Advances in Pharmacology and Therapeutics Journal
Shahid Sadoughi University of Medical Sciences, Yazd, Iran

SHAHID SADOUGHI UNIVERSITY OF MEDICAL SCIENCES

Original Article

Preparation of Thymol Nanoliposome and Solid Lipid Nanoparticle and Evaluation of their Inhibitory Effects on *Leishmania Major* Promastigotes

Running Title: Thymol on Leishmania

Mohsen Zabihi[1], Mahdiyeh Shafaei[1], Vahid Ramezani[2], Tahereh Dara[2], Farzaneh Mirzaie[3*]

[1]Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[2]Department of Pharmaceutics, School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[3]Department of Medical Parasitology and Mycology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran..

A R T I C L E I N F O

Received: 02/07/2022
Accepted: 01/14/2023

Corresponding author
Department of Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.E-mail mzabihi100@gmail.com

Abstract

Introduction: Thymol has an antiprotozoal effect. Nanoparticulate systems are useful carriers for both small and large drug molecules, which can protect them from some chemical and biological damages as well as target drug delivery to specific organs or receptors. In this work, the nano-liposomal system and solid lipid nanoparticles loaded by thymol were prepared and the effectiveness of them were evaluated on *Leishmania major* promastigotes.

Methods: Several formulations of nano-liposomes and solid lipid nanoparticles were prepared, and the amount of thymol loading, in-vitro release profile, particle size, and zeta potential were evaluated. Finally, the best formulations were serially diluted and incubated for 24, 48, and 72 hours on *Leishmania major* promastigotes, which cultured on Novy–MacNeal–Nicolle medium, and the results were analyzed.

Results: The highest loading of thymol in nano-liposomes (92%) was seen in the formulations made with phosphatidylcholine (Called L3), and among the solid lipid nanoparticles, the formulation prepared with glycerol monostearate (S1) had the most entrapment efficiency of thymol (87%). These formulations were selected to evaluate the release rate of thymol. The results showed that S1 has a slower release rate than L3; this may be due to the presence of Glycerol monostearate in solid lipid nanoparticles structure. The best formulations, L3 and S1, were chosen for anti-Leishmaniosis assessment; which showed that all three forms of free thymol, nanoliposomes, and solid lipid nanoparticles inhibited *Leishmania major*. The half maximal inhibitory concentration (IC50) of free thymol, nanoliposome, and solid lipid nanoparticles for a 24-hour incubation are 7.8, 62.5, and 125, respectively, which decrease to 7.8, 7.8, and 15.6 for 48 hours and 7.8, 0.49, and 0 for 72 hours of incubation.

Conclusion: Thymol has a significant effect on the inhibition of *Leishmania major* promastigotes and usage of thymol in the form of liposomes or solid lipid nanoparticles can sustain the drug release and have a lower IC50 during the longer incubation time.

Keywords: *Leishmania major*, Phenol, Thymol, Thyme, Liposomes, Solid Lipid Nanoparticles.

Citation: Zabihi M, Mirzaie F, Ramezani V, Dara T, Shafaei M. Preparation of Thymol Nanoliposome and Solid Lipid Nanoparticle and Evaluation their Inhibitory Effects on *Leishmania Major* Promastigotes. Adv Pharmacol Ther J. 2023;3(1): 49-60.

Introduction

Leishmaniasis is one of the most common zoonotic diseases and is a major public health problem worldwide. The disease usually appears in humans in one of three forms: 1) cutaneous, the most common form, also known as Leishmaniasis; 2) visceral, which is so-called Kala-azar, and is the most serious form; or 3) mucocutaneous, which can lead to the partial or complete destruction of mucous membranes of the mouth, nose, and throat. Leishmaniasis is seen in two conditions: rural (wet) and urban (dry), which are caused by *Leishmania Major* and *Leishmania Tropica*, respectively (1). Manifestations of this disease include small red lesions that gradually get larger and larger, and the center of the wound becomes ulcerated besides occasionally bacterial superinfections. Multiple systemic or topical pharmacological and non-pharmacological treatments for Leishmaniasis have been suggested (2, 3). The drug of choice for Leishmaniasis is Glucantime, which is a pentavalent antimony compound. In addition, there are some alternatives or complementary include paromomycin, amphotericin B, chloroquine, pentamidine, metronidazole, ketoconazole, dapsone, terbinafine, rifampin, and some herbal medicines (4, 5). The low efficacy of drugs, which is associated with the need for long-term treatment of this disease has caused an economic burden on families and communities along with the toxicity and side effects of them. For example, in the case of Glucantime as a first-line treatment for Leishmaniasis, drug resistance and side effects including arrhythmia, elevated liver enzymes, and anemia, are major challenges that threaten patients' health (6).

Thymol is one of the phenolic compounds derived from plants such as thyme (Thymus vulgaris). It has antimicrobial effects on a variety of fungi and bacteria, as well as antioxidation properties. Plants containing thymol, the Thymus genus, are considered in traditional medicine and have many usages, especially for their antimicrobial effects (7).

