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EFFECTS OF TEUCRIUM POLIUM EXTRACT ON CYTOTOXICITY, OXIDATIVE STRESS, AND INFLAMMATORY BIOMARKERS IN AN ORAL CANCER CELL MODE

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ABSTRACT

Background: Oral squamous cell carcinoma (OSCC) is among the most frequent forms of cancer affecting the mouth, with the genesis of this disease being related to oxidative stress and inflammatory processes. The purpose of the present study was to carry out investigation of cytotoxic effects of *Teucrium polium* and its antioxidant, anti-inflammatory and anti-biofilm properties. The alcohol extract was produced from aerial parts of *T. polium* with the help of 70% ethanol. The cytotoxicity was tested on SCC-25 cells using the MTT assay, and the IC₅₀ was calculated. There were conducted measurements of the levels of intracellular ROS, MDA, and GSH, as well as levels of IL-6 and TNF-α with ELISA. The impact of the extract on biofilm formation and eradication of *Porphyromonas gingivalis* was assessed as well. Results: The extract exhibited a cytotoxic effect that was dependent on dose; in the control sample, 100.00% of SCC-25 cells remained viable, but at the dose of 300 µg/mL, the viability decreased to 24.7 ± 2.6%, indicating an IC₅₀ of 170.7 µg/mL. At the same time, progressive decreases in the levels of ROS and MDA were observed, while GSH levels increased with increasing concentrations of the extract. Levels of IL-6 and TNF-α also declined progressively. At the same time, at a dose of 150 µg/mL, the extract was able to inhibit biofilm formation of *P. gingivalis* by 64% and to remove 51% of already formed biofilm.

Keywords: *Teucrium polium*; Oral squamous cell carcinoma; Biofilm; Cytotoxicity; Oxidative stress; Inflammatory biomarkers.

1 INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most frequently occurring malignant tumor in the oral cavity and continues to be a serious health issue (Chamoli *et al.*, 2021). The process of OSCC development involves complex interactions of genetic modifications, chronic inflammation, oxidative stress and environmental influences. Therefore, it is possible to

find new natural agents combining antitumor, anti-inflammatory, oxidative stress suppressing and various other properties for the solution of the problems being investigated (Conway *et al.*, 2018).

The field of cancer biology has significant involvement with oxidative stress, which is caused by excessive production of reactive oxygen species (ROS), lipid peroxidation, and deficiency in endogenous antioxidants (Nakamura *et al.*, 2021). The assessment of reactive oxygen species (ROS) can be performed through dichlorofluorescein-based assays. There are malondialdehyde (MDA) and reduced glutathione (GSH), which are widely accepted as indicators of lipid oxidative damage and cellular antioxidant defense, respectively (Cathcart *et al.*, 1983).

The biological behavior of OSCC is also influenced by inflammatory mediators. In particular, interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) are involved in the inflammatory signaling pathways and have been linked to the tumor microenvironment and progression of oral squamous cell carcinoma (Brierly *et al.*, 2023). Their measurement can provide additional proof for evaluating the anti-inflammatory effects of potential therapeutic agents.

Teucrium polium L. is a medicinal herb highly contented with biologically useful secondary metabolites. Examinations on *T. polium* show antioxidant, anti-inflammatory, antimicrobial, and cytotoxic properties and make it relevant point for studies on bioactive materials (Bahramikia *et al.*, 2022). The effects of plant extracts on cells can be assessed via the tetrazolium-based assays for cell viability such as the MTT assay (Bruggisser *et al.*, 2022).

Apart from its impacts on cancer cells, the oral microbiome has its own importance in the context of oral disease. One significant periodontal pathogen that was studied with respect to chronic inflammation and oral carcinogenesis is *Porphyromonas gingivalis*. The microtiter-plate crystal violet assay gives the opportunity to quantitatively assess biofilm formation and shows a well-known method for quantifying inhibition and removal of bacterial biofilms (Stepanović *et al.*, 2007). That is why, the present research was aimed to study the influence of *T. polium* extract on OSCC cells viability, markers of oxidative stress (ROS, MDA level, GSH), inflammatory biomarkers (IL-6, TNF- α), and biofilm formation by *P. gingivalis*.

