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Research Paper

Pure Thymol, and its Nanoliposome and Nanoparticle Forms, Inhibit *Trichomonas vaginalis* Infection in Culture

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ABSTRACT

Background: There is a rising trend in the use of herbal stem cell remedies among the populace due to the belief that such remedies have all-encompassing health benefits, and without side effects. However, there is little or no scientific data reported on their safety profile. This study addressed the toxicological effects of STC-30, one of the popular polyherbal stem cell remedies used in several countries of the world including Nigeria, Ghana, Australia, among others.

Methods: The inhibitory activity of thymol and the nano-preparations were evaluated qualitatively versus Metronidazole against T. vaginalis in TYI-S33 culture medium. The live T. vaginalis parasites were counted on a hemocytometer, the inhibition rate was assessed and the data analyzed statistically.

Results: Thymol and its two nanopreparations at varying concentrations inhibited T. vaginalis in culture after 24, 48, or 72 hours of incubation. The inhibition of T. vaginalis was also achieved in culture with Metronidazole at 65 µg/ml.

Conclusion: The percent inhibition of T. vaginalis by thymol and its nanopreparations depended on the duration of incubation and the concentration. Thymol and its nanoliposome preparation showed a lower inhibitory effect (IC50) on T. vaginalis than that of the thymol nanoparticles after 24 or 48 hours of treatment. However, the efficacy of the three thymol forms did not significantly differ after 72 hours of treatment.

Keywords: Inhibitory Activity; Nanopreparations; Nanoliposomes; Thymol; Trichomonas Vaginalis Infection

Introduction

Trichomona vaginalis (*T. vaginalis*) is a flagellate protozoan that causes genitourinary tract infections, mainly in women (1). The incidence of this parasitic infection has been reported to be between 4-6% in different parts of the world (1, 2). This infection is transmitted through sexual contact, shared towels and underwears, or via non-sterile medical devices. Factors such as poverty, illiteracy, multiple sexual partners, and aging increase the risk of this infection (3, 4).

In women, this parasite causes vaginitis, inflammation of bladder, foul-smelling mucus, burning, and itching. Similarly, it causes urinary tract inflammation, burning, and prostate inflammation in men (4, 5). In addition, this parasite can cause many other complications, such as preterm delivery, low birth weight, miscarriage, premature rupture of amniotic sac, ectopic pregnancy, postpartum endometriosis, salpingitis, chronic cervicitis, cervical cancer, and reversible infertility (6, 7).

Metronidazole and its analog tinidazole are two popular drugs for treating *trichomoniasis* in humans. Among the risks of using these drugs is their carcinogenicity in animals. About 5% of *T. vaginalis* infections are estimated to be resistant to these two drugs (8, 9). Microbial resistance is one of the main reasons for the failure of treatment with this and

other antibiotic drugs (10, 11). This drug is contraindicated in pregnant women, especially in the first trimester (12). Given the adverse effects of Metronidazole, the need for an alternative drug is warranted to treat *T. vaginalis* infection.

Application of plant-derived formulations in traditional medicine has been popular since they are low-risk, available and inexpensive natural agents, compared to synthetic antibiotics in the treatment of various microbial infections (13, 14). Thymol is a phenolic compound found in plants, such as thyme and its seeds, ajowan (*T. ammi*). Phenolic compounds, such as thymol and its isomer carvacrol, are believed to have anti-bacterial, anti-fungal, and antioxidant properties. They are used as natural food preservatives, and have anti-aging properties in mammalians (15). Their antimicrobial effect is likely due to increased permeability in the cell membrane, which can disrupt the concentration of cations (15).

Since there are problems with pure plants' essential oils, such as instability, evaporation, and decomposition under environmental conditions, a modern approach is to prepare nanoparticle compounds (16). While providing excellent preservation, nanoparticles do not hurt the antimicrobial activity of thymol preparations. Further, due to the small size at nanoscale, the nano-preparations can increase the inactive cell uptake, reduce the transfer resistance, and improve the antimicrobial activity (17).