Thymol is a highly promising, herbal-derived substance for the treatment of Leishmaniasis, and it should be able to reduce drug resistance. In addition, due to concerns about hepatotoxicity induced by standard drugs, thymol may be a good option (8).

Some studies indicated loading of thymol in nanoparticulate systems can improve antimicrobial effects via the exhibition of a sustained release profile, and protection from medium effects which is a promising approach in drug delivery (9).

Nowadays, modern drug delivery systems such as nanoparticles are used because of their ability to increase the stability of active pharmaceutical ingredients, control release, reduce side effects, and increase the bioavailability of the drugs (10). Various nanoparticle systems such as solid lipid nanoparticle, polymeric nanoparticle, liposomes, etc. have advantages and limitations over each other based on the materials used in their formulations as well as the structural properties such as size, zeta potential, and lipophilicity (11). SLN is an alternative carrier system for traditional colloidal structures such as emulsions, liposomes, microemulsions, and polymeric nanoparticles. It

 DOI: https://doi.org/10.18502/aptj.v3i1.12501

has the advantages of previous traditional systems without having many of their defects. SLNs are biocompatible, low toxic, nanosized particles, which can protect the loaded drug from decomposition, modulate drug release, and stay in blood circulation for a long time. These great particularities make SLN a suitable carrier for both lipophilic and hydrophilic drugs (12).

Liposomes are another particulate system used for the encapsulation of numerous substances such as antimicrobials, flavors, antioxidants, and various hydrophobic and hydrophilic compounds. Preparation of these sub-micron foams-shape systems is an interesting and efficient field for drug delivery. Liposomes can protect encapsulated materials from enzymatic, chemical, free radicals, and other destructive situations by forming a barrier around them. They also carry entrapped drugs to targeted organs (13).

The study aimed to prepare liposomes and SLNs loaded by thymol as modern drug delivery systems and to compare their effect on *Leishmania major* in-vitro. Various structural parameters of these nanoparticles were also studied.

Material and Methods

Materials

The standard strain of Iranian rural leishmaniasis agent L. major [MRHO/IR/ER/70] was prepared by the Department of Parasitology, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. Thymol, streptomycin, and penicillin were purchased from Sigma, USA. Phosphatidylcholine was provided from Lipoid, Germany. Polysorbate20 (tween®20), polysorbate80 (tween®80), cholesterol, polyvinyl alcohol (PVA), chloroform, acetone, and ethanol were purchased from Merck, Germany. Glycerin monostearate (GMS), tefose®, and labrafil® were kindly donated by Gattefossé, France. Stearic acid was obtained as a kind gift from Haiyan Company, Iran. NNN and RPMI 1640 culture medium were obtained from Qlab, Canada. All solvents were of analytical grad**.**

Nanoliposomes preparation

The modified thin-film hydration method followed by sonication was used to make nanoliposomes. For this purpose, according to **Table 1**, different amounts of lipids (cholesterol, phosphatidylcholine) and non-ionic surfactant (tween20, tween80) were dissolved in 1ml of chloroform and then, the mentioned amounts of thymol were added to it and completely mixed. Afterward, the mixture was transferred to a rotary evaporator (Büchi, Switzerland) under a vacuum pump to eliminate chloroform and to form a thin layer of film on the inner surface of the glass flask. To hydration of the prepared film, 5ml of phosphate buffer (37-40 °C) was added in the presence of glass beads and was shaken for 10 minutes, until a milky-colored suspension, liposomes, was prepared. The resulting suspension was sonicated for 5 minutes at 4 °C to reduce and homogenize the sizes of liposomes.

Table 1. The experimental compositions of thymol-loaded nano-liposomes (left) and solid lipid nanoparticles (right). In each group, the formulation that had the highest thymol load (L3 & S1) is marked in blue.

NLP Formulation	L1	L2	L3	L4	L5	L6
Phosphatidylcholine (mg)	50	50	50	40	40	40
Cholesterol (mg)	-	-	-	10	-	-
Tween 20(mg)	-	-	-	-	10	-
Tween 80(mg)	-	-	-	-	-	10
Thymol (mg)	10	20	30	10	10	10
chloroform (ml)	1	1	1	1	1	1

SLN Formulation	S1	S2	S3	S4	S5	S6
Acetone (ml)	1	1	1	1	1	1
Thymol (mg)	10	10	10	10	10	10
PVA2% (ml)	10	10	10	10	10	10
GMS (mg)	10	20	50	-	-	-
Tefose® (mg)	-	-	-	10	-	-
Labrafil®(mg)	-	-	-	-	10	-
Stearic acid (mg)	-	-	-	-	-	10

SLN preparation

SLN production was performed based on emulsification and solvent evaporation methods. At first, according to **Table 1**, the required amount of selected lipids (GMS, tefose®, labrafil®, stearic acid) were dissolved in 1ml of acetone for 20-30 min under heating. After the complete dissolution of lipids, 10 mg of thymol was added and truly mixed. To prepare oil in water emulsion, this oily prepared phase was injected dropwise for 2 min into a 10 ml aqueous phase containing PVA 2% as a surfactant. This process is done using a high-shear homogenizer (IKA T 25D, Germany) at 17000 rpm. Finally, the organic solvent was evaporated by a rotary evaporator at 95 rpm and 26 °C.