2 MATERIALS AND METHODS

2.1 Extract preparation

The aerial parts of *Teucrium polium* L. were collected from a place around Al-Shabaka in Najaf Governorate of Iraq (roughly about 110 km from the center of the governorate), where the plant parts are sun-dried, and the leaves are crushed to form fine powder. The obtained powder is subsequently subjected to extraction using 70% ethyl alcohol, after which the extract is filtered and evaporated by means of vacuum distillation.

2.2 Cell Culture

The method was done according to the study by Nematollahi-Mahani *et al.* (2007) with human oral squamous cell carcinoma cells (SCC-25; ATCC CRL-1628). Cells were cultured in DMEM and F-12 (1:1) medium with 10% FBS, 400ng/mL hydrocortisone, and 1% penicillin-streptomycin at 37 degrees Celsius in a humidified environment containing 5% CO₂.

2.3 Cytotoxicity Assay and IC₅₀ determination

The viability of SCC-25 cells was determined through the MTT assay after exposure to the extract at 25-300 µg/mL concentrations for 24 hours.

The final concentration of MTT was 0.5 mg/mL, and the cells were kept for 4 hours. After that, the formed formazan crystals were dissolved in DMSO, and the absorbance was measured at 570 nm. The viability of cells was compared to the control group, and IC₅₀ was calculated using a four-parameter nonlinear regression method.

2.4 Measuring Reactive Oxygen Species (ROS)

The level of intracellular ROS was determined by exposing the cells to 10 µM DCFH-DA for 30 minutes at 37°C in the dark before monitoring fluorescence at approximately 485/530 nm. The results were expressed as a percentage of control values. DCFH-DA has long been known to be the probe of choice for measuring oxidative stress (Mosmann, 1983; Riss *et al.*, 2016).

Cell viability was calculated with respect to the untreated control using:

$$\text{Cell viability (\%)} = (\text{Absorbance of treated cells} \div \text{Absorbance of control cells}) \times 100$$

IC₅₀ denoting the concentration that causes 50% inhibition of cell viability was then calculated from the whole concentration-response curve (Ritz *et al.*, 2015).

2.5 Measurement of MDA and GSH

To determine levels of MDA, the Lipid Peroxidation MDA Assay Kit (Beyotime, S0131) was applied as an indicator for the degree of lipid peroxidation, while the amount of GSH was determined using the GSH and GSSG Assay Kit (Beyotime, S0053) according to the instructions given in the kit. The measurements for MDA were taken at 532 nm, whereas GSH readings were made at 412 nm by means of DTNB reaction, and the results were expressed as per the determination of protein content (Wang & Joseph, 1999).

2.6 Evaluation of IL-6 and TNF- α Levels

The cell culture supernatants were collected 24 hours after the treatment with three different concentrations of the extract, i.e. 50, 100, and 150 $\mu\text{g/mL}$, and the levels of Human IL-6 in the supernatants were measured using sandwich enzyme-linked immunoassays. The ELISA kit (Quantikine-D6050B) with Human IL-6 was purchased from R&D Systems/Bio-Techne. The levels of TNF- α in the supernatants were measured on an ELISA test kit (Quantikine-DTA00D) developed by R&D Systems/Bio-Techne, reading at 450 nm. Calculated concentrations were determined as per the standard curve (Ohkawa *et al.*, 1979).

2.7 Estimation of Reduced Glutathione

Spectrophotometric methods were applied for determining the concentration of reduced glutathione (GSH). The method exploits thiol reactions with the reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

Cellular lysates were prepared in such way that the reduced thiols' oxidation was prevented, and then, the lysate samples were assessed according to the method described by Sedlak and Lindsay (1968). The results were observed spectrophotometrically at the wavelength of approximately 412 nm for the yellow precipitate (5-thio-2-nitrobenzoate) formed in the course of the reaction. The levels of GSH in samples were calculated according to the standard curve for reduced glutathione.

2.8 Total Protein Determination

In order to ascertain the concentration of total protein found in the lysate, we conducted a total protein assay that is defined by the Bradford Method. This method stems from the principle of binding of the Coomassie Blue G-250 dye with the proteins and measuring absorbance at 595 nm (Bradford, 1976). Bovine serum albumin (BSA) was utilized as a standard to calibrate this experiment.