A liposome is a microscopic vesicle consisting of two phospholipid layers surrounding an aqueous component. The lipid bilayer thickness is typically between 3-6 nm, and the resultant liposomes are between 50nm and 50µm in diameter. Due to the amphipathic nature of their constituent elements, liposomes allow for the delivery of hydrophilic or lipophilic drugs. These tiny sac-like structures are like pockets or capsules prepared to carry the drug to different parts of the body. Liposomes have been able to reduce adverse side effects and improve drug stability. They are used as carriers for drugs, enzymes, anticancer, fungicidal, antiparasitic, antiviral, and antibacterial agents (18).

Considering the above facts and reviews, the aim of this study was to investigate the effect of pure thymol and its two nano-preparations on the inhibition of *T. vaginalis* parasites.

Materials and Methods

Measurement and determination of thymol concentration: Thymol (Merck, Germany) was dissolved in ethanol as the solvent. The UV absorption of seven concentrations of thymol solution were read at 276 nm at a concentration of 1, 5, 10, 20, 30, 40, or 50 mg/ml on a UV/vis spectrophotometer (Unico, China), based on the following equation: $Y = 0.0149X + 0.0019$.

Preparation of thymol solid lipid nanoparticles: Solid lipid nanoparticles were prepared using the emulsification solvent and evaporation methods. Thymol and various fats were dissolved in acetone according to Table 1, and dispersed in an aqueous medium containing polyvinyl alcohol. Solid lipid nanoparticles were prepared after evaporation of acetone from the aqueous medium.

Table 1. Specifications of the Solid Thymol Lipid Nanoparticles.

Component	*S1	*S2	*S3	*S4	*S5	*S6
Thymol	10 mg	10 mg	10 mg	10 mg	10 mg	10 mg
Solvent (vol)	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
PV Alcohol	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Fat (mg)	GMS (10)	GMS (20)	GMS (50)	Tefose (10)	Labrafil (10)	SA (10)
Total (vol)	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Thymol (en)	92%	19.32%	16.84%	18.55%	8.72%	100%

* Solid thymol lipid nanoparticles.

GMS, Glycerol monostearate; SA, Stearic acid; PV, Polyvinyl; vol, volume; en, enclosure.

Table 2. Formulation of different nanoliposome forms of thymol.

Group	PTC	PS-20	PS-80	CLS	Thymol	CLF	Conc. (µg/ml)	ENC
L1	50 mg				10mg	1 ml	66.2080	33%
L2	50 mg				20mg	1 ml	78.1140	39%
L3	50 mg				30mg	1 ml	187.020	93%
L4	50 mg			10 mg	10mg	1 ml	2.4697	1%
L5	50 mg		10 mg		10mg	1 ml	5.5100	2%
L6	50 mg	10 mg			10mg	1 ml	29.0671	14%

PTC, Phosphatidylcholine; PS, Polysorbate; CLS, Cholesterol; CLF, Chloroform; Conc, Concentration; ENC, Encapsulation.

Evaluation of thymol release from particles: To investigate the release of thymol from the nanoparticles, 1 ml of nanoparticles was poured in a dialysis bag (cut off =13 kD) and placed in 100 ml

phosphate buffer at pH 7. Then, a 2ml sample was withdrawn at each of the following 14 time points: 1, 2, 5, 10, 15, 20, 30, 45, 60, 120, 180, 240, 300, and 360 min, and a 2 ml phosphate buffer were replaced in the tube. Lastly, the thymol concentration of each sample was read at 276 nm on a UV/vis spectrophotometer.

Preparation of thymol in liposomal form: Thymol nanoliposomes were made by thin layer hydration method as described previously (19). The required amount of phosphatidylcholine, thymol, and the lipid-soluble ingredients were dissolved in chloroform as shown in Table 2. The thin layer was formed by evaporation of the solvent under vacuum in a rotary evaporator at 50 rpm. The lipid layer was hydrated with phosphate buffer. The nanoliposomes were prepared after sonication of the formed large-sized vesicles for 5 minutes at 4°C (Table 2).

Encapsulation efficiency of thymol nanoliposomes: One ml of the prepared nanoliposomes was transferred to the dialysis bag and kept in distilled water at 4°C. Then, they were broken using ethanol, and the amount of encapsulated thymol was measured on a UV spectrophotometer.

Release of thymol from nanoliposomes: To determine the release pattern of thymol from nanoliposomes, 1-ml of each formulation was transferred to the dialysis bag, suspended in 100 ml of phosphate buffer at pH 7, and stirred. Then, 2-ml of the sampled was withdrawn at fixed time points. The sample's thymol concentration was determined on a UV/vis spectrophotometer at λmax 276.

