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Studying the effect of thymol-containing Nano emulsion extracted from thyme on angiogenesis controlling genes VEGF and VEGFR in a human liver cancer cell line

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Abstract

Thyme oil contains thymol (2-isopropyl-5-methylphenol), a naturally occurring, colorless crystalline phenolic compound with a characteristic odor. It is considered an antioxidant. Thymol has low solubility in water and good solubility in oil. Thymol nanoemulsion, dissolved in sesame oil, was prepared by converting it into nanodroplets. From a biological perspective, biological surfaces can effectively adsorb nanoemulsion droplets for more efficient biological activities. Nanoemulsions are spherical particles that act as a carrier for drug molecules, and range in size from 10-1000 nanometers. Because of the anticancer effect of thymol, it can be used as a suitable treatment to prevent angiogenesis in hepatocellular carcinoma. performed a cytotoxicity investigation using the MTT assay, in which a human hepatocellular carcinoma cell line was treated with different concentrations of thymol nanoemulsion. The results indicated that as the concentration increased, cell viability decreased. RNA was then extracted and C-DNA was synthesized. The expression levels of VEGFR and VEGF genes in nanothymol- and free-thymol-treated cells and in untreated cells were then examined. The results showed that the expression of this gene in cells treated with free thymol decreased by 5.33 times compared to untreated cells, but when the cells were treated with nanothymol, the level of gene expression decreased by about 10.02 times.

Keywords: Hepatocellular carcinoma, Nanoemulsion, Thymol, Tumors, VEGF, VEGFR.

Introduction

Thyme is a perennial aromatic plant grown all over the world from the Origanum family[1]. It is considered an antioxidant and antimicrobial. Eating thyme before and during meals protects against toxins and has been used as a main ingredient in medicinal potions and ointments because thyme oil contains thymol (2-isopropyl-5-methylphenol), is a crystalline phenol compound that is found in nature in a colorless crystalline form with a distinctive odor and is an isomer of carvacrol[2]. It is found in plants, especially thyme. It has medicinal activity in treating disorders affecting the respiratory and digestive systems and is considered an antioxidant, antifungal, anti-inflammatory, and antimicrobial agent[3]. In fact, thymol and its derivatives work against cancer through a variety of mechanisms, such as inhibiting cell growth, causing programmed cell death, generating reactive oxygen species inside the cells, removing mitochondrial membrane polarization, and activating the proapoptotic membrane of the mitochondria with the help of mitochondrial proteins such as Bax or Caspase or polymerase ribozyme ADP in a variety of experimental settings. The biological activity of thymol decreases in a watery environment and its solubility is low, so preparing nanoemulsions of thymol and coating it with suitable surfactants can increase its efficiency by converting it into nanoparticles[4]. The spherical dispersion form

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called nanoemulsion or small emulsion has the ability to increase the bioavailability of many active ingredients. The properties of the nanoemulsion include high drug bioavailability, controlled release, protection from degradation, rapid digestion, and good stability[5, 6].

Thymol has low solubility in water and good solubility in oil, which reduces its biological activity and limits its use in the aquatic environment[4]. Therefore, nanoemulsion of thymol was prepared, dissolved in sesame oil, which is also an antioxidant, by converting it into nano-droplets through encapsulation in suitable surfactants. Due to this encapsulation, thymol becomes physically and chemically stable in the aquatic environment. From a biological perspective, nanoemulsion droplets can be effectively absorbed by biological surfaces for more efficient biological activities[6]. Nanoemulsions are spherical particles that act as carriers for drug molecules, ranging in size from 10-1000 nanometers. These carriers are solid particles with a non-crystalline surface and are lipid-loving, thus increasing the therapeutic effectiveness of the drug[7]. Due to thymol's anti-cancer effect, it can be used as a suitable treatment to prevent the formation of blood vessels in liver cell cancer[8]. Liver cancer is the fourth leading cause of death worldwide, claiming the lives of over 800,000 people each year. Around 90% of primary liver cancer cases are hepatocellular carcinoma, followed by intrahepatic cholangiocarcinoma and other primary malignant tumors in the liver[9, 10]. Nearly 90% of hepatocellular carcinomas have known underlying causes, most commonly non-alcoholic fatty liver disease, heavy alcohol consumption, and chronic viral hepatitis. These causes have different geographical distributions. For example, chronic viral hepatitis is more common in East Asia, while heavy alcohol consumption is more prevalent in Europe. There are regional variations in both liver cancer incidence rates and deaths[9, 11]. Vascular invasion is a prominent feature of liver cancer in clinical practice. However, the degree of vascular invasion fluctuates significantly with cancer progression. Inhibiting the formation of new blood vessels may be a potential strategy for liver cancer treatment. Hepatocellular carcinoma is, in fact, a highly vascular tumor primarily nourished by hepatic arteries, while the portal vein mainly supplies regenerative nodules and parenchymal distortion in the natural liver lobule[12]. When diagnosing liver cancer, this characteristic is used as a radiological feature. Angiopoietin and vascular endothelial growth factor have been identified as a family of endothelial lining growth factors that are crucial for liver cancer[13, 14]. One of the most important factors in angiogenesis in cancer cells is VEGF. An increase or decrease in the expression of this gene affects the increase or decrease in the size and number of cancer cells. Increased gene expression enhances tumor formation in humans and animals, while its decrease leads to tumor size reduction as it is a cytokine that has the ability to directly affect body cells, endothelial cells, and astrocytes[15, 16]. In addition, VEGF can function as a growth factor that enhances the proliferation of cancer cells expressing VEGF receptors, in addition to being a vascular endothelial growth factor. Hypoxia and acidity independently regulate VEGF expression in tissues and organs. Cancer-causing mutations, hormones, cytokines, and other signaling molecules (including nitric oxide and mutagen-activated protein kinases) all impact VEGF production[17-19]. To determine the cytotoxicity of the prepared nanoemulsion, the HepG2 fibroblast cell line was cultured Using Mtt test. In this study, one million five hundred cells were counted. In our research, we extracted total RNA from HepG2 cells after 24 hours of treatment with thymol nanoemulsion using triazole reagent and phenol-chloroform method. In this study we aimed to investigate the effects of nanoemulsion thymol on the expression of angiogenesis controlling genes VEGF and VEGFR in a human liver cancer cell line.

