



# In Vitro Study of the Potential Role of Olive Oil Oleuropein in Modulating the 5-FU Cytotoxic Efficacy against the Tongue Squamous Cell Carcinoma

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## Abstract

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**BACKGROUND:** 5-fluorouracil (5-FU) is an anti-cancer drug used to inhibit the proliferation of many different tumor cells. However, some side effects have been noted while using this drug. Oleuropein (OLEU), a natural compound, has been mentioned to have inhibitory effects on various cancers. Hence, its combination with 5-FU would allow its use in significantly lower doses.

**AIM:** The main objective of this study was to assess the cytotoxic effect of OLEU and the chemotherapeutic drug 5-FU on Tongue Squamous Cell Carcinoma Cell Line (HNO-97) and Human Normal Oral Epithelial Cell Line (OEC) by either using each drug separately or in combination.

**MATERIALS AND METHODS:** Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the half-maximal inhibitory concentration (IC50) was calculated. In addition, flowcytometry and an *in vitro* scratch assay were performed to analyze the cell cycle and assess the inhibitory effects of OLEU on the migration of cells, respectively.

**RESULTS:** The MTT assay demonstrated that using OLEU and/or has shown significant inhibitory effects on normal and cancer cell lines, with a favorable impact for OLEU on cancer cell lines rather than normal ones. Furthermore, a substantial increase in the cell inhibitory percentage was reported between the single and the combination treated groups as compared to the non-treated control group. Moreover, cell cycle analysis through flowcytometry showed that OLEU had induced cell cycle arrest at the G0/1 phase, decreased S phase, and G2/M phase either independently or in combination after 24 h and 48 h, when compared with a non-treated control group. Finally, a scratch assay test showed that OLEU could inhibit migration via delayed wound healing.

**CONCLUSIONS:** The present study findings suggest that OLEU can exert an anti-cancer effect on HNO-97 and has the potential to boost 5-FU cytotoxic effects and reduce its adverse effects. Ultimately, OLEU can inhibit cancer migration and metastasis from the initial tumor.

## Introduction

Tongue squamous cell carcinoma (TSCC) is regarded as an aggressive cancer that is frequently associated with a poor prognosis. In other words, 5-year survival rates have remained essentially unchanged over the past 20 years, despite the current advancements in its treatment [1].

The standard treatment modalities against cancer are a series of interventions and approaches that include surgery, radiotherapy, and chemotherapy, which continue to be the mainstay treatments. Some patients are ineligible for surgery either because of advanced local tumor growth, distant metastasis, or severe medical comorbidities. As a result, these patients are frequently advised to pursue chemotherapy as an

induction, adjuvant, neoadjuvant, or palliative treatment modality [2].

Chemotherapy delivers cytotoxic drugs systemically so that they can reach and kill the tumor cells. Nevertheless, most of these drugs can cause serious side effects as they are toxic to other viable cells in the human body. Therefore, these drugs need to be given cautiously at suboptimal levels [3].

The following chemotherapeutic drugs, namely, cisplatin, carboplatin, 5-fluorouracil (5-FU), paclitaxel, and docetaxel, are most commonly used against oral squamous cell carcinoma (OSCC). 5-FU is an anti-metabolite drug that works by inhibiting essential biosynthetic processes or being incorporated into macromolecules, such as DNA and RNA, and inhibiting their normal function. Nevertheless, as with most chemotherapeutic agents, 5-FU causes many side effects, such as neurotoxicity [4],

hand and foot syndrome [5], cardiotoxicity [4], and one of the most common causes of oral mucositis [6]. Therefore, this has led to the development of a series of trials aimed at studying how to encounter this issue.

Recent literature highlights the potential of phytochemicals as a source of therapeutics for certain forms of cancer [7]. In terms of modulating some of the signaling pathways involved in carcinogenesis, such as anti-proliferative, pro-apoptotic, and anti-angiogenic pathways with nominal cytotoxicity [8]. Olive oil, the main fatty component of the Mediterranean diet, consists of monounsaturated fatty acids as well as an elevated content of antioxidant agents. Moreover, olive oil has been reported to protect against the occurrence of many malignant tumors such as breast [9], ovarian [10], colon [11], oral cavity, and pharynx cancers [12] and has also been reported to have anticancer activity against prostate [13] and liver [14] cancers.

