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Original Research Article

Evaluation of the Effects of Thymol Nanoemulsion on Free Thiol Levels and the Expression of Bcl2 and Bax Genes in the Hela Human Cervical Cancer Cell Line

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Abstract: Thymol, a natural monoterpenoid phenol commonly found in thyme extract, possesses numerous therapeutic properties, including antioxidant, antimicrobial, and anti-inflammatory activities. This study aimed to develop a novel approach for cervical cancer treatment by evaluating the effects of a thymol-loaded nanoemulsion on the human cervical cancer cell line, HeLa. The research focused on three key biological markers: the expression of the pro-apoptotic gene *BAX*, the anti-apoptotic gene *BCL2*, which are indicative of the cell's antioxidant capacity. A stable thymol nanoemulsion was synthesized and characterized. Dynamic Light Scattering (DLS) analysis revealed a uniform particle size of 72.6 nm. The cytotoxic effects of the nanoemulsion on HeLa cells were assessed using an MTT assay, which determined the half-maximal inhibitory concentration (IC50) to be 111.96 µM. HeLa cells were then treated with this IC50 concentration to investigate the molecular mechanisms of action. Real-Time Quantitative PCR (RT-qPCR) analysis demonstrated that the thymol nanoemulsion significantly modulated the expression of key apoptotic genes. The expression of the pro-apoptotic *BAX* gene was upregulated by 1.7-fold, while the expression of the anti-apoptotic *BCL2* gene was downregulated to 0.71-fold of the control level. This shift resulted in a significant increase in the BAX/BCL2 ratio, favoring apoptosis. Moreover, a colorimetric assay revealed that treatment with the thymol nanoemulsion caused a significant reduction in the total free thiol content in HeLa cells, from 751.66 µM in control cells to 531.66 µM in treated cells, indicating an induction of oxidative stress. In conclusion, the results of this study suggest that the thymol nanoemulsion effectively reduces the viability of cervical cancer cells by inducing apoptosis through a dual mechanism: (1) activating the intrinsic apoptotic pathway by altering the BAX/BCL2 balance and (2) disrupting the cellular redox homeostasis by depleting free thiol groups. These findings highlight the potential of thymol nanoemulsion as an effective therapeutic agent for cervical cancer.

Keywords: Cervical Cancer, Hela, Thymol, Nanoemulsion, Apoptosis, BAX, BCL2, Oxidative Stress, Free Thiols.

INTRODUCTION

It is estimated that cervical cancer is one of the leading types of cancers in females globally; it constitutes the fourth most prevalent type of cancer amongst females worldwide, making it a serious global health challenge, particularly within resource-constrained nations, due to the lack of access to vaccine and screening programs. High-risk Human Papilloma Virus (HPV) infection is viewed as the main cause of cervical carcinogenesis. The process of programmed cell death known as apoptosis is crucial for the body's biological functions for maintaining cellular homeostasis and the removal of defective cells. Cancer cells are defined by their capability to avoid apoptosis, leading to unlimited multiplication. Apoptosis is controlled by several molecules from the BCL-2 protein family, such as BAX and BCL2, which are pro-apoptotic and anti-apoptotic proteins, respectively. The relationship between these two proteins, specifically their BAX/BCL2 ratio, is vital for deciding the fate of the cells. Thus, the use of apoptosis pathways can be considered a strategy in treating cancers. In addition, cancer cells have been associated with oxidative stress. This is because cancer cells can

survive due to the existence of antioxidant systems that include free thiols such as glutathione. In this regard, low amounts of free thiols lead to higher oxidative stress, leading to the death of cancer cells. Therefore, the number of free thiols indicates the performance of cellular antioxidants. Thymol has several biological activities and acts as an essential compound of thyme essential oil. Thymol exhibits several biological activities, which include antimicrobial, anti-inflammatory, antioxidative, and anticancer activities. However, the main barrier hindering the absorption of thymol is its hydrophobicity. Nanoemulsion can play a significant role in overcoming this problem. In turn, nanoemulsion will enhance the solubility, stability, cell penetration ability, and efficacy of the thymol drug. Given this information, the current study aims to investigate the feasibility of thymol nanoemulsion to exhibit anticancer activity on HeLa cervical cancer cell line. The specific objectives of the study are to evaluate the cytotoxic effect of thymol nanoemulsion, impact of thymol on the expression levels of apoptosis genes (BAX and BCL2), and thymol-induced oxidative stress (total free thiol content).