Materials and Methods

Extracting thyme oil

Thyme oil was extracted by distilling the essential oils [20] through placing 100 grams of finely ground plant leaves, resulting in 1 ml of the required oil, as shown in Fig. 1.

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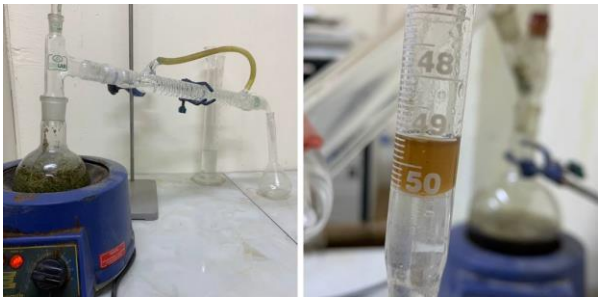

Figure 1. The extraction of thyme oil.

Extracting thymol from thyme oil

Thymol was extracted by hot water method[20] by adding 0.5 mL of 0.1 M sodium hydroxide to 1 mL of the prepared essential oil in a test tube, resulting in an alkaline solution with a pH of 13. Then, 2 mL of distilled boiling water at 100 degrees Celsius was added and mixed for two minutes. After that, the test tube was placed on a stable surface for two minutes, resulting in the formation of a two-phase mixture. The oily phase was separated, and the pH was adjusted to 7 by adding 2M hydrochloric acid. Then, 2 mL of hexane was added to the solution and stirred for 3 minutes to completely dissolve the thymol with the hexane As in Fig. 2 A. The surface phase (hexane + thymol) was isolated, and after evaporating the hexane, pure thymol was obtained, as in Fig. 2 B. To confirm the presence of thymol, the UV was measured and FTIR with the thymol standard data. It has been confirmed that we have successfully prepared thymol.

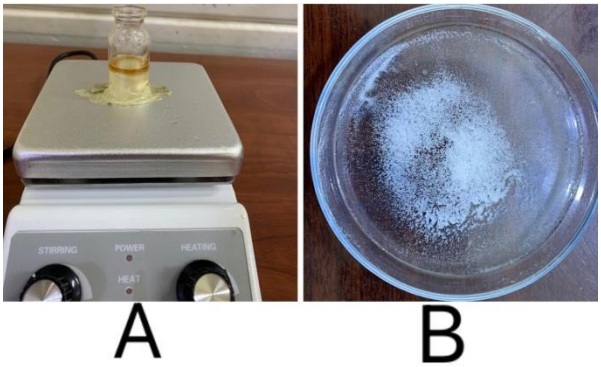

Figure 2. The production of thymol.

Preparation of nanoemulsions containing thymol

1. First, 0.15 g of sesame oil was mixed with 10 mg of thymol extracted from thyme. After adding a magnet to the baker, it was placed on the magnetic stirrer for 20 minutes.
2. Then 0.31g of SP80 was added to the above suspension and stirred for 5 minutes.
3. Next, 1.49g of Tween was added to the above suspension and stirred for 5 minutes.
4. In the next step, 0.50g of ethanol was added to the above suspension and placed on the engine for 15 minutes.
5. Finally, 2.55g of water was added drop by drop into the agitator and the nanoemulsion was placed on the stirrer for 30 minutes. After the end of time, we obtained the nanoemulsion as in Fig. 3.
6. On the day after increasing, the stability of the nanoparticles was determined visually.
7. Using DLS, stability and after centrifugation, the properties of the nanoemulsion were determined.

Dilution of Thymol stock (sequential dilution)

Six dilutions of concentrations of 1, 2, 4, 6, 8 and 10 µl of thymol were prepared in a test tube and each tube was filled up to 3 cc with methanol and pumped with a sampler. First, 100 µL was taken from the first stock and dissolved in 10 cc of methanol, then as calculated for other concentrations, made from it and vortexed.

Cell culture MTT assay

The HepG2 cell line was planted. After 48 hours, the cells were removed from the incubator and the residual material was discarded. They were washed once, and then 300 microliters of trypsin were added. After that, the vial containing trypsin was transferred to the incubator, and after two to three minutes, complete medium was added to neutralize the trypsin. It was then centrifuged at 2000 revolutions per minute for 5 minutes to discard the residual material. 1 ml of complete medium was added to dissolve the cells and create a uniform cell suspension. Ten microliters of the initial suspension were removed and added to 90% ethanol. Neubauer chamber was used to count the cells, and for this purpose, 20 microliters of the cells were placed under the chamber and observed under the microscope. To calculate the cell count, the number of cells in four of the 16 squares on the Neubauer chamber was counted and calculated according to the following formula: $(A+B+C+D)/4*10^4$

In this study, a total of one million and five hundred cells were counted. After calculations, 350 microliters were removed from the same diluted suspension and added to 4650 microliters of medium. After aspiration, 100 microliters were added to each well of a 96-well plate and placed in the incubator for 24 hours. Then, the zinc medium was drained and a nanoemulsion of thymol 10 mg/ml was prepared at concentrations of 200, 100, 50, 25, 12, and 6 micrograms/ml, with 100 microliters of each added to three replicates of cells cultured in 96-well plates.

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After 24 hours, the floating material was gently discarded and 20 microliters of a solution of MTT at a concentration of 0.5 micrograms/microliter were added to each well of a 96-well plate. After three hours, 100 microliters of DMSO were added to each of the wells. Then, to read the absorbance of the samples, a spectrophotometer was used at wavelengths of 570 and 630.

