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Effect of Nanoformulated Thymol, Nanoformulated Doxorubicin and Their Combination on Oral Squamous Cell Carcinoma Cell Line: A Comparative Ex-Vivo Study

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Abstract

Background: *Nanoparticles are a promising technology in drug delivery mechanisms as they are considered the upcoming technological revolution in the 21st century. Squamous cell carcinoma is the most common kind of cancer in the head and neck, with oral squamous cell carcinoma (OSCC) accounting for the majority of cases and an expanding number of scientific research on this subject supports the need to emphasize that there is still much room for improvement. Recently, there has been a noticeable rise in interest in dietary phytochemical consumption for possible cancer chemoprevention. Our study is pioneer in applying nanoformulated thymol as antineoplastic drug to overcome drawbacks of chemotherapeutic drugs. Although Doxorubicin (DOX) is the most preferrable chemotherapeutic drug for the treatment of OSCC, its application is limited due to its various side effects. Accordingly, it is mandatory to explore the outstanding properties of nanoformulated form on HNO-97 cell line.* ***Aim:*** *Investigate the possible synergy between nanoformulated thymol and nanoformulated DOX on the oral squamous cell carcinoma cell line.* ***Material and Method:*** *Lingual cell line (HNO-97) cells were cultured. Serial concentrations of nanoformulated thymol and nanoformulated DOX were prepared. Calculation of minimum inhibitory concentration (IC50) of thymol, DOX, nano-thymol and nano-DOX, followed by investigation of cell proliferation by quantitative real-time polymerase chain reaction (QRT-PCR) through the measurement of C-MYC gene expression was performed.* ***Results****: HNO-97 cells treated with combined therapy of nano-thymol and nano-DOX, drastically reduced C-MYC gene expression .****Conclusion:*** *Based on current findings, combination of nano-DOX and nano-thymol may be a viable supplementary anticancer therapy for OSCC by reducing proliferation of the cells.*

Keywords: *DOX, nano-DOX, thymol, nano-thymol, C-MYC, HNO-97.*

1. Introduction

Head and neck cancer (HNC) is considered a broad term that includes epithelial and mesenchymal malignancies located in the paranasal sinuses, oral cavity, nasal cavity, pharynx and larynx (Pezzuto F. et al., 2015). Squamous cell carcinoma (SCC) represents 90% of head and neck malignancies (Miranda-Filho A. et al., 2020). It is the sixth most common type of malignant tumors as 50 million new cases occur annually worldwide (Mascitti M. et al., 2018).

Treatment of OSCC often incorporates multiple approaches managed by a multidisciplinary team of surgeons and clinical oncologists due to the huge diversity of anatomical sites and pathogenesis. However, conventional treatment protocol includes resection of neoplasms followed by chemotherapy or radiotherapy or both (Solomon B. et al., 2018).

Phytochemicals recently have been gaining attention as hopeful anticancer agents. Thymus vulgaris is a medical plant with several therapeutic properties (Dauqan E. & Abdullah A., 2017). This plant, common in Mediterranean regions, has a long history of use for various medical applications.

Nowadays, thymol and thyme are used in a wide range of pharmacy, food and cosmetic industry. There is a great interest in the formulation of pharmaceuticals and cosmeceuticals based on thymol (Salehi B. et al., 2018). Several studies have evaluated the possible therapeutic uses of this plant and its role in the treatment of disorders affecting the respiratory, nervous and cardiovascular systems (Sharangi A. & Guha S., 2013).

Moreover, this compound also exhibits antioxidant, antimicrobial, anti-inflammatory, antispasmodic activities (Alagawany M et al., 2021), neuropharmacological (Bianchini A. et al., 2017), antidiabetic (Saravanan S. & Pari L., 2016), antihyperglycemic and hypolipidemic effects (Saravanan, S. and Pari, L., 2015); in addition to its role as an immunomodulator and growth enhancer (Salehi,B. et al., 2018).

Thymol and its derivatives have been studied for their anticancer effect (Salehi B. et al., 2018) and have been reported for their ability to exert anticancer activity through different mechanisms of action including apoptosis, suppressing cell growth, producing intracellular reactive oxygen species and depolarizing mitochondrial membrane potential (Kang S. et al., 2016).

Nanoformulated thymol has been mainly used as an antibacterial drug in the agriculture field. Many studies confirmed the superiority of thymol nanoemulsion over conventional thymol in antibacterial activity and promoting plant growth (Kumari et al. 2018; Kumari et al. 2019) Although nano-formulated thymol has proven its powerful antibacterial activity, it was not yet proposed as an anticancer drug.

A new strategic application of phytochemicals with the combination of the potent chemotherapeutic drug has been addressed as it is considered a promising approach in improving the cytotoxicity of the drug and minimizing non-specificity (Patra S. et al., 2021).

