

A Study of the inhibitory effect of Terpinen-4-ol on Amastigote Forms of *Leishmania tropica* within Macrophages of Mouse *in vitro*

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Abstract

It was recorded that Terpinen-4-ol has an anti-parasitic activities, so that it can be noteworthy to intensify further studies about such compound.

The present study aimed to test the effectiveness of terpinen-4-ol on amastigote forms of *Leishmania* parasite in macrophages.

The effect was studied by adding of the increasing concentrations of Terpinen-4-ol in the culture wells containing mouse's macrophages that were previously incubated with the promastigote forms of the parasites for 24 hours. Then, they were incubated for another 24 hours with increasing concentrations of Terpinen-4-ol. Parasites were enumerated into macrophages in wells either treated with Terpinen-4-ol or in control wells.

Treatment with Terpinen-4-ol at concentrations of (0.01%, 0.02%, 0.05%, 0.1%) in (v/v) decreased the viability of the amastigote forms inside macrophages (24.02%), (32.74%), (66.72%) and (100%) respectively, compared to control wells (Distilled water).

The present study showed the activity of Terpinen-4-ol against amastigote forms of *Leishmania tropica in vitro* with a minimal inhibitory concentration (MIC₅₀) level which was 0.0416% (v/v).

Keywords: Terpinen-4-ol, Amastigote forms, Promastigote forms, Macrophages, Viability.

دراسة فعالية Terpinen-4-ol المثبطة لليشمانيات المدارية في المختبر

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الخلاصة

سُجِّلت خواص مضادة للطفيليات لـ Terpinen-4-ol، مما استدعى لتكثيف الدراسات حول هذا المركب. تهدف هذه الدراسة لإختبار فعالية Terpinen-4-ol على ليشمانيات طفيلي اليشمانيه داخل البلاعم. تمت هذه الدراسة بإضافة تراكيز متزايدة من مركب Terpinen-4-ol إلى حفر الزرع الحاوية على بلاعم فأرية والتي حُصِنَت مسبقاً مع مُشَبِّقات الطفيليات لمدة 24 ساعة. بعد ذلك حُصِنَت مع تراكيز متزايدة من Terpinen-4-ol لمدة 24 ساعة أخرى. ثم تم تعداد الطفيليات داخل البلاعم في حفر الزرع الحاوية على Terpinen-4-ol وفي الحفر الشاهدة. أدت المعالجة بـ Terpinen-4-ol بتراكيز (0.01%, 0.02%, 0.05%, 0.1%) (حجم/حجم) إلى تناقص في حيوية اليشمانيات داخل البلاعم بنسبة (24.02%, 32.74%, 66.72%, 100%) على التوالي بالمقارنة مع الحفر الشاهدة التي تحتوي على ماء مقطر فقط. أثبتت هذه الدراسة فعالية Terpinen-4-ol على ليشمانيات المدارية في المختبر عند تركيز أدنى مثبط (MIC₅₀) 0.0416% (حجم/حجم).
الكلمات المفتاحية: ليشمانيات طفيلي اليشمانيه، مشبقات الطفيليات، بالعات فأرية و Terpinen-4-ol.

Introduction

Leishmaniasis is a parasitic disease that is caused by *Leishmania tropica*. It leads to intracellular infection in human after being bitten by a female of sand-fly. The insect belongs to one of more than 30 species of the heart family, which spread in the old and new world. It is considered as the main vector of the disease⁽¹⁾.

Leishmaniasis is endemic in 88 countries⁽²⁾. 95% of the cases are found in south and central America, the Mediterranean basin, the middle east, and central Asia⁽³⁾. According to its clinical forms, leishmaniasis can be divided into three main types: visceral, cutaneous and mucocutaneous⁽⁴⁾. Cutaneous leishmaniasis is one of the most important current public health problems.