RNA extraction

Total RNA of HepG2 cells was extracted by the triazole method and the phenol-chloroform method[21]. For this purpose, 800 µL of cold Run-Plus and 200 µL of cold chloroform were added to the desired samples, the microtube was dipped several times and the microtube was placed on ice for 15 min (during this time the microtube was dipped several times) and the microtube was centrifuged Centrifuge for 15 minutes at 14,000 rpm and 4 °C. At the end of the centrifugation, the microtube was slowly removed from the device and the supernatant was gently removed and transferred to a new 1.5 µl microtube. Then, 500 µL of cold isopropanol was added to microtubules, and the microtube was placed in a freezer at −20 °C for 15–30 min and centrifuged again (15 min, 14,000 rpm and 4 °C). The isopropanol (supernatant phase) was then rapidly eluted and 1000 µl of 75% ethanol and sucker slowly added until the RNA granules separated and the ethanol floated. Centrifugation was performed again (7800 rpm, 8 min, and 4 °C). After this step, the upper phase was drained and the pellets were dried under a hood. Finally, 30–50 µl of DW deionized water was added and after pipette, it was probed by nanodrop. Finally, the RNA was stored at -70.

cDNA synthesis

This step was performed using a bio-editing company (cDNA) synthesis kit. After thawing, the RNA and primers on ice, a mixture of the following was prepared:

• RNA sample: up to 1 microgram
• Random hexagon: 1 µl
• dNTP: 2 µl
• Nuclease empty water: until the size reaches 10

After homogenization, the resulting mixture was incubated at 30° for 10 min and immediately placed on ice. In the next step, the following items were added to the above mixture:

• Reverse transcriptase: 1 µl
• Reaction buffer (5x): 4 µl
• Nuclease-free water: 5 µL

After homogenization, the resulting mixture was incubated at 55°C for 60 minutes and finally placed at 95°C for 10 minutes to inactivate the enzyme[22, 23]. And transfer it to the freezer -20. Then, the primers used for testing were designed in real time by AllelID software and their specificity was verified by Blast software. Prefixes used:

Real-time PCR

Real-time PCR was carried out using the purification of the hood laminar during the examination.[24]

1- 10 microliters of SYBR Green Premix EX mixture in Real Time image in a microtube additive.
2- 0.6 microliters of Forward primer should be added.
3- 0.6 Reverse primer (0.6 microliters) to be added.
4- cDNA by adding a stretch (1 microliter) .
5- The DEPC strainer and add it to a final volume of 7.8 µl pressed.
6- Materials made of hardened mixed aspen.
7- For segmentation and analysis of the ΔΔ CT instrument, it is recommended to use it.

Results and Discussion

FTIR for thymol

After comparing the FTIR spectrum of the prepared thymol with the spectrum of standard thymol from available sources, the similarity between them in terms of the peaks was proven, as a clear peak was found at 3350.35, which belongs to the hydroxyl group of phenol. At 3020, we had a clear peak belonging to the c=c aromatic group. And at 2962.66, appeared. A peak goes back to the aliphatic CH. At frequency 2353.16, we have a peak that goes back to the benzene ring. We have two homologous peaks at 1460.11 and 1421.54, which are due to the presence of two homologous groups of CH_3. And the presence of a peak at 1361.74 goes back to the proverbial group associated with the benzene ring a s in Fig. 3.

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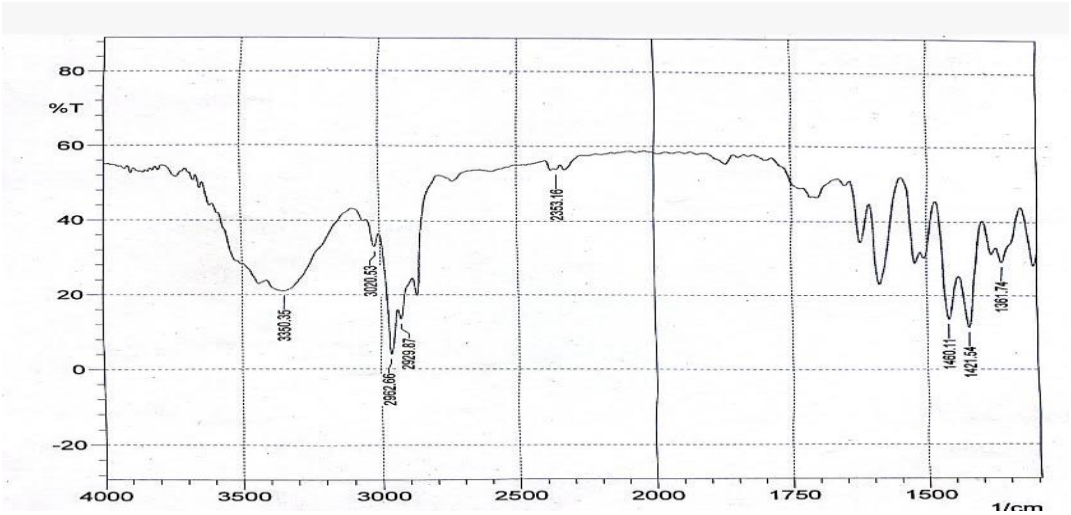

Figure 3. The FTIR spectrum of thymol extracted from thyme.

Checking the particle size of the thymol nanoemulsions using DLS

DLS method was used to measure the volume of nanoemulsion particles, which were subjected to sonication for homogenization before examination. After the examination, it was found that the average particle size (D50) was 72.6. There was only one peak obtained, as shown in Fig. 4, indicating the homogeneity of the nanoemulsion particles and the success in preparing particles of nano size.