One of the most potent chemotherapeutic drugs used for treatment of HNC is Doxorubicin. Doxorubicin is the most commonly used chemotherapeutic drug in the treatment of cancer of the stomach, bladder, breast, thyroid, lung, ovaries, soft tissue sarcoma, Hodgkin's lymphoma and multiple myeloma (Rivankar S., 2014).

However, Doxorubicin has several adverse effects such as cardiotoxicity, myelosuppression, nausea, vomiting, alopecia (Danesi R. et al., 2002), gastrointestinal disorders, stomatitis and bone marrow toxicity (Octavia Y. et al., 2012). All these adversely affect the clinical use of conventional DOX and direct physicians to develop a specific nano-delivery system that can reduce the non-specific cytotoxic effect of this drug and enhance its anti-tumor efficacy (Hu C. & Zhang L., 2012).

Recently, the use of phytochemicals in conjugation with chemotherapeutic agents has increased dramatically due to reduced cytotoxicity, increased biocompatibility, reduced resistance, specificity and reduced systemic toxicity (Dhupal M. & Chowdhury D., 2020).

2. Material And Methods:

The cell line, drug and nanoparticle were all purchased from Nawah scientific Inc. where the experiment was performed in Global lab.

Drug Preparation:

Preparation of Thymol Nanoemulsion:

Antimicrobial nanoemulsion was prepared from 2 w/w% thymol oil (correspond to 20 mg/ml), 2 w/w% surfactant (Tween 80) and 96 w/w% aqueous phase (deionized water). Tween 80 was dispersed in the aqueous phase while the cosurfactant lecithin was added to the oil phase at a level of 0.1 w/w%. A high energy homogenization method was used to prepare the nanoemulsion. Aqueous phase was added drop wise to oil phase under magnetic stirring at 1000 rpm for 30 min. The formed microemulsion was converted into nanoemulsion by homogenization at 20000 rpm for 5 min and temperature increase was minimized by surrounding the sample with an iced water bath.

Preparation of Liposomal Doxorubicin:

We used the pH gradient hydration method to prepare the liposomes. Lecithin, CHOL and DSPE-mPEG (67.9:29.1:3 molar ratios) were dissolved in chloroform and the mixture was warmed to 55˚C. We added Dil to the lipid phase at 0.1 mol% for lipid staining in order to evaluate cellular uptake. The solvent was evaporated under vacuum in a rotary evaporator until a thin-layered film was formed. Once prepared, the film was hydrated with 250 mM of ammonium sulfate. The hydrated film was homogenized at 20000 for 5 minutes in order to decrease liposomal particle size. Doxorubicin 1 mg/ml was loaded into the liposomes.

MTT Cytotoxicity Assay at 24 hours:

Culturing of Human Tongue Squamous Cell Carcinoma Cell Line (HNO-97):

HNO-97 cells were seeded in a 96-well culture plate. An average of 1x104 HNO-97 cells were seeded in 200 µL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% of penicillin G sodium, streptomycin (10 mg) and amphotericin B. Culture plates were incubated at 37 °C in an atmosphere of 5% CO2 for 24 hours. The next day, a serial concentration of DOX, nano-DOX, Thymol and nano-Thymol drugs "100µmol, 10 µmol, 1 µmol ,0.1 µmol ,0.01 µmol", was prepared. In addition, the carrier solvent (0.1% DMSO) was used for control cells. The treated HNO-97 cells were incubated at 37 °C in an atmosphere of 5% CO2 for 24 hours, then the cell proliferation assay was conducted for the calculation of IC50.

Assessment of Cell Viability by Cell Proliferation Assay (MTT):

The cell proliferation assay was performed using the Vybrant® MTT Cell Proliferation Assay Kit, cat no: M6494 (Thermo Fisher, Germany). The HNO-97 cells (8×103 cells per well) were seeded in 96-well culture plates and incubated at 37 °C with 5% CO2 for 48 hours in DMEM media, then 100 µL of media was removed and replaced by new media. Twenty µL of 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1mg/mL) (Invitrogen, ThermoScientific, Germany) was added to each well. The plates were incubated at 37 °C and 5% CO2 for four hours. The MTT solution was removed and 100 µL of sodium dodecyl sulfate with hydrochloric acid (SDS-HCL) was added to the wells. Cell viability was determined by measuring the optical density at 570 nm on a spectrophotometer.

Calculation of half-maximal Inhibitory Concentration (IC50):

After conducting the cell proliferation assay, the percent of viability was determined which represents the HNO-97 cell response to serial doses of each drug formula including 0.01, 0.1, 1.0, 10 and 100 µM of the drugs. IC50 is the half-maximal inhibitory concentration, It indicates how much drug is needed to inhibit a biological process by half. After calculation of IC50 for the drugs, half IC50 and double IC50 were also calculated to be used in the experimental study.