According to the world health organization (WHO), about 1.3 million new cases of cutaneous leishmaniasis are reported annually⁽⁵⁾. In Syria, the manifestations of cutaneous leishmaniasis vary from spontaneous healed lesions to permanent disfigurement⁽⁶⁾. Since 1960, cutaneous leishmaniasis cases are concentrated in Aleppo and Damascus. This is corresponded to the registration of 2,300 new cases annually⁽⁷⁾. The infection rate has significantly increased by the beginning of 2014, reaching 41,000 new cases annually⁽⁸⁾. Leishmaniasis is a hetero-host parasite, that can colonize different hosts⁽⁹⁾. The life cycle of the parasite is divided into two forms: the amastigote form and the promastigote form. The first form is found in humans, while the promastigote form occurs in the sand-fly⁽¹⁰⁾.

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An infected human can carry some types of leishmaniasis for a long time without showing any symptoms, and the incubation period for cutaneous leishmaniasis varies from 1-2 weeks to several months, and sometimes it takes several years⁽¹¹⁾.

Leishmania major (*L.major*) and *Leishmania tropica* (*L.tropica*) are the main Two species of Leishmanial parasite that can cause cutaneous leishmaniasis. The incubation period is less than two months for *L. major* and between 2 to 8 months for *L. tropica*⁽¹²⁾.

Australian tea tree oil (TTO) has antibacterial, antiviral, antifungal activity (including yeast)⁽¹³⁾. TTO has anti-parasitic activity when applied on the skin and mucous membranes⁽¹⁴⁾. Studies have confirmed that the main criteria for TTO are that Terpinen-4-ol should be at least 30%. It has antifungal and antibacterial activity⁽¹⁵⁾, therefore this study was designed to evaluate the effectiveness of this substance as an anti-parasitic agent against amastigote forms of *Leishmania* parasite within macrophages.

Materials and Methods

Preparation of the terpinen-4-ol solution

A concentrated solution of Terpinen-4-ol (4%) was prepared by diluting 0.04 ml of terpinen-4-ol solution (purchased from sigma) using distilled water. The final volume was 1 ml, and it was called the stock solution.

The stock solution was used to prepare solutions with concentrations of (2%, 0.8%, 0.4%) v/v.

Adding 25 μ l of the tube containing Terpinen-4-ol at a concentration 0.4% to get the final concentration of Terpinen-4-ol equal 0.01% in culture wells containing 1 ml of culture medium.

Obtaining mouse's macrophages

Six to eight week-old mice (BALB/C) were used as a source of macrophage. 5 ml of RPMI-1960 medium was injected into the mouse peritoneum and then withdrew the fluid after 10 minutes. The macrophages were counted using Neubauer counting chamber to determine the number of live cells in 1 ml of the previous suspension. And the number of macrophages were calculated according to the following equation:

The number of macrophages in 1 ml = average number of macrophages in 4 white squares $\times 10 \times 1000$ ⁽¹⁶⁾.

Testing the efficacy of Terpinen-4-ol on Amastigote form of *Leishmania tropica* inside macrophages

The effect was studied by adding mouse's macrophages to culture wells. Then, promastigote forms taken from a farm in the process of cultivation were added at a rate of 5 parasites per macrophage, they were previously incubated at 37°C for 24 hours⁽¹⁶⁾.

Then, Terpinen-4-ol was added at final concentrations of 0.01, 0.02, 0.5 and 0.1%, while the same volume of distilled water was added to the control wells and the culture wells were incubated at 37°C for an additional 24 hours. Parasites were counted into 100 macrophages in each well of wells treated with Terpinen-4-ol and in control wells⁽¹⁶⁾.

The calculation of minimum inhibitory concentration (MIC50)

IC₅₀ was calculated using the linear deviation equation, where the concentrations of Terpinen-4-ol were plotted against the number of amastigote forms inside macrophages on the X-Y axes using the same statistical program and the equation were as follows: $Y = a \times X + b$ ⁽¹⁶⁾.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism, version 8.0. ANOVA test to compare more than two groups. The results were considered statistically significant when $P < 0.05$.

Results

Treatment with Terpinen-4-ol at concentrations of 0.01%, 0.02%, 0.05% and 0.1% (v/v) led to a decrease in the viability of the amastigote forms within the macrophages by 24.02%, 32.74%, 66.72% and 100% it respectively as a comparison with the control group.

