RESEARCH ARTICLE

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In Vitro Assessment of the Cytotoxic Effect of 5-Fluorouracil, Thymoquinone and their Combination on Tongue Squamous Cell Carcinoma Cell Line

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Abstract

Background: Tongue cancer is the most prevalent type of oral cancer. Recently, natural compounds have been considered important resources for several anticancer drugs. Thymoquinone (TQ) exhibits a potent anti-cancer effect. 5-Fluorouracil (5-FU) is a chemotherapeutic drug that has been utilized in the treatment of cancer. Recently, combination therapy has gained popularity as a treatment option for patients with cancer. **Objectives:** The present study was carried out to assess the cytotoxic effect of 5-Fluorouracil (5-FU), Thymoquinone (TQ), and their combination on tongue squamous cell carcinoma cell line (HNO-97). **Methods:** Tongue carcinoma cell line (HNO-97) was maintained in cultured flasks and the cells were divided into four groups; group I: control untreated group, group II: HNO-97-treated cells with different concentrations of 5-FU from 0.5 µM/ml to 3µM/ml, group III: HNO-97-treated cells with different concentrations of TQ from 7.25µM/ml to 23.05µM/ml, and group IV: HNO-97-treated cells with both 5-FU and TQ in serial concentrations till (IC_{50}) in a dose of 27.44 µM/ml. Determination of the cytotoxic effect of the tested agents on the HNO-97 cell line was done using methyl thiazole tetrazolium assay, nuclear morphometric analysis, microscopic examination, and annexin-v/ propidium iodide staining assay. **Result:** The findings revealed that the cytotoxic effect of 5-FU, TQ, and their combination on tongue squamous cell carcinoma cell line (HNO-97) was dose-dependent. The microscopic examination revealed that 5-FU, TQ alone, or their combination induced apoptotic cell death. P-value < 0.05 was statistically significant. **Conclusion:** The combination of 5-FU and TQ produced a marked cytotoxic effect on HNO-97 cells.

Keywords: Human tongue squamous carcinoma- 5-Fluorouracil- Thymoquinone- *in vitro*- oral cancer

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Introduction

Oropharyngeal cancer is the 7th most prevalent cancer worldwide, while oral cancer is ranked 18th most prevalent cancer globally [1]. Oral squamous cell carcinoma (OSCC) is considered the most common malignant tumor in the head and neck region accounting for around 90% of all malignancies [2, 3]. The World Health Organization (WHO) reported in 2020 that oral cavity cancer, including tongue cancer, is prevalent in Egypt and is ranked 21st among all types of cancer with a mortality rate of 2.57% of all cancer deaths and 0.15% of total deaths [4]. Based on reports of the International Agency for Research on Cancer, tobacco smoking, and alcohol continue to be one of the highest risk factors [5, 4].

Chemotherapy is one of the most prevalent treatment methods for metastasizing oral cancers, where surgical excision will be insufficient alone as a treatment modality. However, drug toxicity and tumor cell resistance are considered the main obstacles to this choice [6].

The 5-Fluoro-2,4(1H,3H)-pyrimidinedione (5-FU) family, commercially known as Fluorouracil are antimetabolite drugs that work by inhibiting essential biosynthetic processes, or by being incorporated into macromolecules, such as DNA and RNA, resulting in inhibition of their normal function [7, 8]. It is a pyrimidine analogue that was first discovered in 1957 and remains a crucial chemotherapeutic drug for various solid tumors including OSCC [7, 9]. It is the most commonly used drug in treating oral, stomach, breast, and pancreatic cancer, and is considered a cornerstone of chemotherapy [7]. 5-Fluorouracil metabolites in cells cause cytotoxicity by inhibiting thymidylate synthetase enzyme, leading to deoxynucleotide imbalance and increased dUMP levels, causing DNA damage and apoptosis activation [7, 10].