Secoiridoids are the most concentrated phenolic compounds in olive oil, representing 70–90% of the total phenolic compounds [15]. Oleuropein (OLEU), the main phenolic secoiridoid from the olive tree, has several benefits for human health. The previous studies have reported that OLEU possesses antitumor function in various cancer cell lines, including melanoma B16 cells and glioma cells [16], [17].

Among the works of literature, studies reporting the cytotoxic effect of olive oil OLEU on the TSCC are scarce. As a result, the current study sought to assess the cytotoxic effect of olive oil oleuropein on TSCC. Meanwhile, it is hypothesized that the cytotoxicity of 5-FU against TSCC might be modulated when combined with olive oil oleuropein.

## Materials and Methods

### Chemicals

Oleuropein ≥98.0% was purchased from (Sigma-Aldrich-Germany) and dissolved according to the manufacturer's instructions). 5-FU was purchased from S.X. Haipu Pharmaceutical Co., Ltd. as ampoules (500 mg/10 ml), where they were stored at room temperature (25°C). MTT (1–3 (4,

5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich, Germany, and prepared as a 5 mg/ml Phosphate Buffered Saline (PBS) solution and used for the evaluation of cytotoxicity. Trypan blue dye was obtained from Sigma-Aldrich, USA. The dye was used to stain dead cells during the assessment of cytotoxicity. Trypsin/EDTA was purchased from Sigma-Aldrich, USA. It was used at a concentration of 0.25% (w/v) to detach cells. The Cell Cycle (Propidium Iodide) FlowCytometry Kit was purchased from Abcam Waltham, USA, and used for flowcytometric detection of apoptosis.

### Cell lines

The Human Tongue Carcinoma Cell Line (HNO-97) and Human Oral Epithelial Cell Line (OEC) were purchased from Nawah-Scientific Research Center (Almokattam Mall, Cairo, Egypt). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza Group Ltd., Switzerland), which was supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin-Amphotericin B. Cells were incubated at 37°C for 24 h in a 5% carbon dioxide incubator. The experiments were performed at two independent times (24 h and 48 h intervals).

### Cell viability and MTT assay

Cells were seeded in a sterile 96-well cell culture plate at a cell density of  $5 \times 10^3$ – $10 \times 10^3$  cells per well. After 24 h and 48 h of seeding to reach confluence (70–80%), different drug concentrations of 5-fluorouracil (5-FU) and oleuropein (40–300 µg/ml) were applied to different cell lines (OEC and HNO-97) and then incubated in the CO<sub>2</sub> incubator for (24 h and 48 h). Using a negative control of medium only without cells as a blank was considered. The medium was removed and replaced with a freshly prepared culture medium without phenol red. 10 µl of the 12 mM MTT stock solution were prepared as described above for each well, including a negative control of 10 µl of the MTT stock solution added to 100 µl of the medium. The cells were then incubated at 37°C for 2–4 h to allow the cells to be labeled with MTT. After incubation, 85 µl was removed and 50 µl of DMSO was added to each well and mixed by the pipette. Cells were incubated again at 37°C in a CO<sub>2</sub> incubator

**Table 1: Growth inhibition percentage of different concentrations of 5-FU and OLEU on OEC cells and HNO-97 cells for 24 h and 48 h**

Growth inhibition % (Mean ± SD)								
Cell line	OEC				HNO-97			
Utilized agent	5-FU		OLEU		5-FU		OLEU	
Duration	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
Dose								
40 µg/ml	12 ± 1.3	29 ± 5.8	17 ± 6.4	21 ± 6	21.4 ± 7.4	36.3 ± 5.7	22 ± 3.4	27 ± 6
80 µg/ml	17.6 ± 5.6	31.6 ± 7.4	14.7 ± 9	23 ± 7.3	25 ± 8	4.5 ± 5.6	24 ± 4.8	31.2 ± 3.4
120 µg/ml	22 ± 9.5	37 ± 6.4	18.6 ± 5.5	25.5 ± 8	27 ± 6.6	44.5 ± 4.5	26 ± 3	36.1 ± 5.4
160 µg/ml	33 ± 13.6	41 ± 1.7	27.8 ± 7	29.2 ± 7.3	31.3 ± 9	50 ± 5.5	32 ± 2	40.3 ± 5.7
200 µg/ml	54 ± 11.3	55 ± 2	31.5 ± 4	36 ± 7.6	50 ± 6	54.2 ± 5.2	38 ± 4.7	44.6 ± 4.5
250 µg/ml	57.3 ± 16.5	61 ± 3.7	44.8 ± 6.7	46 ± 6.6	56.4 ± 2.2	62.2 ± 2.2	44 ± 3	57.7 ± 4.5
300 µg/ml	63 ± 9.1	74 ± 7.7	48 ± 4.5	49.3 ± 4	63 ± 9.5	73.5 ± 4	53.7 ± 2.3	66.6 ± 2

SD: Standard deviation.

for 10 min. All samples were well mixed again and absorbance was gained at 540 nm (BioTek ELx-USA). The IC<sub>50</sub> (the half maximal inhibitory concentration) values were calculated using GraphPad Prism software (version 6.0).