Assessment of the Drugs Cytotoxic Effect on HNO-97 Cells:

Following the calculation of IC50 of each drug formula, the HNO-97 cells were treated with a panel of concentrations half IC50, IC50 and double IC50 for nano-DOX, nano-thymol and the two drugs in combination. The assessment of cell viability was tested at three-time intervals including 12, 24 and 48 hours.

Culture plates were incubated at 37 °C in an atmosphere of 5% CO2 for 24 hours to reach the 70% confluence. On the next day, a panel of three concentrations of nanoformualed DOX (IC50: 0.607, half IC50:0.304, double IC50: 1.214) and nanoformualed thymol (IC50: 0.948, half IC50:0.474, double IC50: 1.9) was prepared. In addition, the carrier solvent (0.1% DMSO) was used for control cells. The treated HNO-97 cells were incubated at 37 °C in an atmosphere of 5% CO2 for 12, 24 and 48 hours. They were treated with the panel of concentration for each drug individually and the two nano-formulated drugs in combination.

Real-Time Quantitative PCR:

The expression of C-MYC as a proliferative oncogene was measured in treated cells at three-time intervals (12, 24 and 48 hours) after treatment with a panel of three concentrations (half IC50, IC50 and double IC50) with nano-thymol and nano-DOX individually and their combination. The gene expression was measured by quantitative real-time polymerase chain reaction (QRT-PCR) technique.

C.1. Total RNA Extraction and Purification: Total mRNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

C.2. Reverse Transcription: cDNA was synthesized by reverse transcription reaction using QuantiTect II RT Kit (Qiagen, Hilden, Germany) using Biometra thermal cycler, analytikjena, Endress+Hauser, Germany.

C.3. Gene Expression Analysis: The quantification of C-MYC gene expression levels was amplified from mRNA using a QuantiTect primer assay. All samples were analyzed using the 5 plex Rotor-Gene PCR Analyzer. The 2ΔΔCt method was conducted for the analysis of gene expression levels, using ACTB as an endogenous reference control for normalization purposes.

Statistical Analysis

The collected data was revised, coded, tabulated and introduced to a PC using Statistical Package for Social Science (SPSS 24 for windows; SPSS Inc, Chicago). All graphs were plotted by GraphPad Prism Software 8.4.2 (San Diego, US).

3. Results And Discussion

1-MTT Cytotoxicity Assay Results:

1.A. Measurement of IC50 of each drug at 24 hours by cell proliferation assay (MTT):

MTT assay revealed that at 24 hours the IC50 of nano-formulated DOX was 0.607 µmol/mL, while the half IC50 was 0.304 µmol/mL and the double IC50 was 1.214 µmol/mL

The IC50 of nano-formulated thymol was 0.948 µmol/mL, while the half IC50 was 0.474 µmol/mL and the double IC50 was 1.90 µmol/mL. The IC50 was calculated using GraphPad Prism Software 8.4.2 (table 1).

Table 1: Calculated IC50 of the two tested drug formulas in HNO-97 cell line after treatment for 24 hours:

	nano-DOX	**nano-thymol**
IC50	**0.607**	**0.948**

Assessment of the Cytotoxic Effect of Nano-Chemicals:

Following the calculation of IC50 of each drug formula, the HNO-97 cells were treated with a panel of concentrations (half IC50, IC50 and double IC50) for nano-DOX, nano-thymol and the two drugs in combination. The assessment of cell viability was performed at three-time intervals, 12, 24 and 48 hours. The cell viability of the three tested formulations (nano-DOX, nano-thymol and nano-DOX/nano-thymol), are presented at three-time intervals (12, 24 and 48 hours) with different concentrations (IC50, half IC50, and double IC50) in tables (2,3 and 4).

At three-time intervals (12, 24 and 48 hours) MTT cytotoxicity assay revealed that the combined drug formula had the highest cytotoxicity with the double IC50 concentration (19.37%), (15.52%) and (11.87%) respectively, while the nano-thymol had the lowest cytotoxicity with the half IC50 concentration (74.6%), (63.31%) and (61.58%) respectively.

Table 2: MTT assay results for HNO-97 cells treated with a panel of three different concentrations of nano-formulated DOX, nano-thymol and their combination after 12 hours.

	Untreated control cells	nano-DOX (µmol/ml)			nano-thymol (µmol/ml)			nano-DOX/ nano-thymol (µmol/ml)		
		½ IC50	IC50	2xIC50	½ IC50	IC50	2xIC50	½ IC50	IC50	2xIC50
Viability (%)	99.6	65.2	46.8	32.5	74.6	48.7	39.0	42.9	34.6	19.37

Table 3: MTT assay results for HNO-97 cells treated with a panel of three different concentrations of nano-formulated DOX, nano-Thymol and their combination after 24 hours:

