COMPARISON EFFECT OF ALCOHOLIC EXTRACT OF LEAF AZADIRACHTA INDICA ON THE VIABILITY OF LEISHMANIA TROPICA PROMASTIGOTE IN VITRO

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ABSTRACTS

Cutaneous leishmaniasis, is a serious parasitic infection that must be detected and treated because it can cause irreversible scarring and long-term psychological effects. The aim of this study is to evaluate the effect of the alcoholic extract of the neem plant, Azadirachta indica, on the culture medium containing cutaneous leishmaniasis parasite (promastigote), to select the minimum inhibitor concentration MIC of the plant comparing with control group treated with pentostam drug. Stained smears and culture media (RPMI-1640) are used to diagnose cutaneous Leishmania. The viability of Leishmania tropica promastigotes was investigated under five different concentrations (30µg, 60µg, 90µg, 120µg, and 150µg) of each agent after 5 days and the results give a significant differences P < 0.05 between concentration. The results showed that the 150 µg/ml concentration was the most active on the promastigotes viability to reach (zero) promastigote/ml after three days, while the (60 µg and 30 µg/ml) showed no change in the numbers until the end of the experiment. The MIC for the alcoholic extract was determined, which was (90 µg /ml) which appeared significant effect P <0.05 comparing with using pentostam. This study investigated that alcoholic extract of neem has an effect on Leishmania tropica promastigotes in vitro.

Keywords: cutaneous leishmaniasis, promastigotes, Azadirachta indica.

I. INTRODUCTION:

Leishmaniasis is a parasitic infection that spreads in more than 92 countries, including Iraq, Iran, Brazil, Afghanistan, Syria, India, Bangladesh, and Sudan, and more than one million cases of leishmaniasis are recorded each year (WHO, 2020). Leishmaniasis has three clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL) (Assafa et al., 2006). The most frequent type is CL (Iantorno et al., 2017). Leishmania sp. is an essential protozoan that lives inside the host's phagocytic cells (Singh, 2006), and it is found in the reticulo-endothelial system of vertebrates (John and Petri, 2013). It has two main forms with different morphology, the anterior flagellum promastigote in the sandfly and the amastigote form in the mammalian host. Leishmaniasis is transmitted to humans through the bite of an infected female sandfly Phlebotomus spp. As well as, The sandfly transmits two types of CL: Phlebotomus papatasi, which causes Leishmania major, and Phlebotomus sergenti, which causes Leishmania tropica (Hamarsheh et al., 2012). The cutaneous lesions caused by L. tropica are characterized by papules and nodules which may progressively develop into an ulcerative form with an ulcerated, erupting cracker-like pimple. And, The cutaneous leishmaniasis is not treated, it can progress to possibly fatal mucocutaneous leishmaniasis (McGhee et al., 2021). The Pentostam (sodium stibogluconate) and meglumine antimoniate are first-line anti-leishmanial treatments, although they have a long list of adverse effects (Sundar et al., 2012), and due to its toxicity, high price, and resistance to parasites (Al-HAlbosiy et al., 2020), it led to the idea of looking for alternative therapies with fewer side effects and toxicity (De Smet, 1997). Plants have been used to treat many diseases, including diseases caused by parasites, because they are safe, have fewer side effects, or are non-toxic, more efficient, and less expensive.

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Azadirachta indica, the neem plant, is native to the Indian subcontinent and belongs to the Meliaceae plant family. Because of its wide variety of biological characteristics, the neem tree is commonly known as the village pharmacy, in addition to, The bark, leaves, fruits, seeds, and twigs of the neem tree have several applications (Agrawal et al., 2020). The neem tree has been widely used since ancient times and has a variety of health-promoting properties (Koul et al., 2014). The neem plant consists of various types of components such as quercetin, azadirachtin and a number of liminoids and nimbosterol in different parts of plants respectively. The leaves contain a mixture of compounds such as nimbin, nimbanene, 6-desacetylnimbinene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol, various amino acids, and nimbiol (Hossain et al., 2011). Neem extracts show an anti-parasitic effect for various types of parasites (Takeda et al., 2008). The present study aims to investigate the effect of alcoholic extract of neem on the viability of Leishmania tropica promastigotes in vitro.

II. MATERIALS AND METHODS

1- Parasite samples
The cutaneous leishmaniasis parasite was obtained from patients with cutaneous leishmaniasis in Salah El-Din General Hospital during the month of September 2020. Stained smears with Giemsa were used to diagnose the amastigote stage, Figure 1.

