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MiR-155 Regulation via the Synergistic Effect of *Tamarix aphylla* and *Artemisia herba alba* Extracts with 5-Flurouracil (5-FU) in Head and Neck Cancer Cell Line (HNO-97)

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Abstract

THE chemotherapeutic drug 5-Fluorouracil (5-FU) is commonly used to treat head and neck squamous cell carcinoma (HNSCC). Nonetheless, the difficulties and toxicity associated with this medicine have considerably impacted its clinical use. As a result, investigating an alternate therapeutic method that decreases 5-FU toxicity while having a synergistic effect on head and neck squamous cell carcinoma (HNSCC) is a viable option for action. *Tamarix aphylla* (D1) and *artemisia herba* alba (D2), both naturally occurring products, have been shown to suppress the development of several cancer cells, including HNSCC. This study aims to look at the combined effect of D1 and D2 and 5-FU on head and neck squamous cell carcinoma (HNSCC) and determine the apoptotic activity of this combination. The viability of cells treated with monotherapy and combination therapy was assessed using the MTT test. The researchers used flow cytometry to investigate the impact of apoptosis induction and cell cycle arrest on cells. In addition, the current study used real-time PCR to examine the expression of miR-155 and a set of specified genes. We have shown that the natural compounds D1 and D2 boost miR-155. So, our study shows that D1 and D2 may have an anti-tumorigenic effect on HNO-97 cells via a novel method. Furthermore, this study sheds light on the cellular routes through which natural compounds exert their effects.

Keywords: Synergistic Effect, Neck and Head Cancer, MiR-155, HNO-97.

Introduction

Head and Neck squamous cell carcinoma (HNSCC) is a cancer that poses a considerable risk to the patient's life and has a high mortality rate [1]. Head and neck cancer (HNC) is the sixth most common disease worldwide, with an annual incidence of about 660,000 cases and a fatality rate of 325,0002. Squamous cell carcinoma accounts for approximately 90% of all Head and Neck Cancers (HNCs). These carcinomas arise from the epithelial linings of the oral cavity, pharynx, and larynx [2].

Substantial studies have been undertaken on the potential anticancer activities of natural compounds with a broad spectrum of chemical variety for over fifty years. The community's collective efforts have yielded significant results, easing the incorporation of natural products into clinical practice, and exposing unique therapeutic opportunities. However, there are still challenges ahead. The landscape of cancer therapy has shifted dramatically, with an increased dependence on modern technology [3].

MicroRNAs (miRNAs) are a non-coding RNA that is around 22 nucleotides long and regulates gene expression at the post-transcriptional stage. According to previous studies, they can influence up to 60% of mRNA by participating in biological processes such as the cell cycle, apoptosis,

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proliferation, and stress response [4]. The pathogenic changes in the processes are visible at all stages of neoplastic growth. MicroRNA analysis is carried out on each plane using the data provided. The human genome contains around 2,000 microRNAs; however, not all have been described so far [5]. Current research focuses on examining the role of microRNA in facilitating the detection of surgical margins during surgical interventions and creating diagnostic markers that may aid in this process [6].

HNSCC provides an excellent chance to reap the benefits of using miRNAs (miRNAs) as novel diagnostic tools. The high heterogeneity of HNSCC and the need for quick and accurate diagnosis have stimulated substantial investigation and evaluation of miRNA's potential utility in diagnosing cancer, predicting treatment outcomes, and assessing treatment efficacy. The first point of significance is identifying miRNAs' roles as oncogenes and suppressor genes in HNSCC [7].

The aim of this study is to study the effect of *Tamarix aphylla* and *Artemisia herba alba* Extracts with 5-Flurouracil (5-FU) in Head and Neck Cancer Cell Line (HNO-97)

Material and Methods

Plant Selection and Collection

Tamarix aphylla (D1) and *artemisia herba alba* (D2) samples were taken from Jeddah, Saudi Arabia, in October 2021. The research used Shabestani et al., [8] processes and instructions to collect and prepare plant materials, which will be discussed further in the following example. The complete plant specimens were collected, desiccated under standard environmental circumstances, and stored in a dehydrated place before use. The plant material was thoroughly washed with water before being air-dried under dark ambient circumstances. The dried plant material was then mechanically ground using an electric grinder, producing a coarse powder. Powders weighing 50 g were extracted with solvents of different polarities, including methanol and hot water. The powders were subjected to a solvent-soaking method at ambient temperature for 48 hours, using 150 milliliters of solvent. The plant extracts were obtained and filtered through the Whatman No.1 filter paper. The leftovers were submerged in a 150-milliliter dichloromethane solution for 24 hours before being filtered again. Samples were dehydrated and stored in glass containers at -20ºC. Before the experiment, the samples were reconstituted in a 1% dimethyl sulphoxide (DMSO) solution, Ali et al., [9].

Nawah Scientific Inc. (Mokatamm, Cairo, Egypt) provided human oral squamous cell carcinoma (HNO-97) cell lines and culture conditions. The cells were kept in RPMI media (Biowest- Nuaillé - France) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 µg/mL) (Gibco-Thermo Fisher Scientific, New York, USA). The cells were cultivated under controlled conditions in a humidified incubator at 37°C/ 18hr. and 5% CO2.

The Cell Cytotoxicity Assay

The Cell Cytotoxicity Assay (MTT Assay) was conducted in HNO-97 cells to evaluate the antiproliferative activity of medicines (D1, D2) and 5-FU from EBEWE PHARMA (Unterach am Attersee, Austria) in both monotherapy and combination treatment models. HNO-97 cells were seeded at a healthy density of 5 x 10^3 cells and serially diluted twice with either 5-FU (6.25 - 100 g/ml) or D1 & D2 (62.5 - 1000 g/ml). Following that, the cells were cultured for 72 hours. The absorbance of formazan crystals in DMSO at 570 nm was measured with a microplate reader. GraphPad Prism 8.0 was used to calculate the IC50 (half maximum inhibitory concentration). Following IC50 determination, cells were treated in triplicate with D1 & D2 and 5-FU at a constant ratio (5:1). The IC50 and alternate doses that were bigger and lower than the IC50 of monotherapy were used in combination treatment9. The IC50 of the combined treatment was used to calculate the combination index (CI) and dose reduction index (DRI).

