Cytotoxic, Cytogenetics and Immunomodulatory Effects of Thymol from *Thymus vulgaris* on Cancer and Normal Cell Lines in Vitro and in Vivo

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The abstract is in Arabic:

The abstract is: استخلص مركب الثامية من الأوراق الحافية لنبات الزعتر *thymus vulgaris* باستخدام نبات الزعتر وكريستال الصوديوم المائي بطرقية كروموتروكرافيا السائل عالي الكفاءة بالاعتماد على زمن الاحتباس لمركب الثامية القياسي ومستخلص الزعتر. استعملت تقنيات تراكيزية من مركب الثامية المستخلص (244,122,61,30.5,15) نانوغرام/مل لدراسة فعاليتها في خزن حلويات سرطانيين للإنسان (خلايا سرطان عنق الرحم البشري, Hep, خلايا سرطان الحنجورة البشري, HeLa) حيث حضنت الخلايا الخلوية بدرجة منوية لمدة 72 ساعة، حيث بيئة النتائج اختلاف حساسية الخلايا الخلوية تجاه السمية الخلوية لمركب الثامية الذي أظهر تأثيرًا محسوسًا في جميع التراكيز المستعملة تجاه الخلايا الخلوية. بينت النتائج، السمية العالية(87.25%) لمركب الثامية تجاه سرطان عنق الرحم البشري وسمية متوسطة (51.45%) تجاه سرطان الحنجورة البشري وسمية قليلة (20.94%). Tجاه الخلايا الطبيعية لجنين الجذيف Ref، ذُكرت خطة عالية لمركب الثامية عند التركيز 30.5 نانوغرام/مل. بصورة عامة، ملاحظة ان بقاء الخلايا الخلوية المعدلة على جميع المادة المستخدمة. بالإضافة إلى أن خلايا سرطان عنق الرحم البشري أظهرت تأثيرًا عاليًا تجاه مركب الثامية مقارنة مع الخلايا الخلوية الأخرى. أظهرت النتائج ارتباط الثانياً التأثير السمي الوراثي لمركب الثامية عند حققه داخل البروتين في الفئران المختبرة بتركيز (2.15, 2, 5) ملغم/كيلوغرام من وزن الجسم وتتمثل بمعامل الانقسام الخلوية (MI) وبين النتائج اختلافات معنوية (0.05 > P) بين التركيزات العالية لمركب الثامية ومعامل السحبة إذ أدى مركب الثامية انخفاضًا معنويًا في معامل الانقسام الخلوية (MI) في التركيز (5) ميليغرام/كيلوغرام من وزن
Cytotoxic, Cytogenetics and Immunomodulatory Effects of Thymol from *Thymus vulgaris* on Cancer and Normal Cell Lines in Vitro and in Vivo

Reema

الجسم مقارنة بمعامل السيطرة. بينت النتائج عدم وجود تأثير لمركب الثималь في تحفيز كريات الدم البيضاء مقارنة مع معامل السيطرة.

**ABSTRACT**

Thymol was extracted from leaves of *thymus vulgaris*, by using NaOH and diethylether. High performance liquid chromatography (HPLC) carried out to identify thymol in the aliquaut extract of thymus vulgaris depended on retention time of thyme extract and standard of thymol. The cytotoxic activity of thymol against two human cancer cell lines HeLa (Human epithelial cervical cancer), Hep (Human larynx epidermoid carcinoma) and one normal cell line Ref (Rat embryonic fibroblast) were estimated in vitro. The grown cells in 96 multi well plates were treated with different concentrations of thymol (15, 30.5, and 61,122,244) ng/ml and incubated at 37c° additional period 72hr. Cancer and normal cell lines elicited various degrees of sensitivity to the cytotoxic effect of thymol .thymol exhibited significant differences (P<0.05) at all concentrations against three human cell lines. The results showed highest toxicity (87.25%) 0f thymol on HeLa cell line, a moderate cytotoxicity (51.45%) on Hep-2, and slight toxicity (20.94%) on normal cell line (Ref), furthermore the highest toxicity was recorded at concentration 30.5 ng/ml for all cell lines. In general, a dose-dependent decrease the survival of the three cell lines was observed, in additional to He La cell line was showed higher sensitivity against thymol than the other cell lines. Also the study was conducted to investigate the effect of thymol intraperitoneally administration in mice at doses of (1.25, 2.5, 5) mg/kg body weight by determining the cytogenetic analysis represented by (mitotic index). The results showed significant differences (P<0.05) between highly doses of thymol and untreated control. Thymol decreased the mitotic index (MI) in high concentrations (5.0) mg/kg when compared with control. The results a showed no effect of thymol in stimulation of leukocytes compared with the negative control.
INTRODUCTION