MATERIALS AND METHODS

Materials and Equipment

A comprehensive list of materials was utilized, including thymol, sesame oil, Tween 80, and SPAN 80 for nanoemulsion synthesis. For cell culture and biological assays, HeLa cells, RPMI medium, FBS, antibiotics, MTT reagent, and DMSO were used. RNA extraction and RT-qPCR involved TRIzol, chloroform, a cDNA synthesis kit, and SYBR Green master mix. The total thiol assay was performed using a commercial kit (Kia Zist, Iran). Key equipment included a probe sonicator, DLS analyzer, incubator, microplate reader, and a real-time PCR system.

Synthesis of Thymol Nanoemulsion

A thymol-loaded nanoemulsion was prepared using an oil-in-water (O/W) spontaneous emulsification method followed by high-energy homogenization.

1. **Oil Phase:** Thymol (10 mg) was dissolved in sesame oil (0.15 g).
2. **Surfactant/Co-surfactant:** The oil phase was mixed with the surfactant SPAN 80 (0.31 g) and co-surfactant Tween 80 (1.49 g), along with ethanol (0.50 g).
3. **Emulsification:** Deionized water (2.55 g) was added dropwise to the mixture under constant magnetic stirring for 30 minutes to form a coarse pre-emulsion.
4. **Homogenization:** The pre-emulsion was sonicated to reduce droplet size and create a stable, translucent nanoemulsion.

Physicochemical Characterization

- **Particle Size (DLS):** The mean hydrodynamic diameter (d50) and size distribution of the nanoemulsion droplets were measured using Dynamic Light Scattering.
- **Encapsulation Efficiency (EE):** The EE was determined by separating the free thymol from the encapsulated thymol via centrifugation. The concentration of free thymol in the supernatant was quantified by spectrophotometry at its λmax (273 nm) against a standard curve. The EE was calculated as:

Equation 3.1: Encapsulation Efficiency

EE (%) = [(Total Thymol - Free Thymol) / Total Thymol] × 100 (3.1)

Cell Line and Culture Conditions

The human cervical adenocarcinoma cell line HeLa was obtained from the National Center for Genetic and Biological Resources of Iran. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin and maintained in a humidified incubator at 37°C with 5% CO_2.

In Vitro Cytotoxicity Assessment (MTT Assay)

The MTT assay was performed to determine the IC50 of the thymol nanoemulsion. HeLa cells were seeded in 96-well plates (10,000 cells/well). After 24 hours, they were treated with various concentrations of the nanoemulsion (5, 10, 20, 40, 80, 150, and 300 µM) for 24 hours. Cell viability was assessed by measuring the absorbance of the dissolved formazan product at 570 nm. The IC50 was calculated from the resulting dose-response curve.

Gene Expression Analysis via RT-qPCR

HeLa cells were treated with the IC50 concentration of the thymol nanoemulsion. Total RNA was extracted from treated and untreated cells using the TRIzol-chloroform method. One microgram of high-quality RNA was then reverse transcribed into cDNA. The mRNA expression levels of *BAX* and *BCL2* were quantified, using *GAPDH* as the reference gene.

Table 3.1: Primer Sequences for RT-qPCR

Gene Name	Sequence (5' → 3')	Type
BAX	TCAGGATGCGTCCACCAAGAAG	Forward
	TGTGTCCACGGCGGCAATCATC	Reverse
BCL2	ATCGCCCTGTGGATGACTGAGT	Forward
	GCCAGGAGAAATCAAACAGAGGC	Reverse
GAPDH	GTCTCCTCTGACTTCAACAGCG	Forward
	ACCACCCTGTTGCTGTAGCCAA	Reverse

The qPCR reaction was set up using SYBR Green master mix. The relative gene expression was calculated using the $2^{-}\Delta\Delta CT$ method.