Investigation of the Effects of Combination Agents

The median effect technique was applied to HNO-97 to determine the combined effects of D1 and D2 with 5-FU. Before measuring the combined effect, the MTT test was used to estimate the IC50 of the drugs, including the single and combination agents. The CI was used to classify pharmacological interactions as synergistic (CI < 1), additive (CI = 1), or antagonistic (CI > 1). The combination index (CI) was then calculated using the Chou-Talalay equation [10].

$$CI = \frac{IC_{50} \text{ of drug 5-FU in Combination}}{IC_{50} \text{ of drug 5-FU in Monotherapy}} + \frac{IC_{50} \text{ of drug D1 (D2) in Combination}}{IC_{50} \text{ of drug D1 (D2) in Monotherapy}}$$

The current study used a technique to calculate the Dose Reduction Index (DRI), which measures how much D1, D2, and 5-FU dosage can be lowered in combination therapy versus monotherapy. Cell Apoptosis Detection with the Annexin V/PI Assay: This study compared the synergistic effects of combination treatment versus monotherapy on apoptosis. In brief, HNO-97 cells were subjected to IC_{50} monotherapy and combination treatment levels for 72 hours. Negative control cells were grown under comparable conditions. To evaluate DNA content with fluorescence-activated cell sorting (FACS), an aliquot [10^5 cells/100 µl] of cell suspension was combined with 1 µl of FITC-conjugated annexin-V and 2.5 µl of Propidium Iodide PI (250 µg/ml). The analysis was conducted

with an Accuri C6 plus Flow Cytometer (Becton Dickinson, Sunnyvale, CA, USA).

$$DRI = \frac{IC_{50} \text{ of drug (D1, D2 OR 5-FU) in Monotherapy}}{IC_{50} \text{ of drug (D1, D2 OR 5-FU) in Combination}}$$

The study evaluated changes in cell cycle stages following monotherapy and combination treatment. Generally, 2 x 10^6 HNO-97 cells were planted into a T75 cm^2 flask and incubated for 24 h. Cells were treated for 72 hours with the IC50 of monotherapy and combination therapy. Negative control cells were grown under comparable conditions. The cells were trypsinized, collected, washed, stained, and incubated at room temperature for 30 minutes with 500 µl of PI. The cell cycle was measured with an Accuri C6 plus Flow Cytometer (Becton Dickinson, Sunnyvale, CA, USA).

In vitro wound healing (scratch) assay HNO-97 cells (3 x 10^5 cells/well) were planted in 24-well plates and allowed to grow as a monolayer for 24 hours. Next, vertically hold a sterile 20-200 µl pipette tip and scratch a cross in each well. To remove unattached cells, wash with 500 µl PBS and shake at 500 rpm for 5 minutes. After that, 500 µl of fresh medium with or without diluted medications (monotherapy or combination) was added and incubated for 48 hours. Before imaging, the plate was washed with 500 µl of pre-warmed PBS and gently shaken for 30 seconds. The pre-warmed medium or sample was reapplied, and photos were taken. Every 24 hours, the scratch closure was examined and photographed with an Olympus inverted microscope (1X70-Japan) at four times magnification.

Gene Expression Assay using Quantitative Real-Time PCR (RT qPCR)

The study used real-time quantitative PCR (RT-qPCR) to determine the expression levels of numerous messenger RNAs (mRNAs) associated with miR-155 and target genes implicated in various biological pathways. The HNO-97 cell line was treated with drugs, either monotherapy or in combination and total RNA was extracted using Quiagen's RNeasy® Mini Kit. In addition, the miRNA was extracted using Quiagen's miRNeasy® Mini Kit. The RNA samples, which included Total RNA and miRNA concentration and purity were measured using the NanoDrop 2000/2000c software (Thermo Scientific),and were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit, yielding complementary DNA (cDNA). The resulting cDNA was kept at -20°C for further analysis. The 20 µl qPCR reaction mixture included 2 µl (10 pmol/l) of forward and reverse primers for the target genes, 2 µl of cDNA template, and 10 µl of SYBR green PCR master solution (Thermo Scientific, Lithuania). Each of the 40 cycles showed a gradual increase. Table 1 shows primer sequences for several genes, which were produced using the primer designing tool available at https://www.ncbi.nlm.nih.gov/tools/primer-blast/. The Ct values for each target gene (GAPDH) were normalized using the control gene's cycle threshold (Ct) value, glyceraldehyde 3-phosphate dehydrogenase.

The study used the comparative Ct technique to evaluate the expression of many target genes under investigation. The Livak approach was used to compare the relative expression of the target gene before and after medication treatment.

Statistical Analysis

The data was analyzed using SPSS (Statistical Package for Social Research) version 23.0 software. Descriptive statistics were calculated using the Mean ± Standard deviation (SD) method. One of the following tests was used to establish the significance of the differences between the groups. "Unpaired" refers to something that does not match or connect with another corresponding item. The student's t-test is a popular statistical tool that compares the means of two sets of parametric numerical data. Furthermore, the One-way ANOVA, or analysis of variance, is used to compare parametric numerical data across more than two groups. Post-hoc Tukey analysis is frequently performed after this.

Results

The effect of mono- and combination therapy on cell inhibition

The monotherapy inhibitory impact of the medicines D1 and D2 was first investigated on OEC cells. The results revealed that D2 had almost little inhibitory effect on normal cells (OEC) within the studied dose range for 24 hr., 48 hr., and 72 hr. After 72 hours, D1 and 5-FU showed an inhibitory action with IC50 values of 609.7 ± 55.74 and 34.26 ± 5.91 µg/ml, respectively (Fig.1).

Both monotherapy and combination treatments inhibited HNO-97 cell growth dose-dependently (Fig. 2). (Table 2). D1 proved to have a robust inhibitory effect throughout 24 hours, 48 hours, and 72 hours, with the most significant influence occurring after 72 hours. IC_{50} = 372.6 ± 49.109 µg/mL. At the same time, D2 had a mild inhibitory impact for 24 hours. And 48 hours. However, it produced an inhibiting effect after 72 hours. IC50: 998.2 ± 68.231 µg/ml. After 24 hours, 5-FU did not show an inhibitory effect. However, it showed an inhibitory effect after 48 and 72 hours, with an IC_{50} of 12.02 ± 4.135 µg/ml.

Based on the monotherapy IC_{50} results, a constant ratio of 5-FU and D1 and D2 (1:5) was used to execute combination treatment with a schedule that

included the IC_{50} values and higher and lower dosages. Combining D1 and D2 with 5-FU inhibited HNO-97 cell growth more effectively than either agent alone, with IC 50s of 42.161 ± 2.681, 124.68 ± 7.467, and 9.112 ± 1.223 for D1 and 11.298 ±2.819 for D2 µg/ml, respectively, after 72 hours.

Synergistic Effects of Combination Therapy

To calculate the CI value for combination against HNO-97 cells, the Chou Talalay equation [9] was used. D1 and D2 had mean CI values of 0.808 ± 0.157 and 0.938 ± 0.155, indicating a synergistic impact of combination therapy on HNO-97 cells. Furthermore, the mean DRI of D1, D2, 5-FU with D1, and 5-FU with D2 in combination treatment was 8.85 ± 0.311, 7.948 ± 512, 1.348 ± 0.299, and 1.134 ± 0.415, demonstrating D1 approximately ninth- and D2 about eighth-fold dose reduction as compared to monotherapy, respectively. At the same time, when combined with D1 or D2, 5-FU doses were approximately half those of when used alone (Fig. 2, 3).

The Annexin V/PI assay determined whether monotherapy or combination treatment induced cellular apoptosis. The flowcytometry data (Table 3 & Fig. 4) revealed that the combination-treated cells (D1+5-FU) had the highest proportion of apoptotic cells (47.1%), compared to the 5-FU treatment (36.2%), D1 (36.7%), and D2 (27.7%) alone. The results also demonstrated a significant influence between D1 and D2 ($p<0.0001$) for D1 better effect, as well as between the two combination treatments (D1+5-FU & D2+5-FU) *($p<0.0001$)* for D1+5-FU better effect. The D2 and combined-treated cells (D2+5-FU) had the smallest percentage of necrotic cells (3.5% and 3.9%, respectively).