5-Fluorouracil, an analogue of uracil with a fluorine

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atom at the C-5 position in place of hydrogen, rapidly enters the cell using the same transport mechanism as uracil. It is converted intracellularly to several active metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP). These active metabolites disrupt RNA synthesis and the action of thymidylate synthase (TS) [7, 9, 8].

Moreover, the toxic side effects that accompany therapeutic dosages of 5-FU have limited its systemic use in tumor therapy [6]. Therefore, recent treatment strategies use phytochemicals along with chemotherapy to reduce its side effects and cell toxicity [11]. On the other hand, Thymoquinone (2-Isopropyl-5-methyl-1, 4-benzoquinone) is a bioactive constituent of the volatile oil of black cumin (Nigella sativa L.) seeds that have been extensively utilized traditionally in the Middle East and Southeast Asian countries owing to its various health promoting-capacities [11].

Several studies have revealed the exploration of different molecular pathways for different cellular mechanisms for the therapeutic potentiality of TQ in the management of different types of metastatic tumors [12].

Multiple molecular targets are acted upon by TQ through different pathways, and also it shows its actions via many cellular mechanisms like proliferation inhibition, induction of apoptosis, cell cycle interruption, and prevention of angiogenesis and cellular metastasis [13].

Treatment with TQ led to the generation of reactive oxygen species (ROS), down-regulated Bcl-2, upregulated Bax, and activated caspase 3 and caspase 8, all of which induced apoptosis. It also exhibited apoptosis by down regulation of the proliferation of p38 mitogen-activated protein kinases MAPK pathway [14]. Thymoquinone (TQ) exhibits antiproliferative effects on various cancer cell lines of the ovary, larynx, lung, colon, breast, osteosarcoma, and myeloblastic leukemia. It induces cell cycle arrest which subsequently induces apoptosis and inhibits the invasion of metastasis via modulating the ecto-mesenchymal transition [11].

Thus, the current study aimed to assess the effect of 5-FU, TQ, and their combination on the tongue squamous cell carcinoma cell line.

Materials and Methods

The research ethics committee of the Faculty of Dentistry, Minia University approved the study protocol (approval number: 66/2022).

Cell line, culture and treatment protocol

The human tongue squamous carcinoma cell line (HNO-97) was obtained from the Cell Culture Department - Nawah Scientific lab- Cairo, Egypt. HNO-97 cells were obtained from Cytion in a frozen vial (reference number: 300129). The cell line was licensed to CLS.

Study design and treatment protocol

HNO-97 cells were divided into four different groups: group I (Control): Untreated HNO-97 cells, group II: HNO-97 cells treated with different concentrations (0.5 μM/ml to 3μM/ml) of 5-FU, group III: HNO-97 cells treated with various concentrations of TQ (7.25μM/ml to 23.05μM/ml), and group IV: HNO-97 cells treated with both 5-FU and TQ in serial concentrations till (IC_{50}) in a dose of 27.44 μM/ml (All cells were incubated for 24 h).

Drug Preparation

5-FU with a chemical formula of 5-Fluoro-2,4(1H,3H) -pyrimidinedione was dissolved in dimethyl sulphoxide (DMSO) (Sigma, USA) with the aid of an orbital shaker until obtaining a clear solution. TQ 98% (Sigma), obtained from the Cell Culture Department - Nawah Scientific, was dissolved in normal saline (0.9% NaCl). The combination solution was obtained from equal weights of 5-FU and TQ (50:50). The viability of HNO-97 cells treated with 5-FU, TQ, and their combination were determined 24 h post-treatment.