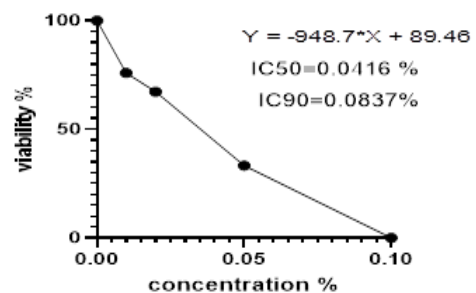


Figure 1. The IC₅₀ and IC₉₀ of Terpinen-4-ol on the viability of amastigote forms *in vitro*.

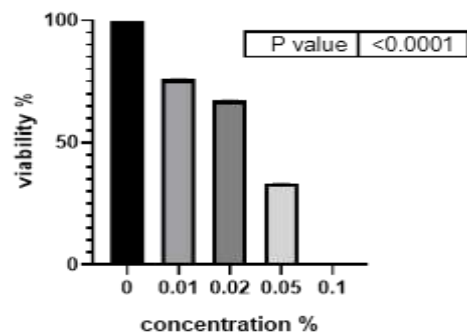


Figure 2. Viability of amastigote forms after treatment it by different concentrations of Terpinen-4-ol.

Table1. The inhibitory effect of Terpinen-4-ol on the proliferation of amastigote forms of parasites in mouse macrophages.

Concentration% of Terpinen-4-ol	The Number of parasites in 100 macrophages Mean \pm SD	Inhibition of parasites proliferation% within treated macrophages compared with the control Mean \pm SD
0	1298 \pm 12	0
0.01	1021 \pm 6.6	24.02 \pm 3.02
0.02	873 \pm 4.6	32.74 \pm 1.02
0.05	432 \pm 1.2	66.72 \pm 0.6
0.1	0	100

Discussion

Several recent studies support the anti-inflammatory activity of Australian tea tree oil (TTO). They have shown that TTO affects many inflammatory factors both *in vitro* and *in vivo*. Terpinen-4-ol plays the main role in this effect⁽¹⁷⁾. Terpinen-4-ol is a strong bactericidal agent. It has anti-fungal properties and an inhibitory activity against *staphylococcus aureus*. It has been reported that the combination of this natural substance and traditional medicines may help in the treatment of yeast resistance and bacterial infection⁽¹⁸⁾. Many recent reports have praised that terpinen-4-ol has antitumor effects by selectively inducing cell death through cell cycle arrest in human melanoma cells. Furthermore, terpinen-4-ol has been shown to induce a dose-dependent cytotoxic response against large cell lung cancer cells⁽¹⁸⁾.

Terpinen-4-ol reduce the infection rate of host cells, and decrease *protozoa* survival rate. Also, they increase phagocytic and lysosomal activities and nitric oxide levels in treated host by preventing bioenergetics imbalance in the spleen of treated host and they modulate immune activity by increasing IgG and pro-inflammatory cytokine (TNF α , IFN- γ , IL-1, IL-4, and IL-6) levels and decreasing anti-inflammatory IL-10 level in treated host⁽¹⁹⁾.

The results of this study declared a decrease in the number of *Leishmania* parasites inside macrophages with an increase in the concentrations of Terpinen-4-ol with IC₉₀ of 0.08%. Francesca and colleagues recorded similar results to the results obtained in this study when studying the activity of Terpinen-4-ol against azole-susceptible and resistant human pathogenic *Candida* species *in vivo*, as IC₉₀ was 0.06%⁽²⁰⁾.

The present study was differed from the study of Mikus and his colleagues in the value IC₉₀ which was 335.9 μ g/ml⁽²¹⁾, while in our study it was 837 μ g/ml. The reason for this difference may be due to the difference in the incubation period. Which was 72 hours in the study of Mikus and his colleague⁽²¹⁾, while in the current study it was 24 hours.

According to our knowledge this study could be the first one that record the effect of Terpinen-4-ol on amastigote forms of *L. tropica* within macrophages.

Conclusion

The present study was concluded that Terpinen-4-ol has activity on amastigote forms of *Leishmania tropica in Vitro*.

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