Effect of Mono- and Combination Therapy on the Cell Cycle of HNO-97

A flow cytometer was used to analyze the DNA content of control (untreated) and treated cells to determine the effect of mono- and combination therapy on HNO-97 cell cycle arrest. The results (Table 4 and Fig. 6) demonstrated that the combined medicines induced cells with the highest G0/1 induction (D1+5-FU & D2+5-FU). Cells treated with 5-FU showed significant reductions in S phase and G2/M phase cell proportions compared to (D1+5-FU) at the S phase *($p<0.0001$)* and both medications at the G2/M phase *($p<0.0001$)*. In contrast, D1 and D2 monotherapy medicines caused cells to be best arrested in the S phase by lowering cell proportion and in the G2/M phase by decreasing cell proportion, respectively. In the combined therapy, cells treated with D1+5-FU were best arrested during the S phase by lowering cell proportion, but cells treated with D2+5-FU were best arrested at the G2/M phase. The results show that D1 and D2 had a substantial effect on decreasing the proportion of S phase cells *($p<0.0001$)* for D1 and reducing the fraction of G2/M phase cells *($p<0.0001$)* for D2. There was a significant difference *($p<0.0001$)* between the effects of (D1+ 5-FU) and (D2+5-FU) during the G2/M phase, with (D2+5-FU) having a more significant effect.

Data were presented as mean ± SD. Significant differences were found when compared to control *(***$p < 0.001$)*, D1+5-FU *(#$p < 0.0001$)*, and D2+5-FU *(@$p < 0.0001$)*.

Effect of Mono- and Combination Therapy on HNO-97 Gene Expression Levels

Fig. 5 depicts the relative gene expression levels of various genes used to estimate mono- and combination therapy's anti-tumor efficacy. This study found that both mono- and combined therapies upregulated miR-155 expression, with the combined therapy (D1+5-FU) or (D2+5-FU) achieving the highest significant expression level *($p<0.0001$)* compared to the control. There was no significant difference between the two combined therapies. 5-FU significantly increased miR-155 expression in the control and combined therapies *($p<0.001$, $p<0.0001$, and $p<0.0001$,* respectively) (Fig. 5a).

Regarding the apoptotic genes

CASP-3 and CASP-9 expression levels increased non-significantly for D1 and significantly for D2 (*$P<0.001$ & $P<0.01$*, respectively) and significantly for both combined therapies *($P<0.0001$ & $P<0.001$)* for D1+55-FU and (*$P<0.0001$ & $P<0.0001$*) for D2+5-FU. 5-FU had the most significant effect compared to control and combined therapy *($P<0.0001$, $P<0.0001$, and $P<0.0001$,* respectively) (Figure 5b).

TP53, AIFM1, and TP3 levels increased considerably in all examined groups, including mono- and combined therapy. 5-FU monotherapy had the most excellent effect compared to control and both combined therapies *($P<0.0001$, $P<0.0001$, and $P<0.0001$,* respectively). D1 and D2 significantly increased TP3 *($P<0.001$ and $P<0.0001$,* respectively) but had no significant influence on each other. The combination of medications resulted in considerable elevation of TP53 *($P<0.0001$ & $P<0.0001$)*, with a significant interaction *($P<0.003$)* for D1+5-FU. Compared to 5-FU, AIFM1 shows a lower non-significant effect for the control and a substantial impact for the combined medications *($P<0.0001$ and $P<0.0001$*, respectively). D2 showed a substantial rise *($P<0.0001$)* compared to D1, which generated a non-significant increase. There was a significant difference between the two monotherapies *($P<0.0001$)*, indicating D2's superior effect. AIFM1 levels increased significantly with both medicines *($P<0.0001$)*, suggesting a more robust effect with D2+5-FU (Fig. 5c).

The results of BAX and BCL2 demonstrated a detrimental effect on both genes, with BAX

significantly decreasing in all groups, mono- and combined therapy *(p<0.0001)*. BCL2 levels increased considerably in both monotherapy *(p<0.0001)* and combined therapy (D1+5-FU) *(p<0.01)* (Fig. 5d).

For autophagy genes

The expression levels of m-TOR and LC3A were significantly increased by both monotherapies, D1 and D2, *(P<0.01 & P<0.0001) & (P<0.01 & P<0.0001),* respectively, but D1 had a more significant effect than D2 *(P<0.0001)*. The same results were obtained for the combined drugs, in which m-TOR and LC3A were significantly increased *(P<0.0001 & P<0.0001) & (P<0.0001 & P<0.0001)* respectively, but (D1+5-FU) had a more significant effect than (D2+5-FU) *(P<0.0001)*.

For the proliferating genes

D1 and D2 significantly reduced c-MYC and KI-76 expression levels *(P<0.0001 & P<0.001 & (P<0.0001 & P<0.0001),* respectively), with D1 having a more significant effect than D2 *(P<0.0001 & P<0.0001)*, while the combined therapies had a more significant decrease in c-MYC and KI-76 expression levels *(P<0.0001 & P<0.0001) & (P<0.0001 & P<0.0001),* respectively, with no significant difference between them. 5-FU significantly lowered c-MYC *(P<0.0001)* and did not affect the combined therapy. In contrast, KI-76 was considerably reduced *(P<0.0001)* with a significance to the combined therapies *(P<0.0001 and P<0.0001,* respectively) (Fig. 5f).

Effect of Mono- and Combination Therapy on Wound Healing (Scratch) Assay with HNO-97

The scratch experiment was carried out on HNO-97 cells to examine the effect of the various formulation medicines on cell motility. Following the formation of a mechanical scratch, indicated in yellow, on confluent cell monolayers, untreated cells could repair the wound within 24 hours, with complete closure occurring within 48 hours, as shown in (Fig.7). In contrast, cells treated with the monotherapies, either D1 or D2, displayed a failure to mend the wound, with the cells failing to fill the gap within 24 hours. Furthermore, after 48 hours, it was discovered that the 5-FU treatment reduced the cells' ability to mend the wound and close the gaps and decreased their consistency with one another, as seen in Fig.7. The cells treated with the combination medicines showed a rise in the percentage of dead cells, resulting in cellular integration loss and intercellular gap widening. (Fig. 7).

Discussion

Combination treatment is a therapeutic method that uses the beneficial effects of pharmacodynamic interactions, whether synergistic or additive, between two or more medications. Synergistic interactions are known to lead to more effective treatments. Introducing two drugs at lower dosages in combination therapy causes interactions with different biological pathways. As a result, treatments based on substances with a synergistic or additive impact and administered in combination are typically less harmful than monotherapy [11]. When compared to monotherapy, combination therapy has shown several advantages. These advantages include lower medication concentrations and toxicity, increased efficacy, targeting numerous molecular pathways, and sensitizing cells to treatment [12]. As a result, the current study aimed to assess the combined impact of D1 and D2 with the chemotherapeutic drug 5-FU using a synergistic method.