2- Culture medium RPMI -1640
I- Components of the culture medium:
1- RPMI- 1640, a ready-made liquid culture medium developed by Moore and his group in 1967 at the Roswell Park Memorial Institute, from which the acronym RPMI came. This medium contains quantities of amino acids, vitamins and bicarbonate and is suitable for the growth of a wide range of cells (Moore et al., 1976).

2- Fetal calf serum (Sundar et al., 2001).

3- Antibiotic: Gentamycin.

II- Prepare the culture medium:
1- 5 ml of RPMI 1640 + 0.5 ml of calf fetal serum was added to the vacuum tube.

2- The antibiotic Gentamycin was added with the antifungal Nystatin and the dose was calculated according to the manufacturer's instructions for each antibiotic.

3- Cultivation of the parasite
from the edge of the ulcer, 0.1 mL of saline was injected subcutaneously by using 1 mL medical syringe and then the fluid was withdrawn again with a little blood, then, the samples were injected into the culture medium under sterile conditions(Al-Alousi, 1979). The culture media was incubated at 26-28 °C. After 24 hours, it was examined to ensure that it was no contamination and to detect the presence of the parasite (Mohebali et al., 2004), And after three days of cultivation, the promastigote of Leishmania was seen, Figure 2.

4- Counting the number of parasites
1- One ml of culture medium containing Leishmania parasite was added into a tube 9 ml of distilled water and shaken well to be diluted in a ratio of 1:10.

2- Hemocytometer chamber was used to calculate the number of parasites by using light microscope 500X, and according to the following equation (Mobarak et al., 2011):

\[ \text{Total number of promastigotes in ml} = \text{the number of promastigotes in 64 small square of haemocytometry x 25 x dilution degree x 10^3.} \]

4- The plant
A- Collection and preparation:
Leaves of neem were taken from a plantation in Salah al-Din Governorate. The genus and species of the plant were confirmed by plant taxonomists in the Department of Biological Sciences - College of Science - Tikrit University. The leaves were washed with distilled water and dried well. Then they were crushed by an electric crush into a powder formula and kept in an airtight container and left until extracted.

B- Alcoholic extraction:
The alcoholic extract of neem was obtained by using a Soxhlet apparatus and ethanol 70% (Azwanida, 2015). Then, the extract was dried in the oven at 30° temperature, then collected and put in sterile tube until uses.

C- Preparation of extract solutions
After evaporation of the solvent (ethanol) from the dry extract of neem leaves, (30µg), (60µg), (90µg), (120µg) and (150µg) were weighed to be used in the experiment. Each one of them was dissolved in 1 ml of ethanol to obtain five concentrations and to calculate the value of the MIC.

5- Effect of alcoholic extract
One ml of cultured medium with (5 x 10^5/ml) promastigotes were added to all test agents and left at 26C⁰. Each concentration was prepared in triplicate. The parasites were counted for five days using haemocytometer. The number of promastigotes were calculated as follow:

\[ GI = \frac{N}{x} \times 100 \]

No.

6- Effect of pentostam drug(control group)
Pentostam (5 µg / 1 ml) was added to the culture medium containing approximately 5 x 10^5 and left at 26C⁰ (Berman and Lee, 1983; Meaad and Ban, 2017). The number of parasites was counted daily for five days.

III. RESULTS AND DISCUSSION

In the current study, several concentrations of alcoholic extract of neem plant (30 µg, 60 µg, 90 µg, 120 µg and 150 µg/ml) were used. The promastigote was treated at each concentration and their numbers were calculated every 24 hours, through which the (MIC) for the alcoholic extract was determined, which was (90 µg /ml), Table 1. The results of the current study also showed a high significant inhibitor activity (P< 0.05) of the alcoholic neem extract on promastigote (8.1 x 10^5 cells/ml) at the concentration of (150 µg/ml) after three days from the start of the experiment comparing with other concentrations(60 µg and 30 µg/ml) where the average number of inhibition was (3.1 x 10^6 and 5.04 x 10^5 cells/ml) respectively, which showed no change in numbers until the end of the experiment, Table 1 and figure 3.

The MIC for the alcoholic extract was determined, which was (90 µg /ml) which has a significant difference comparing with pentostam, Table 2 and figure 4.