It is worth mentioning that formulations of liposomes and SLNs with other amounts of ingredients were also prepared, but since nanoparticles were not formed, they are not mentioned here.

Thymol standard calibration curve

Different concentrations of thymol in the range of 1 to 50 µg/ml in water were prepared at pH 7.4 and their absorption was measured by spectrophotometer at a wavelength of 276.3 nm and the calibration curve was plotted.

entrapment efficiency

Liposomes: The amount of thymol loaded in liposomes (entrapment efficiency) was measured by the direct method. Briefly, 1 ml of the prepared liposomes was transferred to a dialysis bag and immersed in 100 ml of distilled water for 4 hours at 4° C to separate the unloaded thymol from liposomes. The leftover liposomes inside the dialysis bag were transferred to a 10-ml balloon, using ethanol 70%, to destroy the structure of liposomes and release the encapsulated thymol (14).

SLN: To measure the amount of loaded drug in SLNs, the produced nanoparticles were centrifuged at 20,000 rpm, for 45 minutes, at 4°C and subsequently washed three times with distilled water. Then the precipitated nanoparticles separated and dissolved in a mixture of acetone/alcohol in a 1:1 ratio.

The amount of extracted thymol from liposomes or SLNs was quantified by UV spectrophotometer

at 276.3 nm and the entrapment efficiency was calculated using the following equation (15):

$$Entrapment\ Efficiency\ = \frac{amount\ of\ extracted\ thymol\ from\ nanoparticles}{amount\ of\ thymol\ used} \times 100$$

In vitro release

The dialysis bag method was used to investigate the release profile of thymol from liposomes and SLNs. Briefly, 1 ml of the prepared formulation was placed in a dialysis bag with a cut-off of 13 kD. To provide a sink condition, a pitcher containing 100 ml of phosphate buffer solution (p=7) was used as a receptor medium, and the containing dialysis bag was immersed in it. The set was placed in a bio-incubator (Heidolph, Titramax 1000, Germany) to mimic biological conditions at 37 ºC and a rate of 100 cycles/min (16). At predetermined time intervals within 24 hours, 3 ml of buffer solution was sampled and replaced with 3 ml of fresh buffer. The amount of thymol released was quantified by UV spectrophotometry at 276.3 nm.

Determination of particle size, and zeta potential

The size and zeta potential of liposomes and SLNs were Measured by photon correlation spectroscopy (Zetasizer Nano ZS, Malvern, U.K.). The formulated liposome was dispersed in the deionized water in a 1:20 ratio and then poured into the sample dispersion unit. The samples were analyzed by a He-Ne 633 nm laser, at 25 °C and with an angle of 90°. The same procedure was performed to measure the size and zeta potential of the SLN. All measurements were done in triplicate (17).

The proliferation of *Leishmania major* in culture medium

The standard strain of Iranian rural leishmaniasis agent L. major [MRHO/IR/ER/70] was cultured in NNN two-phase culture medium and then the harvested parasites were transferred to RPMI 1640 medium for mass proliferation. Because if the cells suddenly enter a large amount of this medium, there is a high possibility of their death; when the cells proliferated in the NNN, some of the supernatants of the medium containing the cells were removed and added to a small volume of RPMI 1640 medium under sterile conditions. The medium was supplemented with 10-20% of fetal bovine serum (FBS), 100 µg/ml penicillin, and 100 mg/ml streptomycin and kept diagonally in an incubator at 37°C. A Hemocytometer slide (Neobar slide) was used daily to determine the number of cells. When the number of promastigotes in the logarithmic phase reached 1000,000/ml, in other words, each tube contained 10,000/ml, the effects of thymol and the formulated nanoparticles on parasites were evaluated (18).

In vitro promastigote assay

The effects of thymol, thymol-SLN, and thymol-nano-liposomes on the viability of *Leishmania major* promastigote were investigated by the Thermo Fisher method. Five groups were examined including 1) the control group: which was treated with phosphate-buffered saline; 2) the standard group: 0.65 µM of amphotericin B; 3) the

Thymol group: thymol in 10 concentrations from 250 to 0.488 µg/ml which prepared via two-fold serial dilution; 4) Nanoliposome group and 5) SLN group that the concentrations of loaded thymol were equal to the amounts of group 3. All tests were done in triplicate. The parasites were incubated in 2ml microtubes in the presence of the above-mentioned treatments at 25 ° C for 24, 48, and 72 hours. Samples were stained with trypan blue and the number of living parasites, moving cells, of each microtube were counted under a light microscope on the Neubauer chamber (Thermo Fisher method) (19). The growth inhibition of each group was calculated by the following equation: GI=(C-T)100/C, in which "C" is the number of viable cells in the negative control group and "T" is the number of viable cells in the test group.