Methyl Thiazole Tetrazolium (MTT) Cytotoxicity Assay

For MTT assay, 1.2 – 1.8 × 103 HNO-97 cells were pre-cultured in 96 micro-titer plates (5×10^4 cells/mL) and treated with various concentrations of 5-FU, TQ, and their combination for 24 h. Then, 10 μL of MTT (0.5mg/ml stock) solution was added to each well and incubated at 37°C for 24 h. Next, the medium was gently aspirated and replaced by 100 μL/well of DMSO to dissolve the purple Formosan crystals. The absorbance was measured at 570 nm using a MR5000 Dynatech spectrophotometer (Dynatech Laboratories Inc., Chantilly, VA). All readings were taken in triplicate. The obtained data were analyzed using the Master Plex Reader Fit program to determine the pre-inhibitory concentration (IC) 50, IC_{50}, the half-maximal IC_{50}, and post IC_{50} of 5-FU, TQ, and their combination (IC_{50}) for 24 h.

Microscopic Examination

The steps of cell maintenance and subculture protocol were repeated, but the cells were dispensed in a 25 ml total volume to get a larger quantity of cells for cytological examination. Microscopic examination was done using a digital video camera (C5060, Olympus, Japan) attached to a light microscope (BX60, Olympus). The photomicrographs were assessed for the presence of morphological criteria of apoptosis.

Nuclear Morphometric Analysis

The photomicrographs were analyzed using Image J software ver 1.27z (NIH, USA). Following automatic correction of the brightness and contrast of images, they were converted into 8-bit grayscale type, and phase color coding of the area of interest was done automatically. The color threshold was adjusted to select the HNO-97 cell nuclei. For method standardization for all analyzed images, efforts were made to minimize the operator guided in favor of the automatic threshold throughout this step. The nuclei surface area and circularity were automatically measured, and the nuclear area factor (NAF) was calculated using the formula: NAF = Circularity × Object area.

Annexin-V Propidium Iodide Staining Assay

Apoptosis and necrotic cell populations were assessed by the Annexin V- FITC apoptosis detection kit (Abcam Inc., Cambridge, UK) coupled with 2 fluorescent channels flow cytometry. After treatment with the test compounds for the specified duration, cells (105 cells) were collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Next, 0.5 ml of Annexin V-FITC/PI solution was added to the collected cells and then incubated for 30 min in the dark at room temperature following the manufacturer's protocol.

The stained cells were injected via the ACEA Novocyte™ flow cytometer (ACEA Biosciences Inc., CA, USA) and analyzed for FITC (λex/em 488/530 nm) and PI (λex/em 535/617 nm) fluorescent signals using FL1 and FL2 signal detector, respectively. For each sample, 12,000 events were obtained and positive FITC and/or PI cells were measured by quadrant analysis and calculated using ACEA Novo Express™ software (ACEA Biosciences Inc.).

Statistical Analysis

Experimental data were tested for normal distribution by Shapiro-Wilk test then statistically analyzed by SPSS software version 16.0 using One-way Analysis of Variance (ANOVA) and Chi-square test to determine the significance of differences between groups. Data were expressed as mean ± standard deviation (SD). Statistically significant was considered when $P< 0.05$.

Results

Methyl Thiazol Tetrazolium Cytotoxicity Assay

The cytotoxic effect of 5-FU, TQ, and their combination on HNO-97 tongue carcinoma cells were assessed for 24 h. Collected data revealed that the cytotoxicity was dose-dependent.

The mean viability percentage of the treated cells decreased compared to control cells as the drug concentrations increased from 0.5 µM/ml to 3µM/ml in 5-FU and from 7.25µM/ml to 23.05µM/ml in TQ. The IC_{50} values were 2 µM/ml, 12.5 µM/ml, and 27.44 µM/ml for 5-FU-treated cells, TQ-treated cells, and the 5-FU/TQ combined treated cells, as shown in Tables (1, 2, and 3).

Microscopic Examination

Control cells

Microscopic findings revealed that control HNO-97 cells were almost rounded with minimal folding in both the cellular and nuclear membranes. Cells showed malignancy criteria such as hyperchromatism and nuclear pleomorphism (Figure 1a).