### Flowcytometry assay (cell cycle analysis)

After trypsinization, HNO-97 and OEC cells were centrifuged at 500×g for 5 min, washed twice, resuspended in 1 ml of 1X PBS, fixed by absolute ice-cold ethanol, and then incubated at 20°C for 24 h. After twice PBS washes, cells were re-suspended in propidium iodide (PI) solution containing 200 µl PI, 100 µl RNase A, and 5 ml PBS, incubated in darkness for 20–30 min at room temperature, and then run on a flowcytometer (Accuri C6 plus FlowCytometer) (Becton Dickinson, Sunnyvale, CA, USA).

Half maximal inhibitory concentration (IC<sub>50</sub>) for cytotoxic agents Concentrations of 5-FU and OLEU that inhibit 50% of cells (IC<sub>50</sub>) was calculated by the use of XLfit5 software (IDBS) and expressed in µg/mL at 95% confidence intervals.

### In vitro scratch assay

For both 2 cell lines, they were divided into four groups: control group (without treatment). Oleuropein IC<sub>50</sub> treated group, 5-FU IC<sub>50</sub> treated group, and combined (OLEU IC<sub>50</sub>+5-FU IC<sub>50</sub>) treated group.

Cells were seeded into a 6-well tissue culture plate at a density such that after 24 h growth, they reached 70–80% confluence. Once at the confluence (usually after 18–24 h), the cell layer was scrapped in a straight line using a 1 mm pipette tip. The tip was kept perpendicular to the bottom of the well. Another line was scratched perpendicular to the first line to create a cross in each well. After the scratch, the cell monolayer was gently washed to remove the detached cells, and then they were replenished with fresh medium. Images at 4× and 10× magnification were taken with an inverted microscope. 6-well plate which was then placed in the incubator and imaged on an inverted microscope after 24 h and 48 h, respectively, until cells migrate to meet in the middle toward the opening to close the scratch [18].

### Statistical analysis

The statistical analysis of data was done using the SPSS version 22 (Statistical Package for the Social Sciences) program (SPSS, Inc., Chicago, IL). All experimental values were presented as mean ± SD. The data were analyzed using ANOVA, and then a paired t-test was used to analyze the difference between the groups.  $p < 0.05$  were considered statistically significant.

## Results

### Effect of OLEU and 5-FU on the viability of cell lines

The MTT assay displayed dose-dependent and significant cytotoxic activity for OLEU and 5-FU on OEC and HNO-97 when used independently. In addition, OLEU has a significant and selective inhibitory effect on HNO-97 cells rather than on OEC cells (Table 1). Furthermore, combinations of (IC<sub>25</sub>, IC<sub>50</sub>, and IC<sub>90</sub>) concentrations for both drugs (5-FU and OLEU) revealed significant inhibitory percentages on OEC and HNO-97 lines after 24 h and 48 h. Meanwhile, the comparison of the doses of the combination on both OEC Cells and HNO-97 Cells has shown a higher significant inhibitory effect for the IC<sub>25</sub> combination for HNO-97 Cells than OEC Cells for 24 h and also a higher significant inhibitory effect for combinations of IC<sub>25</sub> and IC<sub>90</sub> for HNO-97 Cells for 48 h (Table 2).

**Table 2: Growth inhibition percentage of different combination concentrations on OEC and HNO-97 cells for 24 h and 48 h**

Growth inhibition % (Mean ± SD)				
Cell line	OEC		HNO-97	
	24 h	48 h	24 h	48 h
Combined doses				
IC <sub>25</sub>	20.44 ± 5.6	26.7 ± 0.51	30.21 ± 7.28	42.45 ± 6.8
IC <sub>50</sub>	53 ± 9.98	58.47 ± 2.05	56.28 ± 4.80	64.13 ± 8.34
IC <sub>90</sub>	61 ± 3.12	64 ± 5.7	66.03 ± 9.5	74.38 ± 10.3

SD: Standard deviation.