2.9 Statistical Analysis

The experiments were carried out in triplicates and results were expressed as mean $\pm$ SD. The data were analyzed with One-way ANOVA followed by Tukey's post-hoc test for comparisons amongst groups and a significance level of $p < 0.05$ was considered statistically significant. The IC₅₀ was calculated using nonlinear regression analysis with four parameters.

3 RESULTS

3.1 Cytotoxicity of *Teucrium polium* extract

According to the findings in table (1) and figure (1), the cytotoxicity of *Teucrium polium* extract was observed to be concentration-dependent. The viability of cells decreased from $100.0 \pm 3.2\%$ of cells in the control to $91.9 \pm 3.4\%$, $82.7 \pm 3.8\%$, $68.9 \pm 3.3\%$, $54.8 \pm 3.6\%$, $43.2 \pm 2.9\%$, $31.9 \pm 2.8\%$, and $24.7 \pm 2.6\%$ when the extract is assessed at 25, 50, 100, 150, 200, 250, and 300 $\mu\text{g/mL}$ respectively. The IC₅₀ value is approximately 170.7 $\mu\text{g/mL}$.

Table 1: Cytotoxic effect of *Teucrium polium* extract on OSCC cells

Extract concentration ($\mu\text{g/mL}$)	Cell viability (%)	Cytotoxicity (%)
0 (Control)	100.0 ± 3.2	0.0
25	91.9 ± 3.4	8.1
50	82.7 ± 3.8	17.3
100	68.9 ± 3.3	31.1

150	54.8 ± 3.6	45.2
200	43.2 ± 2.9	56.8
250	31.9 ± 2.8	68.1
300	24.7 ± 2.6	75.3

IC₅₀ ≈ 170.7 µg/mL.

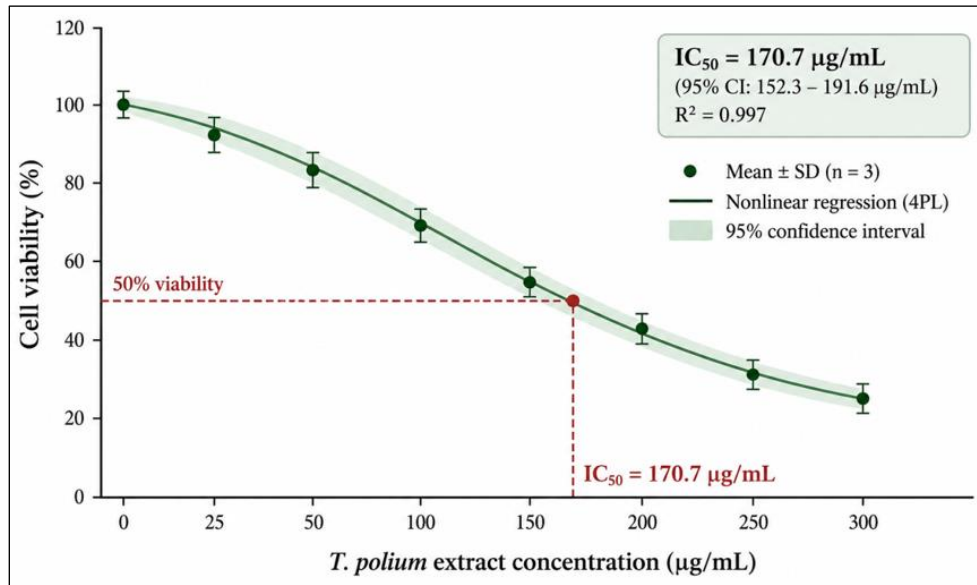


Figure 1: Cytotoxic effect of *Teucrium polium* extract on OSCC cells

3.2 Impact on Intracellular ROS Levels

Data shown in Table (2) reveal that intracellular levels of reactive oxygen species (ROS) significantly decrease when extract concentration rises. In the control group, the percentage of ROS was $100.0 \pm 5.3\%$, while at higher concentrations of 50 µg/mL, 100 µg/mL, and 150 µg/mL, reductions to $88.5 \pm 4.8\%$, $72.7 \pm 4.2\%$, and $57.9 \pm 3.9\%$ were observed, respectively.