Particle size measurement: The nanoliposomes' particle size and solid lipid nanoparticles (SLNs) were measured using a dynamic light scattering method (Zetasizer, NanoZS, Malvern) after dilution with an aqueous medium at 25°C.

Proliferation of *T. vaginalis*: This parasite strain was isolated from vaginal secretions of a woman with *trichomoniasis* at Isfahan University of Medical Sciences and stored in a freezer at minus 70°C. The parasites were cultured in the required amount in TYIS-33 medium for mass proliferation.

Exposure of *T. vaginalis* to the products: Based on previous studies, after the parasite strain reached a concentration of 10000 per ml in the logarithmic phase, it was exposed to the 10 concentrations (0.5-25 µg/ml) of each of the thymol nanoparticle forms, liposomal structures (formulations L3 and L6), and pure thymol. In addition, the 10 samples were infected with drug-free nanoparticle and liposomal forms, plus one sample for each of Metronidazole and controls. The experiment was repeated three times, and the inhibitory effects of the interventions were examined three times on the growth of *T. vaginalis* within 24, 48, and 72 hours. This experiment consisted of 468 samples in total. All groups were kept at 25°C for 72 hours, and the live *T. vaginalis* parasites were counted at 24, 48, or 72 hours on a hemocytometer.

The growth inhibition for each of the thymol concentrations, nanoparticle and liposomal forms was calculated in the presence of the parasites, using the following formula: GI = [(a-b) / a]. Where, "GI" was growth inhibition, "a" was the number of the live parasites in the control sample, and "b" was the number of the live parasites in the sample containing a specific thymol product.

Statistical analysis: Using Excel software, version 2019, the inhibitory concentration (IC_{50}) was calculated for thymol nanoparticle and liposomal forms, and pure thymol. The results were presented as the means and standard deviations. The statistical data analyses were performed, using SPSS version 21, one-way ANOVA and Tukey's post hoc test at a 95% confidence interval.

Results

The anti-*Trichomonas* activity of the formulations was evaluated on S_1, S_6, L_3 and L_6 formulations. The S_1 and S_6 formulations were tested for entrapment efficiency at 92% (containing stearic acid) and 100% (containing glycerol monostearate), respectively. Also, the L_3 and L_6 formaulations were evaluated for entrapment efficiency at 93% and 14%, respectively.

As shown in Figure 1, the contamination of glycerol mono-stearate in S_1 enhanced the release of thymol from nanoparticles compared to that of the stearic acid. This trend was also noted when Tween 20 was used in the L_6 formulation. Adding this substance to nanoliposomes changed the liposomes membrane fluidity and the entrapment of thymol as a lipid-soluble agent. The added fluidity led to a decreased lipid bilayer capacity and entrapment efficiency. Thymol was released more rapidly from the S1 formulation compared to that of S6 (Figure 1). However, the remaining thymol in liposome containing polysorbate 80, with an entrapment efficiency of 14%, showed a kinetic pattern similar to liposome without surfactant (L_3). See Figure 2.

Figure 1 compares the thymol release from the nanoparticle form in formulations S1 and S6. Figure 2 compares the concentration of thymol release from the liposomal forms in L3 and L6 preparations. The results on the empty nanoparticle system and nanoliposomes showed that neither group had a lethal effect on *T. vaginalis* parasites. However, Metronidazole at 65µg/ml completely inhibited the parasites at the three time points of 24, 48, and 72 hours.

The liposomal nanoparticles (L3 & S1) were used to inhibit the growth of *T. vaginalis*. As seen in Figure 3, increasing the inhibitory effect of thymol as the concentration increased, the differences in the inhibitory effect of thymol against *T. vaginalis* were significant in most of the concentrations (P <0.01).

This indicated the effect of raising the thymol concentration on the inhibition of the parasite growth. The IC_{50} for thymol was obtained at different times using the calculation formula, 10.022, 6.728, and 6.607 µg/ml at 24, 48, and 72 hours, respectively. See Figure 6.