	Untreated control cells	nano-DOX (µmol/ml)			nano-thymol (µmol/ml)			nano-DOX/ nano-thymol (µmol/ml)		
		½ IC50	IC50	2xIC50	½ IC50	IC50	2xIC50	½ IC50	IC50	2xIC50
Viability (%)	99.87	58.57	41.90	26.72	63.31	40.13	35.31	30.21	23.28	15.52

Table 4: MTT assay results for HNO-97 cells treated with a panel of three different concentrations of nano-formulated DOX, nano-Thymol and their combination after 48 hours:

	Untreated control cells	nano-DOX (µmol/ml)			nano-thymol (µmol/ml)			nano-DOX/ nano-thymol (µmol/ml)		
		½ IC50	IC50	2xIC50	½ IC50	IC50	2xIC50	½ IC50	IC50	2xIC50

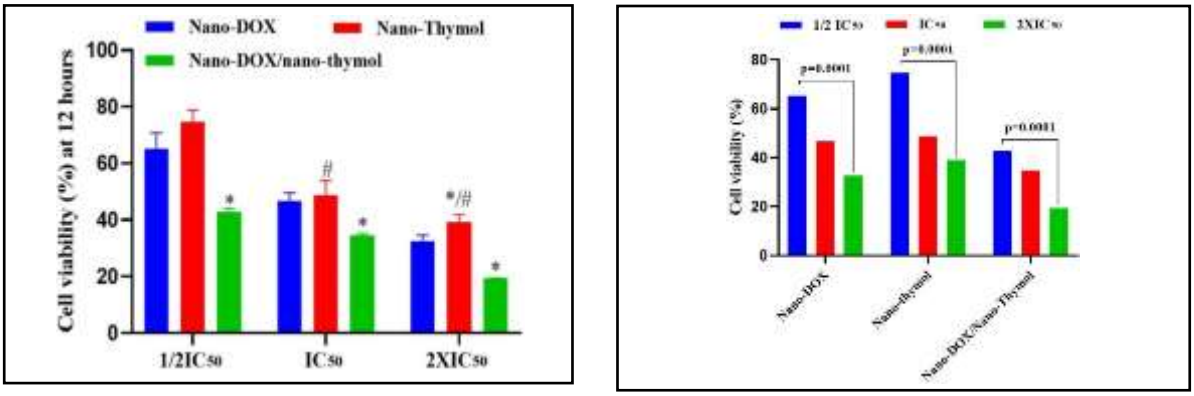
Viability (%)	100.13	50.96	37.55	21.19	61.58	35.09	23.66	19.67	16.72	11.87

1.C. Pair-wise Comparison of the MTT Cytotoxicity and Viability Percentage:

The cytotoxic effect of each individual dose (IC50, half IC50, double IC50) of the three formulations (nano-DOX, nano-thymol and combined nano-DOX/nano-thymol) was compared at three-time intervals (12, 24 and 48 hours).

At 12- and 24-hours similar results were achieved, as the results revealed a highly significant difference on comparing the mean values of viability percentage upon application of half IC50 of the three formulas ($P < 0.001$), the maximum cytotoxic effect was achieved on comparing the mean value of viability percentage upon treatment with the combination of nano-DOX / nano-thymol.

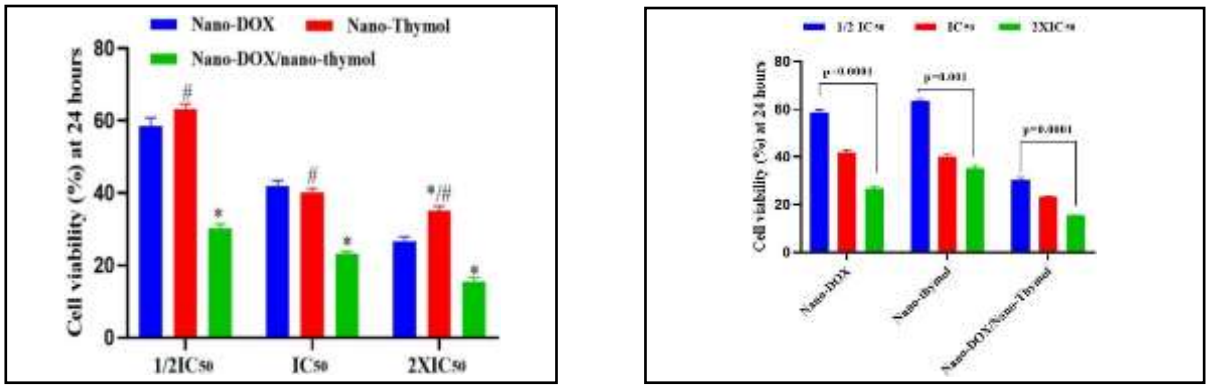
However, at 12 hours a high significant difference was obtained on comparing the mean values of viability percentage of the nano-DOX and nano-thymol when treated with half IC50 and mild significant difference on comparing the mean value of viability percentage of cells treated with the IC50 doses. Meanwhile, nano-thymol showed the least cytotoxic effect at the IC50 dose compared to the nano-DOX or the combined formula. These data are presented in (figure 1).



