Anti-*Leishmania Tropica* Activity of *Candida Abicans* Proteinase

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Absract

Cutaneous leishmaniasis is the most common form of leishmaniasis. It is a skin infection caused by a single-celled parasite that is transmitted by sand fly bites. Although the cutaneous form of the disease is often self-limiting, it does result in significant scarring and can spread to more invasive, mucocutaneous disease. Leishmaniasis is endemic in 88 countries on five continents. The pentavalent antimonials meglumine antimonate (85 mg Sb/mL) for intramuscular administration and sodium stibogluconate (100 mg Sb/mL) for intravenous and intramuscular administration have been used for decades for the treatment of New World cutaneous leishmaniasis, and are the gold standard for other new investigational drugs. Cutaneous leishmaniasis has been treated in patients of all ages with a wide range of physical methods, including cauterization, surgical excision, cryotherapy and the application of local heat. The antibacterial activity of proteinase was determined against different types of bacteria isolated from patients and healthy individuals. The most sensitive bacteria were *Lactobacillus* spp. While *Pseudomonas aeruginosa* is the most resistant. Candidal proteinase, also have an anti-*leishmania donovani* activity (on the promastigote stage)[15] . Thus this study was conducted to evaluate the effect of proteinase enzyme on promastigote stage of *L. tropica*, *in vitro* and the possibility to be used in the future as an anti-leishmanial drug.

Introduction

Cutaneous leishmaniasis is the most common form of leishmaniasis. It is a skin infection caused by a single-celled parasite that is transmitted by sand fly bites. There are about 20 species of Leishmania that may cause cutaneous leishmaniasis. Some Leishmania species are closely linked to humans and are therefore found in cities (Leishmania tropica), whereas some are more traditionally associated with animal species and are therefore

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considered zoonoses (Leishmania major). Although the cutaneous form of the disease is often self-limiting, it does result in significant scarring and can spread to more invasive, mucocutaneous disease. Therefore, treatment may be considered to prevent these complications [1].

Leishmaniasis is endemic in 88 countries on five continents. There are 1-1.5 million cases of cutaneous leishmaniasis reported yearly worldwide. There has been a sharp increase in recorded cases over the last 10 years [2]. Based on geographical distribution, cutaneous leishmaniasis is divided into Old World and New World leishmaniasis [3].While the Old World species mostly cause benign and often self-limiting cutaneous disease, the American species(the New World Leishmaniasis) cause a broad spectrum of conditions from benign to severe manifestations, including mucosal involvement. The response to treatment varies according to the species. The proposed choice of local or systemic treatment of cutaneous leishmaniasis is guided by the risk of developing mucosal disease.

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Pentamidine, an aromatic diamidine, is toxic for a number of protozoa and fungi including *Leishmania*, *Pneumocystis carinii* and African trypanosomes. The mechanism of action has not been established.

The imidazoles and the structurally related triazoles were introduced as antifungal drugs, but also have an antileishmanial activity.

Itraconazole has also been used, but data are scarce and controversial.

Fluconazole was studied in a randomized, double-blind, placebo-controlled trial in Iran. It was well tolerated and showed promising results in *Leishmania major* leishmaniasis [7].

Cutaneous leishmaniasis has been treated in patients of all ages with a wide range of physical methods, including cauterization, surgical excision, cryotherapy and the application of local heat.

The extarcellular proteolytic activity is one of the several hydrolytic enzyme activities described for *C.albicans* [8]. Aspartyle proteinase is one of these enzymes; it plays an important role as a virulent factor [9]. This enzyme is secreted by pathogenic species of *candida in vivo* during infection [10, 11]. The enzyme is secreted in vitro when the organism is cultured in the presence of exogenous proteins, usually bovine serum albumin, as the nitrogen source [12]. Proteinase production is believed to enhance the ability of the organism to colonize and penetrate host tissue and evade the host immune system [13].

The antibacterial activity of proteinase was determined against different types of bacteria isolated from patients and healthy individuals. The most sensitive bacteria were *Lactobacillus* spp. While *Pseudomonas aeruginosa* is the most resistant [14]. Candidal proteinase, also have an anti-*leishmania donovani* activity (on the promastigote stage)[15] . Thus this study was conducted to evaluate the effect of proteinase enzyme on promastigote stage of *L. major in vitro* and its possible to be used in the future as an anti-leishmanial drug.

Materials and methods

***Candida albicans* strain :**

The standard strain of *Candida albicans* (ATCC 10230) was cultured on Sabouraud' s agar, then inoculated in 100 ml Sabouraud' s broth, supplied by the Biotechnology and Molecular Biology Department/ Baghdad University/Iraq.The culture was incubated in a shaker incubator at 37^0c for 24 hrs.

Two Erlynmyer's flasks of 250 ml containing 50 ml of wheat bran medium, PH 8.5, were inoculated with 1 ml of *C. albicans* broth culture. The flasks were incubated for 72 hours at 37^0c in a shaker incubator [16].The cultures were harvested with 100 ml of 0.1M KH_2PO_4(pH 7.2) and centrifuged at 1500Xg for 30 minutes . The volume of the supernatant was measured and the enzyme activity was determined by using 1% hemoglobin (Oxide) , pH2.0, as a substrate .

Determination of proteinase enzyme activity:

The activity was determined according to Murachi (1970) as follows:

Two glass tubes , contained 1.9 ml of 1% hemoglobin , pH 2.0, and the other with 1.9 ml of 1% hemoglobin , pH 2.0 . 0.1 ml of the sample (proteinase) was added to one of the tubes. Both tubes were incubated in a water bath at 35^0C for 10 minutes.