Table 2: Effect of *T. polium* extract on intracellular ROS levels

Group	ROS (% of control)
Control	100.0 ± 5.3
Extract 50 µg/mL	88.5 ± 4.8
Extract 100 µg/mL	72.7 ± 4.2
Extract 150 µg/mL	57.9 ± 3.9

3.3 Effect of MDA and GSH

Table 3: Effect of *T. polium* extract on MDA and GSH

Group	MDA (nmol/mg protein)	GSH (µmol/g protein)
Control	3.83 ± 0.21	8.66 ± 0.39
Extract 50 µg/mL	3.32 ± 0.19	9.29 ± 0.42
Extract 100 µg/mL	2.78 ± 0.18	10.19 ± 0.46
Extract 150 µg/mL	2.23 ± 0.16	11.08 ± 0.48

The data in Table (3) and Figure (2) indicate that the extract resulted to a steady decline of the MDA levels as well as a parallel rise of the GSH levels. The levels of MDA started from 3.83 ± 0.21 nmol/mg protein in the control group and fell to 3.32 ± 0.19 , 2.78 ± 0.18 and 2.23 ± 0.16 nmol/mg protein at concentrations of 50, 100 and 150 $\mu\text{g/mL}$ respectively. In contrast, the levels of GSH showed incremental rises starting from 8.66 ± 0.39 and reaching 9.29 ± 0.42 , 10.19 ± 0.46 , and 11.08 ± 0.48 $\mu\text{mol/g}$ protein respectively.

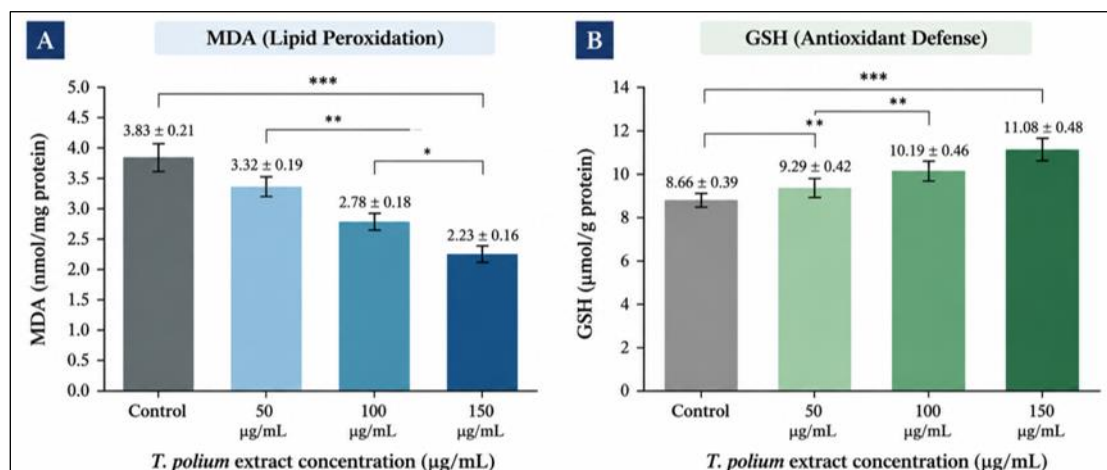


Figure 2: Effect of *Teucrium polium* extract on MDA and GSH levels in OSCC cells

3.4 Impact on Inflammatory Biomarkers

The results shown in Table (4) and Figure (3) demonstrated that using the alcoholic extract caused IL-6 and TNF- α levels to decreased progressively with increasing extract concentration relative to the control group. Examples include the reduction of IL-6 level from 48.7 ± 3.3 pg/mL recorded in the control group to 41.8 ± 2.9 , 32.6 ± 2.6 , and 24.9 ± 2.3 pg/mL when the concentrations of alcoholic extract were 50, 100, and 150 $\mu\text{g/mL}$, respectively. In a similar manner, TNF- α levels decreased dramatically: from 36.9 ± 2.8 pg/mL to 31.9 ± 2.3 , 25.8 ± 2.2 , and 19.9 ± 1.9 pg/mL.