*: statistical significance compared to the nano-DOX, #: statistical significance compared to the combined nano-DOX/nano-Thymol.

Figure 1: Bar chart showing high statistically significant difference ($p<0.0001$) on comparing the cytotoxic effect of the three different formulations (nano-DOX, nano- Thymol and combined) on HNO-97 cells using three different concentrations (half IC50, IC50 and double IC50) after 12 hours treatment.

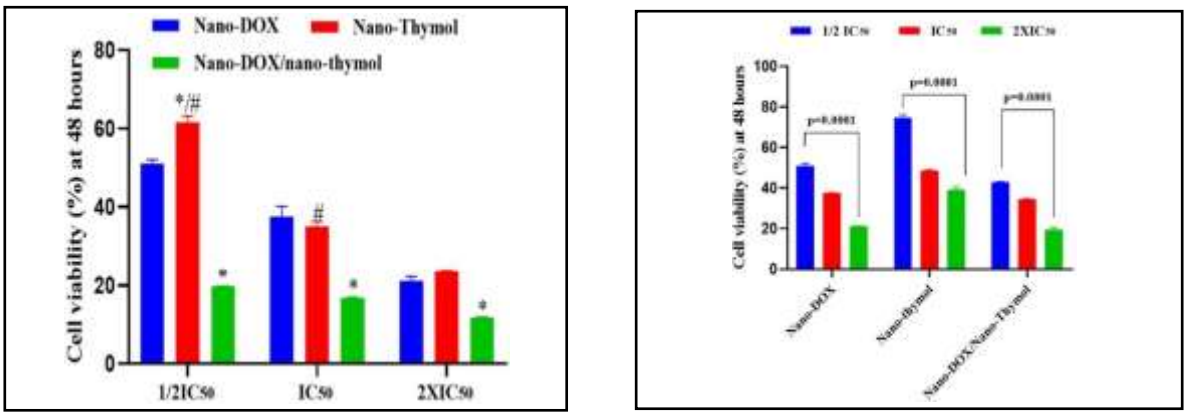
While at 24 hours; no significant difference was found on comparing the mean values of viability percentage of the nano-DOX and nano-thymol when using IC50 doses. Meanwhile, nano-thymol showed the least cytotoxic effect at the IC50 dose compared to the nano-DOX or the combined formula. These data are presented in (figure 2).



*: statistical significance compared to the nano-DOX, #: statistical significance compared to the combined nano-DOX/nano-thymol.

Figure 2: Bar chart showing high statistically significant difference ($p<0.0001$) on comparing the cytotoxic effect of the three different formulations (nano-DOX, nano-thymol and combined) on HNO-97 cells using three different concentrations (half IC50, IC50 and double IC50) after 24 hours treatment.

At 48 hours, the results revealed a highly significant difference between the mean values of viability percentage for half IC50, IC50 and double IC50 of the three formulas at 48 hours ($p < 0.001$), the maximum cytotoxic effect was achieved upon treatment with the combination of nano-DOX / nano-thymol formula at half IC50, IC50 and double IC50 respectively. Yet, at double IC50 there was no statistically significant difference on comparing the mean viability percentage of the combination and the nano-DOX and a mild statistically significant difference on comparing between them when using IC50 dose. These data are presented in (figure 3).



*: statistical significance compared to the nano-DOX, #: statistical significance compared to the combined nano-DOX/nano-thymol.

Figure 3: Bar chart showing high statistically significant difference ($p<0.0001$) on comparing the cytotoxic effect of the three different formulations (nano-DOX, nano-Thymol, combined) on HNO-97 cells using three different doses (half IC50, IC50 and double IC50) after 48 hours treatment.

2- Quantitative Real-time Polymerase Chain Reaction (QRT-PCR) Results:

The C-MYC gene expression was evaluated in both untreated and treated HNO-97 cells using quantitative real-time polymerase chain reaction (QRT-PCR). The cells treated with three different doses represent the half IC50, IC50 and double IC50, and the gene expression was measured after treatment of the cells at three times intervals (12, 24 and 48 hours). The C-MYC gene expression presented in fold change (FC) compared to untreated cells. The expression of gene is presented in (table 5).