### Effect of OLEU and 5-FU on cell cycle

The effects of the two drugs (5-FU or OLEU) on the cell cycle phase's distribution of both cell lines (OEC and HNO-97) after 24 h and 48 h are listed in Tables 3 and 4. The utilized cytotoxic drugs either applied singly or in combination lead to cell cycle inhibition. They reduced cells in the S phase and G2/M phase and increased cells in the G0/1 phase during the two periods of the study (24 h, 48 h) (Figures 1 and 2).

Statistically, significant differences were found between the control group and treated groups at all phases of the cell cycle for the two periods of the study (Tables 3 and 4). Nevertheless, no significant difference was found between control and treated cell lines for 24 h and 48 h.

### The qualitative effectiveness of OLEU IC<sub>50</sub>, 5-FU IC<sub>50</sub>, and drugs combination on cell migration assay

While 5-FU (IC<sub>50</sub>) has nearly the same inhibitory effect on either OEC or HNO-97 cells, preventing them from healing the wound, an uplifted effect was found from the combined drug (OLEU IC<sub>50</sub> and 5-FU IC<sub>50</sub>) on both cell lines. On the other hand, OLEU had a more inhibitory effect on HNO-97 cells than on OEC cells (Figures 3 and 4).

**Table 3: Cell cycle phases distribution on OEC cells after on drugs treatment after 24 h and 48 h**

Time	OEC Cells	OLEU		p-value	5-FU		p-value	Combined		p-value
		Control	Treated		Control	Treated		Control	Treated	
24 h	G0/1 Phase	45 ± 3.4	71 ± 4.1	0.006**	45 ± 3.4	80.2 ± 2.6	0.001**	45 ± 3.4	88.2 ± 2.5	0.001**
	S Phase	42.4 ± 3	13 ± 0.8	0.003**	42.4 ± 3	1.8 ± 0.4	0.002**	42.4 ± 3	1.9 ± 0.55	0.002**
	G2/M Phase	4.2 ± 1.3	1.0 ± 0.3	0.01**	4.2 ± 1.3	0.2 ± 0.12	0.033*	4.2 ± 1.3	0.3 ± 0.14	0.04*
48 h	G0/1 Phase	42.2 ± 2.2	85 ± 3.3	0.005**	42.2 ± 2.2	80.8 ± 3	0.006**	42.2 ± 2.2	80.3 ± 2.2	0.004**
	S Phase	29 ± 1.3	4.0 ± 1.2	0.001**	29.0 ± 1.3	0.6 ± 0.17	0.001**	29.0 ± 1.3	0.5 ± 0.1	0.001**
	G2/M Phase	7 ± 0.9	1 ± 0.24	0.004**	7.1 ± 0.9	0	0.005**	7.1 ± 0.9	0	0.005**

\*p &lt; 0.05, \*\*p &lt; 0.01.

**Table 4: Cell cycle phases distribution on HNO-97 cells after drugs treatment after 24 h and 48 h**

Time	HNO-97 Cells	OLEU		p-value	5-FU		p-value	Combined		p-value
		Control	Treated		Control	Treated		Control	Treated	
24 h	G0/1 Phase	48.3 ± 1.7	72.2 ± 3	0.009**	48.3 ± 1.7	76 ± 1.4	0.0001***	48.3 ± 1.7	90.4 ± 2.4	0.0001***
	S Phase	40.3 ± 1.7	10.4 ± 0.9	0.0001***	40.3 ± 1.7	6.4 ± 1.2	0.001**	40.3 ± 1.7	1.1 ± 0.26	0.001**
	G2/M Phase	8.0 ± 0.8	1.8 ± 0.36	0.002**	8.0 ± 0.8	0.6 ± 0.14	0.005**	8.0 ± 0.8	0.2 ± 0.06	0.003**
48 h	G0/1 Phase	32 ± 2.1	75.1 ± 2.2	0.0001***	32 ± 2.1	88.3 ± 1.2	0.0001***	32 ± 2.1	80.7 ± 1.2	0.0001***
	S Phase	53 ± 0.9	6.5 ± 1	0.001**	53 ± 0.9	1.9 ± 0.6	0.0001***	53 ± 0.9	0.6 ± 0.2	0.0001***
	G2/M Phase	6.3 ± 1.4	0.8 ± 0.11	0.021*	6.3 ± 1.4	0.2 ± 0.07	0.017*	6.3 ± 1.4	0.1 ± 0.02	0.016*

\*p &lt; 0.05, \*\*p &lt; 0.01.