Figure 3 compares the average growth inhibition percentage of *T. vaginalis* at different thymol concentrations for 24, 48, and 72 hours. Figure 4 demonstrates the rise in the inhibitory effect of thymol nanoparticles by increasing its concentration. The difference in the inhibitory effect of thymol nanoparticles on *T. vaginalis* was statistically significant at the 3.59 µg/ml concentration and above within 24 hours, and the 3.29 concentration and above within 48 hours. The inhibition was evident at all concentrations within 72 hours (P <0.01). The IC_{50} for thymol nanoparticles within 24, 48, and 72 hours was 64.202, 16.471, and 9.55 µg/ml, respectively. See Figure 6.

Figure 4 compares the mean growth inhibition of *T. vaginalis* at different concentrations of thymol nanoparticles during three time points of 24, 48, and 72 hours. Similar to other preparations, the effect of thymol liposomal form increased with raising the concentration. The difference in the inhibitory effect of the liposomal form of thymol on *T. vaginalis* was significant (P<0.01) at the thymol concentration of 2.99 µg/ml and above within 24 hours, and at all concentrations within 48 and 72 hours. See Figure 5. The IC_{50} concentrations for the liposomal thymol at 24, 48, and 72 hours were 11.86, 7.41, and 4.71µg/ml, respectively. See Figure 6.

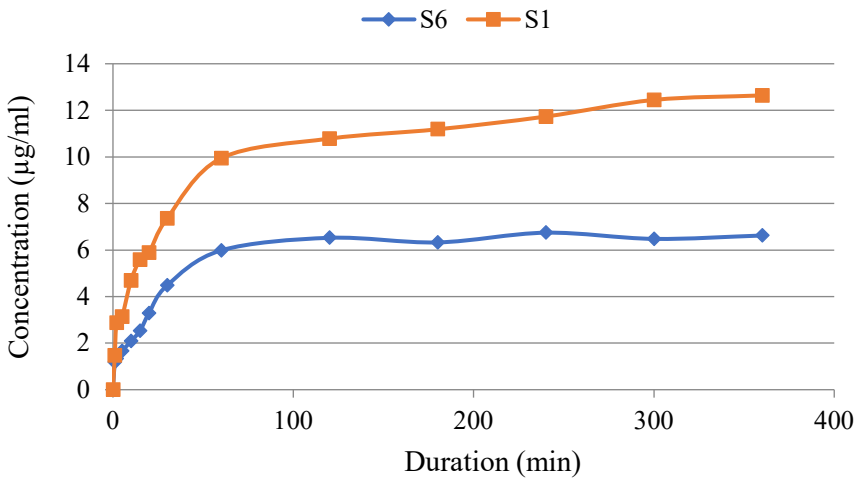

Figure 1. Comparison of thymol release from its nanoparticle form S1 and S6 formulations.

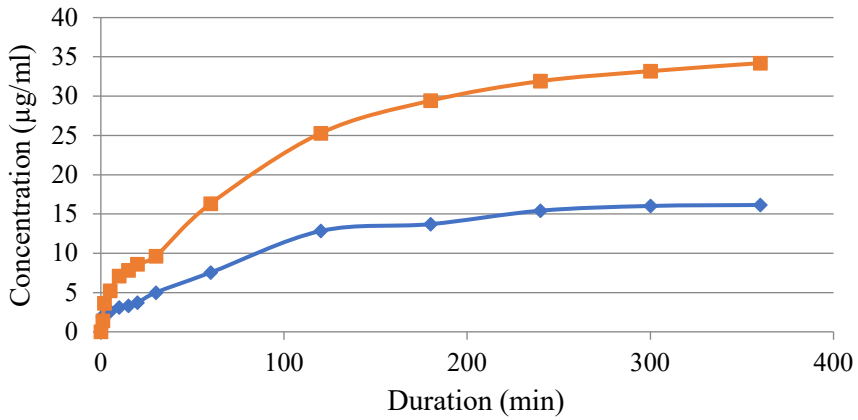

Figure 2. Comparing thymol release concentration from its liposomal form (L3 & L6). The red line is phosphatidylcholine; the blue line is Tween-20.

