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In Vitro Activity of *Mentha longifolia* Leaves and *Pimpinella anisum* Seeds Against a Clinical Strain of *Trichomonas Vaginalis*

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ABSTRACT

Trichomoniasis is one of the most common urinary-genital tract disease. The causal agent of trichomoniasis is flagellate protozoan called *Trichomonas vaginalis*. Metronidazole and tinidazole are the drug of choice for trichomoniasis treatment. However, carcinogenic and embryo-teratogenic effects of the mentioned drugs and resistance to metronidazole has been reported. According to cytotoxic effects of medicinal herbs including *Mentha longifolia* and *Pimpinella anisum* on microbial organisms and their traditional use in sexually transmitted diseases the in vitro activity of the *Mentha longifolia* and *Pimpinella anisum* against a clinical strain of *Trichomonas vaginalis* was investigated. Concentrations of aqueous extract of *Mentha longifolia* leave (100-250 µg/ml) and *Pimpinella anisum* seed (100-500 µg/ml) were obtained. *Trichomonas vaginalis* isolated from patient with urogenital complications was cultivated in TYI-S-33 medium. After treatment of *T. vaginalis* (1×10^6) with various concentrations of extracts, the minimum inhibitory concentration (MIC) was calculated and data were analyzed by repeated measures using SPSS software. Aqueous extract of *Mentha longifolia* decreased the average number of live parasites, in accordance of the time and concentration manner. The MIC of *Mentha longifolia* at 24 and 48 h were 250 and 200 µg/ml, respectively. The percent of growth inhibition under various concentration of *Pimpinella anisum* showed only 73.7% and 86.9% at 500 µg/ml concentration after 24 and 48 hours, respectively. The present study demonstrate the higher anti-*Trichomonas* activity of *Mentha longifolia* in comparison to *Pimpinella anisum.* There is a clear need to isolate effective fragments of *Mentha longifolia* in future and expand the data for in vivo evaluation including cell culture and animal models study.

1. Introduction

Trichomonas vaginalis is a parasitic protozoan with a predilection for human urogenital tract and causative agent for vaginitis, cervicitis and urethritis in females (Garcia, 2006). There are serious health consequences in trichomoniasis such as adverse pregnancy outcome, increased risk of infertility and cervical carcinoma (Gutierrez, 2000). Interestingly, there are several studies indicating the association of trichomoniasis infection in men and benign hyperplasia and prostate cancer (Petrin et al., 1998; Rezaeian et al., 2009).

The prevalence of trichomoniasis in Iran has a range from 0.1 to 15% due to various methods of detection and different examined population (Rezaeian et al., 2009). It should be mentioned

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there are many unreported cases of trichomoniasis in the region, because of chronic pattern and mild symptoms in over 50% of women (Rezaeian et al., 2009). The drug of choice for therapy is 5-nitroimidazole compounds including metronidazole and tinidazole (Ryu and Min, 2006). In Iran the only available 5-nitroimidazole drug is metronidazole, however the main drawback to metronidazole is wide adverse effects including nausea, gastrointestinal disturbance, leukopenia and headache (Sobel and Sobel, 2015). Besides, there are evidences of emerging resistance to metronidazole as Center for Disease control (CDC) reported that 6% of *Trichomonas vaginalis* isolated from patients showed resistant to metronidazole (Sena et al., 2014).

There were reports regarding teratogenic and carcinogenic effects of metronidazole. To this end, natural products derived from medicinal plants are a priority objectives (Sobel et al., 1999). Accordingly, the world health organization (WHO) estimates that approximately 80% of the world population mainly use plant based remedies for various diseases. Indeed, the potential of natural products such as *Mentha longifolia* (Lamiaceae) and *Pimpinella anisum* in the treatment of diseases especially sexually transmitted disease is evident (Kose et al., 2015; Palmeira-de-Oliveira et al., 2013; Saeidi et al., 2014). Noteworthy, in Iran medicinal plants are an attractive approaches as an alternative options to the chemical drugs in various diseases (Bakht et al., Shaheen and Shafi, 2014; Kose et al., 2015; Saeidi et al., 2014). *Mentha longifolia* (Lamiaceae) and *Pimpinella anisum* has been used in medicine due to their cytotoxic effect in bacteria and fungi (Bakht et al., 2014; Eissa et al., 2014; Kose et al., 2015). *Pimpinella anisum (anise)* belonging to Umbelliferae family is an endemic plant which has been used in Iranian traditional medicine, *anise* showed antibacterial effects on various microorganisms (Ghoshegir et al., 2014) . It should be mention that there were no previous reports of anti-*trichomonas* activity of *Mentha longifolia* and *Pimpinella anisum* in Iran and around the world.