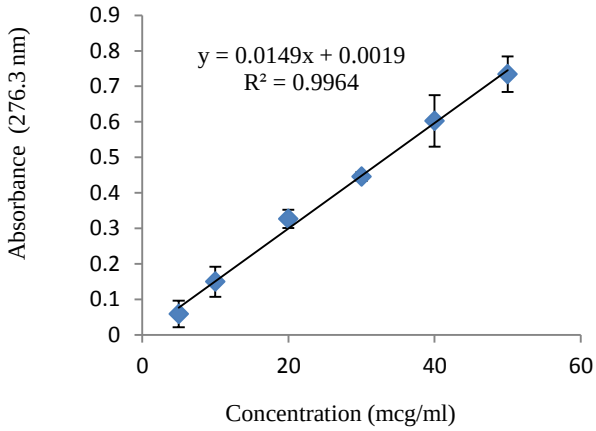
Statistical analysis

The data analysis was performed by SPSS 26 software using one-way ANOVA followed by the Tukey test, and P<0.05 was considered as the level of significance.

RESULTS

In this study, 6 formulations of thymol in the form of nano-liposomes and 6 formulations of solid lipid nanoparticles were prepared and their characteristics were evaluated **(Table 1)**. At first, the standard calibration curve of thymol was obtained in the range of 1 to 50 µg/ml using concentration vs. UV absorbance; that is equal to Y=0.0149X + 0.0019 and the correlation coefficient (R2) of 0.9964 **(figure 1)**.

Figure 1. Thymol standard calibration curve in water; λ=276.3,pH=7.4



Entrapment efficiency

Liposomes and SLNs were analyzed for their entrapment efficiencies and a formulation of each group that had the highest EE was selected to evaluate in-vitro drug release. In the liposomes group, the highest EE (92%) was shown in formulation L3, which contained phosphatidylcholine without any surfactant. The formulation L5, formulated with phosphatidylcholine and polysorbate 20, was in the second line of EE (41%). In the category of SLNs, formulations S1 and S6 were shown higher EE than others: (87% and 76% respectively).

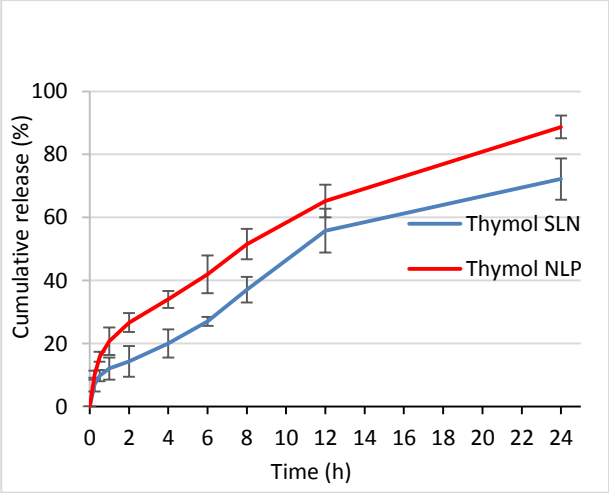
Cumulative drug release

In vitro release of thymol-loaded NLPs and SLNs in phosphate buffer pH 7 was studied in a USP type II dissolution apparatus over 24 hours and data from the percentage of thymol released from the nanoparticles are shown in **Figure 2**. Nanoliposome (formulation L3) containing phosphatidylcholine exhibited more drug release than SLN (formulation S1) within 24 hours. The maximum percentage of release in NLP and SLN

 DOI: https://doi.org/10.18502/aptj.v3i1.12501

were 88.73% and 72.20% respectively. It may be attributed to the lipophilicity of the SLN matrix and the affinity of thymol to the SLN component. It should be noted that the liposome released a little more than 20% of thymol in the first hour, while this number was about 12% in the case of SLN. The presence of thymol in the release medium at the first hour of the experiment indicates the accumulation of thymol on the surface of the nanoparticles and their sudden release upon entering the release medium which is called the burst release (20).

Figure 2. Cumulative release of thymol from nano-liposomes and solid lipid nanoparticles at 37°C during 24 h.



Particle size and zeta potential

The size and PDI of the L3 formulation were 402.1± 24.5 and 0.18±0.21. The Zeta potential was -19.65± 2.1 mV. These characteristics of the S1 formulation were 91.7nm ±15.4, 0.23±0.03, and -16.2± 1.8 mV respectively. The sizes of selected formulations were less than a micron. Dispersion indicated a monomodal size distribution profile and the PDIs were less than 0.25 representing low polydispersity of the particles. The zeta potential of L3 and S1formulations were between -15 to -20mV, which is an essential factor of the repulsion effect between nanoparticles with a similar charge. This can prevent particle aggregation and formulation instability (20).