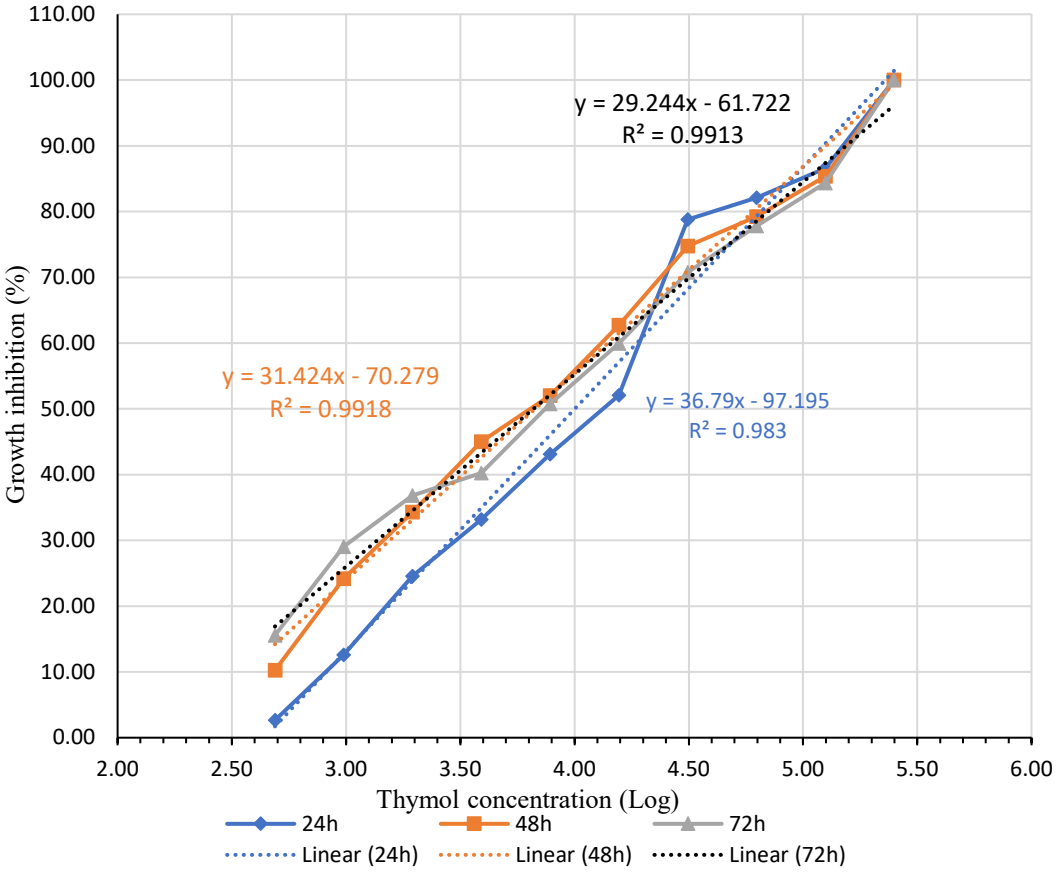

Figure 3. Comparison of the average growth inhibition percentage of *T. vaginalis* at varying concentrations of thymol over three durations, 24, 48, and 72 hours.