Table 4: Effect of *T. polium* extract on inflammatory biomarkers

Group	IL-6 (pg/mL)	TNF- α (pg/mL)
Control	48.7 ± 3.3	36.9 ± 2.8
Extract 50 $\mu\text{g/mL}$	41.8 ± 2.9	31.9 ± 2.3
Extract 100 $\mu\text{g/mL}$	32.6 ± 2.6	25.8 ± 2.2
Extract 150 $\mu\text{g/mL}$	24.9 ± 2.3	19.9 ± 1.9

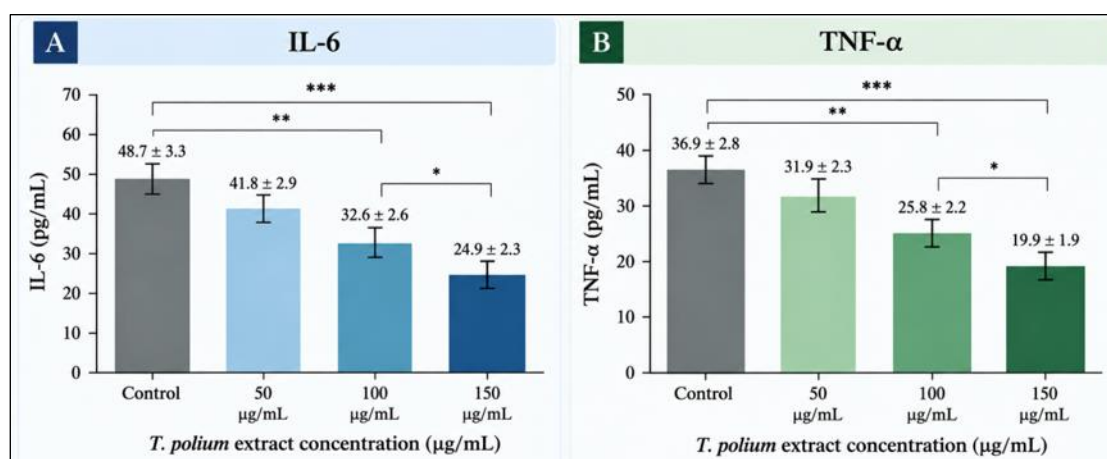


Figure 3: Effect of *Teucrium polium* extract on inflammatory biomarkers in OSCC cells

3.5 Antibiofilm Activity of *Teucrium polium* Extract

As is shown in Table 5, there is a gradual decline in the biofilm formation by *P. gingivalis* with increased doses of *T. polium* extract. The OD₅₇₀ of the control group was 1.00 ± 0.03 , while for the concentrations of 50 µg/mL, 100 µg/mL, and 150 µg/mL it was 0.79 ± 0.04 , 0.58 ± 0.04 , and 0.36 ± 0.03 accordingly. The corresponding percentage of inhibition of biofilm formation for these concentrations was 21.0%, 43.0%, and 64.0% consecutively. The extract has also shown increasing efficiency in eliminating pre-formed biofilms with its percentage of removal equaling 13.0%, 31.0%, and 51.0% for the same dosages.

Figure 4 also demonstrates that the relationship is indeed dose-dependent, as the effectiveness of the extract in biofilm suppression is better than in elimination of existing biofilms.

Table 5: Antibiofilm activity of *Teucrium polium* extract against *Porphyromonas gingivalis*

Treatment group	Biofilm formation (OD ₅₇₀)	Biofilm inhibition (%)	Preformed biofilm (OD ₅₇₀)	Biofilm eradication (%)
Control	1.00 ± 0.03	0.0	1.00 ± 0.04	0.0
Extract 50 µg/mL	0.79 ± 0.04	21.0	0.87 ± 0.05	13.0
Extract 100 µg/mL	0.58 ± 0.04	43.0	0.69 ± 0.04	31.0
Extract 150 µg/mL	0.36 ± 0.03	64.0	0.49 ± 0.04	51.0