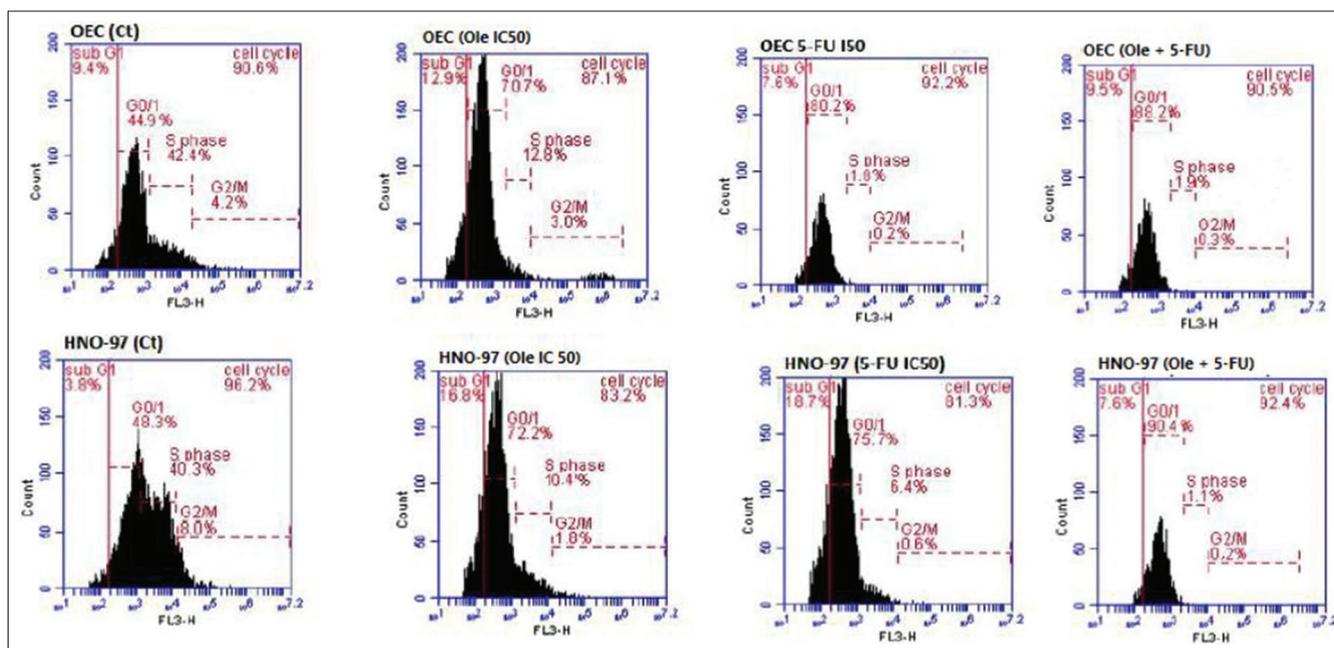


Figure 1: Effects of 5-FU, OLEU, and their combinations on the cell cycle progression of different cells after 24h. A representative histogram shows cell cycle distribution phases in normal cells (OEC) and cancer one (HNO-97)

## Discussion

Several epidemiological studies have found a link between olive oil consumption and a decreased risk of cancer progression and mortality [19]. The major phenolic component in olive oil is OLEU, where there is considerable evidence that it has anti-cancer properties in various forms of cancer [20]. Therefore, OLEU is an attractive compound as a therapeutic molecule due to its vast array of benefits, such as low toxicity in normal cells and a wide spectrum of activities in cancer-related pathological pathways [21]. In the present study, the anti-migratory and antiproliferative effects of OLEU together with its modulatory effect on 5-FU cytotoxicity on OEC and HNO-97 cell lines were investigated.

The cell cytotoxicity assay (MTT assay) demonstrated that OLEU and 5-FU alone or in combination have produced a significant inhibitory

effect on both OEC and HNO-97 cell lines, with a favorable impact for OLEU on cancer cell lines rather than the normal one, in which there was a significant increase in the cell inhibitory percentage between the single and the combination treated groups as compared to the non-treated control group. This was in agreement with many trials that found the inhibitory effects of the OLEU on breast cancer [22], colorectal cancer [23], as well as blood cell cancer [24]. The inhibitory effects of OLEU could be explained by Hassan *et al.* 2012 and Fernández-Arroyo *et al.* 2012, who reported that OLEU could inhibit the proliferation and motility of cells [25] and induce cell cycle arrest [26].