HNO-97 treated cells

HNO-97-treated cells showed modifications in the nuclear morphology that corresponded to the morphological parameters of apoptosis. These parameters are; nuclear and cellular membranes irregularities, peripheral condensation of chromatin against the nuclear membranes, membrane blebbing, and nuclear shrinkage fragmentation. Such criteria were clear in 5-FU-treated HNO-97 cells and TQ-treated HNO-97 cells, but they became more obvious in the combined treatment of both drugs. (Figure 1 b, c, d).

In addition to apoptotic criteria, some cells revealed nuclear alterations that resembled the morphological hallmarks of necrosis including nuclear and cellular swelling, cell membrane rupture, and increased eosinophilia of the cytoplasm. These criteria exhibited with the combined treated cells, and TQ-treated cells, and increased with the 5-FU-treated cells (Figure 1 f, g). In 5-FU-treated HNO-97 cells, TQ-treated HNO-97 cells, and the combined treatment of both drugs, the presence of secondary necrotic cells with both necrotic and apoptotic characteristics including cytoplasmic swelling and nuclear fragmentation (Figure 1 e, f, g).

Nuclear Morphometric Analysis Results

The NAF mean values indicated a decrease in the HNO-97 treated cells with different concentrations of TQ and the combined treatment of both drugs as compared to untreated cells (control) due to the decrease in the nuclear circularity and shrinkage of cells, while the NAF mean values of the 5-FU-treated group showed a gradual increase of the mean values compared to the control group due to swelling of cells indicating more necrosis than apoptosis Tables (4, 5).

There was a statistically significant difference between NAF in different groups ($P<0.001$, Effect size= 0.534).

Table 1. The Mean Viability Percentage of 5-Fluorouracil Treated HNO-97 Cells with low concentration (0.5µM/ml), medium concentration (1µM/ml), and high concentration (3µM/ml) for 24 h incubation.

Sample	HNO-97 cells treated with 5-FU for 24h incubation					
Conc. (µM)	0.5	1 Pre IC_{50}	1.5	2.08 IC_{50}	2.5	3 Post IC_{50}
Viability %	80.25%	74.52%	55.60%	50%	46.80%	25.27%

5-FU, 5-Fluorouracil, IC: inhibitory concentration.

Table 2. The Mean Viability Percentage of Thymoquinone Treated HNO-97 Cells with Low Concentration (7.25µM/ml), medium concentration (12.52µM/ml), and high concentration (23.05µM/ml) for 24 h incubation.

Sample	HNO-97 cells treated with TQ for 24h incubation					
Conc. (µM)	7.25 Pre IC_{50}	8.57	12.52 IC_{50}	15.78	19.23 Post IC_{50}	23.05
Viability %	75.25%	57.74%	50%	40.92%	25.63%	31.18%

TQ, thymoquinone; IC, inhibitory concentration.