Considering the adverse effect of metronidazole, the present study was aimed to determine the effect of *Mentha longifolia* and *Pimpinella anisum* on *Trichomonas vaginalis* isolated from a female patient with urogenital complication.

2. Materials and Methods

2.1. Parasite and cultivation in TYI-S-33

Trichomonas vaginalis parasite was isolated from vaginal discharge of women with symptomatic trichomoniasis (Heidari et al. 2013). Confirmation regarding the presence of *T. vaginalis* was done using direct wet mount and culture. *T. vaginalis* were cultured axenically in the Tripticase (Sigma-Aldrich, Germany), Yeast extract (Merck, Germany), iron-serum (TYI-S-33) medium for 24-48 h according to previous studies (Diamond et al., 1995). The medium was supplemented with 10% heat inactivated bovine serum, vitamins (Merck, Germany) and streptomycin (Merck, Germany) and incubated at 37^{O}C (Diamond et al., 1995). Organisms in the logarithmic phase of growth were harvested, centrifuged and suspended on fresh medium for experiments. The criteria used for viability was eosin tests. An initial inoculum of $1x10^{6}$ trophozoites/ml was incubated in the presence of the extracts.

2.2. Preparation of plant extracts

Mentha longifolia leaves and *Pimpinella anisum* seeds were collected from the Ray area of Iran and identified and approved by a local herbalist (Mohammad Kamalinejad) from the School of Pharmacology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Plants were chosen regarding their traditional application in the treatment of venereal disease (Furst and Zundorf, 2015). Aqueous extracts of two endemic plant including *Mentha longifolia* leaves and *Pimpinella anisum* seeds were obtained using maceration technique as previously described (Rogers et al., 2014; Shreaz et al., 2011). Briefly, plants were air dried, powdered and suspended in sterile distilled water for several hours. The extracts were centrifuged and the supernatants filtered through 0.22 filters. The concentration range of the extracts were from 100-250 µg/ml for *Mentha longifolia* and 100-500 for *Pimpinella anisum.*

2.3. Anti-Trichomonas activity of the plants

On thousands microliter of trichomonads with the same volume of extracts were thoroughly mixed and the micro tubes kept at 37^{O}C for 24 and 48 hours. Eosin dye was used to determine the extract effectiveness. Same volume of eosin 0.1% and parasite suspensions was then mixed. Positive and negative controls were metronidazole (0.1 µg/ml) and culture medium, respectively. Experiments were performed in triplicate.

2.4. Determination of the Minimum Inhibition Concentration (MIC) of the plant extracts

Results were recorded as the percentage of living organisms using exclusion test by eosin (0.1%). The percent of growth reduction were calculated for each concentration. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of plant extract in which no lived parasite was detected.

2.5. Statistical analysis

The data were analyzed using repeated measures of SPSS. The P value was set at 0.05 for significance level.

3. Results

The results of the present research are presented as the percentage of growth reduction of *Trichomonas vaginalis* by comparison of the number of parasites found in the tests to the number of parasites in control. The present research, showed the effectiveness of the extract of *Mentha longifolia* by remarkable trichomonocidal effect (Fig 1), since the aqueous extract obtained from *Mentha longifolia* leaves totally abolished the trophozoite viability. This extract was active in a dose dependent manner. The MIC of *Mentha longifolia* at 24 h and 48 h were 250 and 200 µg/ml, respectively (Fig 2, Table 1). However, it should be mention that the concentrations 100-250 µg/ml failed to completely destroy the parasites (Table 1). The concentration 150 g/ml, 8.4×10^4 viable *T. vaginalis* were detected at the end of the incubation period. Viable trophozoites within the control media did not show any relevant changes during the test. Average number of viable trophozoites revealed increase with the decreasing concentrations of the aqueous extract of *Mentha longifolia.*

However, the percent of growth inhibition under various concentration of *Pimpinella anisum* showed only 73.7% and 86.9% at 500 µg/ml concentration after 24 and 48 hours, respectively. The effect of *Pimpinella anisum* on the viability of *T. vaginalis* is shown in Table 2 and fig 3.