Equation 3.2: The $2^{-}\Delta\Delta CT$ Method

Fold Change = **2 ^ -[(CT_Target - CT_GAPDH)_Treated - (CT_Target - CT_GAPDH) Control]** (3.2)

Measurement of Free Thiol Group Levels

The total free thiol content in cell lysates was measured using a commercial kit based on the Ellman's method. This colorimetric assay relies on the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with free sulfhydryl (-SH) groups to produce a yellow-colored compound, 2-nitro-5-thiobenzoate (TNB), which is quantified by measuring its absorbance at 405 nm. The concentration of thiols in the samples was determined by comparing their absorbance to a standard curve prepared using a known concentration of a thiol standard (e.g., GSH). Cell lysates from both treated and untreated HeLa cells were prepared and analyzed according to the manufacturer's protocol.

Results and Data Analysis

Characterization of the Thymol Nanoemulsion

- **Physical Appearance and Stability:** The synthesized formulation was a stable, translucent, yellowish liquid, showing no signs of phase separation or precipitation after preparation, indicating successful emulsification.
- **Particle Size and Homogeneity:** DLS analysis was performed to determine the size distribution of the nanoemulsion droplets. The results showed a mean particle diameter (d50) of 72.6 nm. The DLS plot exhibited a single, sharp peak, confirming the presence of a monodisperse and homogeneous population of nanoparticles. This uniform and small size is optimal for enhancing bioavailability and cellular interaction.
- **Encapsulation Efficiency:** The efficiency of encapsulating thymol within the nanoemulsion was calculated to be **77%**. This high value confirms that the formulation method was effective in loading a significant amount of the active compound, which is crucial for delivering a therapeutic dose.

Cytotoxic Effects on the HeLa cell Line

The MTT assay was used to assess the impact of the thymol nanoemulsion on the viability of HeLa cells. The cells were treated with a range of concentrations for 24 hours.

Table 4.1: Cell Viability of HeLa Cells after 24h Treatment

Concentration (µM)	Mean Cell Viability (%) ± SD
0 (Control)	100 ± 0.0
5	95.2 ± 4.5
10	88.1 ± 5.1
20	79.5 ± 4.8
40	68.3 ± 6.2
80	57.6 ± 5.5
150	44.0 ± 4.9
300	21.7 ± 3.8

The results demonstrate a clear dose-dependent cytotoxic effect. As the concentration of the thymol nanoemulsion increased, the viability of the HeLa cells decreased significantly. A non-linear regression analysis of the dose-response curve yielded an IC50 value of 111.96 µM. This concentration, which causes 50% inhibition of cell growth, was selected for all subsequent molecular experiments.

Modulation of Apoptotic Gene Expression (BAX and BCL2)

To investigate the molecular mechanism behind the observed cytotoxicity, the expression levels of the key apoptotic regulatory genes, *BAX* (pro-apoptotic) and *BCL2* (anti-apoptotic), were quantified using RT-qPCR. HeLa cells were treated with the IC50 concentration (111.96 µM) of the thymol nanoemulsion for 24 hours.

The results, normalized to the *GAPDH* reference gene, revealed a significant shift in the expression of these genes, favoring an apoptotic state:

- **BAX Expression:** The expression of the pro-apoptotic *BAX* gene was increased by 1.7-fold in the treated cells compared to the untreated control group (p=0.01).
- **BCL2 Expression:** The expression of the anti-apoptotic *BCL2* gene was decreased to 0.71-fold of the control level, representing a 29% reduction (p=0.01).

Analysis of the BAX/BCL2 Ratio:
The ratio of BAX to BCL2 expression is a more potent indicator of the cellular commitment to apoptosis than the individual gene levels.

- **Control Group:** BAX/BCL2 Ratio = 1.0 / 1.0 = 1.0
- Treated Group: BAX/BCL2 Ratio = 1.7 / 0.71 = 2.39

Treatment with the thymol nanoemulsion caused a 2.39-fold increase in the BAX/BCL2 ratio. This dramatic shift strongly indicates that the nanoemulsion pushes the cellular balance towards mitochondrial-mediated apoptosis.

Melt Curve Analysis:
Melt curve analysis for *BAX*, *BCL2*, and *GAPDH* each produced a single, sharp peak, confirming the high specificity of the amplified products and the absence of non-specific amplification or primer-dimers. This validates the accuracy of the RT-qPCR results.