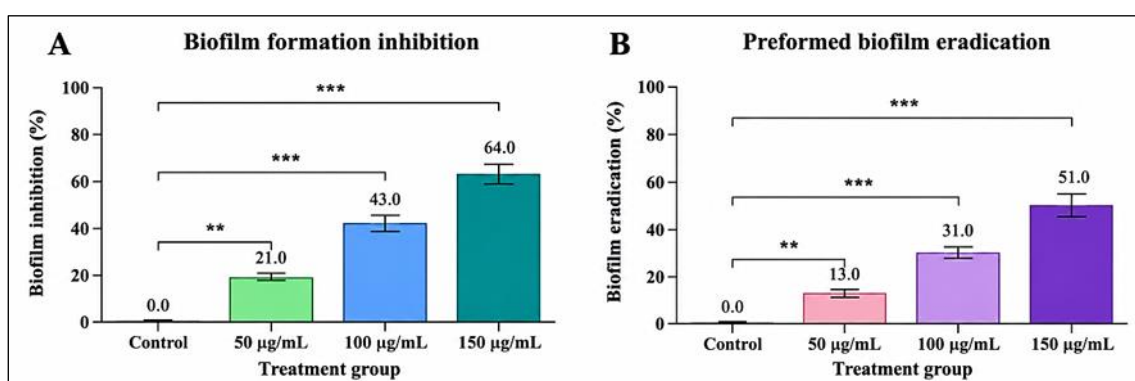


Figure 4: illustrates the antibiofilm properties of extract of *Teucrium polium* against *Porphyromonas gingivalis*. (A) Suppression of biofilm formation and (B) destruction of already formed biofilms. The data are presented in the form of mean $\pm$ SD (n = 3).

4 DISCUSSION

The extract derived from *T. polium* exhibited cytotoxicity against SCC-25 cells proportionate to the concentration of the extract with the viability being lowered to $24.7 \pm 2.6\%$ at a concentration of 300 µg/mL, while the IC₅₀ value was found to be 170.7 µg/mL. This observation corresponds with earlier reports of the cytotoxic effects of *T. polium* extracts on cancer cell lines and highlights the ability of the plant to act as a source of phytochemicals and bioactive compounds (Nematollahi-Mahani *et al.*, 2007; Elmasri *et al.*, 2016).

This extract reduced the levels of both ROS and MDA and raised those of GSH, indicating a reduction in the oxidative state. This observation is in accordance with the known antioxidant properties of *T. polium* attributable mostly to its phenolic and flavonoid composition (Sharififar *et al.*, 2009; Bahramikia *et al.*, 2022). Since DCFH-DA reads the overall oxidative activity inside the cell, these findings are to be viewed as redox modulation rather than any alterations in a particular species of ROS (Wang & Joseph, 1999).

The level of IL-6 and TNF- α was noted to decrease consistently as the concentration of the extract increased. This indicates that both cytokines play a role in the inflammatory signaling associated with OSCC and give credence to the

anti-inflammatory property of the extract observed in previous studies on preparations of *T. polium* (Brierly *et al.*, 2023).

Also, the extract stopped the formation of the biofilm of the genus *P. gingivalis* by 64%, and destructed 51% of the biofilm already formed, which reflects a better power of the extract to prevent biofilm formation than to destroy already developed biofilm. The given data support the data about the antimicrobial and antibiofilm activity of *T. polium* constituents reported in the literature (Elmasri *et al.*, 2016; Bahramikia *et al.*, 2022). On the whole, the data obtained suggest the necessity to conduct further studies of *T. polium* as the source of bioactive substances with multiple usage in dentistry.

5 CONCLUSION

In conclusion, the extract of *T. polium* has shown a complex action on OSCC cells, markers of oxidative stress, inflammatory markers, and biofilms indicating its potential as a plant product with complex biological activity.

The extract of *T. polium* showed significant dose-dependent toxicity on OSCC cells, and its action was accompanied by alterations in oxidative stress and inflammation indicators.

The extract was able to decrease the levels of ROS and MDA and increase the level of GSH with a progressive decrease in IL-6 and TNF- α . The extract also possessed anti-biofilm activity and was able to destroy already formed biofilms of *Porphyromonas gingivalis*.

STATEMENTS ON COMPLIANCE WITH ETHICAL STANDARDS

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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