4. Discussion

Trichomoniasis is considered as a neglected disease mainly due to poor understanding of pathogenesis factors. According to WHO report, trichomoniasis affects approximately 174 million new cases annually (Arab-Mazar and Niyyati, 2015; Rogers et al., 2014; Sena et al., 2014). In Iran, the only available 5-nitroimidazole drug is metronidazole, however there are several reports in this region regarding resistance. Due to adverse effect of chemical drugs such as metronidazole, use of plant- based compounds could be an alternative approach for treatment of Trichomonasiasis. In Iran traditional healers prescribe maceration of the leaves and seeds of lamiacea family specially *Mentha longifolia* and *Pimpinella* seeds to be taken orally for treatment of urogenital infectious organisms (Kose et al., 2015). The traditional use of the leaves of *Mentha longifolia* was mainly for recovery from several diseases including sexually transmitted diseases such as bacterial vaginitis. The present study revealed that *M. longifolia* could be an appropriate candidate for further phytochemical analysis as percent of growth inhibition was %100 at 200 and 250 µg/ml after 24 and 48 hours, respectively. This is important because it showed an efficient capacity in destroying the parasites at low concentrations. Previous researchers demonstrate 1,8 cineole as the most important component of *M. longifolia* (LaGow, 2004). Interestingly a recent research on *Eucalyptus* leaves revealed a high anti-*Trichomonas* activity. These researchers highlighted 1,8 cineole as a vital component of *Eucalyptus* for abolishing trophozoites viability. Indeed, 1,8 cineole is a common component between both plants and it may lead to high anti-*trichomonas* activity (Robles-Zepeda et al., 2011). It should be mention that there were no previous research regarding trichomonicidal

effect of *M. longifolia* in Iran and worldwide. Other researchers reported the effectiveness of several plants on *Trichomonas* including *Stachys*, however it was not able to destroy *Trichomonas* after 72 hours (Youse et al., 2012). On the other hand, there are some promising reports regarding anti-*trichomonas* activity of herbal medicines (Gamberini et al., 2015; Kaur and Arora, 2009; Robles-Zepeda et al., 2011). Further analysis is highly recommended with shorter incubation periods and analysis of active component of *M. longifolia.*

Pimpinella anisum (Anis) is an endemic growing plant in Iran and it is widely used for gastrointestinal disturbance. However there are reports regarding its cytotoxic effect to microbial population including *Streptococcus* and *Bacillus* (Robles-Zepeda et al., 2011).

It is worthy to mention the main antimicrobial component is Trans-anethole. Indeed previous researchers reported that trans-anethole could lead to death of various microorganisms (Gamberini et al., 2015; Kaur and Arora, 2009). The present research revealed the decline of parasite average number by increasing the concentration, however the parasite decrease percent was not significant and thus the mentioned extract may not be as effective as *M. longifolia*. The highest percent of growth reduction of the mentioned extract was 86% at 500 µl/ml concentration after 48 hours. It should be mention that aqueous extracts were examined, since traditional healers mainly prepare their remedies as infusion or maceration.

Altogether, there is a clear need for further analysis regarding isolation of active component of *M. longifolia* extract, cytotoxicity effect and in vivo tests should be in priority for further researchers.

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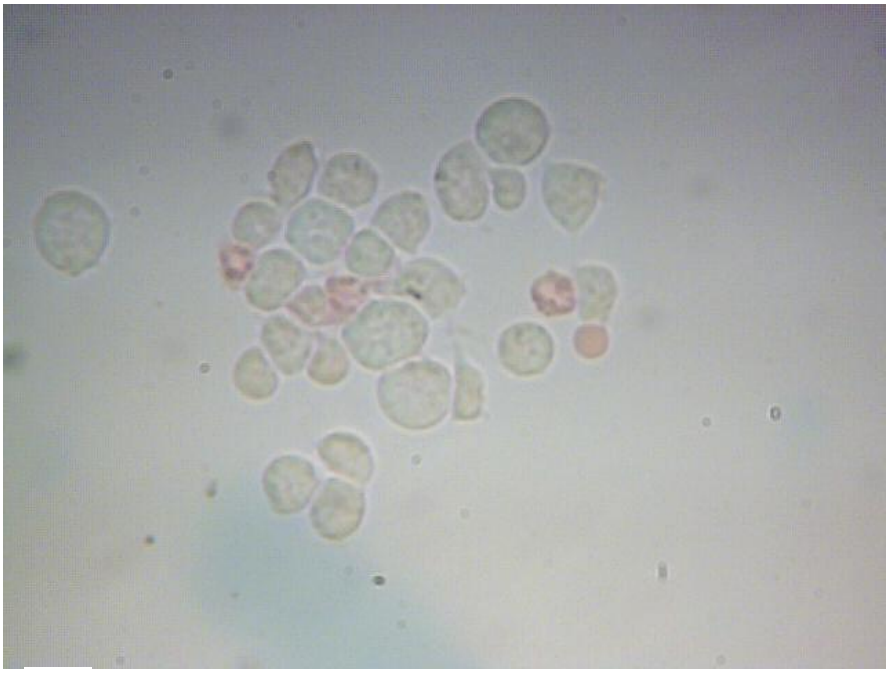