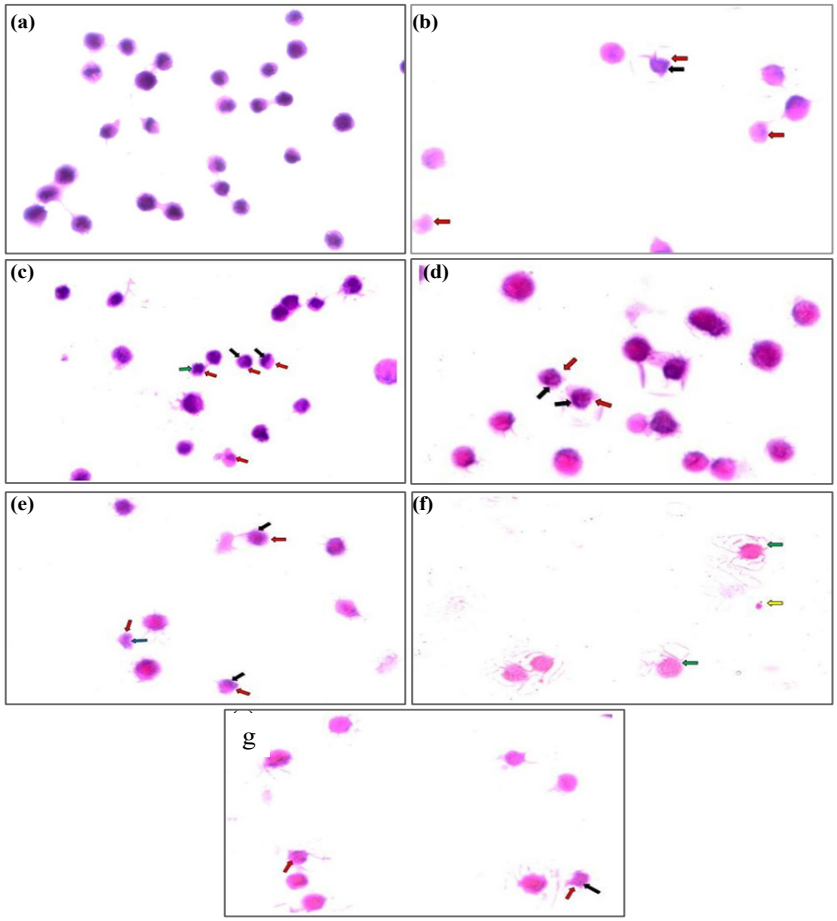

Figure 1. (A) A photomicrograph showing malignant cells with pleomorphism and nuclear hyperchromatism, (B) A photomicrograph showing shrunken cells (red arrows) and peripheral condensation of chromatin (black arrow), (C) A photomicrograph showing shrunken cells (red arrows), irregular cellular and nuclear membranes (green arrow) and peripheral condensation of chromatin (black arrows), (D) A photomicrograph showing shrunken cells (red arrows), and peripheral condensation of chromatin (black arrows), (E) Peripheral condensation of chromatin (black arrows) and membrane blebbing (blue arrow), (F) A photomicrograph showing swollen necrotic cells (green arrows) and apoptotic body (yellow arrow), (G) A photomicrograph showing shrunken apoptotic cells with shrunken nuclei (red arrow) and peripheral condensation of chromatin (black arrow). (HE stain, Original magnification 100X).

Table 3. The Mean Viability Percentage of the Combined Treatment of 5-fluorouracil and Thymoquinone Treated Cells with Low Concentration (13.23µM/ml), medium concentration (27.44µM/ml), and high concentration (45µM/ml) for 24 h incubation.

Sample	HNO-97 cells treated with 5-FU/TQ combination for 24h incubation					
Conc. (µM)	13.23 Pre IC50	27.44 IC50	30	35	45 Post IC50	50.16
Viability %	75.11%	50%	41.32%	34.65%	25.28%	20.26%

5-FU/TQ, combined treatment of 5-fluorouracil and thymoquinone

Pair-wise comparisons between groups revealed that 5-FU IC_{75} showed the statistically significantly highest mean NAF. Combination group treatment showed the statistically significantly lowest mean NAF.

Apoptosis assessment by Annexin V propidium iodide staining Assay

HNO-97 cells were treated with 5-FU and TQ in three different doses and their combination and then stained with APC-Annexin. (Figure 2) showed that 5-FU induced both apoptosis and necrosis of HNO-97 cells in a dose-dependent manner. Indeed, 5-FU caused a significant increase in the number of necrotic and apoptotic cells in comparison with the TQ-treated group.

The combination treatment showed the highest percentage of apoptotic cells in comparison with the 5-FU and TQ treatment alone, it reached approximately 65%, compared to the highest dose of 5-FU treatment which reached 30% in the highest dose. TQ treatment showed a percentage of apoptotic cells approximately 45% and 8% necrotic cells (Figure 2).