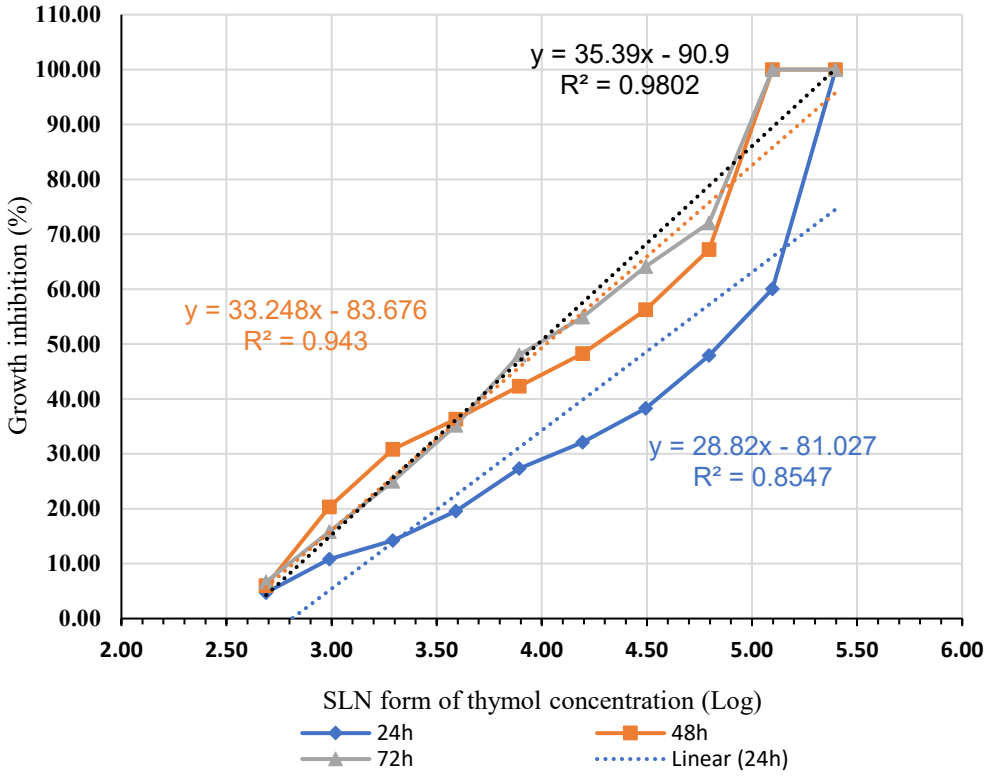

Figure 4. Comparison of the mean growth inhibition of *T. vaginalis* at varying concentrations of thymol nanoparticles over three durations: 24, 48, and 72 hours.

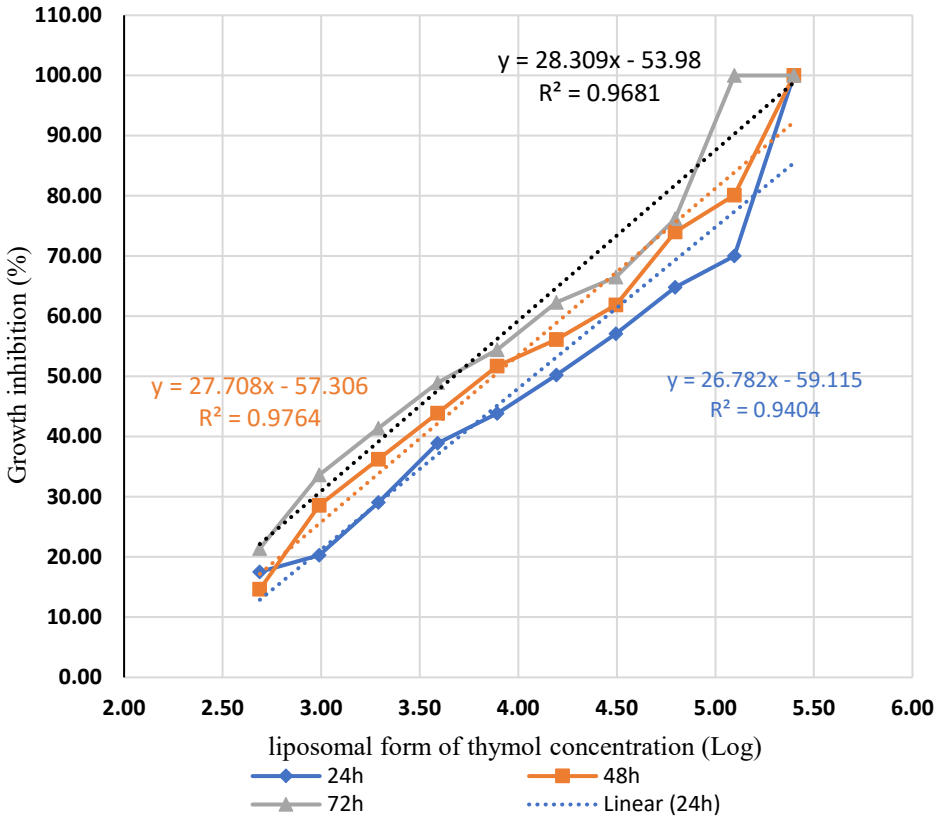

Figure 5. Comparison of the mean percentage of growth inhibition of *T. vaginalis* at varying concentrations of the liposomal thymol over three durations: 24, 48, and 72 hours.