Figure 1. Eosin stained smear showing lethal effect of *Mentha longifolia* on *T. vaginali*

Table 1. Effect of aqueous extract of *Mentha longifolia.* on the invitro growth of *T. vaginalis* for Different incubation periods

Dosage of treatment µg/ml	24h			48h			P-Value	
	Average Count of Parasite in 1 ml*	percentage of growth reduction	Standard deviation	Average Count of Parasite in 1 ml*	percentage of growth reduction	Standard deviation	Concentration effect	Interaction of time-concentration
100	53.65	------	13.7	15.6	-------	3.1	<0/001[1]	<0/001[1]
150	40.55	24.4	10.1	8.4	46.1	1.5	<0/001[2]	<0/001[2]
200	12.8	68.4	2.1	0	100	0	<0/001[3]	<0/001[3]
250	0	100	0	0	0	0		
Negative control	552.1	--------	31.8	872.1	----------	65.9		
Interaction of time-concentration							<0/001	<0/001

[1.] Comparison of 100 and 150 µg / ml concentration through the analysis of repeated measures
[2.] Comparison 150 and 200 µ g / ml concentration through the analysis of repeated measures
[3.] Comparison 200 and 250 µ g / ml concentration through the analysis of repeated measures
[*] Data: $\times 10^4$

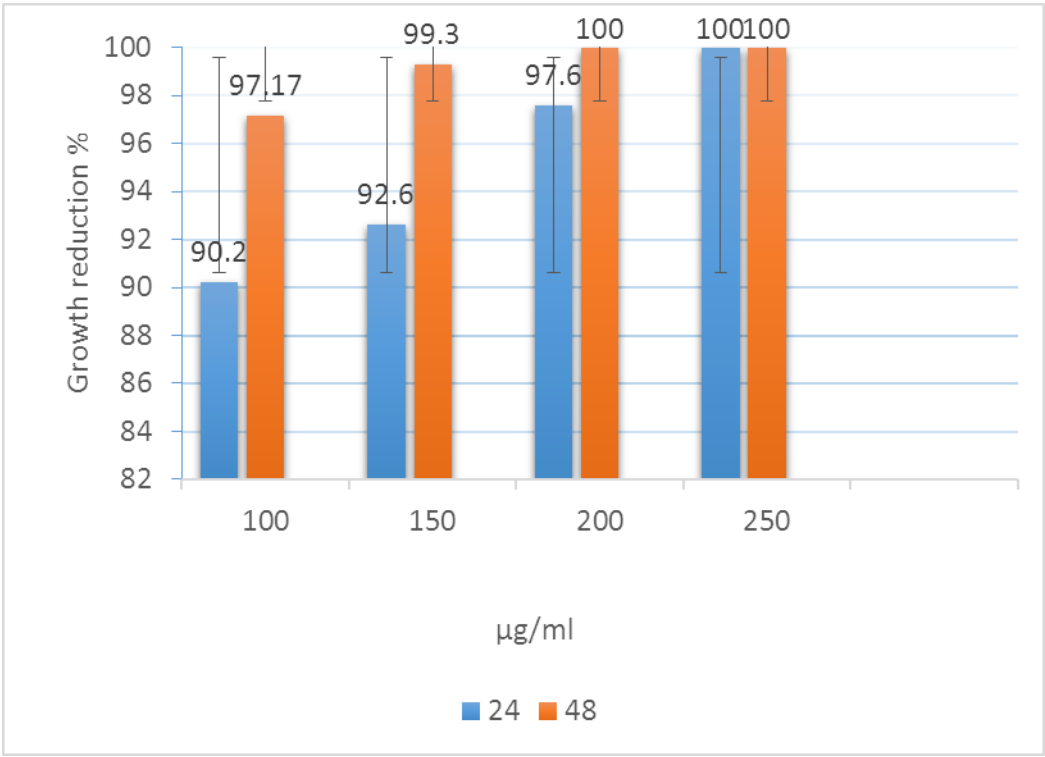

Figure 2. Percentage of growth inhibition of *Trichomonas vaginalis* at 24 and 48 h after exposure to different concentration of *Mentha longifolia* seed extract