Thyme has a wide spectrum of pharmacological properties. In fact, it has been reported that the essential oil of *Thymus vulgaris*, the most studied species of thyme, has antibacterial, antifungal and antioxidant activities (1, 2). Additionally, dietary supplementation with thyme oil maintained significantly higher superoxide dismutase and glutathione peroxidase activities and total antioxidant status (3,4,5). At non-toxic concentrations, thyme extract was also identified as a natural antimutagen with the possibility of enhancement of error-free DNA repair (6).

Furthermore, thyme extract has been shown to induce a considerable stimulation of leucopoiesis and also an elevation of thrombocyte count in blood (7). In addition, there is evidence that thymol, a constituent of the essential oil, could be involved in the stimulation of active proliferation of pulp fibroblasts (8).

The use of medicinal plants for the treatment of diseases is as old as mankind. Essential oils and their components are becoming increasingly popular as naturally occurring bioactive agents (9). Thyme, the most popular medicinal plant in Morocco, has been used in traditional medicine for thousands of years in African and European countries, particularly in the Mediterranean basin (10).

Recent investigations suggested that thyme extracts are anti-mutagenic due to potent antioxidant properties of thymol (11). Thyme leaf extracts and its phenolic compounds thymol and carvacrol contain substance induces enzymes of both phase I and phase II biotransformation of xenobiotic substance in the mice liver (12). Another study conducted by (13) showed that phenolic chemotype (thymol and carvacrol) possesses stronger anti-oxidant properties than the non-phenolic linal oil in *Thymus vulgaris* (14).

Systemic therapy with cytotoxic drugs is the basis of most effective treatments of disseminated cancers. However, the tumors responses to chemotherapeutic regimens vary, and failures are frequent owing to the emergence of drug resistance. Additionally, the induction of tumor cell resistance to one drug often results in coordinate resistance to other structurally and functionally unrelated drugs, and this defines the multiple drug resistance phenotypes (15).

It is expected that drug resistance may be circumvented by the rational design of new non-cross-resistant agents, by novel delivery or
combinations of known drugs and by the development of other treatments that might increase the activity of - or reverse resistance to - known antineoplastic agents (16).

The present study was planned with the aim to evaluate the antitumor potentials of thymol from leaves of thymus vulgaris in vitro by using two cancer Hela (Human epithelial cervical cancer), Hep (Human larynx epidermoid carcinoma) and one normal Ref (Rat embryonic fibroblast) cell lines and estimate the cytogenetic analysis of mitotic index (MI) in mice bone marrow cells, furthermore its immunomodulation effect on leukocytes.

**MATERIALS AND METHODS**

All the chemicals were obtained from Sigma Chemical Co. (USA) and BDH abundances for each of the populations (England), and the fresh plant was obtained from Jordan.

**Extraction of thymol from Thyme leaves:**

Air dried leaves of thymus vulgaris were grinded, then 15g of leaves powder were extracted with 300ml of 5% NaOH, then filtrated with wattman no. 1. The filtrate was mixed with diethylether(2:1)(v:v) in separating funnel to removed non phenolic constitutes and upper layer was acidified with concentrated acid (HCL) pH 5.7, then the product obtained by cooled the solution in -20c and collected crystallized granules from internal walls of flask to use in further experiments (17).

**Identification of thymol by High performance liquid chromatographic quantization (HPLC):**

The thyme leaves extract and standard compound were dissolved in DMSO; both samples were analyzed by HPLC separation with column Luna 5u C18, 250 × 4.6) mm internal diameter (id). The mobile phase was acetonitrile (ACN) 100% with a flow rate of 0.5 ml/min. Injection volume for sample and standard solution was 10 μl. The pH was adjusted to 3.5. The detection occurred at UV light at 305 nm wave length.

**1- Cell growth and cytotoxicity assays:**

**- Cell culture:**

HeLa (Human epithelial cervical cancer), Hep (Human larynx epidermoid carcinoma) and Ref (Rat embryonic fibroblast) cell lines were obtained from...
Iraq Center for Cancer and Medical Genetics Research (ICCMGR)–Almustansiriya University. cells were cultured in 15 ml of RPMI-1640 media containing 10% Fcs in T-25 tissue culture flask and incubated at 37c for 24-48 hr.to complete confluent monolayer by changing the media with a new fresh medium daily.