In vitro promastigote assay

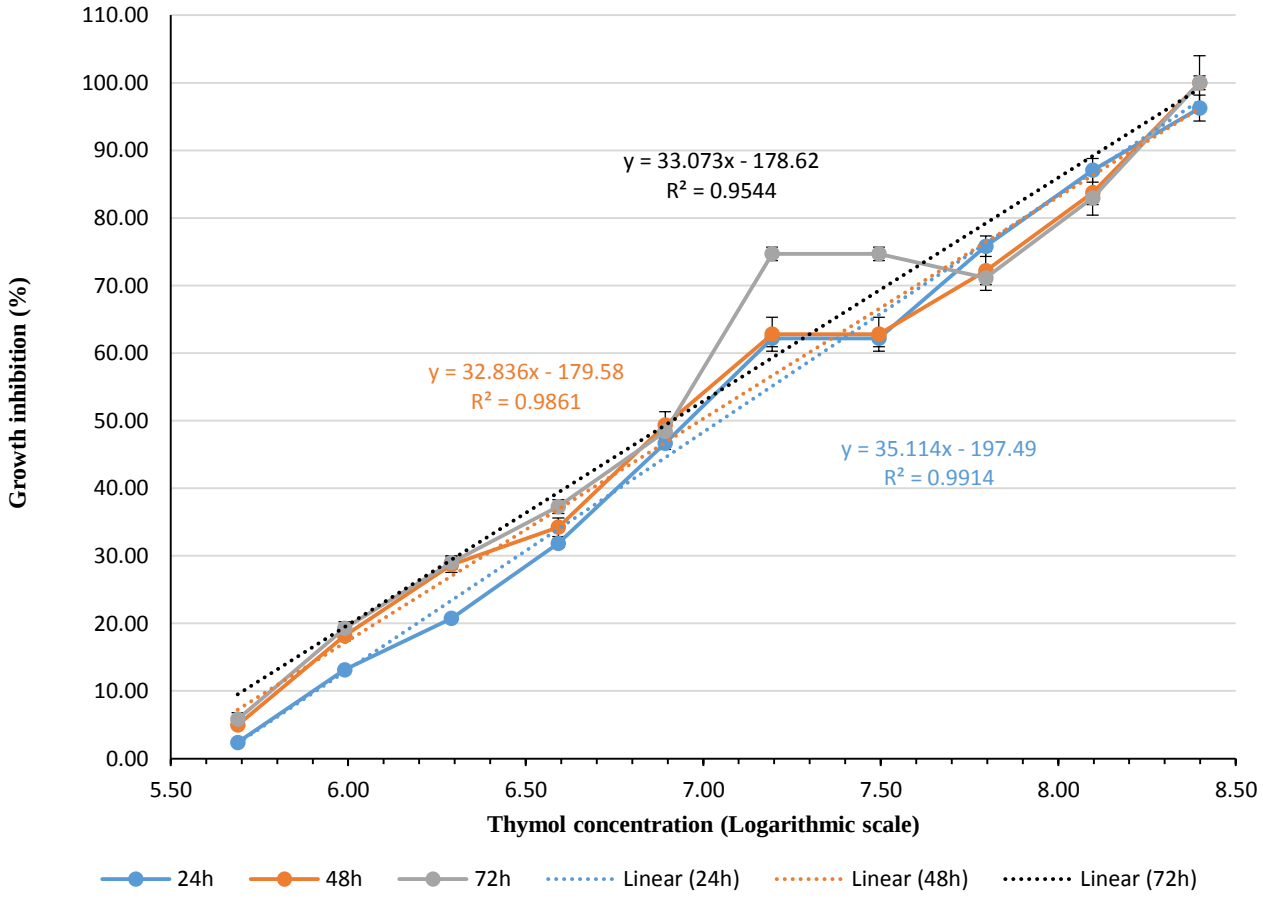
The effects of the mentioned formulations of thymol on *Leishmania major* promastigotes in comparison with free thymol and amphotericin B indicated that amphotericin B (0.65µg/ml) caused complete inhibition of *Leishmania major* in vitro. All forms of thymol including free thymol, thymol nano-liposomes, and thymol SLN at specific doses were able to inhibit *Leishmania major* during incubation for 24, 48, and 72 hours. This inhibitory effect of free thymol and NLP and SLN was maximized at concentrations of 250, 125, and 250 µ/ml in three incubation times of 24, 48, and 72 hours, respectively. Increasing the concentration in all three types of thymol formulations increased their inhibitory effect on *Leishmania major*, which on a logarithmic scale caused a typical sigmoid curve **(Figure 3,4, 5).**

Regarding the free form of thymol, there was no significant difference between the incubation time of 24, 48, and 72 hours in different concentrations in the rate of inhibition of *Leishmania major* growth **(Figure 3,6)**. However, concerning the form of NLP and SLN in each of the concentrations used, with increasing incubation time, the rate of inhibition of Leishmania growth also increased **(Figure 4, 5, 6).**

By comparing the IC50 of the products used, it was found that with 24 hours of incubation, free thymol (7.81 µg/ml) with a huge difference compared to NLP (62.5 µg/ml) and SLN (125 µg/ml), had the greatest inhibitory effect on

Leishmania major growth. The pharmaceutical form of nano-liposomes also has a much greater inhibitory effect than thymol SLN. However, with increasing the incubation time to 48 and 72 hours, the effectiveness of SLN (15.62) and thymol nano-liposomes increased more than free thymol, respectively, so that in 48 hours, there was a significant difference between free thymol (7.81) and nano-liposomes (7.81). Thymol SLN (15.62) still has significantly less inhibitory effect than the other two forms of thymol. With 72 hours of incubation, the inhibitory effect of both nano-liposomes (0.49) and SLN (0.98) is significantly greater than the free form of thymol (7.81), and there is no significant difference between the two forms of nano-liposomes and thymol SLN (**Figure 6**, P <0.05)

Figure 3. Growth inhibitory effect of free thymol on *Leishmania major* during 24, 48, 72h; The more increase in the concentration of thymol shows the more inhibitory effect



 DOI: https://doi.org/10.18502/aptj.v3i1.12501

Figure 4. Inhibition of *Leishmania major* growth by thymol nano-liposomes during 24, 48, 72 h incubation.