The usual strategy for treating different types of cancer, such as head and neck cancer, is to utilize a mix of chemotherapy medications. However, this type of therapy is known to have severe adverse outcomes. Using bioactive natural substances with anticancer characteristics in combination therapy can improve chemotherapy efficacy while reducing detrimental adverse effects. Furthermore, natural bioactive chemicals have higher structural variety, bioactivity, and complexity than manufactured medications. They can also inhibit previously thought-to-be intractable targets. Moreover, it is critical that most natural bioactive substances, whether secondary metabolites or signaling molecules are inherently directed toward physiologically relevant pathways.

Furthermore, there is a limited overlap between molecular signaling pathways aiming at natural goods and manufactured medications. This feature not only represents the possibility of identifying novel therapeutic targets for head and neck cancer, but it can also reduce the costs associated with developing new agents by utilizing pre-existing natural compounds, providing an additional option for combination therapy [13]. Furthermore, individuals undergoing the Pembrolizumab chemotherapy regimen [14], which is the first-line treatment of head and neck squamous cell carcinoma, frequently experience a range of adverse effects affecting the heartbeat, neurological, respiratory systems, and vision problems, such as double vision, blurry vision, sensitivity to light, eye pain, and changes in eyesight [15].

The TPF regimen (docetaxel + cisplatin + 5-FU) is a conventional chemotherapy combination. This regimen has been linked to undesirable toxic symptoms such as stomatitis, diarrhea, nausea, vomiting, neuropathy, and high toxicity [16]. The current study investigated the potential synergistic connection between 5-FU and D1 and D2 HNO-97 head and neck cancer cells. As previously shown [17], tumor growth heavily depends on cancer cells' proliferative capacity. Our findings show that various

5-FU, D1, and D2 concentrations suppress HNO-97 cell proliferation dose-dependently.

Furthermore, the examination of interactions revealed that combination therapy had a synergistic effect on HNO-97 cells, with a CI value below one. The IC_{50} value of 5-FU dropped by one factor: from 12.02 µg/ml to 9.112 µg/ml with D1 and 11.298 µg/ml with D2. This lowering is thought to be beneficial in reducing the high toxicity and negative consequences associated with 5-FU treatment. These findings were like previous research that found olive oil Oleuropein has a synergistic effect with 5-FU on HNO-97 [18], and diosmetin had a synergistic activity with 5-FU on colorectal cancer cells, HT-29 and HCT116 [19]. The combination therapy results provide another advantage by allowing numerous medications to target the heterogeneous character of tumors, boosting the likelihood of eradicating cancer cells, including cancer stem cells. This is particularly noteworthy because cancer stem cells have been linked to medication resistance and recurrence following remission [20].

Apoptosis, or imbalance of cellular proliferation and programmed cell death, is a significant contributor to the genesis and progression of cancer. Apoptosis is essential for the stability of normal tissue [21]. Thus, treatment strategies focused on triggering apoptosis show promise in slowing the progression of colorectal cancer. The current study suggests that the treated cells exhibited apoptotic characteristics, such as early apoptosis-associated membrane blebbing and late apoptosis-related chromatin condensation. Annexin V is a calcium-dependent phospholipid-binding protein with a strong affinity for PS. It is widely used with PI, a fluorescent dye, to detect cells undergoing apoptosis or necrosis [12]. To give additional quantification of apoptotic HNO-97 cells arising from monotherapy and combination treatment, Annexin V/PI staining was applied to the cells, which were then examined by flow cytometry. The use of combination therapy resulted in a considerable increase in apoptotic cells, with 47.1% for (D1+5-FU) and 36.5% for (D2+5-FU). This contrasts with cells treated just with 5-FU, which had a lower percentage of apoptosis (36.2%). HNO-97 cells treated with 5-FU had a more significant proportion of necrotic cells (10.9%) than cells treated with a combination of 3.9% (D2+5-FU) but not 13.5% (D1+5-FU).

According to reports, chemotherapeutic medications cause apoptosis and other types of cell death, such as necrosis, leading to inflammation. As a result, it is not a recommended strategy for cancer treatment [22]. As a result, administering 5-FU in combination with either D1 or D2 has a favorable effect via triggering the apoptosis route while not affecting the necrosis pathway compared to administering 5-FU alone. These findings were consistent with a study that found that oleuropein had a more significant effect on cell apoptosis than necrosis in HNO-97 cells and is more suited for combination therapy than monotherapy [23].

Reducing cancer cell proliferation by cell cycle arrest at various stages, which may result in cell death, is an essential factor to consider when fully and accurately defining the combination interaction mechanism [24]. Several researchers have reported that cell cycle obstruction influences the induction of the synergistic cytotoxic impact. In the current study, monotherapy and combined therapy elicit cell cycle arrest by increasing the G0/1 phase, with the combined therapy having a more significant effect and decreasing the S phase and G2/M phase. This finding is consistent with oleuropein's influence on cell cycle arrest17 and demonstrates its impact on breast cancer cells [25].

Another study found that colorectal cancer cells increased in the G2/M phase following a combination therapy (diosmetin + 5-FU). More research is needed to evaluate whether combo therapy induced M phase arrest or inhibited the G2/M transition. The study of phosphor-CDK1 expression levels and the B/CDK1 complex can help achieve this goal, as it is commonly accepted that reducing the synthesis of the cyclin B/CDK1 complex during cell cycle progression results in G2/M phase arrest [26].

The scratch assay is a widely acknowledged in vitro tool for studying cell migration in two dimensions [27]. Cancer is becoming more common when malignant cells spread beyond the primary tumor location, penetrating basement membranes and endothelial barriers and populating distant organs such as lymph nodes and bone marrow [28]. Because cellular migration, invasion, and adhesion play an essential part in cancer treatment, a thorough understanding of this mechanism is required. Cancer cell propagation to distant areas, followed by elimination, contributes significantly to cancer morbidity and mortality [24].

The study carried out a scratch assay to observe the independent or combined anti-migratory effects of D1, D2, and 5-FU as either mono-or combined therapy on HNO-97 cells indicated a significant decrease in the ability of cell migration, which is consistent with the findings of Choupani et al. [26] in their study involving the application of oleuropein and doxorubicin to a breast cancer cell line. Abdulla et al. [18] in their anticancer effect of oleuropein on HNO-9.

Regarding oleuropein's autonomous anti-migratory effect, the current study discovered that D1 and D2 significantly influenced HNO-97 cells. This finding is consistent with the research conducted by Wang et al. [27], who investigated the functions of cytotoxic alkaloids in biological mechanisms associated with cellular migration on

U2OS human osteosarcoma cancer cells, and Przychodzen et al. [28], who elucidated the capacity of oleuropein, either alone or in combination with 2-methoxyestradiol, to inhibit proliferation and migration in osteosarcoma cell lines.

Numerous studies have found aberrant miRNA expression in several types of human cancer, including head and neck cancer. These studies were conducted [29-34]. In the present study, it was reported that miR-155 expression was decreased in the head and neck cancer cell line (HNO-97) (Control group) and upregulated upon the mono- and combined treatment, which was more effective in the combined therapy than the mono-therapy and was and that was in agreement with Lerner et al. [34], who reported the downregulation of miR-155 in patients with head and neck cancer in both blood and tissue samples and the UM-SCC-1 cell line. The results revealed a statistically significant increase in proliferation rates for cells transfected with miR-146a and miR-155 inhibitors. Cells transfected with mimic miR-155, on the other hand, had a lower proliferation rate.