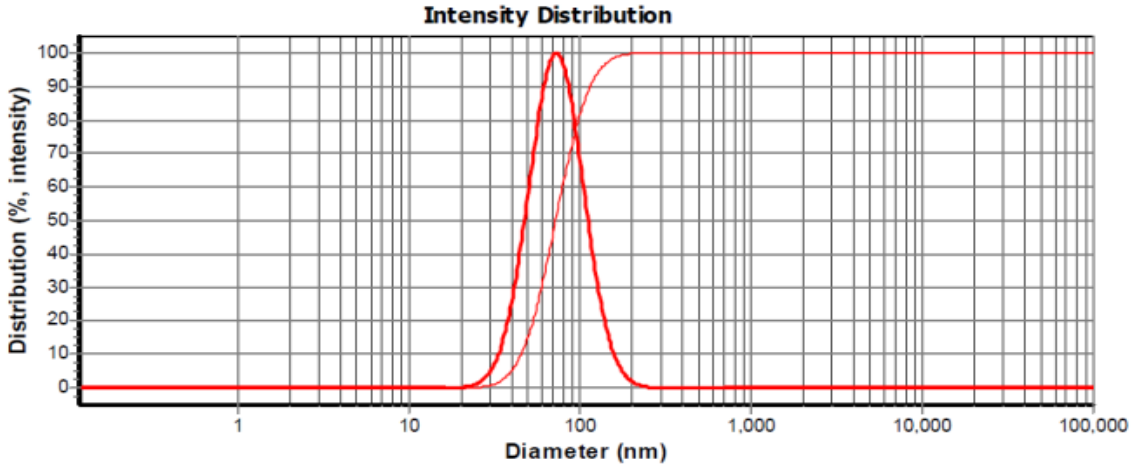

Figure 4. DLS test.

Determining the wavelength for thymol absorption.

To know the wavelength of thymol, UV technology was used to obtain the absorption spectrum (10 mg/ml) of thymol dissolved in methanol, and the maximum wavelength was 273 nanometers. Excel software processors were used to draw the peak as shown in Fig. 5.

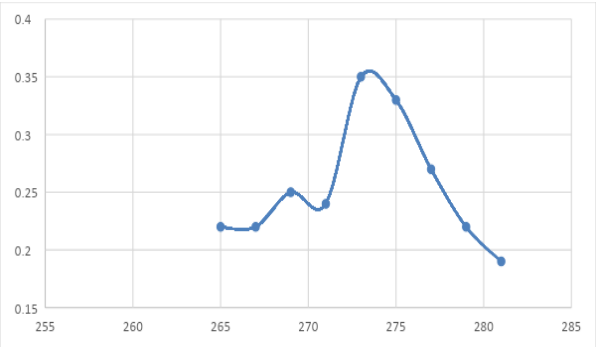

Figure 5. Determine the length of the thymol nanoemlusions attraction wave.

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Measuring the absorption coefficient of the nanoemulsion and determining the packaging efficiency.

The efficiency of the packaging refers to the method of preparing the nanoparticles to contain thymeol inside the nanocarrier. Ideally, the goal is to achieve a higher drug accumulation using lower doses. The packaging efficiency expresses the ratio of the amount of drug in the particles to the total basic drug quantity used in the manufacturing process, expressed as a percentage. To verify the amount of the packaged drug, the nanoemulsion was placed in a cooled centrifuge at 4 degrees Celsius for 30 minutes at a speed of 13,000 cycles, and the floating material was separated and its optical absorption was read at 273 nanometers. Using the packaging equation, the percentage efficiency of the packaging of the nano thymeol emulsion was obtained, and the percentage was 80%.

Draw the calibration curve of thymol nanoemulsion.

After determining the maximum wavelength (λmax) and matching it with the available sources, to obtain the relationship between drug concentration and the amount of absorptivity read from the device, by making different concentrations and reading their absorption as in Table 2. at 273 nanometers, the calibration curve was plotted and the linear curve was obtained as in Fig. 6.

Table 2. The concentration of thymol with absorbance.

Thymol concentration (ug/ml)	Absorption
1	0.11
2	0.13
3	0.19
4	0.23
6	0.23
8	0.28
10	0.35

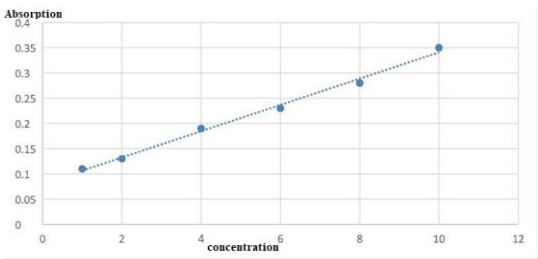

Figure 6. Absorption diagram of thymol.

Cell morphology.

HepG2 cell lines were examined morphologically They have a morphology similar to epithelial cells and have a high proliferation rate. HepG2 cells are used for studying cytotoxicity as shown in Fig. 7.

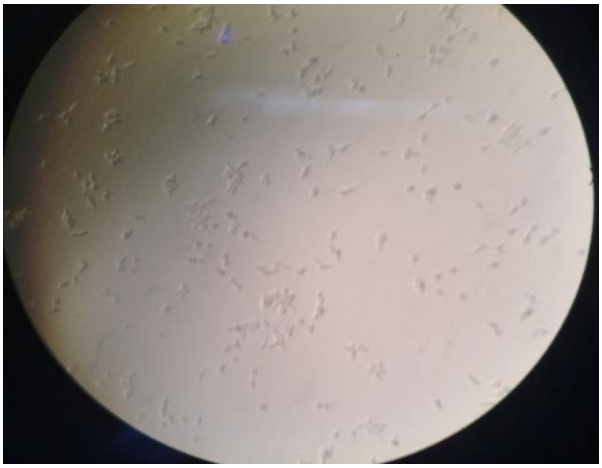

Figure 7. Morphology of HepG2 cell lines.