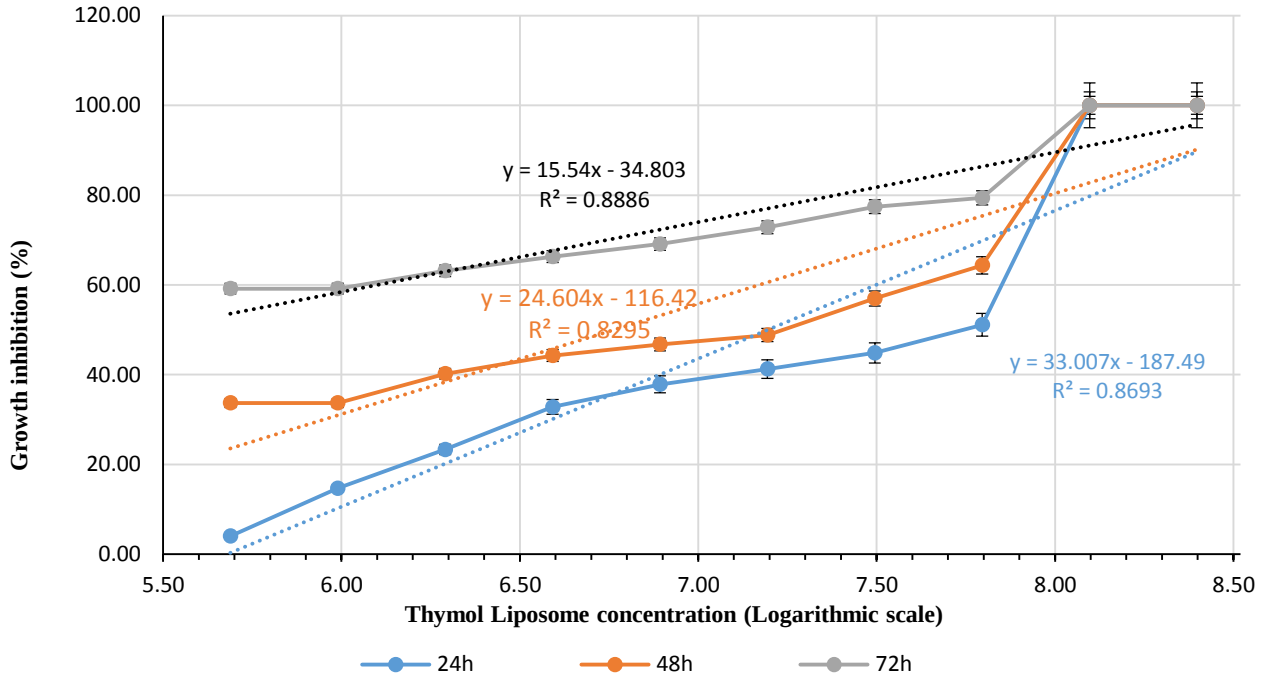


Figure 5. Inhibition of *Leishmania major* growth by thymol solid lipid nanoparticles during 24, 48, 72 h incubation.