In the present study, it was found that treatment of both OEC cells and HNO-97 cells with 5-FU combined with OLEU displayed a synergistic effect in inhibiting their proliferation. Moreover, it induced a more selective effect on HNO-97 cells than on OEC cells, suggesting that administration of oleuropein

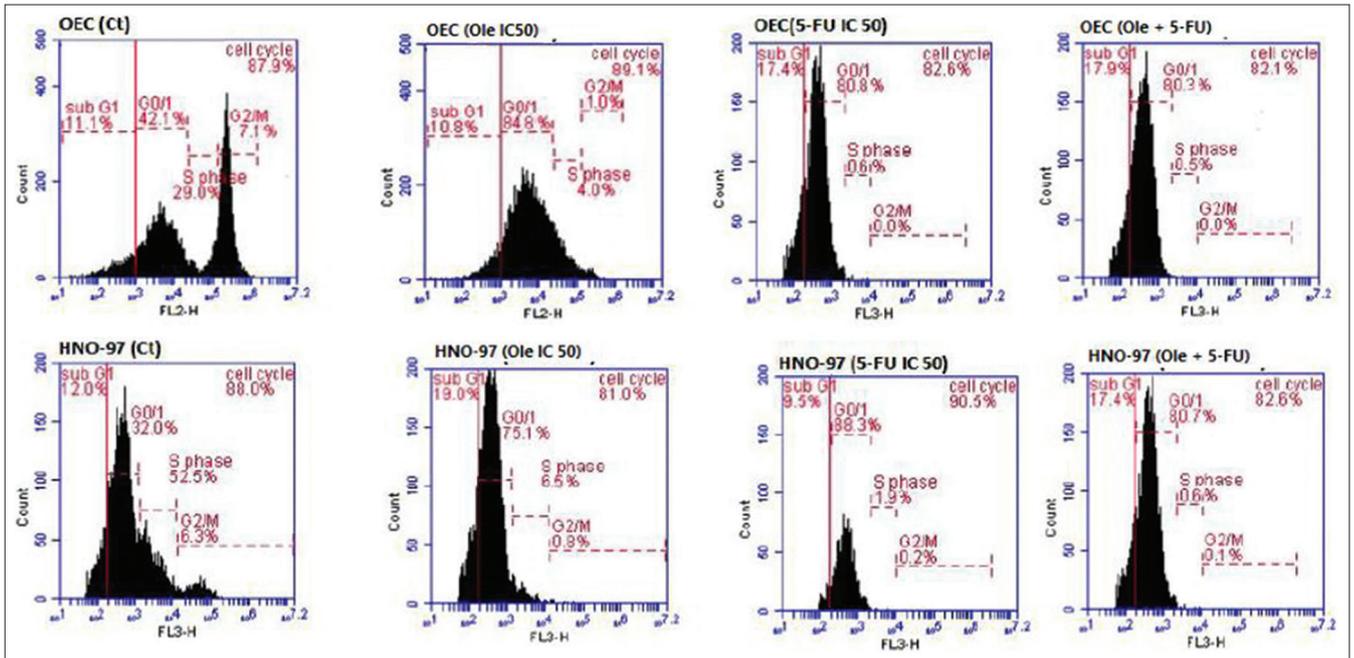


Figure 2: Effects of 5-FU, OLEU, and their combinations on the cell cycle progression of different cells after 48 h. A representative histogram shows cell cycle distribution phases in normal cells (OEC) and cancer one (HNO-97)

together with the standard chemotherapeutic drug may decrease its cytotoxic side effects. This finding was

in agreement with Sheriff *et al.*, 2018, and Hashemi Sheikhsabani *et al.*, 2021, who found the synergistic