Apoptosis is carefully monitored to cause cellular death. Cellular proliferation and apoptosis balance is essential for optimal cell function and tissue homeostasis36. Cancer formation is caused by an imbalance between cell proliferation and apoptosis, which results in uncontrolled proliferation and defective apoptosis of the cells. HNC is a frequent malignant malignancy characterized by dysregulated cellular proliferation and apoptosis [35]. An et al. [36] found that using the cellular mechanism to induce programmed cell death is a highly effective technique for cancer treatment. The current study found that most apoptotic genes were upregulated after mono- and combined therapy treatment. TP53 and AIFM1 expression levels were also upregulated, which was consistent with Emam et al., [37], who found that both genes were upregulated on different cell lines, HepG II, MCF-7, and Caco-2, after treatment with natural Exopolysaccharides (EPS). Furthermore, the BAX with the downregulation of BCL2 coincided with Emam et al. [37] regarding the lowered BCL2, still not for BAX, which has been downregulated. Furthermore, as R Vidhyalakshmi et al. reported, the expression level of Bcl-2 was reduced. In contrast, the expression level of TP53 was elevated in the MCF-7 breast cancer cell line due to the action of extracted bacterial EPS40, whereas BCL2 was downregulated in the colorectal cancer cell line, as Alajez, [38] reported the activating the caspase cascade is critical for inducing programmed cell death, generally known as apoptosis. This study investigated the activation of caspases 8, 9, and 3/7 in HCT116 cells to determine the apoptotic pathway triggered by monotherapy and combination therapy. The combined therapy caused an apoptotic impact by activating caspases 3 and 9. The previously reported data suggest that combination therapy may be a more advantageous treatment option because it requires less 5-FU to have a similar therapeutic outcome to monotherapy, hence reducing 5-FU toxicity. The results of this study show that the use of combination therapy induces apoptosis via separate apoptosis mechanisms. Further protein expression research is required to determine the many pathway targets of combination treatment underpin these activities. That was consistent with [39], which reported the activation of Caspase-3 by Doxorubicin-loaded silica nanoparticles in the HNO-97 cell line18, for the activation of Caspase-3 in colorectal cancer cells (HCT116), for the overexpression of Caspase-9 in hepatocellular carcinoma cells (HepG II). Autophagy is downregulated in various tumour forms, indicating that it can reduce tumourigenesis43. LC3A and m-TOR were important autophagic genes that were decreased in control untreated HNO-97 cells. They then significantly increased better in the combined therapy than in the monotherapy, which was consistent with Emam et al., [37], who found that LC3A was significantly increased in HepG II cells but not m-TOR, which was decreased after EPS treatment. The nuclear protein KI-67 proliferative gene and proto-oncogene c-myc were overexpressed in many cancer cells. Madden et al., [39] and Zhang, et al., [40] and that was reported in the present study and then were significantly decreased in the mono- and combined therapy better in the combined therapy than monotherapy and were by Emam, et al., [37], suggest a better effect for the combined therapy better than the monotherapy on HNO-97.

Conclusion

The present study suggests that the combined treatment of D1, D2, and 5-FU has a synergistic effect on HNO-97 head and neck cancer cells. Combination therapy may increase 5-FU efficacy while decreasing its adverse side effects, as indicated by a high DRI in HNO-97 cells. The combination of 5-FU with D1 and D2 may promote apoptosis and stop the progression of HNO-97 cells in the G0/1 and S phases.

Recommendation

Additional research is necessary to evaluate the amalgamation's fundamental mechanism of action and validate the anti-tumorigenic properties of this integration in a suitable animal model.

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Ethical consent

No ethical consent is required.

Conflicts of interest

The authors state that they have no competing interests. Authors'

Contributions

The publication's content is the writers' responsibility. Only those who participated in the initial scientific research are eligible to be authors: AAE, MMA, and NTE contributed to the study's conception and design. AAE and NTE conducted most of the experiments, and AAE and MMA analyzed the results. AAE wrote the first draft of the manuscript. All authors revised the paper. All authors read and approved the final manuscript.

TABLE 1. Primer Sequences for Different Studied Genes

Gene	Forward Primer (F) Reverse Primer (R)	Product Size (bp)	Annealing Temperature (^{o}C)
miRNA155	**Forward:** TTAATGCTAATCGTGATAGGGGT **Reverse:** ATATGTAGGAGTCAGTTGGAGGC	55	61
TP53	**Forward:** CATAGTGTGGTGGTGCCCTATGAG **Reverse:** CAAAGCTGTTCCGTCCCAGTAGA	172	63
AIFM1	**Forward:** CAGAAAAAGGCCGCGTTATCT **Reverse:** ATACAATCAGTACCCTGGCCCC	160	59
RB-1	**Forward:** CCTCTCGTCAGGCTTGAGTT **Reverse:** ACAGATTCCCCACAGTTCCT	180	59
Caspase-9 (CASP9)	**Forward:** TTCCCAGGTTTTGTTTCCTG **Reverse:** CCTTTCACCGAAACAGCATT	143	61
Caspase-3 (CASP3)	**Forward:** CTCGGTCTGGTACAGATGTCGA **Reverse:** CATGGCTCAGAAGCACACAAAC	177	60
BCL2	**Forward**: CTGCACCTGACGCCCTTCACC **Reverse**: CACATGACCCCACCGAACTCAAAGA	119	61
BAX	**Forward:** CGGGTTGTCGCCCTTTTCTA **Reverse:** TGGTTCTGATCAGTTCCGGC	82	60
m-TOR	**Forward:** GCCCAGGCCGCATTGTCTCTAT **Reverse:** GCAGTAAATGCAGGTAGTCATCCAGGTT	84	62
LC3A	**Forward:** GCCTTCTTCCTGCTGGTGAAC **Reverse:** AGCCGTCCTCGTCTTTCTCC	91	58
c-MYC	**Forward**: AGGGAGATCCGGAGCGAATA **Reverse**: GTCCTTGCTCGGGTGTTGTA	156	60
KI-67	**Forward:** GTGGTTCGACAAGTGGCCTT **Reverse:** ACCCCTTCCAAACAAGCAGG	224	61
GAPDH	**Forward:** TGCACCACCAACTGCTTAGC **Reverse:** GGCATGGACTGTGGTCATGAG	87	59

TABLE 2. IC $_{50}$ of D1, D2, and 5-FU as Monotherapy or Combination Therapy in HNO-97 Cells.