There was a statistically significant difference between groups (P=0.001, Effect size = 0.322). The statistically significantly highest prevalence of necrosis was observed with 5-FU (IC_{75}) followed by 5-FU (IC_{50}) while the lowest prevalence of necrosis was observed with the control group. The statistically significantly highest prevalence of late apoptosis was observed with the combination group

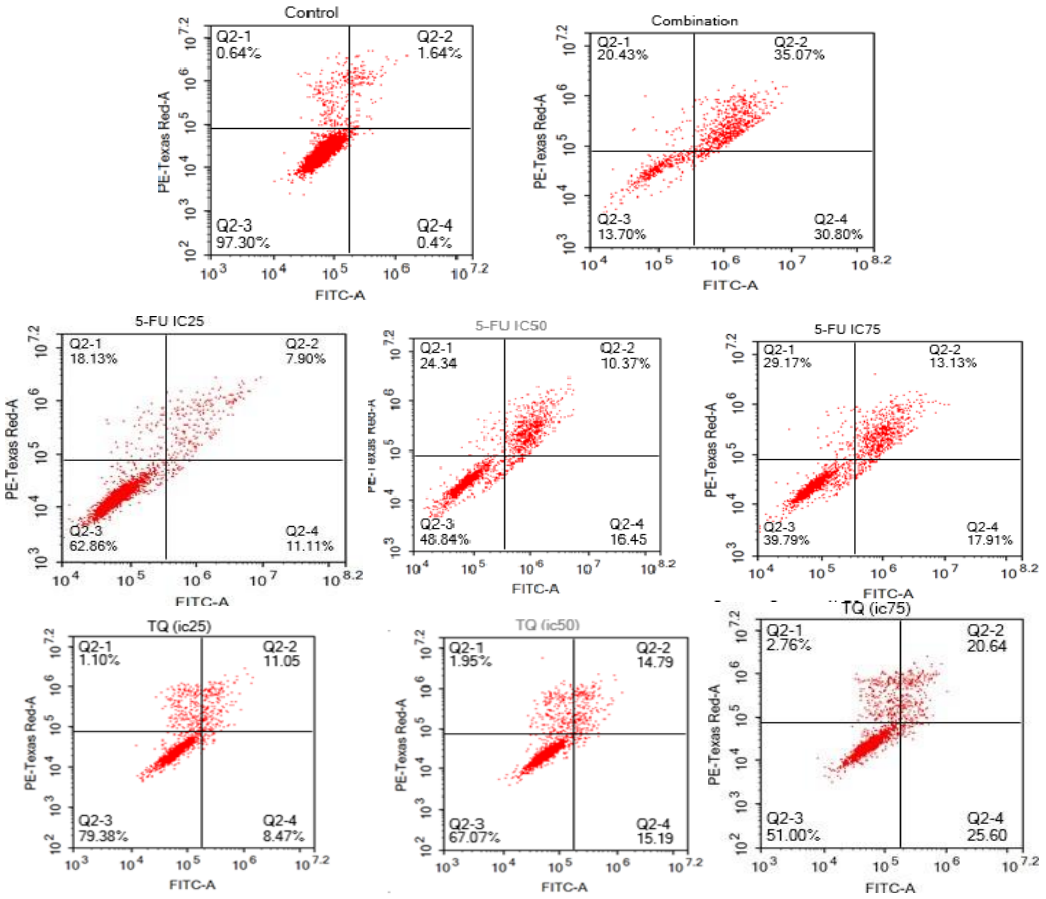

Figure 2. Histograms of Annexin V Apoptosis assessment of Control and All Treatment Groups

Table 4. Descriptive Statistics for Comparison between NAF in Different Groups

	NAF		P-value	Effect size (Eta squared)
Group	Mean	SD		
5-FU IC50	10020.4 B	1945.6	<0.001*	0.534
Control	9192.6 B	2927.3		
Combination	5901.7 D	1475.5		
TQ IC25	7080.4 C	2098.9		
TQ IC50	8361.7 B	800.1		
5-FU IC75	14119.7 A	4076.1		
TQ IC75	8595.7 B	2238.9		
5-FU IC25	9783.8 B	2029.3		

*, indicates P ≤ 0.05, Different superscripts indicate significant difference between groups

followed by TQ (IC_{75}) while the lowest prevalence of late apoptosis was observed with the control group. The statistically significantly highest prevalence of intact cells was observed with the control group followed by TQ (IC_{25}) while the lowest prevalence of intact cells was observed with the combination group. The statistically significantly highest prevalence of early apoptosis was observed with the combination group followed by TQ (IC_{75}) while the lowest prevalence of early apoptosis was observed with the control group.