Table 5: C-MYC gene expression in HNO-97 cells treated with three different concentrations of nano-thymol, nano-DOX and their combination at three-time intervals (12,24 and 48):

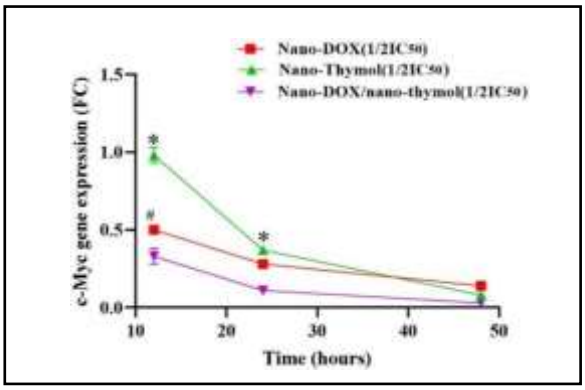
Duration	Drug	Concentration	Conc. (µmol/ml)	FC (C-MYC)	P-Value
12 hours	Untreated control cell			1.00	P<0.0001 (h.s)
	nano-DOX	½ IC50	0.304	0.51	
		IC50	0.607	0.35	
		2x IC50	1.214	0.15	
	nano- Thymol	½ IC50	0.474	0.98	
		IC50	0.948	0.58	
		2x IC50	1.90	0.41	
	nano-DOX/ nano-Thymol			0.33	
				0.24	
				0.12	
24 hours	Untreated control cell			1.00	P<0.0001 (h.s)
	nano-DOX	½ IC50	0.304	0.28	
		IC50	0.607	0.15	
		2x IC50	1.214	0.09	
	nano- Thymol	½ IC50	0.474	0.46	
		IC50	0.948	0.37	
		2x IC50	1.90	0.24	
				13.45	

	nano-DOX/ nano-Thymol			19.29	
				29.45	
48 hours	Untreated control cell			1.00	P=0.08 (n.s)
	nano-DOX	½ IC50	0.304	0.14	
		IC50	0.607	0.08	
		2x IC50	1.214	0.05	
	nano- Thymol	½ IC50	0.474	0.22	
		IC50	0.948	0.08	
		2x IC50	1.90	0.03	
	nano-DOX/ nano-Thymol			0.03	
				0.02	
				0.01	

The two-way ANOVA test was used to compare the mean value of C-MYC gene expression in HNO-97 cells treated with half IC50 dose of each drug individually at the three-time intervals. The XY plot was used to present the change in C-MYC expression versus time. The obtained results revealed a significant difference in the C-MYC gene expression in HNO-97 cells when treated with half IC50 of each drug at 12,24 and 48 hours. As detected by the two-way ANOVA test, a statistically significant difference was detected in the C-MYC expression at the different times of measurement.

As shown in figure 4, a significant decrease was detected in C-MYC gene expression in cells treated with all three drugs formulas when using the half IC50 with increase duration of incubation from 12 hours to 48 hours. At 12 and 24 hours, a significant difference ($p<0.001$) in the C-MYC gene expression was detected on comparing the gene expression in the cells treated with the combined nano-DOX/nano-thymol (mean: 0.33) and nano-DOX (mean:0.51). In addition, a significant difference was detected on comparing gene expression in treated cells with nano-thymol and the combined drugs ($p<0.001$).

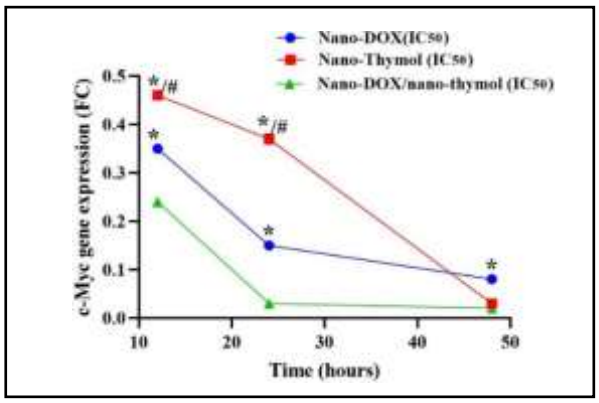
The C-MYC gene expression was significantly lower in cells treated with half IC50 of nano-thymol ($p<0.01$). No significant difference was detected on comparing the expression of C-MYC in HNO-97 cells treated with the three tested drugs at 48 hours ($p>0.05$).



*: statistical significance compared to the nano-DOX, #: statistical significance compared to the combined nano-DOX/nano-thymol.

Figure 4: XY plot presents the change in C-MYC gene expression in HNO-97 cells treated with half IC50 of nano-DOX, nano-Thymol and the combined nano- DOX/nano-thymol at 12, 24 and 48 hours.