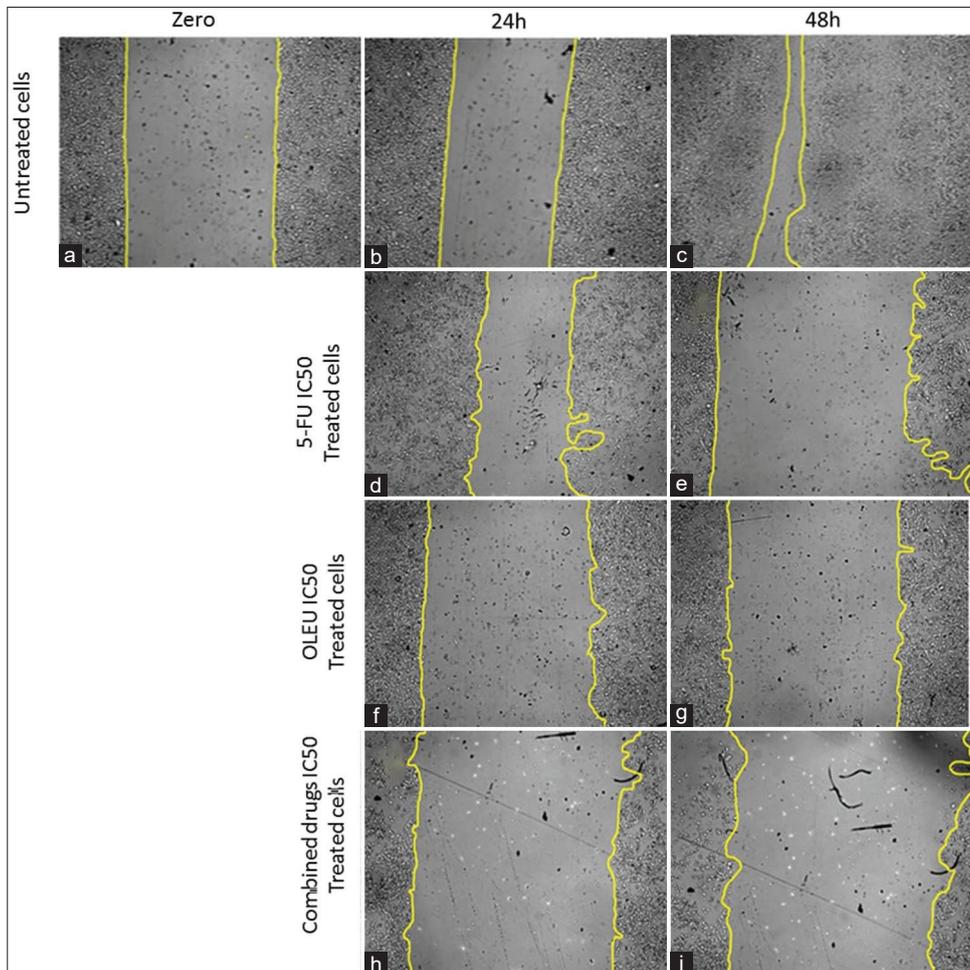


Figure 3: Qualitative analysis of cell migration assay on OEC cells

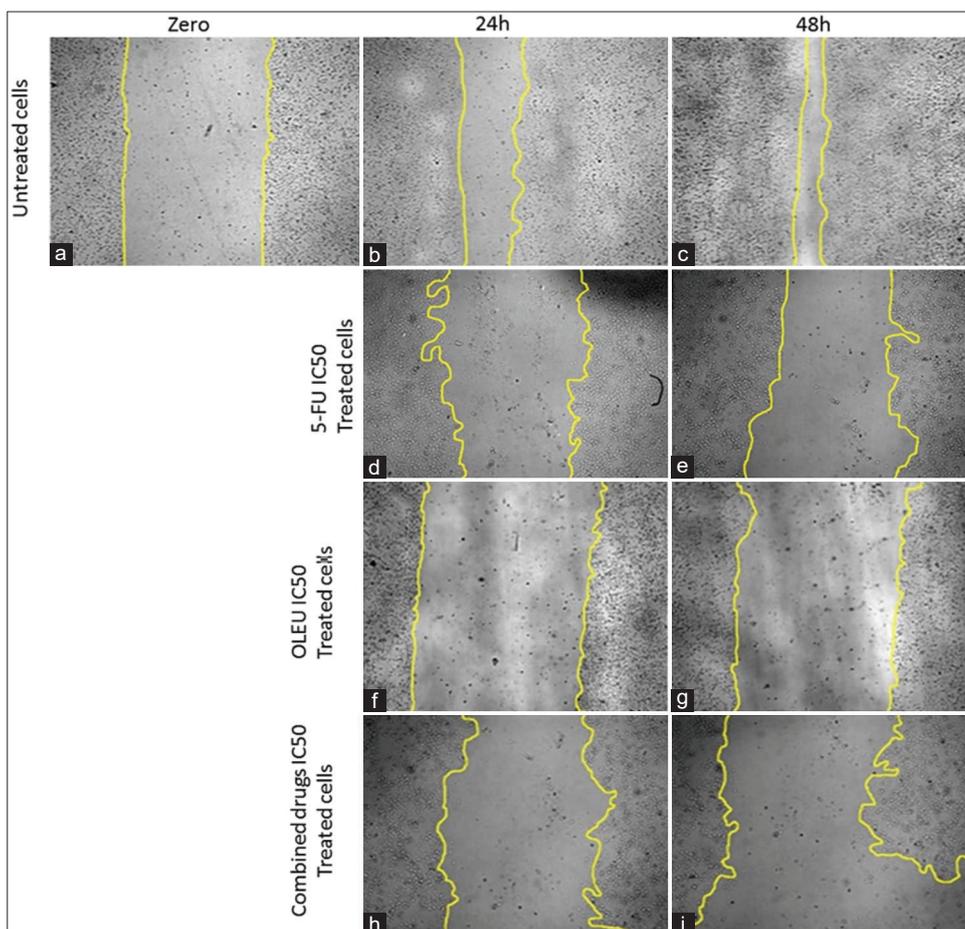


Figure 4: Qualitative analysis of cell migration assay on HNO-97 cells

effect of oleuropein when applied with cisplatin against cancer cell lines of the liver [27] and ovaries [28].

It has also been shown that OLEU induced cell cycle arrest at the G0/1 phase, decreased S phase, and G2/M phase either independently or in combination after 24 h and 48 h. This finding is consistent with Messeha *et al.* 2020, who demonstrated that OLEU induces cell cycle arrest by increasing the G0/1 phase and inhibiting the S phase in breast cancer cells [29]. This finding could be explained by Notarnicola *et al.* (2011), who revealed that OLEU could induce cancer cell cycle arrest, leading to a change in the population of cells at different stages. Moreover, they revealed that the cell cycle is halted during DNA damage to allow time for damaged DNA to be repaired. When substantial DNA damage occurs, the apoptotic process is activated, and cells die [28].

The wound healing technique (scratch assay) is a well-established *in vitro* tool for exploring two-dimensional collective cell migration [31]. Progression of cancer occurs when tumor cells expand from the initial tumor, infiltrate across the basement membranes and endothelial walls, and populate distant tissues, such as bone marrow and lymph nodes [32]. Thorough knowledge of this process is essential in the battle against cancer because of the importance of cell migration, invasion, and adhesion. Furthermore, the spread of cancer cells to other parts of the body and

their subsequent elimination is a major factor in cancer-related morbidity and mortality [33].

A scratch assay was performed to detect OLEU and 5-FU anti-migratory effects independently or in combination on OEC and HNO-97 cells. The assay results showed significantly decreased cell migration ability, which comes in agreement with these results following Choupani *et al.* 2019 who applied OLEU and doxorubicin to the breast cancer cell line [34].