-Cytotoxicity assay:
Cells growth were detected from the surface of flask and collected by trypsin /EDTA solution.
The trypsin activity was stopped by adding fresh media, then 200µl of cell suspension seeded at required density (1x10 cell/ml) in 96 micro-plates, and incubated for 24hr. in humidified atmosphere supplement with 5% at 37c°. The cells were treated with 200µl of five concentrations (15, 30.5, 61,122,244) ng/ml prepared in DMSO: SFM of thymol for additional period 72hr. (in tripicales).
After the end incubation periods, the procedure done according to (18,19).

2- Experimental Animals:
twenty male albino mice purchased from the Biotechnology Research Center/ AL-Nahrain University, were used in this study and their ages were ranged between (8-12) weeks and weighting range from(20-25) gm, maintained in controlled animal house at 25c, 12 hr. artificial light/12hr. dark. Before carrying out experiments, the animals were kept for 5 days prior to dosing in animal house of biology department –Baghdad university to allow for their acclimatization for the laboratory conditions. The were divided into 4 groups, and each group was putted in a separate plastic cage. The animals were fed with a suitable quantity of water and complete diet.

Administration of Experimental Animals:
The groups of animals used in this experiment were injected intraperitoneally (I.P) with one dose only of thymol concentrations(1.25,2.5,5)mg/Kg B.W. sacrificed after 24h.

Group1: Negative control, injected with (0.25ml) of DMSO.
Group 2: Animal in this group injected I.P with (0.25 ml) of thymol (1.25mg /Kg) B.W.
Group 3: Animal in this group injected .IP with (0.25 ml) of thymol (2.5mg /Kg) B.W.
Group 4: Animal in this group injected i.p with (0.25 ml) of thymol (5mg/Kg) B.W.

Chromosomal preparation from somatic cells of the mouse bone marrow:
All this experiment was done according to (20).

3- Immunomodulating effect of thymol:
Immunization procedure:
This procedure was done according to the method described by (21)
Five animals unless control was injected with 1 mg/ml of thymol intraperitoneally, after one week, each animal was injected with the same dose. The animals were killed after one week of the last dose. Blood smear was done for each animal to study the blood picture (Lymphocyte, Monocyte, Granulocyte), after stained by Giemsa stain.

The percent of leukocytes calculation:
The percent of leukocytes determined in microscopically examination for animals were compared to control group using the following formula (22), (L= leukocytes):

\[
\frac{\text{L ratio in experiment}}{\text{L ratio in control}} \times 100
\]

Statistical Analysis:
Statistical analyses were done using SPSS (version 17) program. Mean and standard deviation were descriptive measures of quantitative data using the analysis of variance test (ANOVA) for independent samples. P-value, \( p<0.05 \) were considered significant test (23).

RESULTS AND DISCUSION
The HPLC was used to detect the presence of thymol in the leaves extract. The result showed a retention time of the standard was 3.309 min and 3.292 min for the sample. Figure (1-A, 1-B). the results gave a white crystals with aromatic odor.
Fig. – 1: Chromatographic resolution by HPLC of thymol standard (A) and thymol extracted from leaves of thymus vulgaris (B), in column Luna 5u C_{18} by mobile phase acetonitrile (ACN) 100% with flow rate 0.5 min/ml, 305nm.

1- Cytotoxicity assays:
To investigate the effect of thymol on two cancer (HeLa, Hep) cell lines and normal cell line (Ref), HeLa, Hep and Ref, the cell cultures exposed to different concentrations of thymol.
The results showed a significant differences (p<0.05) in percentage of inhibition on Hela cell line dependet on concentration of thymol ,thus the percentage of inhibition ranged from(87.25-74.06)% , and the concentration ranged from(30.5-244)ng/ml, the highest inhibition 87.25% recorded at concentration 30.5 ng/ml after 72hr of exposure.(figure 2)

The results showed a significant differences (p<0.05) in percentage of inhibition on Hep cell line dependent on concentration of thymol ,thus the percentage of inhibition ranged from(51.45-28.94)% , and the concentration ranged from(30.5-244)ng/ml, the highest inhibition 51.45% recorded at concentration 30.5 ng/ml after 72hr of exposure.(figure 3)

The results showed a significant differences (p<0.05) in percentage of inhibition on Ref cell line dependet on concentration of thymol ,thus the percentage of inhibition ranged from(20.94-10.20)% , and the concentration ranged from(30.5-244)ng/ml, the highest inhibition 20.94% recorded at concentration 30.5 ng/ml after 72hr of exposure.(figure 4)

This results deal with (24) that thymol was inhibit the growth of HeLa cell line.