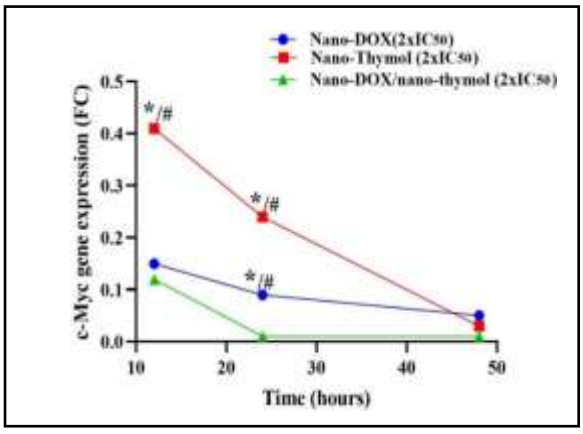
A statistically significant decrease was detected in C-MYC gene expression in cells treated with all three drugs when using the IC50 with increase duration of incubation from 12 hours to 48 hours. At 12 and 24 hours, a significant difference ($p < 0.001$) in the C-MYC gene expression was detected on comparing gene expression in the treated cells with the combined nano-DOX/nano-thymol (mean: 0.24), nano-DOX (mean: 0.35) and cells treated with nano-thymol (mean: 0.58). Similar results were obtained for the C-MYC gene expression at 24 hours. However, at 48 hours, no statistically significant difference was detected on comparing gene expression in the nano-thymol treated group compared to the group treated with combined drugs ($p = 0.08$). The results are presented in (figure 5).



*: statistical significance compared to the nano-DOX, #: statistical significance compared to the combined nano-DOX/nano-thymol.

Figure 5: XY plot presents the change in C-MYC gene expression in HNO-97 cells treated with IC50 of nano-DOX, nano-Thymol and the combined nano- DOX/nano-Thymol at 12, 4 and 48 hours.

A statistically significant decrease was detected in C-MYC gene expression in cells treated with all three drugs when using the double IC50 with increase duration of incubation from 12 hours to 48 hours. At 12 hours, a significant difference ($p < 0.05$) in the C-MYC gene expression was detected on comparing the combined nano-DOX/nano-thymol (mean: 0.12), nano-DOX (mean: 0.15) and cells treated with nano-thymol (mean: 0.41). The lowest C-MYC expression level in HNO-97 cells was detected in the group treated with combined nano-DOX/nano-thymol. However, no significant difference was detected on comparing the C-MYC gene expression in HNO-97 cells treated with either nano-DOX or nano-thymol for 12 hours. At 24 hours, a statistically significant difference was found on comparing the gene expression in HNO-97 cells treated with nano-DOX and nano-thymol separately compared to the combination of both drugs ($p < 0.01$), as well as between nano-DOX and nano-thymol. On the other hand, no significant difference was detected on comparing the C-MYC gene expression in HNO-97 cells after being treated for 48 hours with double IC50 of any of the tested drugs ($p > 0.05$). The results are presented in (figure 6).



*: statistical significance compared to the nano-DOX, #: statistical significance compared to the combined nano-DOX/nano-thymol.

Figure 6: XY plot presents the change in C-MYC gene expression in HNO- 97 cells treated with double IC50 of nano-DOX, nano-Thymol and the combined nano- DOX/nano-Thymol at 12, 24 and 48 hours.

Phytochemicals are organic substances derived from naturally occurring plants. They are mostly made up of compounds that are non-nutritive and are present in grains, vegetables and fruits (Tuorkey, 2015). The anti-cancer effect of phytochemicals is mostly mediated through the induction of cell-cycle arrest or apoptotic cell death (Zhang et al., 2019). Nanotherapies have been widely used to overcome the lack of selectivity of anticancer drugs, multi-drug resistance (MDR) and the low aqueous solubility of anticancer drugs (Jabir et al., 2012).

The combination of these nanomaterials in cancer drug design can achieve a balance between increasing efficacy and reducing the toxicity of drugs (Jin et al., 2020). In the present study, studying the cytotoxic effect, cell proliferation and apoptosis of nano-formulated thymol and nano-formulated DOX and

comparing their effect on oral squamous cell carcinoma cell line (HNO-97) as a model of HNC was performed. In addition, the synergy between nanoformulated thymol and nanoformulated DOX was investigated.

In the present study, MTT assay results showed that the drugs have a cytotoxic effect on cultured HNO-97 cells. The calculated for DOX and nano-DOX IC50 after 24 hours was 1.219 µmol/ml and 0.607 µmol/ml, respectively. Consequently, we calculated the half IC50 and double IC50, where the half IC50 and double IC50 of DOX was 0.6095 µmol/ml and 2.438 µmol/ml respectively, while the half IC50 and double IC50 of nano-DOX were 0.304 µmol/ml and 1.214 µmol/ml respectively.

After 12 hours, MTT assay revealed that nano-DOX had the highest viability of 65.2 at half IC50 and the lowest viability (32.5) at double IC50, while nano-thymol had the highest viability (74.6) at half IC50 and the lowest viability (39) at double IC50. The combination of both drugs had the highest viability (42.9) at half IC50 and the lowest viability (19.37) at double IC50.

After 24 hours, MTT assay revealed that nano-DOX had the highest viability (58.57) at half IC50 and the lowest viability (26.72) at double IC50, while nano-thymol had the highest viability (63.31) at half IC50 and the lowest viability (35.31) at double IC50. The combination of both drugs had the highest viability (30.21) at half IC50 and the lowest viability (15.52) at double IC50.