Treatment	Drug	HNO-97 Cells IC $_{50}$ (µg/ml)
Monotherapy	D1	372.6 ± 49.109
	D2	998.2 ± 68.231
	5-FU	12.02 ± 4.135
Combination Therapy	D1	42.161 ± 2.681
	D2	124.68 ± 7.467
	5-FU+D1	9.112 ± 1.223
	5-FU+D2	11.298 ±2.819

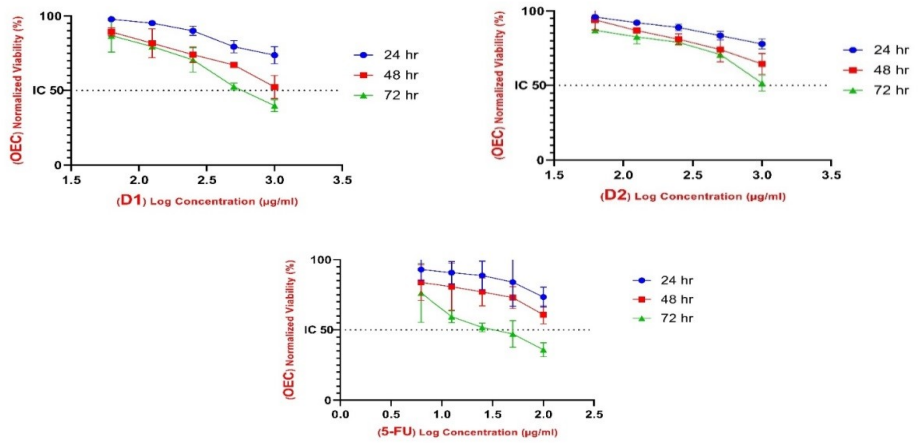

Fig. 1. Cell Growth Inhibitory Effect of D1, D2, and 5-FU as Monotherapy in OEC.

Data were expressed as mean ± standard deviation (SD).

TABLE 3. Apoptosis Induction Effect of Mono- & Combined Therapy on HNO-97 Cells

	Control	D1	D2	5-FU	D1+5-FU	D2+5-FU
Viable Cells	94.8 ± 0.872^{bcdef}	52.5 ± 1.216^{acef}	68.8 ± 4.036^{abdef}	52.9 ± 1.587^{acef}	39.4 ± 1.946^{abcdf}	59.6 ± 1.47^{abce}
Early Apoptosis	0^{bcdef}	35.1 ± 1.113^{ace}	25.5 ± 1.539^{abdef}	34.6 ± 0.953^{ace}	44.9 ± 1.442^{abcdf}	35.4 ± 0.721^{acf}
Late Apoptosis	1.4 ± 0.3	1.6 ± 0.264	2.2 ± 0.361^{f}	1.6 ± 0.529	2.2 ± 0.435^{f}	1.1 ± 0.264^{de}
Necrosis	3.8 ± 0.435^{bde}	10.8 ± 1.473^{acef}	3.5 ± 0.529^{bde}	10.9 ± 1.249^{acef}	13.5 ± 0.854^{abcdf}	3.9 ± 0.264^{bde}

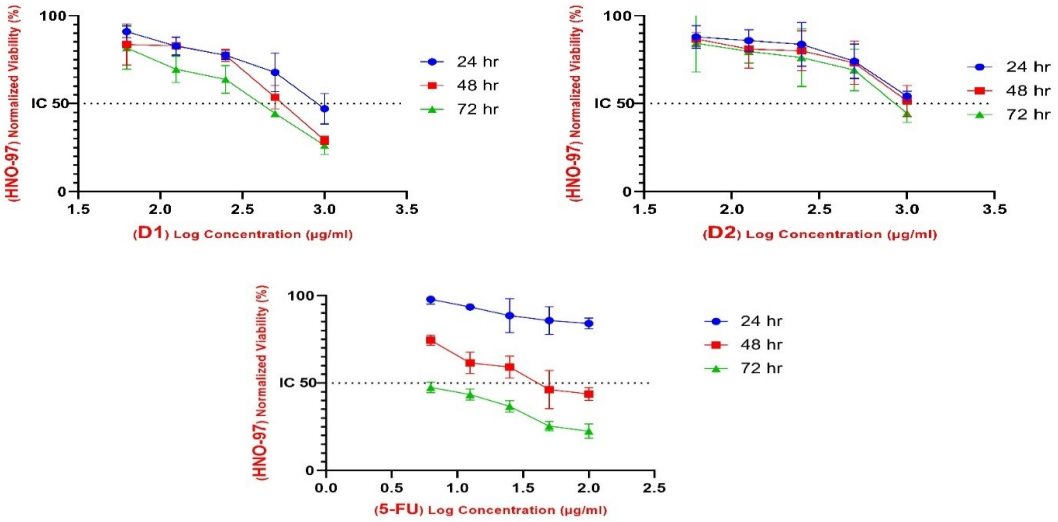

Fig. 2. Cell Growth Inhibitory Effect of D1, D2, and 5-FU as Monotherapy in HNO-97.

Data were expressed as mean ± standard deviation (SD).

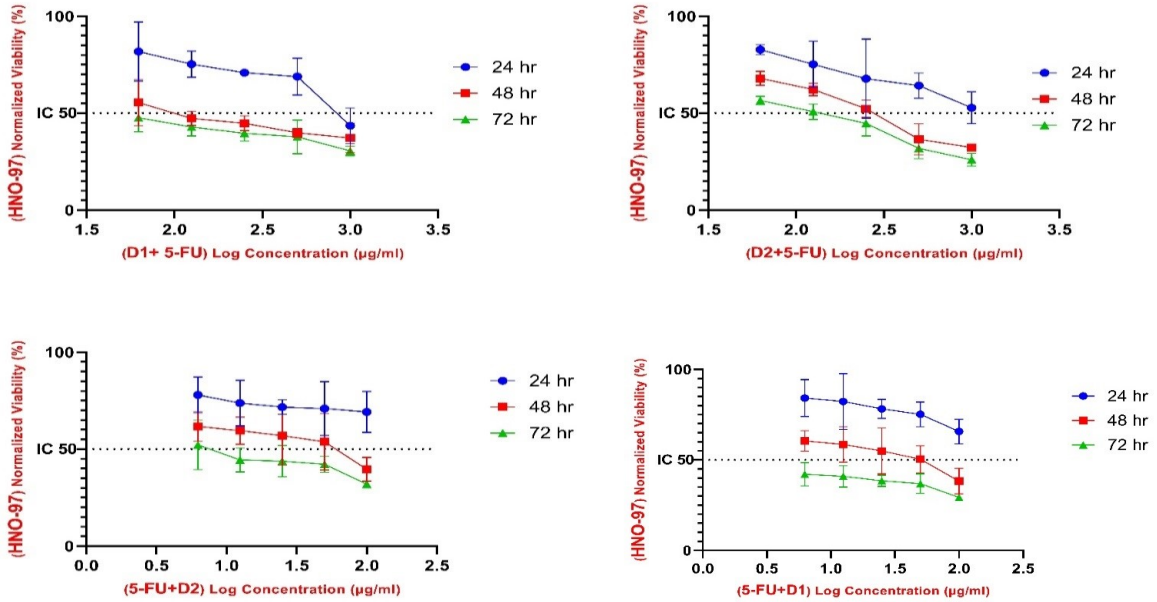

Fig. 3. Cell Growth Inhibitory Effect of the Combined Therapy in HNO-97.

Data were expressed as mean ± standard deviation (SD).