Concerning the independently anti-migratory effect of OLEU, in the current study, it was found that OLEU had more effect on HNO-97 cells. This was in agreement with Przychodzen *et al.* 2019, who clarified the anti-proliferative and anti-migratory potential of OLEU with 2-methoxyestradiol separately or in combination on osteosarcoma cell lines [35].

## Conclusions

Oleuropein (OLEU) has a synergistic anticancer effect with 5-FU on HNO-97 cell lines and can reduce 5-FU-induced side effects by reducing its effective dose. Furthermore, unlike the chemotherapeutic effect of

5-FU, which has a non-selective effect on either cancer or normal cells, OLEU has a significant and selective inhibitory effect on HNO-97 cells for cell viability, its effect on the cell cycle distribution phases for cancer cells, and its ability to reduce cell migration and invasion. Finally, OLEU could inhibit cancer progression and expansion from the initial tumor and prevent OEC and HNO-97 cells from healing wounds effectively. Therefore, OLEU could reduce the ability of cells to migrate or move, and hence, it inhibits infiltration across basement membranes and endothelial walls and populates distant tissues such as bone marrow and lymph nodes.

## Declarations

### *Ethics approval and consent to participate*

Not applicable: This article does not include any human or animal studies with human or animal subjects, simply Human Tongue Carcinoma Cell Line (HNO-97) and Human Oral Epithelial Cell Line (OEC) were purchased from Nawah-Scientific Research Center (Almokattam Mall, Cairo, Egypt).

### *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' Contributions

Resources, data analysis, and writing original draft: Mouna A. Abdullah; review and editing: Mohamed I. Mourad; Supervision: Mahmoud F. Elsherbeny, Azza Abbas I. Elsisssi.

All authors read and approved the final manuscript.

## Disclosure Statement

No potential conflict of interest was reported by the authors.

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