Investigation of cytotoxicity

MTT assay is a non-radioactive colorimetric assay which is used to measure the cell viability in response to a variety of cytotoxic stimuli. The assay is based on the reduction of yellow, water soluble tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) within metabolically active cells. The reduction of the tetrazolium salt occurs by the action of mitochondrial dehydrogenases present only in viable cells, yields a purple formazan product which can be quantified spectrophotometrically

HepG2 cells were seeded in 96-well plates and then 10 mg/ml nano-thymol emulsion was made at concentrations of 200, 100, 50, 25, 12, and 6 µg/ml and 100 µl of each was added to three replicates of sorted cells. It was added in the 96-well home plate. After two hours of MTT solution, 20 µL was added to each well of the 96-well plate, and after three hours, 100 µL of DMSO was added to each well. Then, to read the absorbance of the samples as shown in Table 3. below, the quotation device was used at wavelengths 570 and 630. The concentration was calculated as IC_{50} = 38. The results indicated that with increasing concentration of thymol nanoemulsion, cell viability decreased. The IC_{50} value was 47.91 as in Fig. 7.

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Table 3. Investigation of cytotoxicity of thymol nanoemulsions on HepG2 cells.

	control	200	100	50	25	12	6
Absorbance	0/415	0/02	0/043	0/109	0/291	0/34	0/394
Absorbance	0/391	0/08	0/083	0/166	0/242	0/376	0/341
Absorbance	0/395	0/064	0/103	0/133	0/23	0/309	0/315
Mean	0/40033	0/0546	0/0763	0/1293	0/2543	0/34166	0/35
POC	100	13/655	19/06744	32/30641	63/53039	85/34555	87/42714

IC_{50}: The median inhibitory concentration is the concentration at which half of the cells are alive and the other half are dead.

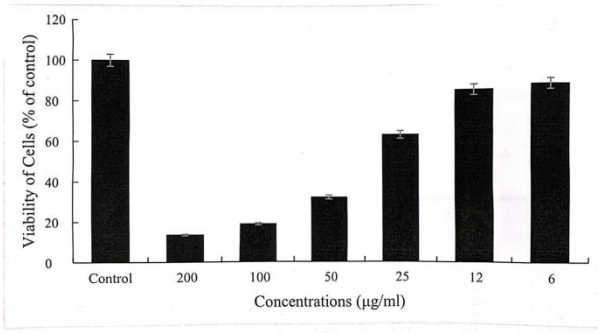

Figure 7. Examining the cytotoxicity of thymol nanoemulsion on HepG2 cells.

Blast results of designed primers

The results indicated that the designed primers have high specificity and bind only to human VEGF and VEGFR genes as in Figs. 8 and 9.

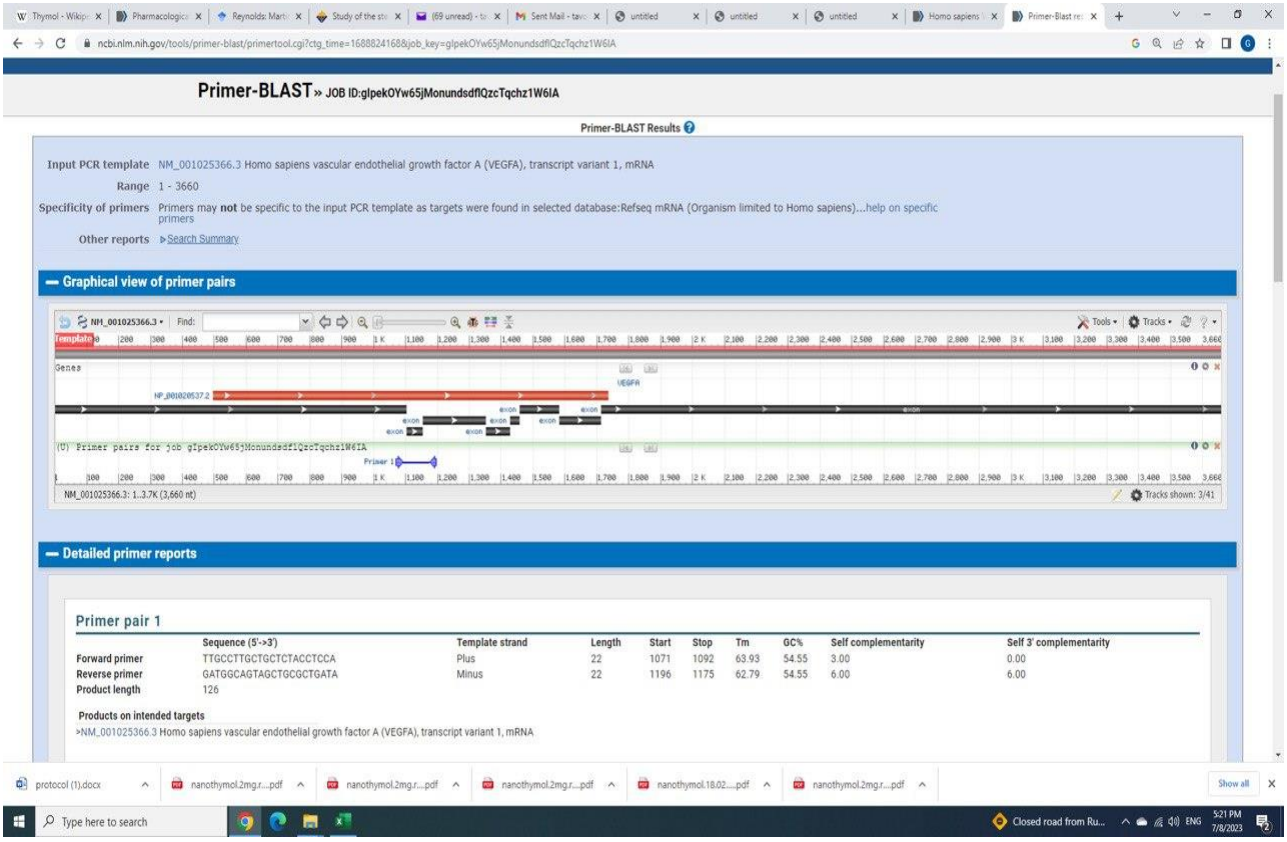

Figure 8. Primers designed for VEGF bind specifically to the target gene.