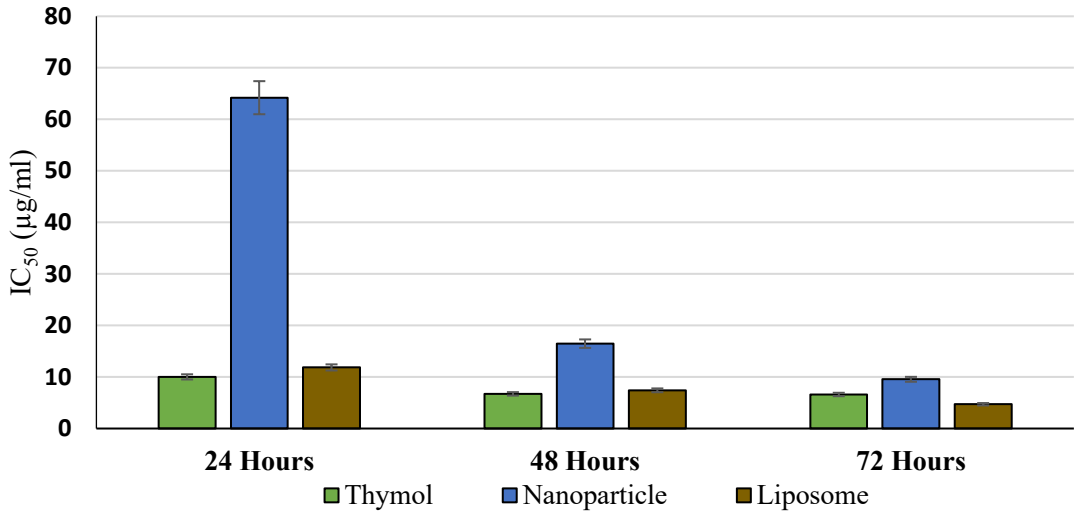

Figure 6. Comparison of the IC_{50} efficacy of thymol, and its nanoparticle and liposomal forms against *T. vaginalis* at varying treatment durations.

Figure 5 compares the mean percentage of growth inhibition of *T. vaginalis* at different concentrations of the thymol liposomal form during three time points of 24, 48, and 72 hours. The results demonstrated that the average percentage of growth inhibition did not differ significantly by increasing the exposure time from 24 to 48 and 48 to 72 hours. In addition, the average rate of growth inhibition of

the pure thymol at different time points was almost similar compared to those of the nanoparticle and liposomal forms. See Figure 6. As reflected in Figure 6, the efficacy (IC_{50}) of pure thymol is compared to those for the nanoparticles, and liposomal forms against *T. vaginalis* at different exposure times.

Discussion

Herbal medicines are inexpensive due to ample availability of raw materials and greater patient acceptance (20), hence, the popularity of plants products as alternative therapies in today's healthcare (21). Although some herbal products can inhibit the growth of one or more protozoan species, very few of them kill the organisms (22). Metronidazole is an anti-protozoan drug by breaking the DNA strands; while the mechanism of thymol action against parasites is known to permeate and disrupt cell membranes and the surface cations, thus inhibiting the vital parasite activities (23).

Metronidazole is the suggested drug of choice for *trichomoniasis*, although it has its side effect and lead to microbial resistance (24). Patients with *trichomoniasis* sometimes have to undergo a multi-stage, long-term treatment due to recurrence of the infection. In such cases, the application of an alternative agent, such as thymol are known to be beneficial to patients (25, 26) This justifies the increased popularity and search for effective natural alternative medicines (26). Therefore, it seems that products from thymol-containing plants are likely to be appropriate treatment choices in these cases. However, further clinical studies are warranted to establish the efficacy of the plants products. In this context, studies have already shown the anti-*trichomoniasis* effect of thymol-containing compounds. Thymol is also effective in relieving itching in the genital area, which increases the importance of its use in such clinical cases (27, 28).