Three ml of trichloroacetic acid (TCA) were added to both tubes. 0.1 ml of proteinase was added to the control tube. Both tubes were centrifuged at 1500Xg for 15 minutes. The activity (unit/ml) of the supernatant was measured by a spectrophotometer at 280 nm[17].

Precipitation of proteinase with 0.5% ammonium sulphate:

Ammonium sulphate (29.11gm / 100ml) was added to the crude suspension gradually with stirring . The suspension was centrifuged at 300Xg for 15 minutes. The precipitate was dissolved in 5 ml of 0.1 M of acetate buffer at 4°C, pH 5.0. Enzyme activity was measured for both supernatant and the dissolved precipitate as described previously. The protein concentration was determined according to the Biuret method [18].

Precipitation of proteinase enzyme with 50- 75 % ammonium sulphate :

Ammonium sulphate (29.11 gm/ 100ml) was added gradually to the supernatant of the crude suspension with stirring[17]. The suspension was centrifuged at 3000Xg for 15 minutes and the precipitate was dissolved in 5 ml of 0.1M acetate buffer , pH 5.0 [14] . The suspension was dialyzed against 2 liters acetate buffer of 0.1 M, pH5.0 at 4°C over night. A further dialysis was done using the same conditions [18]. The activity of the enzyme and the protein concentration were determined .

The parasite:

Leishmania donovani (MHOM/SU/58/OG) was used . The parasite was stored in liquid nitrogen and maintained by continuous passage in Balb/ C mice. Promastigotes were cultivated in RPMI medium [10]. On the day of the experiment, when the promastigotes were at the logarithmic growth phase, they were adjusted to 1x 10^6 cells/ ml in RPMI, supplemented with 10%fetal calf serum (FCS). The crude enzyme (1ml) was added at different concentrations (3 tubes for each concentration) (whole, diluted 1:2 with distilled water, 1:4, 1:8). The total promastigotes count was determined each day for six days.

Results

Table (1) shows the results of the purification process of proteinase enzyme.

Table(2)shows the number of *L.tropica* promastigotes after the addition of proteinase. The enzyme had no lethal effect on the parasite in any concentration.

Table (1): Purification of proteinase enzyme from *C.albicans*.

Step	Volume(ml)	Activity (unit/ ml)	Protein (mg/ml)
Crude	50	100	5
Precipitation by 50-75% Ammonium sulphate	13	215	3

Table (2): Number of *L. tropica* promastigotes after the addition of proteinase enzyme from *C.albicans*.

Day	1	2	3	4	5	6
Control	6×10^6 5.5×10^6 5.8×10^6 5.8×10^6	15×10^6 14×10^6 14.5×10^6 14.5×10^6	25×10^6 23×10^6 24×10^6 24.2	40×10^6 39×10^6 38×10^6 39×10^6	53×10^6 51×10^6 50×10^6 51.3×10^6	60×10^6 55×10^6 58×10^6 57.6×10^6
Concentrated	Zero	Zero	Zero	Zero	Zero	Zero
1/2	5×10^6 4.5×10^6 5×10^6 4.8×10^6	10×10^6 8×10^6 9×10^6 9×10^6	12×10^6 13×10^6 11.5×10^6 12.2×10^6	7×10^6 7.5×10^6 6.5×10^6 7×10^6	5×10^6 4.8×10^6 5.3×10^6 5×10^6	1×10^6 1.5×10^6 2×10^6 1.5×10^6
1/4	10×10^6 7×10^6 6.5×10^6 7.7×10^6	20×10^6 24×10^6 21×10^6 21.7×10^6	20×10^6 22×10^6 19×10^6 20.3×10^6	29×10^6 29.5×10^6 30×10^6 29.5×10^6	38×10^6 38×10^6 40×10^6 38.7×10^6	50×10^6 40×10^6 43×10^6 43.3×10^6
1/8	11×10^6 6×10^6 10×10^6 9×10^6	30×10^6 29×10^6 28.5×10^6 29.2×10^6	35×10^6 32×10^6 33×10^6 33.3×10^6	30×10^6 28×10^6 32×10^6 30×10^6	31×10^6 30×10^6 31.5×10^6 30.8×10^6	40×10^6 45×10^6 44×10^6 43×10^6

Disscussion and Conclusions

Treatment with antimonials will heal lesions faster and prevent relapse, local dissemination, mucosal disease (usually), and transmission [19]. Not all lesions require treatment. Old Word disease tends to be self-healing, and systemic treatment seldom is used New World lesions more often require systemic treatment. Reasons to treat Cutaneous Leishmaniasis are:

1. Cosmetically unacceptable lesions.
2. Chronic lesions.
3. Large lesions.
4. Lesions in immunosuppressed patients.
5. Lesions over joints.
6. Mucosal disease.
7. Multiple lesions.
8. Nodular lymphangitis.
9. Worsening lesions

Pentavalent antimony remains the treatment of choice, but it have a high incidence of side effects, which are reversible [20]. Side effects included aching, arthralgia, fatigue, gastrointestinal upset, elevation of amylase, lipase, and liver enzyme levels, leucopenia, anemia, and electrocardiography abnormalities.

In this study we tried to use *Candida albicans* proteinase as a drug of choice against *L.tropica*, we found that the concentrated enzyme have a good effect as the result obtained in other study which also revealed the anti-*leshmania dnovani* activity of this enzyme [21]. There is another study revealed the antibacterial activity of this enzyme such as *E.coli*, *Kleibseilla* spp., *Enterobacter* spp., *Lactobacillus* spp. [14], but in the same time the enzyme showed no activity against *Pseudomonas aeruginosa*, because this bacteria is highly resistant as known and as the case of our parasite. From this study we concluded that this enzyme may be used as an alternative drug of choice of the chemical ones (in

particularly those toxic drugs) in the future, by using a heavy growth of *Candida albicans* culture and after the purification process of this enzyme.

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