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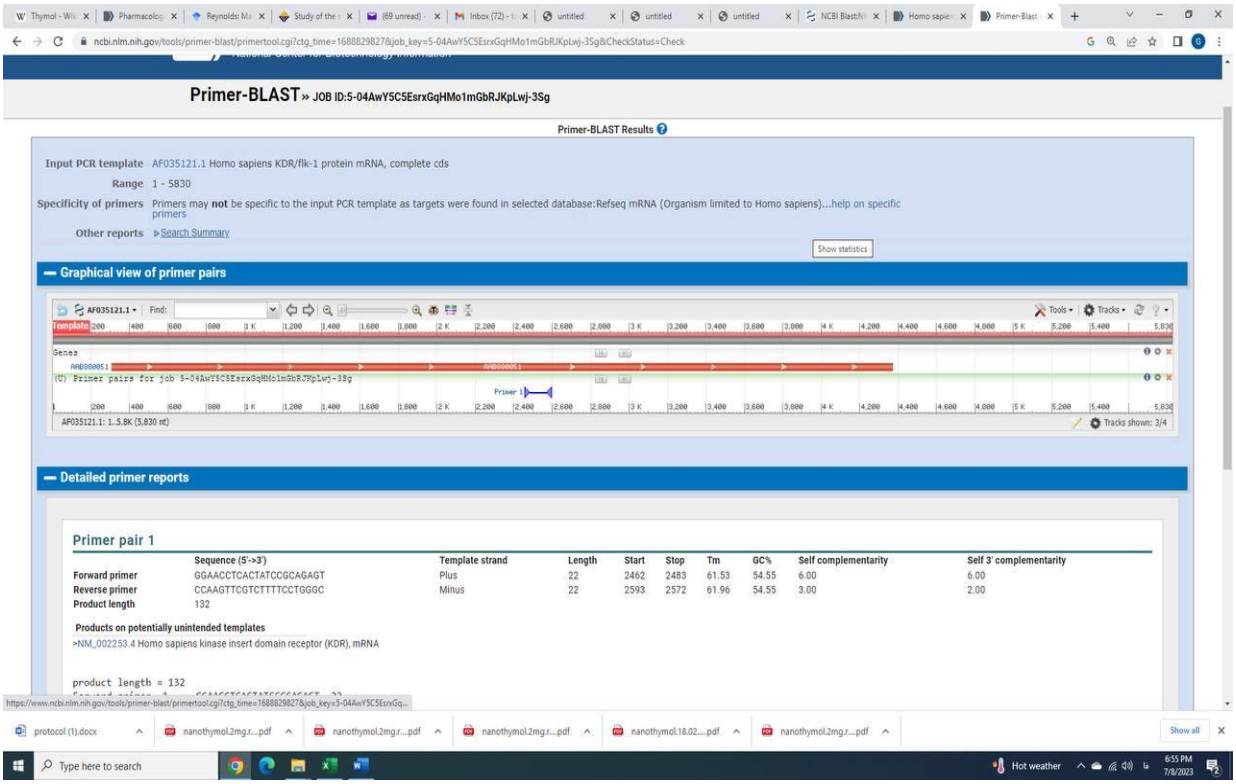

Figure 9. The primers designed for VEGFR are specifically connected to the target gene.

The results of investigating the level of VEGFR and VEGF gene expression in HepG2 cells treated with thymol nanoemulsion compared to the control

Real-time PCR results of VEGF genes of HepG2 cells treated with thymol nanoemulsion and free thymol and cells without treatment showed that the expression of this gene in cells treated with free thymol decreased 5.33 times compared to the control, but when the cells treated with thymol nanoemulsions, the level of VEGF gene expression decreased by 10.02 times as in Fig. 10.

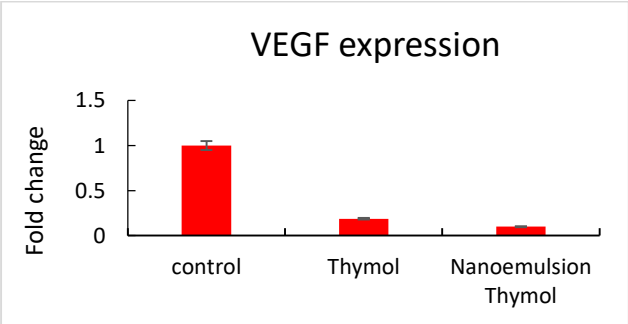

Figure 10. VEGF expression in HepG2 cells treated with thymol and thymol nanoemulsion and control.

Real-time PCR results of VEGFR genes of HepG2 cells treated with thymol nanoemulsion and free thymol and cells without treatment showed that the expression of this gene in cells treated with free thymol decreased 1.30 times compared to the control, but when the cells treated with thymol nanoemulsions, the expression level of VEGFR gene decreased about 8.1116 times as shown in Figs. 11, and 12.

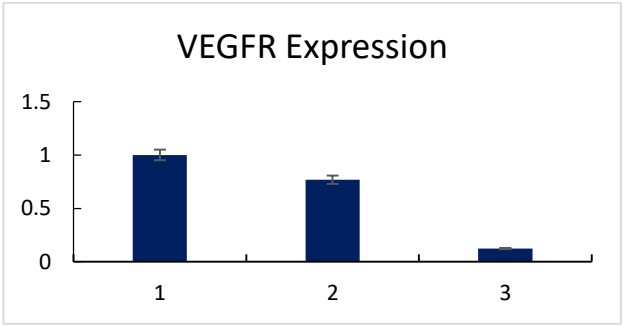

Figure 11. VEGFR expression in HepG2 cells under thymol control treatment.