Discussion

Oropharyngeal cancer holds the position of being the seventh most prevalent form of cancer globally, whereas oral cancer is ranked as the eighteenth most common cancer worldwide. OSCC, the preponderant malignant tumor affecting the head and neck region, which encompasses the oral cavity, constitutes approximately 90% of all diagnosed cases of malignancies [3]. The frequency

Table 5. Frequencies (n), Percentages (%) and Results of Chi-square Test for Comparison between Apoptosis in Different Groups

Groups	Control		5-FU (IC_{25})		5-FU (IC_{50})		5-FU (IC_{75})		Thymoquinone (IC_{25})		Thymoquinone (IC_{50})		Thymoquinone (IC_{75})		Combination	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Necrosis	32	0.6	906	18.1	1217	24.3	145	29.2	55	1.1	97	2	138	2.8	102	20.4
Late apoptosis	82	1.6	395	7.9	518	10.4	656	13.1	552	11.1	739	14.9	103	20.6	175	35.1
Intact cells	4865	97.3	3143	62.9	2442	48.8	198	39.8	396	79.5	335	67.8	255	5	685	13.7
Early apoptosis	20	0.4	55	11.1	822	16.4	895	17.9	418	8.4	759	15.3	128	25.6	154	30.8

, Significant at P ≤ 0.05; P-value <0.001; Effect size (v): 0.322

of OSCC has witnessed an upward trend in numerous nations, particularly among the younger demographic [11, 15]. Consequently, OSCC was the primary focus and chosen lesion in the present investigation.

Regarding the management of OSCC, conventional treatments have been linked to significant morbidity because of the engagement of crucial anatomical structures, adverse effects, and therapeutic resistance [3].

Consequently, there exists a pressing necessity for innovative alternative therapies for such a medical condition. Thankfully, multiple avenues of evidence have proposed the potential use of dietary constituents derived from fruits and vegetables, as they have exhibited noteworthy efficacy against oral cancer over the past few decades [16]. These bioactive dietary phytochemicals, which occur naturally, are not toxic and do not have significant adverse effects. As a result, they are considered as potential options for treating cancer. Taking into consideration the significant benefit of utilizing these substances in cancer treatment. Therefore, this study aims to explore a novel approach that is both natural and less invasive, which has gained popularity in recent years [17].

Nigella sativa (NS) has been described as the "miracle herb of the century" and is also known as black cumin, black seed [16]. The most pharmacologically active compounds isolated from NS are TQ, dithymoquinone, thymol, and thymohydroquinone [18]. TQ, the most efficacious constituent of NS, has been the subject of extensive study and has been determined to possess a broad spectrum of pharmacological attributes, encompassing antimicrobial (specifically antibacterial, antiviral, anthelmintic, and antifungal) properties; anti-inflammatory effects, analgesic properties, inhibition of histamine release, antihypertensive capabilities, hypoglycemic effects, anticarcinogenic potential, antioxidant activity, and hepatoprotective qualities [11].

Previous investigations have also brought to light the utilization of thymoquinone in conjunction with other pharmaceuticals, such as 5-FU and cisplatin, as a chemotherapy-enhancing agent or to attain a concomitant effect [18]. In this investigation, the MTT assay was employed, It was performed as it is one of the dependable techniques for evaluating cellular viability. Furthermore, it is renowned for its discerning detection of cellular proliferation, as it quantifies the rate of cellular growth through the utilization of a linear correlation between cellular activity and absorbance [19].