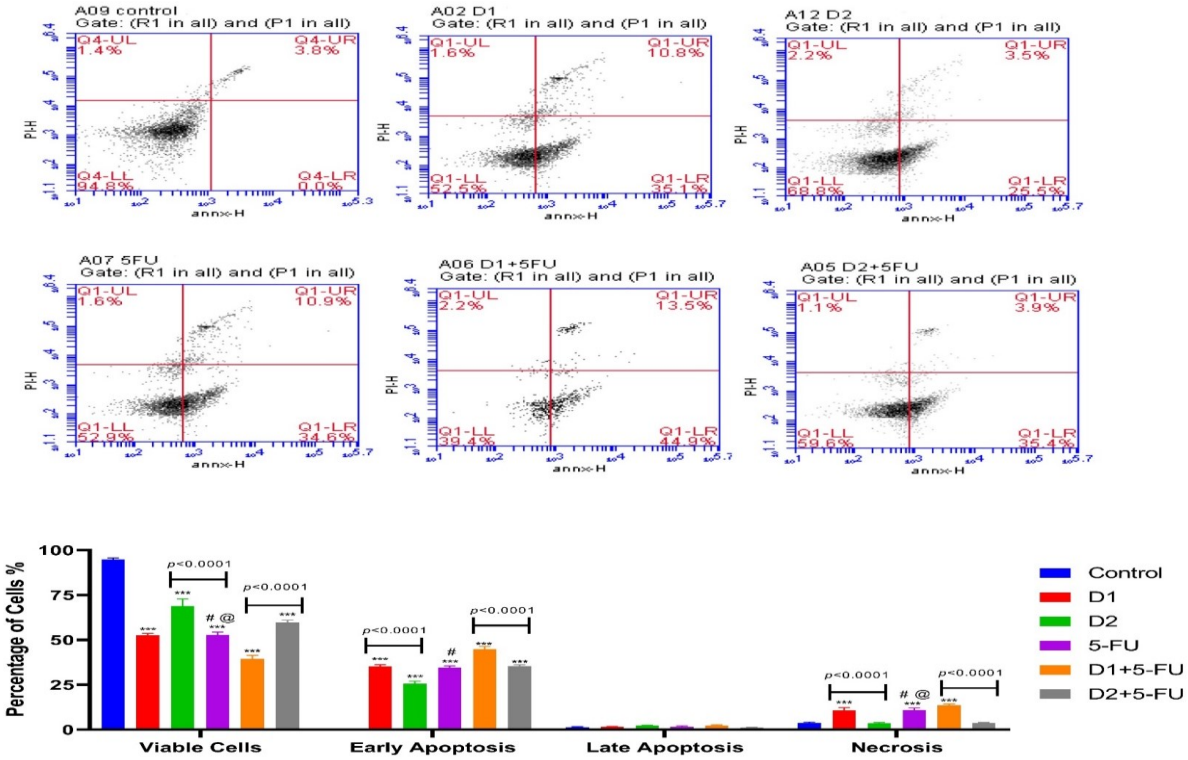

Fig. 4. Flow cytometry Charts and Quantification of Apoptosis in HNO-97 cells after 72 hours of Monotherapy and Combination Treatment.

Data were expressed as mean ± standard deviation (SD). ***p < 0.001 indicates a significant difference compared with control, # p < 0.0001 indicates a significant difference compared with D1+5-FU & @ p < 0.0001 indicates a significant difference compared with D2+5-FU.

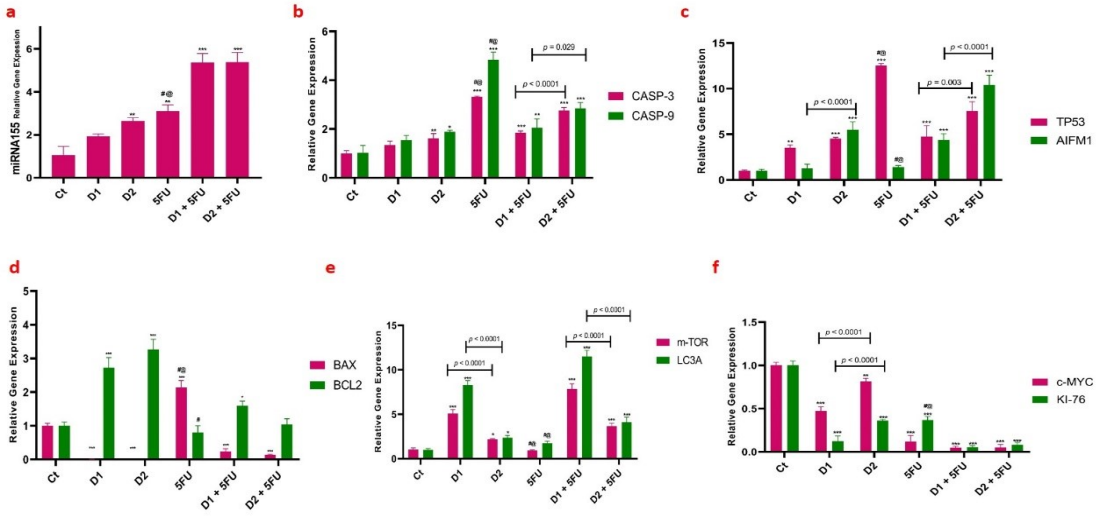

Fig. 5. Cell Cycle Phases Distribution Effect of Mono- & Combined therapy for 72 hr. on HNO-97 Cells. Data were expressed as mean ± standard deviation (SD). ***p < 0.001 indicates a significant difference compared with control, # p < 0.0001 indicates a significant difference compared with D1+5-FU & @ p < 0.0001 indicates a significant difference compared with D2+5-FU.