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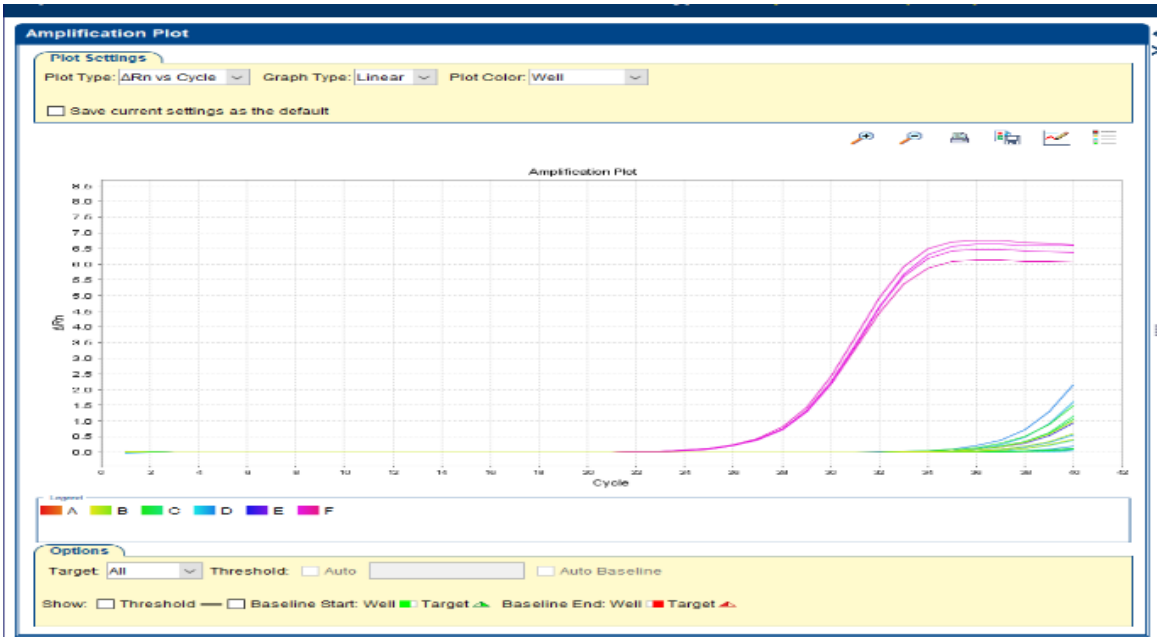

Figure 12. Graph of VEGF and VEGFR gapdh Cts.

Melt curve analysis was used to determine the melting temperature (Tm) is the temperature at which 50% of the DNA is double-stranded and 50% is dissociated into single-stranded DNA. The software plots a melt curve based on the fluorescence of the dye with respect to change in temperature. Using the melt curve, the software calculates the melting temperature (Tm). It was found to be equal to 83.18 as shown in Fig. 13.

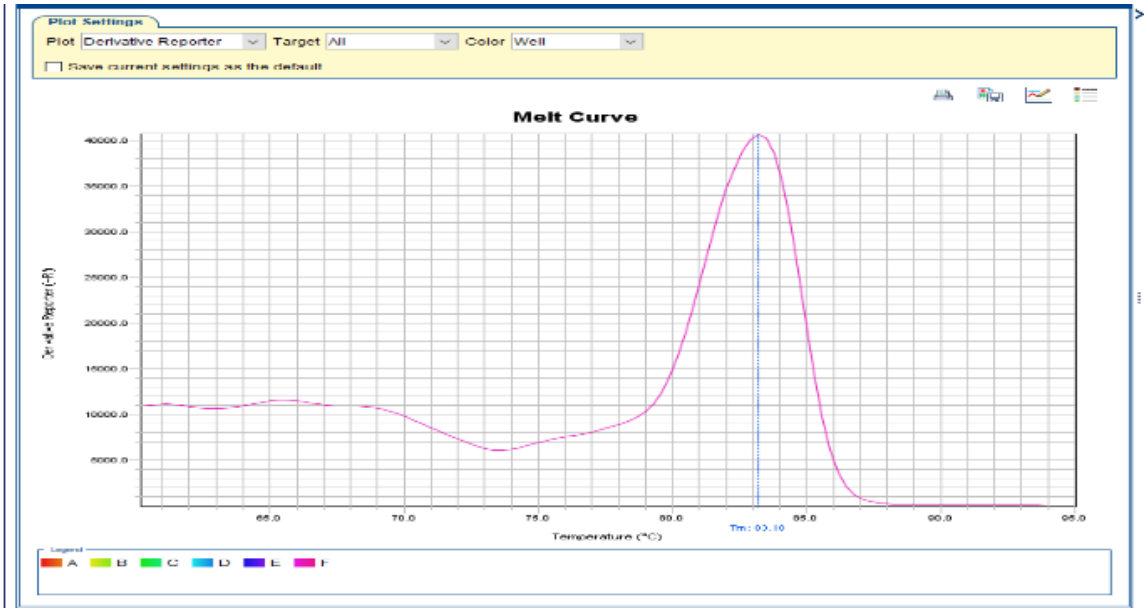

Figure 13. GAPDH melt curve.

The specificity of PCR products were determined by the melting curve profiles. The melting point of every amplicon depends on its sequence, length, , allowing us to use melting curve profiles to identify DNA products. As the temperature was slowly increased, the fluorescence of the sample was monitored to determine the melting temperature profiles. When the PCR reaction was finished, the temperature was first reduced from 95 ºC to 58 ºC at –0.1 ºC per second and then increased again to 95 ºC. The specific melting point for VEGF is 83.33 as in Fig. 14.