Impact on Cellular Free Thiol Levels
To determine if the thymol nanoemulsion induced oxidative stress, the total level of free thiol groups in the cell lysates was measured.

Table 4.2: Free Thiol Levels in Treated vs. Control HeLa Cells

Treatment Group	Mean Free Thiol Concentration (µM) ± SD
Control (Untreated)	751.66 ± 35.4
Thymol Nanoemulsion (111.96 µM)	531.66 ± 28.9

The results show a significant decrease in the free thiol content in the cells treated with the thymol nanoemulsion compared to the control group ($p<0.05$). The level of free thiols, a primary indicator of the cell's antioxidant capacity, was reduced by approximately 29.3%.

DISCUSSION

This study successfully demonstrated that a thymol-loaded nanoemulsion possesses potent anticancer activity against the HeLa cervical cancer cell line. The findings provide a clear, dual-mechanism explanation for this activity: the induction of apoptosis via modulation of the BCL-2 family proteins and the promotion of oxidative stress via depletion of cellular antioxidants.

The synthesized nanoemulsion, with a particle size of 72.6 nm and an encapsulation efficiency of 77%, proved to be an effective delivery vehicle for the hydrophobic thymol. The small, uniform particle size likely contributed to the robust cytotoxic response observed in the MTT assay (IC50 = 111.96 µM) by facilitating enhanced cellular uptake and bioavailability.

The core of this study lies in its mechanistic investigation. The observed 1.7-fold increase in *BAX* expression and the simultaneous 29% decrease in *BCL2* expression are highly significant. This alteration dramatically shifts the cellular balance, increasing the BAX/BCL2 ratio by 2.39-fold. This is a classic hallmark of the activation of the intrinsic apoptotic pathway. By increasing the prevalence of the pro-apoptotic BAX protein and reducing the protective anti-apoptotic BCL-2 protein, the thymol nanoemulsion effectively lowers the threshold for mitochondrial outer membrane permeabilization (MOMP), committing the cancer cells to a path of programmed cell death. These results are in strong agreement with previous studies, such as that by Zeng *et al*., (2020), which also implicated the BAX/BCL-2 axis in thymol-induced apoptosis in colorectal cancer.

Furthermore, this study uniquely connects thymol's action to the induction of oxidative stress in cervical cancer cells. The significant 29.3% reduction in free thiol levels is a direct indicator that the cell's primary antioxidant defense system is being overwhelmed. The depletion of glutathione and other thiol-containing molecules leaves the cell vulnerable to damage from ROS. This state of heightened oxidative stress is itself a powerful trigger for apoptosis, often acting

upstream of the mitochondrial pathway. It is plausible that the induced oxidative stress is one of the initial signals that leads to the observed changes in BAX and BCL2 expression.

Therefore, the thymol nanoemulsion appears to attack HeLa cells on two synergistic fronts: it directly modulates the genetic machinery of apoptosis while simultaneously creating a pro-oxidant intracellular environment that makes cell death inevitable. This dual mechanism is highly desirable in an anticancer agent, as it may be more effective and less prone to resistance than agents that target only a single pathway.

CONCLUSION

Based on the results obtained in this thesis, the thymol nanoemulsion is an effective agent for reducing the survival of cervical cancer cells. The molecular investigations revealed that the activation of the intrinsic apoptotic pathway, evidenced by a significant increase in the BAX/BCL2 ratio, and the disruption of the cellular oxidant-antioxidant balance, evidenced by a marked decrease in free thiol levels, are the primary mechanisms underlying this effect. Therefore, thymol, particularly when delivered via an advanced nanoemulsion system, represents a promising and effective compound for further investigation in the fight against cervical cancer.

Recommendations

1. Assess the anticancer potential of thymol nanoemulsion on various cancer cells.
2. Examine other cellular mechanisms associated with apoptosis, angiogenesis, and metastasis.
3. Conduct animal experiments to determine the efficacy and bioavailability of thymol nanoemulsion.
4. Study the combined effect of thymol nanoemulsion with other existing chemotherapeutics like cisplatin and paclitaxel.

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