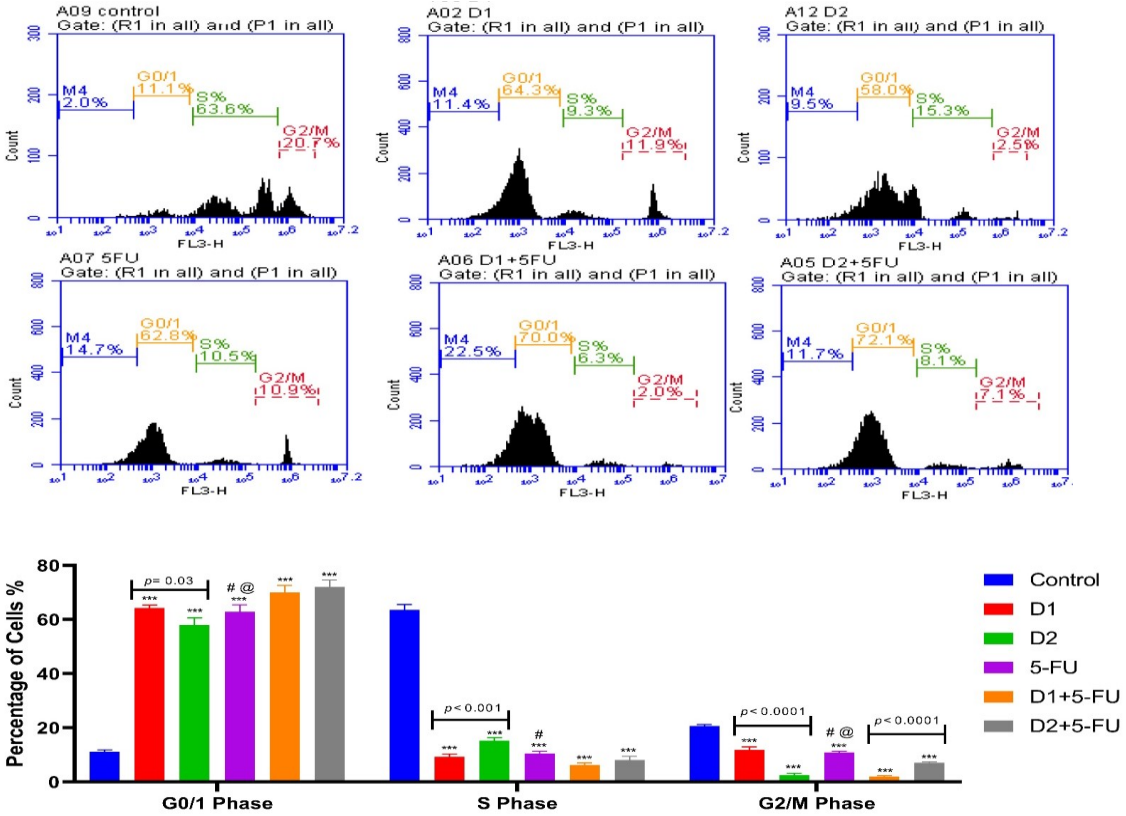

Fig. 6. Effect of Mono- Combined Therapy on the Gene Expression Level of: (a) miR-155, (b) CASP-3 & CASP-9, (c) TP53 & AIFM1, (d) BAX & BCL2, (e) m-TOR & LC3A, (f) c-MYC & KI76 on HNO-97 Cells.

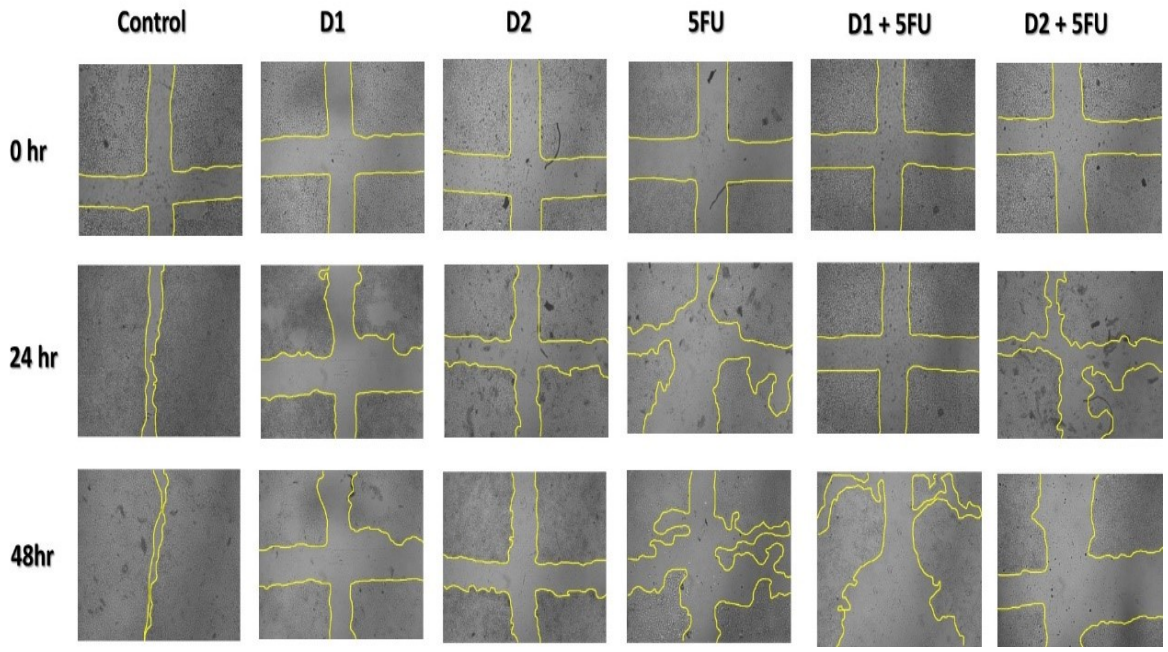

Fig. 7. Qualitative Analysis of Mono- and Combined Therapies on Wound Healing Assay on HNO-97 Cells.

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تنظيم MiR-155 من خلال التأثير التآزري لمستخلصات *Tamarix aphylla* و*Artemisia Herba Alba* مع Flurouracil (5-FU)-5 في خط خلايا سرطان الرأس والرقبة (HNO-97)

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[3] قسم علوم الحركة والصحة، كلية علوم الرياضة، جامعة جدة، جدة، المملكة العربية السعودية وقسم صحة الغذاء، كلية الطب البيطري، جامعة الوادي الجديد، مصر.

الملخص

غالبًا ما يستخدم عامل العلاج الكيميائي، 5-فلورويوراسيل (5-FU)، لعلاج سرطان الخلايا الحرشفية في الرأس والرقبة (HNSCC). ومع ذلك، فإن التطبيق السريري لهذا الدواء قد تأثر بشكل كبير بالمضاعفات والسمية المرتبطة به. ولذلك، فإن البحث في نهج علاجي بديل يقلل من سمية 5-FU ويظهر في الوقت نفسه تأثيرًا تآزريًا ضد سرطان الخلايا الحرشفية في الرأس والرقبة (HNSCC) يمثل خيارًا قابلاً للتحقيق. تم إثبات التأثيرات المثبطة لنبات *Tamarix aphylla* في صورة (D1) والشيح العشبي ألبا في صورة (D2)، وهي منتجات تحدث بشكل طبيعي، على تكاثر الخلايا السرطانية المختلفة، بما في ذلك خلايا HNSCC. يهدف هذا البحث إلى دراسة التأثير المشترك لـ D1 وD2 و5-FU على سرطان الخلايا الحرشفية في الرأس والرقبة (HNSCC) والتحقيق في نشاط موت الخلايا المبرمج لهذا المزيج. تم تقييم جدوى الخلايا الخاضعة للعلاج الأحادي والعلاج المركب باستخدام اختبار MTT. استخدمت الدراسة قياس التدفق الخلوي لفحص آثار تحريض موت الخلايا المبرمج وتوقف دورة الخلية على الخلايا. وكذلك استخدمت الدراسة الحالية تفاعل البوليميراز المتسلسل في الوقت الحقيقي لفحص التعبير عن miR-155 ومجموعة من الجينات المحددة. هنا، قمنا بتوثيق أن المنتجات الطبيعية، D1 وD2، تعزز miR-155. لذا، خلصت هذه الدراسة إلى أن D1 وD2 قد يكون لهما تأثير مضاد للأورام على خلايا HNO-97 من خلال آلية تم تحديدها حديثًا. بالإضافة إلى ذلك، يوفر هذا البحث رؤى قيمة حول المسارات البيولوجية التي تستخدمها المنتجات الطبيعية لممارسة آثارها.

الكلمات الدالة: MiR-155، سرطان الرأس والرقبة، HNO-97، التأثير التآزري .