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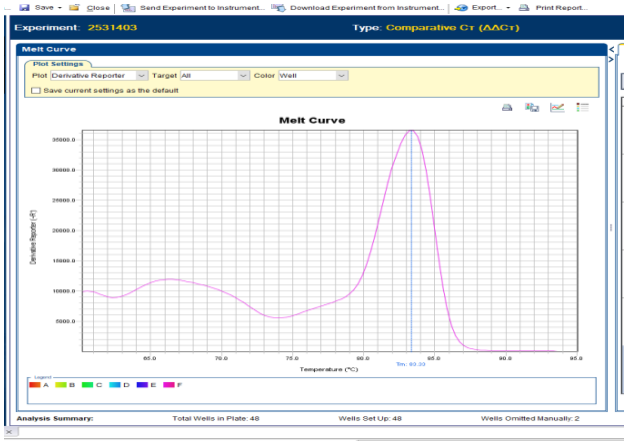

Figure 14. VEGF melt curve.

The specific melting point for VEGFR It is 83.03 as in Figure 15

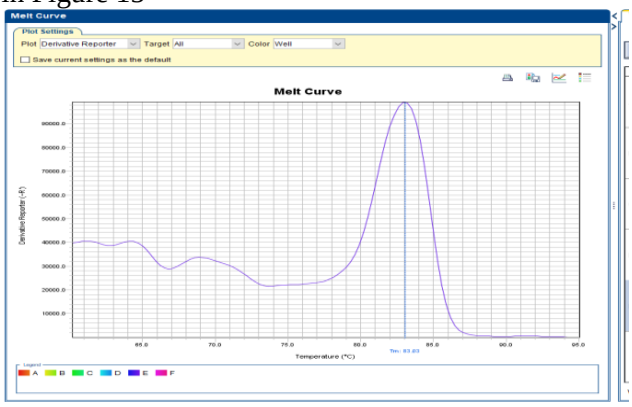

Figure 15. VEGFR melt curve.

Conclusion

This study indicates that were able to extract thyme oil from the thyme plant, and then were able to extract thymol from the oil. Next, prepared thymol nanoemulsion and studied the ability of our prepared thymol nanoemulsion to reduce the amount of VEGF and VEGFR in a line of human hepatocellular carcinoma cells treated with thymol nanoemulsion. The results showed that the expression of this gene in cells treated with free thymol decreased by 5.33 times compared to untreated cells, but when the cells were treated with nanothymol, the level of gene expression decreased by about 10.02 times. Therefore, thymol nanoemulsion could be a suitable treatment against angiogenesis in this cancer. A study could be conducted in the future to study other molecular mechanisms involved in HCC under the influence of thymol nanoemulsion. Also, the protein concentration of VEGF and VEGFR in HepG2 cells could be studied under the influence of thymol nanoemulsion.

Authors' Declaration

- Conflicts of Interest: None.
- I hereby confirm that all the Figures and Tables in the manuscript are mine. Besides, the Figures and Images, which are not mine, have been given permission for re-publication attached with the manuscript.
- No animal studies are present in the manuscript.
- No human studies are present in the manuscript.
- No potentially identified images or data are present in the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee at Islamic Azad University, Tehran, Iran

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دراسة تأثير المستحلب النانوي المحتوي على الثيمول المستخرج من الزعتر على الجينات المتحكمة في تكوين الأوعية الدموية VEGF و VEGFR في خط خلايا سرطان الكبد البشري.

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الخلاصة

يحتوي زيت الزعتر على الثيمول (2-إيزوبروبيل-5-ميثيلفينول)، وهو مركب فينولي بلوري عديم اللون وذو رائحة مميزة. يعتبر مضاد للأكسدة. الثيمول ذو قابلية منخفضة للذوبان في الماء وقابلية ذوبان جيدة في الزيت. تم تحضير مستحلب الثيمول النانوي المذاب في زيت السمسم عن طريق تحويله إلى قطرات نانوية. حيث أن في الدراسات السابقة أجريت العديد من الدراسات البحثية لدراسة تأثير الثيمول على سرطان المعدة وتأثيره على خلايا الورم الأرومي الدبقي. لقد قاموا بدراسة تأثير VEGF على نمو وهجرة الخلايا السرطانية في الكبد. من منظور بيولوجي، يمكن للأسطح البيولوجية أن تمتص قطرات المستحلب النانوي بشكل فعال من أجل أنشطة بيولوجية أكثر كفاءة. المستحلبات النانوية هي جسيمات كروية تعمل كحامل لجزيئات الدواء، ويتراوح حجمها بين 10-1000 نانومتر. بسبب تأثير الثيمول المضاد للسرطان، يمكن استخدامه كعلاج مناسب لمنع تكوين الأوعية الدموية في سرطان الخلايا الكبدية. أجرينا تحقيقًا في السمية الخلوية باستخدام اختبار MTT، حيث تمت معالجة خط خلايا سرطان الكبد البشري بتركيزات مختلفة من مستحلب الثيمول النانوي. أشارت النتائج إلى أنه مع زيادة التركيز، انخفضت صلاحية الخلية. ثم تم استخراج الحمض النووي الريبي (RNA) وتم تصنيع C-DNA. تم بعد ذلك فحص مستويات التعبير عن جينات VEGFR وVEGF في الخلايا المعالجة بالنانوثيمول والثيمول الحر وفي الخلايا غير المعالجة. وأظهرت النتائج أن تعبير هذا الجين في الخلايا المعالجة بالثيمول الحر انخفض بمقدار 5.33 مرة مقارنة بالخلايا غير المعالجة، ولكن عندما عولجت الخلايا بالنانوثيمول انخفض مستوى التعبير الجيني بنحو 10.02 مرة.

الكلمات المفتاحية: سرطان الخلايا الكبدية، المستحلب النانوي، الثيمول، الأورام، VEGF، VEGFR.