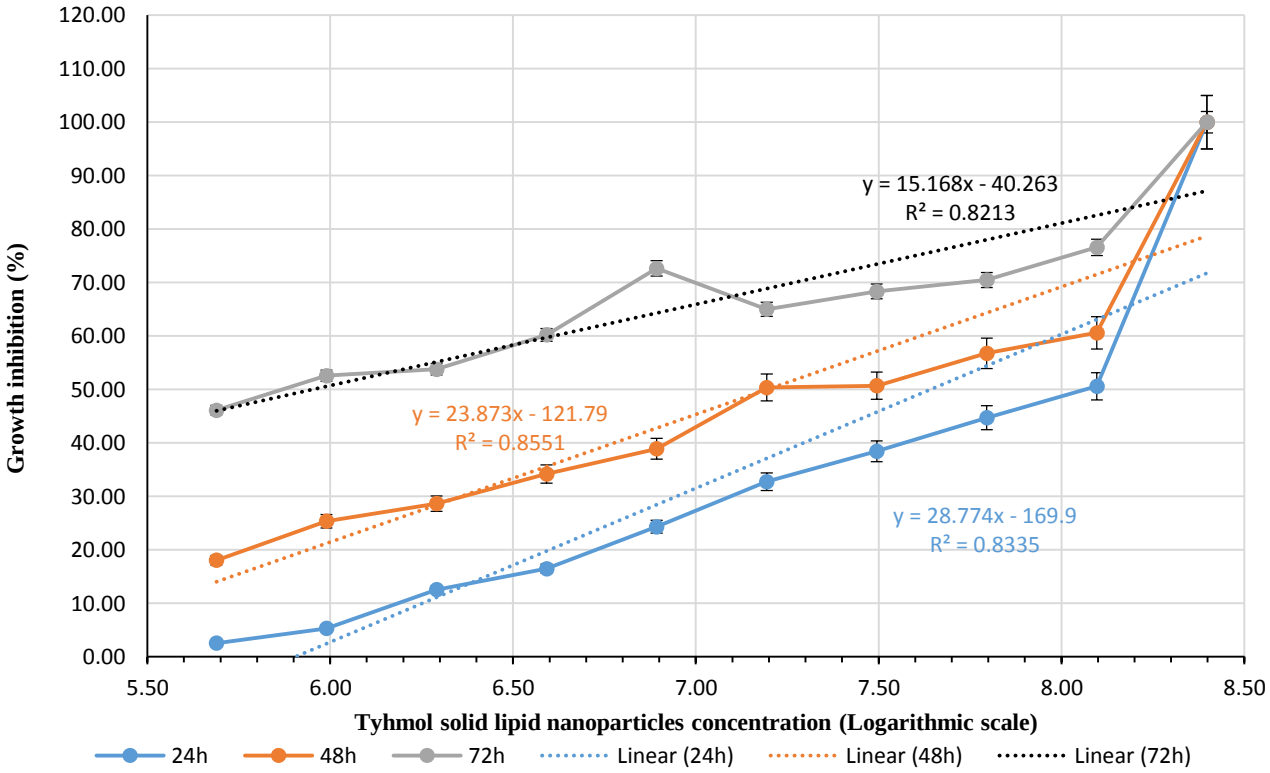
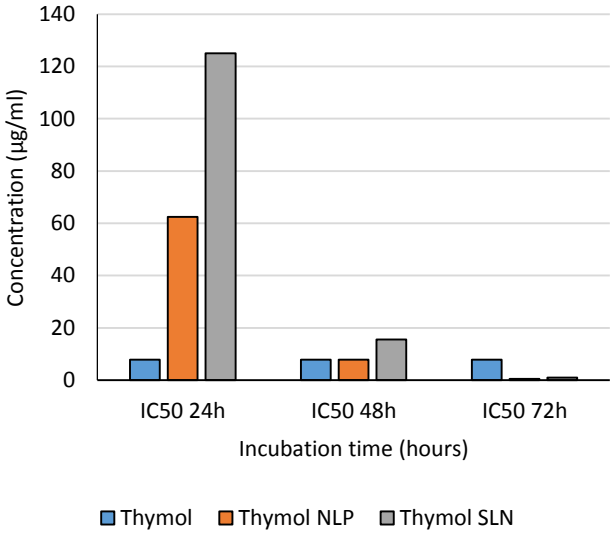


Figure 6. IC50 of thymol in forms free, nano-liposomes and solid lipid nanoparticles against *Leishmania major* promastigotes.



Discussion

According to the results of this study, thymol can inhibit the *Leishmania major* promastigote in vitro. In this study, amphotericin B was able to inhibit this protozoan up to 100%, as in other studies, the inhibitory effect of amphotericin B on *Leishmania major* was seen (21).

A study by Robledo et al. in 2005 showed that thymol and its derivatives had potential anti-leishmaniasis properties and could be used as a new drug in the treatment of Leishmaniasis (22). However, the present study was performed only on *Leishmania major* and found that thymol in all tested conditions can cause complete inhibition of *Leishmania major* promastigotes which is comparable to amphotericin B.

A study by Saedi et al. in 2016 showed that the most important constituents of thyme are thymol (41.8%) and carvacrol (28.8%), and according to these cases, for anti-leishmaniasis activity, the amount of IC50 of the primary extract and the methanolic extract was 3.2 and 9.8 µg/ml for promastigote form and 3.8 and 34.6 µg/ml for amastigote form, respectively (23). Considering the inhibitory effect of thymol on *Leishmania major* and that thymol is the main fraction of thyme, the inhibitory effect of thyme on Leishmania can be considered due to thymol and thymol derivatives in this plant, but more research is needed on this plant and its other fractions to confirm it definitively.

Observing the inhibitory effect of nanoliposome and SLN forms of thymol on *Leishmania major*, it was found that the carriers can prolong the release of thymol in the culture medium over time, which finally can completely inhibit *Leishmania major* promastigotes. The greater inhibitory effect of free thymol compared to nano-liposomes and SLN with 24 h of incubation and observation of the release profiles of thymol nanoparticles indicates that the release of thymol from the nano and microcarriers is gradual; while the inhibitory effect of these nanoparticles rises by increasing the incubation time to 48 and 72 h. Indicating that the nanocarriers cause a stable and controlled release of thymol. However, the inhibitory effect of thymol without a carrier remains almost constant with increasing incubation time. On the other hand, this gradual and controlled release of thymol from SLN is more evident than in thymol nano-liposomes.