In general, a dose-dependent decrease in survival of the three tumor and normal cell lines was observed. However, thymol exhibited stronger cytotoxicity at concentration 30.5 ng/ml towards three human cell lines. The inhibition effect of thymol on Ref cell in significant manner lower than other cell line may be due to high cytotoxicity of thymol toward cancer (HeLa, Hep) cell line than normal cell line (Ref).

This results were came in agreement with (25) that thyme essential oil, which contains carvacrol and thymol, as the major components have an important in vitro cytotoxic activity against tumor cells .The molecular mechanism of the observed cytotoxicity is unknown, but owing to their lipophilic nature, plant volatile compounds appear to accumulate in the cell membrane and increase its permeability, resulting in leakage of enzymes and metabolites (26,27).
Fig. -2: Percentage of Cytotoxicity represented by inhibition rate (IR) in HeLa cell line treated with different concentrations of thymol.

Fig. -3: Percentage of Cytotoxicity represented by inhibition rate (IR) in Hep cell line treated with different concentrations of thymol.

Fig. -4: Percentage of Cytotoxicity represented by inhibition rate (IR) in Ref cell line treated with different concentrations of thymol.
2- Cytogenetic analysis of thymol on mitotic index:

The effect of thymol administrated (I.P) in three concentration (1.25, 2.5, 5) mg/kg B.W. in animal groups were studies by estimating the mitotic index in mice bone marrow cells.

In Table (1) shows significant differences (P<0.05) in MI of bone marrow cells between animal groups depended on doses of thymol in comparison to negative control animals.

Under normal experimental conditions, white mice had a mitotic index of (49.92) % in their bone marrow cells (table 1) this considered as a negative control. Table (1) was showed that low-dose of thymol don’t caused a significant reduction (p<0.05) in MI (1.25 and 2.5) mg/kg B.W. (49.61% and 48.86%) respectively, while high dose have shown significant reduction in MI (5.0) mg/kg B.W. (47.35%) in comparison with negative control. These results were came in agreement with (28) that Thymol decreased the MI at the higher concentration without dose-dependent effect. The present work demonstrated the bad effect of this spice when used in large quantities, induced DNA damage (29) and inhibit cell proliferation (30) and this may be due to the poisonous effect of aromatic compound including phenolic compounds and terpene-phenolic derivatives which are poisonous. This conclusion is in consistence with those offered by (31).

Table -1: Cytogenetic effect of thyme extract in comparison with control (0.0) on mouse bone marrow cell.

<table>
<thead>
<tr>
<th>Concentration of thyme extract mg/kg</th>
<th>Mitotic index (mean±Std. deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>49.92±0.096</td>
</tr>
<tr>
<td>1.25</td>
<td>49.61±0.204</td>
</tr>
<tr>
<td>2.5</td>
<td>48.86±0.357</td>
</tr>
<tr>
<td>5.0</td>
<td>47.35±0.294</td>
</tr>
</tbody>
</table>

Significant different (P< 0.05) .Each value represents Mean± standard deviation

3- Immunomodulation examination:

The results in vivo procedure were showed; no effects of thyme extract were seen in stimulation of leukocytes compared with the control. These
results were in agreement with (32) that no effects were seen in the thymus, spleen, lymph nodes, white cell counts, red cell counts, haemoglobin counts, or hematocrits following the dosing of rats with 1000 or 10000mg/kg of food grade thymol for 19 weeks administration.

It can be concluded that thymol have strong antitumor activity against HeLa cell line and decreased mitotic index at the higher concentration without dose-dependent effect; also the results showed no effect of thymol in stimulation of leukocytes compared with the negative control.

**REFERENCES**


7- Von Ardenne, M. and Reitnauer, P.G.” The elevation of the leukocyte and thrombocyte counts produced by a thyme extract in the peripheral blood as compared to that caused by 2-cyanoethylurea”. *Pharmazie*; 36: 703-705(1981).


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