Although the Pentostam’s mechanism of action is through inhibiting the enzyme phosphofructokinase, which helps this enzyme determine the concentration of glucose in the anaerobic metabolism of glucose, which occurs in Leishmania at a high rate, in addition to the resistance of cutaneous leishmaniasis against Pentostam according to what was mentioned (Ozbilgin et al., 2020). But, the activity of the alcoholic neem extract is attributed to the presence of terpenes, which are responsible for the anti-parasitic activity, as well as the presence of limonoids, especially gedunin, dihydrogedunin, nimbolide and nimidin, which had a biological role against the malaria parasite (Shumutterer, 2002).

These results agreement with (Shumutterer, 2002; Roy and Saraf, 2006) who reported these components possess biological activity against the same parasite. Azadirachtin, which belongs to the limonoid group, has an effect against the eggs of Haemonchus contortus through the ability to penetrate its wall (Azra et al., 2019). Limonoids have an effective role in inducing the programmed death of the living cell of the parasite through inhibiting the enzyme topoisomerase II or through the dysfunction of mitochondria and the production of reactive oxygen compounds and thus the death of the parasite (Mitra et al., 2000). Khalid et al. (1998) reported that the limonoids compound in the plant of Khaya senegalensis has biological activity against the same parasite, and
using the alcoholic extract of neem plant in vitro as a possible future alternative against malaria (Zuleta-Castro et al., 2021). Other studies (Panday et al., 2018; Abbas et al., 2006) mentioned that neem leaf powder has a very high effect against the number of eggs of the coccidiosis parasite after infection in chickens.

![Leishmania sp. amastigotes isolated from dermal ulcer and stained with Giemsa stain at 100x](image1)

![Leishmania sp. promastigotes from culture at 500x](image2)

Table.1: Results of the mean number (cell/ml) and standard error of live parasites after adding different concentrations of alcoholic extract/In vitro.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Number of promastigote /Day</th>
<th>Mean concentration ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcoholic extract of neem</td>
<td>control 1 2 3 4 5</td>
<td></td>
</tr>
<tr>
<td>150 µg</td>
<td>5x10⁴ 4x10⁴ 3x10⁴ 0 0</td>
<td>8.18 x10⁴ ± 6.50 x10⁴</td>
</tr>
<tr>
<td>120 µg</td>
<td>4x10⁵ 7x10⁴ 7x10⁴ 3x10⁴ 0</td>
<td>1.17 x10⁵ ± 7.68 x10⁴</td>
</tr>
<tr>
<td>90 µg</td>
<td>5x10⁵ 2x10⁵ 2x10⁵ 8x10⁴ 0</td>
<td>2.1 x10⁵ ± 9.04 x10⁴</td>
</tr>
<tr>
<td>60 µg</td>
<td>5x10⁵ 4x10⁵ 4x10⁵ 1x10⁵ 9x10⁴</td>
<td>3.1 x10⁵ ± 8.92 x10⁴</td>
</tr>
<tr>
<td>30 µg</td>
<td>6x10⁵ 7x10⁵ 6x10⁵ 4x10⁵ 3x10⁵</td>
<td>5.04 x10⁵ ± 5.70 x10⁵</td>
</tr>
</tbody>
</table>

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The significant differences at $(P < 0.05)$

Note: ns: No significant; * $P<0.01$; ** $P<0.004$; ***$P<0.0003$; **** $P<0.0001$

Figure.3: Results of the average number (cells/ml) after adding different concentrations of alcoholic extract/in vitro between concentrations.

Table.2: Results of the mean number (cell/ml) and standard error of live parasites between MIC concentrations of alcoholic extract and Pentostam/In vitro.

<table>
<thead>
<tr>
<th>Treat.</th>
<th>Con. μg/ml</th>
<th>control</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5 Mean concentration ± standard error</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>90 μg</td>
<td>5x10^5</td>
<td>6.91x10^5</td>
<td>2x10^5</td>
<td>2x10^5</td>
<td>8x10^5</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>5 μg</td>
<td>5x10^5</td>
<td>8.53x10^5</td>
<td>8x10^5</td>
<td>7.43x10^5</td>
<td>6.82x10^5</td>
<td>6.4x10^5</td>
</tr>
</tbody>
</table>

Mean days ± standard error

The significant differences at $(P < 0.05)$

B: Pentostam (100μl)/ml

The significant differences at $(P < 0.05)$
REFERENCE


Figure 4: Results of the average number (cells/ml) of live parasites between MIC concentrations of alcoholic extract and Pentostam/In vitro.


