmi *et al.*, 2026).

The findings obtained from ROC analysis lend additional evidence to the possible role of salivary biomarkers as discriminators between healthy individuals and OSCC patients. However, results should be interpreted with caution because ROC analysis was not aimed at assessing the discrimination between OSCC and OPMD and other inflammatory diseases of the oral cavity. Thus, further investigations are needed to validate these findings in larger cohorts and account for confounding factors (e.g., age or smoking).

The ethanolic extract of the *Glycyrrhiza glabra* showed that it produces cytotoxicity that is concentration-dependent and increases the number of cells undergoing apoptosis in OSCC cells. These findings are consistent with recent findings supporting the use of plant-derived products as potential candidates for the development of anti-cancer drugs, however, the exact mechanism through which the extract promotes apoptosis cannot be established based solely on the findings made in this study (Ren *et al.*, 2024; Ramsridhar *et al.*, 2025).

Findings established that a combination of inflammatory, oxidative, and miRNA profiling offers useful biological information along the healthy-OPMD-OSCC spectrum while *Glycyrrhiza glabra* extract exhibit preliminary anticancer activities against OSCC. Further studies need to be conducted to confirm the results obtained and find a rationale for their application in clinical practice.

5 CONCLUSION

The results of this study indicate that oral potentially malignant disorders and oral squamous cell carcinoma are related to coordinated changes in salivary inflammatory, oxidative, and molecular indicators. The simultaneous evaluation of IL-6, IL-8, MDA, GSH, miR-21, and miR-31 yields a multidimensional expression linked to the healthy-OPMD-OSCC continuum and proves its possible usefulness as a non-invasive indicator. Moreover, ethanolic extract demonstrated significant anti-cancer property by exhibiting cytotoxic effect and apoptosis-inducing potential against OSCC cells. Hence, it provides initial evidence that the plant could be a source of bioactive anticancer drugs. Further investigation on larger sample size with independent biomarker validation and mechanistic studies of its effects would be necessary.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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