In 2018 study by Pivetta et al. also showed that gel-containing thymol nanostructured lipid carriers (NLCs) in an animal model with dermatitis and mice with imiquimod-induced psoriasis improved their wound and inflammation

 DOI: https://doi.org/10.18502/aptj.v3i1.12501

compared with the negative control group. Their study showed that the formulation of thymol in nanoparticles form and the encapsulation prosses are effective methods to control the release of the active substance and increase the stability of the drug as well as reduce its toxicity (20). The results of the present study indicate the complete superiority of carriers, especially SLN nanocarriers in controlled release and increase drug efficacy during the incubation time.

Conclusion

Thymol, like amphotericin B, can inhibit *Leishmania major* promastigotes in vitro, and thymol-containing nano-liposomes and solid lipid nanoparticles give the product complete superiority in controlled drug release and increase drug stability over free thymol. Nano-liposomal carriers have a high ability to trap, load, and release thymol more rapidly, whereas thymol-containing solid lipid nanoparticles provide more controlled, longer, and efficient release.

Conflict of interest: There is no conflict of interest to be declared by the authors.

Funding: No

Acknowledgments: Thanks to our esteemed colleagues in the laboratory department of Shahid Sadoughi School of Pharmacy in Yazd, who helped us in this study.

Authors' contributions: MZ, FM, MS and VR conceived the study, participated in the design, MZ, MS and TD revised the final manuscript for important intellectual content. All authors read and approved the final version of the manuscript.

References

1. De Brito RCF, Aguiar-Soares RDdO, Cardoso JMdO, Coura-Vital W, Roatt BM, Reis AB. Recent advances and new strategies in Leishmaniasis diagnosis. Appl Microbiol Biotechnol. 2020;104(19):8105-8116.
2. Al-Gindan Y, Kubba R, Omer A, El-Hassan A. Br J Dermatol . 1988;118(6):851-4.
3. Currie MA. Br Med J. 1983; 287(6399): 1105–1106.
4. Sadeghian G, Ziaei H, Sadeghi M. Ann Acad Med Singap. 2008;37(11):916-8.
5. Sridharan K, Sivaramakrishnan G. Acta Trop . 2021;220:105944.
6. Khajedaluee M, Yazdanpanah MJ, SeyedNozadi S, Fata A, Juya MR, Masoudi MH, et al. Epidemiology of cutaneous leishmaniasis in population covered by Mashhad University of Medical Sciences in 2011. medical journal of mashhad university of medical sciences. 2014;57(4):647-54.
7. Nabavi SM, Marchese A, Izadi M, Curti V, Daglia M, Nabavi SF. Food Chem. 2015;173:339-47.
8. Youssefi MR, Moghaddas E, Tabari MA, Moghadamnia AA, Hosseini SM, Farash BRH, et al. Molecules. 2019;24(11):2072.
9. Wang T, Luo Y. Int J Mol Sci. 2018;19(10):3112.
10. Dara T, Vatanara A, Meybodi MN, Vakilinezhad MA, Malvajerd SS, Vakhshiteh F, et al. Colloids Surf B Biointerfaces . 2019;178:307-316.
11. Sun J, Bi C, Chan HM, Sun S, Zhang Q, Zheng Y. Colloids Surf B Biointerfaces . 2013;111:367-75.
12. Venishetty VK, Chede R, Komuravelli R, Adepu L, Sistla R, Diwan PV. Colloids Surf B Biointerfaces . 2012;95:1-9.
13. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, et al. Nanoscale Res Lett. 2013;8(1):102.
14. Methods Mol Biol. 2010;605:29-50.
15. Ghadiri M, Fatemi S, Vatanara A, Doroud D, Najafabadi AR, Darabi M, et al. Int J Pharm . 2012;424(1-2):128-37.
16. Sadegh Malvajerd S, Azadi A, Izadi Z, Kurd M, Dara T, Dibaei M, et al. ACS Chem Neurosci. 2019;10(1):728-739.
17. Wongsagonsup R, Shobsngob S, Oonkhanond B, Varavinit S. Zeta potential (ζ) analysis for the determination of protein content in rice flour. Starch-Stärke. 2005;57(1):25-31.
18. Shirzad H, Khorami S, Soozangar N, Yousefi M, Darani HY. World J Vaccines, 2012, 2, 105-108
19. Javed B, Raja NI, Nadhman A, Mashwani Z-u-R. Understanding the potential of bio-fabricated non-oxidative silver nanoparticles to eradicate Leishmania and plant bacterial pathogens. Applied Nanoscience. 2020;10(6):2057-67.
20. Pivetta TP, Simões S, Araújo MM, Carvalho T, Arruda C, Marcato PD. Colloids Surf B Biointerfaces. 2018 ;164:281-290.

21. Mehrizi TZ, Ardestani MS, Molla Hoseini MH, Khamesipour A, Mosaffa N, Ramezani A. Nanomedicine (Lond). 2018;13(24):3129-3147.
22. Robledo S, Osorio E, Munoz D, Jaramillo LM, Restrepo A, Arango G, et al. Antimicrob Agents Chemother. 2005; 49(4): 1652–1655.
23. Saedi Dezaki E, Mahmoudvand H, Sharififar F, Fallahi S, Monzote L, Ezatkhah F. Pharm Biol. 2016;54(5):752-8.

 DOI: https://doi.org/10.18502/aptj.v3i1.12501