To assess the advantageous impact of TQ on OSCC, it held paramount importance to employ a benchmark chemotherapeutic medication to contrast its outcomes with those of thymoquinone and the amalgamation treatment cohorts. The selection of 5-FU was made due to its esteemed reputation as one of the most potent anti-neoplastic agents that effectively combated a broad range of carcinomas [18]. In the present study, the synergistic therapy involving 5-FU and TQ manifested the most potent cytotoxic impact, accompanied by a minimal proportion of viable and actively dividing cells.

This positive outcome was consistent with findings from previous studies demonstrating that 5-FU and TQ had a synergistic effect in the treatment of breast cancer cell line invitro, compared to each drug alone, in colorectal cancer cells, and in gastric cells, respectively, both in vitro and in vivo [20-22]. These findings were also consistent with the results of Ndreshkjana et al, who reported a synergistic cytotoxic effect of both 5-FU and TQ on resistant colorectal cancer cells in terms of apoptosis and reducing the angiogenic capacity of tumor cells [23].

This work revealed that TQ had an anticancer effect by increasing apoptosis in a dose-dependent manner. These findings are consistent with those of Shabani et al. [24], Xu et al. [25], Chu et al. [26], Salim et al. [27] and El-Mahdy et al. [28] who reported similar results in human cholangiocarcinomas, human squamous cell carcinoma, breast cancer MCF-7 cells, Murine leukemia cells invitro and in vivo and myeloblastic leukemia cells, respectively.

Similar to this, additional phytochemicals including Graviola extract and cucurbitacin E had a potent cytotoxic effect on OSCC and HNSCC cell lines that was concentration-dependent [29]. The current study employed a novel method based on morphometric analysis to calculate the NAF by measuring the nuclear surface area and nuclear circularity using image analysis software.

This approach can provide quantitative data on both necrosis and apoptosis in addition to the morphological changes of the nucleus [30]. Nuclear surface area and nuclear circularity, two added important apoptotic morphological criteria, are multiplied to determine NAF [31]. The results of the nuclear morphometric study showed that, in comparison to control cells, the mean values of nuclear surface area and nuclear circularity of the cells decreased following TQ treatment at all concentrations and the combined treatment as well.

The groups treated with thymoquinone at varying doses and the combined treatment showed a statistically significant difference in NAF mean values when compared to the control group, according to the NAF statistical data. These findings suggest that apoptosis can be directly predicted by the NAF calculation, particularly when it comes to nuclear alterations associated with early apoptosis, such as nuclear shrinkage and irregularity. When compared to the well-known chemotherapeutic agent 5-FU, the current study showed a high success rate, promising TQ activity against OSCC cells, and a clear synergism of the combination treatment of both drugs. TQ had an anticancer impact with increasing dose, however, 5-FU has proven a stronger anticancer effect. With decreased toxicity and adverse effects, this study clarifies the prospect of employing TQ as a supplemental treatment for traditional cytotoxic medicines.

In conclusion, thymoquinone had an anti-cancer effect on HNO-97 tongue carcinoma cells in a dose-dependent manner, using the combined treatment of both drugs induced a significant cytotoxic effect on HNO-97 cells

Author Contribution Statement

DE, SE, AE participated in study design. DE, SA, ME participated in experimental work and drafted the manuscript. DE, SA carried out the MTT assays. SE, AE, ME performed the statistical analysis. All authors read and approved the final manuscript.

Acknowledgements

Approval

The research is a part of a doctoral thesis that will be submitted to Faculty of Dentistry, Minia University. Publishing a paper is a prerequisite for thesis defense. It is approved by supervisors, Prof. Dr. Sherif Farouk El-Gayar, Amr Helmy El-Bolok, Sabreen Gamal Ammar & Marwa Mokbel ElShafei.

Ethical Declaration

The research ethical committee of the Faculty of Dentistry, Minia University approved the protocol of the study (approval number: 66/2022)

Data Availability

Data is available upon request.

Conflict of Interest

None.

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