Table 2. Effect of aqueous extract of *Pimpinella anisum* on the in vitro growth of *T. vaginalis* for Different incubation periods

Dosage of treatment µg/ml	24h			48h			P-Value	
	Average Count of Parasite in 1 ml*	percentage of growth reduction	Standard deviation	Average Count of Parasite in 1 ml*	percentage of growth reduction	Standard deviation	Concentration effect	Interaction of time-concentration
100	315.4	-----	25.8	153.1	------	4.7	<0/001[1]	<0/001[1]
200	183.6	41.7	7.3	135.4	11.5	3.2	<0/001[2]	<0/001[2]
300	153.7	16.2	2.4	125.7	7.7	12.5	0.005[3]	0.005[3]
400	150.9	1.8	3.3	118.3	5.8	4	0.011[4]	0.011[4]
500	145	3.9	10	114	3.6	7.7		
Negative control	552.1	-----	31.8	872.1	-------	65.9		
Interaction of time-Density							<0/001	

1. Comparison of 100 and 200 µ g / ml concentration through the analysis of repeated measures
2. Comparison of 200 and 300 µ g / ml concentration through the analysis of repeated measures
3. Comparison of 300 and 400 µ g / ml concentration through the analysis of repeated measures
4. Comparison of 400 and 500 µ g / ml concentration through the analysis of repeated measures

* Data ×104

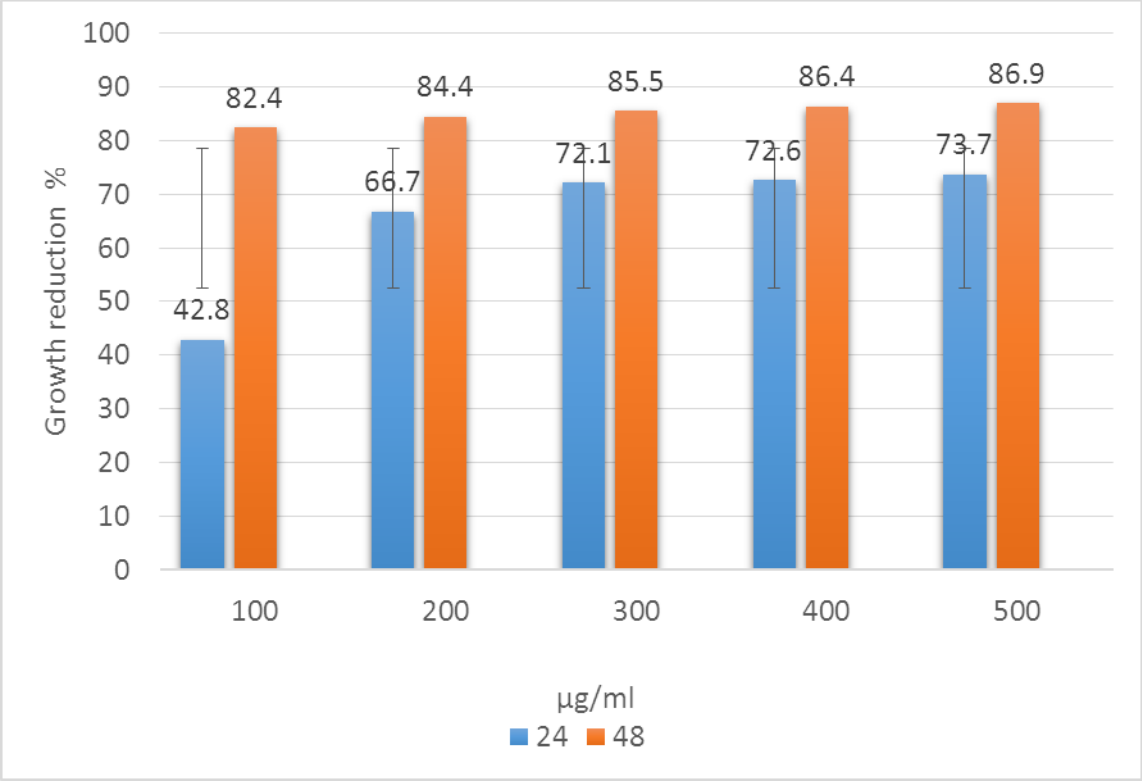

Figure 3. Percentage of growth inhibition of *Trichomonas vaginalis* at 24 and 48 h after exposure to different concentration of *Pimpinella anisum* seed extract

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