In a study by Golmakani, *et al*., the effect of a vaginal cream containing thymol was evaluated versus that of Metronidazole (29). The results demonstrated that the success rate of a wet smear-based treatment was 51.9% in the thyme group versus 88.9% in the Metronidazole counterpart (30). The results of the present study demonstrate that the three plant products; i.e., pure thymol, the nanoparticle and liposomal forms effectively inhibit the growth of *Trichomonas*, the inhibitory effect of which increases by raising its concentration. Although the inhibitory effect increased slightly over longer treatment periods, this effect was not significant on the parasite inhibition within the duration used in the current study. It was also shown that the average percentage of growth inhibition did not differ significantly by increasing the treatment duration from 24 to 48 and to 72 hours. In addition, the average inhibition rate of the parasite's growth by thymol alone over different treatment periods with the nanoparticle and liposomal forms was almost the same.

It was also revealed that the three forms of thymol used at higher concentrations produced similar inhibition in the parasites compared to that of Metronidazole at 65 µg/ml. The liposomal formulation caused 100% growth inhibition of the parasites when the treatment time increased to 72 hours, which was significantly better than that of thymol alone. Based on the findings of one study by Wattanasatcha (31), nanoparticles and nanoliposomes did not seem to significantly affect the growth inhibition of the parasite relative to thymol alone despite the additional cost and difficulty for their preparation (31). This study evaluated the anti-bacterial effect of thymol nanoparticles, and showed that they had lower MIC and MBC than pure thymol extract against *Staphylococcus aureus*, *E-Coli*, and *Pseudomonas aeruginosa*. This study also showed that the use of nanocapsule form improved the duration of drug effect, i.e., the nanoparticle form suppressed bacterial life up to 3 months while the extract only suppressed it for 2 to 4 weeks (31).

In another study (32), herbs containing thymol and carvacrol have been used, and demonstrated that thymol effectively inhibited *Trichomonas* parasites experimentally at two concentrations of 0.1 and 0.01% as did Metronidazole. Furthermore, similar to the present study findings, increasing the exposure period from 24 to 48 and 72 hours did not significantly affect the antiparasitic power of the thyme extract. It seems that the liposomal and nanoparticle forms of thymol can stabilize and increase the duration of drug effect. The efficacy of the liposomal was more than the thymol alone within72 hours, as evident by the current study's findings. However, we found no significant differences among the three forms, i.e., thymol alone, nanoparticle form, and liposomal thymol at different concentrations. Pivetta, *et al*. also showed that thymol nanoparticles and encapsulation effectively released the active ingredient, which increased the stability of the drug and reduced its toxicity (33, 34). Therefore, it appears that the nanoparticle and liposomal and encapsulation forms of thymol, can improve the drug stability.

In line with the present study, Ardestani, *et al*. showed that thymol at 4000 µg/ml within 24 hours inhibited the growth of *T. vaginalis* by 91%. In that study, the IC_{50} values for thymol over 24 and 48 hours were 195.9 and 91.85 µg/ml, respectively. Similar to our findings, Ardestani, *et al*.'s study showed that the effect of thymol was dependent on its concentration and treatment duration. Although the impact of increasing the treatment duration from 24 to 48 hours was small, the average percentage of growth inhibition by increasing the thymol

concentration from 125 to 4000 µg/ml had a significant impact (35).

Conclusions

Based on our findings, the percent inhibition of *T. vaginalis* by pure thymol versus its nanoliposome and nanoparticle forms depended on the duration and the concentration. Thymol and its nanoliposome form showed a lower inhibitory effect (IC_{50}) on *T. vaginalis* than that of the thymol nanoparticles over 24 or 48 hours of treatment. However, the efficacy and the IC_{50} values of the three thymol formulations did not significantly differ after 72 hours of treatment.

Ethical Considerations

This study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. (Code: IR.SSU.MEDICINE.REC.1398.180).

Compliance with Ethical Guidelines

No animals were used in this study. The *T. vaginalis* strain was used in the culture medium. During the study, ample hygienic guidelines were used to prevent the spread of this protozoan to humans and the laboratory environment. At the completion of the study, all of the remaining protozoan samples were discarded safely with other infectious wastes at the isolated disposal area of the School of Pharmacy for regular disposal.

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Authors' Contributions

Conceptualization, supervision, and methodology: MZ and FM; Investigation: SJ, MZ, FM & VR; Writing the original draft of the manuscript: SJ, HG; Review & editing: HG, MZ; Data collection: SJ, FM and VR; Data analysis: SJ and MZ; Funding Administration: MZ and FM.

Conflict of Interest

The authors declare no known competing financial interests or personal relationships that could have influenced the work reported in this article.

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