After 48 hours, MTT assay revealed that nano-DOX had the highest viability (50.96) at half IC50 and the lowest viability (21.19) at double IC50.while nano-thymol had the highest viability (61.58) at half IC50 and the lowest viability (23.66) at double IC50.The combination of both drugs had the highest viability (19.67) at half IC50 and the lowest viability (11.87) at double IC50.

These results are in agreement with the study conducted by Abbas et al. (2021), who performed a study comparing free DOX with LGN/- DOX microemulsion (Lignin-Stabilized Doxorubicin Microemulsion) and found that LGN/DOX microemulsion had a dose dependent inhibitory effect and a significant reduction in the number of MCF7, C152 and HUVEC cell lines compared with untreated cells.

Compared to control untreated HNO-97 cells, there was a concentration and duration-dependent decline in the viability percent among the three groups, as the group treated with half IC50 showed the highest viability percentage compared to the groups treated with IC50 and the double IC50, while the group treated with double IC50 showed the least viability percentage. Additionally, nano-DOX generally showed less cell viability compared to nano-thymol, while the combined drug formula showed the least cell viability (the highest cytotoxic effect), especially after 48 hr.

The cytotoxic properties of nano-Dox could be attributed to the ability of the nano-DOX to enter the cancer cells easily. A reduction in the dose of such a chemotherapeutic drug will definitely reduce the associated unfavourable side effects in OSCC patients. Although both DOX and nano-DOX in the current study reduced the viability of the HNO-97 cell line in a dose-dependent manner, the better outcomes of nano-DOX indicate a more effective cytotoxic anticancer effect. Several mechanisms might be responsible for this action, including the production of ROS and the induction of DNA damage through the inhibition of type II topoisomerases, which inhibit DNA and RNA synthesis. Also, it was found that DOX has the capacity to suppress the PI3K/Akt pathway, which is crucial in activating Hedgehog signalling proteins that are responsible for cancer cell proliferation, migration and metastasis (Smolensky et al., 2017).

Concerning C-MYC gene expression, the result of QRT-PCR revealed that the control HNO-97 cells showed a moderate amount of C-MYC gene expression. This may be related to the normal occurrence of limited proliferation in cell culture as a result of limited nutrition, toxic byproducts accumulation, cell-cell inhibition, growth factor deprivation and other stress inducers (Hsu et al. (1995); Miller and Zachary (2017)).

In the current study, QRT-PCR assessment of C-MYC expression in HNO-97 cells treated with ascending concentrations of nano-DOX, nano-thymol and their combination revealed that in each duration 12, 24 and 48 hours, a concentration dependent decrease in the level of gene expression with increasing the time, was observed.

This data was confirmed by the statistical results which showed a statistically significant difference in gene expression on comparing the results of the three drugs. The lowest C-MYC gene expression level was seen in the group of HNO-97 cells treated with combined drugs, while the highest C-MYC gene expression level was seen in the group of HNO-97 cells treated with nano-thymol.

A significant decrease was detected in C-MYC gene expression in cells treated with any of the drugs when using the half IC50 with increase in duration of incubation from 12 to 48 hours.

On the other hand, when using IC50 at 12 hours, a statistically significant difference ($p < 0.001$) was detected on comparing the C-MYC gene expression in HNO-97 treated cells with the combined drugs, nano-DOX and nano-thymol separately. Similar results were obtained for the C-MYC gene expression at 24 hours. however, at 48 hours, a statistically significant difference was detected on comparing the gene expression in the groups treated with nano-thymol and the combined drugs ($p \leq 0.05$).

Regarding gene expression using double IC50 at 12hours, a statistically significant difference ($p < 0.05$) was detected on comparing the C-MYC gene expression in the cells treated with the combined drugs, nano-DOX and nano-thymol separately. However, no statistically significant difference was detected on comparing the gene expression in cells treated with either nano-DOX or nano-thymol for 12 hours. At 24 hours, a statistically significant difference was obtained on comparing the gene expression in cells treated with nano-DOX and nano-thymol separately compared to the combination of both drugs ($p < 0.01$). On the other hand, no statistically significant difference was detected on comparing the C-MYC gene expression in HNO-97 cells after being treated for 48 hours with double IC50 of any of three drugs ($p > 0.05$).

Our results showed that the combined therapy of nano-DOX and nano-thymol had the lowest gene expression, this could be explained by the highest cytotoxic effect of the drug combination on HNO-97 cell line as proved by reduction of C-MYC gene expression.

4. Conclusion:

According to the obtained data, it could be concluded that nano-thymol alone has an proliferation-inhibiting effect on HNO-97 cells. However, combined nano-DOX and nano-thymol have a more powerful inhibiting effect. Taken together, combined nano-DOX and nano-thymol may constitute a promising treatment modality for HNC that can allow for minimizing drug dose with consequent reduction of the grave concomitant side effects.

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