

Diagnostic of visceral leishmaniasis by polymerase chain reaction PCR in experimental mice infected which treatment with *Viscum album* extract and Pentostam.

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Abstract

Visceral leishmaniasis (VL), or kala-azar, is an infection of the reticuloendothelial system, Fatalities due to leishmaniasis are associated with visceral disease. the *Leishmania donovani* is a causative agent for this disease. In the current study, white male mice of the Balb /C strain were experimentally infected with the *leishmania donovani* promastigotes and were then treated with a *Viscum* extract and compared with the Pentostam. The aim of this study was to evaluate the sensitivity and specificity of polymerase chain reaction (PCR) in the detection of *Leishmania* DNA in blood samples of infected mice. as well as, evaluation the ability of *Viscum album* extract to treatment the visceral leishmaniasis. The genetic study gave excellent results in the diagnosis of infection throughout the treatment period compared to the positive and negative control . Using the specific LdI primer , the 700bp of the parasite was diagnosed. This Broad band was shown in the positive control samples, in the pantostam treatment groups, and the muscular injection with *viscum* extract in the first 15 days and in the oral dosage group in the first 20 days of initiation. While the band did not appear in any other. The current study has shown promising results in the use of *V. album* in the treatment of visceral leishmaniasis and also gave impressive results in the diagnosis of infection PCR.

Keyword: *Viscum album*, alcohol extract, *Leishmania donovani*, PCR.

Introduction

Leishmaniasis, a neglected tropical disease prevalent in 98 countries [1], The leishmaniasis are parasitic diseases which are endemic in many countries in the tropics and subtropics. Approximately 350 million people are considered to be at risk of contracting the disease. Visceral leishmaniasis (VL), also known as kala azar, accounts for an estimated 75,000 deaths annually [2]. Experimental hosts, such as laboratory mice, are largely used to study the immunobiology of these parasites and to screen the efficacy of newly developed drugs and vaccines [3].

Viscum album is a hemi-parasitic plant that grows on various trees. It has a long tradition as medicinal plant, being used in traditional medicine as a popular remedy for hypertension, vascular disease, epilepsy, arthritis and rheumatism for centuries. Nowadays, *Viscum* extracts are widely used as complementary therapy for cancer patients [4]. because of compounds it that contain bioactive . They are safe to use; they contain low toxicity levels. Many studies on medicinal plants used in the treatment of leishmaniasis *in vitro* have shown excellent and effective results in the treatment of leishmaniasis and most of the biologically active natural substances are alkaloids, quinones, terpenes, monoclonal terpenes, saponins, and flavonoids. These compounds are among the basic compounds contained in the *V. album* [5]. the alkaloids have recently drawn attention due to their antitumor activities. Further mechanistic studies indicate that alkaloids derivatives inhibit DNA topoisomerases [6]. The DNA of *leishmania* contains the topoisomerase enzyme, which is an astounding target for anti-leishmaniasis drugs, and most anti-leishmania treatments rely on inhibitors of this enzyme. Studies

have shown that alkaloids have an important role in inhibiting the topoisomerase enzyme [7]. alkaloids isolated from *V. album* fruits found to inhibit DNA topoisomerase, thus interfere with DNA synthesis in *Leishmania donovani* promastigotes and amastigotes. These properties have important implications for rational chemotherapy of leishmaniasis [8].

Whatever, PCR technology is one of the most advanced technologies used globally in parasite modeling in general, because of its high accuracy and sensitivity [9]. The advantage of this method is that it is characterized by ease and speed and lead to wonderful results in terms of sensitivity and privacy in the diagnosis of *Leishmania donovani*. [7]. In the field of *leishmania*, DNA has been isolated and relayed electronically using PCR technology ,one of the most important and most used methods, and this method was able to compete and dream was many of the traditional methods used in the past specifically for different types of parasites causing cutaneous leishmaniasis , visceral leishmaniasis diagnosis [10]. The LdI primer is used to diagnose *Leishmania donovani* in a location close to the 700bp (700 base pairs) . This primer can detect the infection even if it is due to a small amount of parasite, which can detect 1fg of parasitic DNA of the Indian strain and this value is equivalent to approximately 0.1 of the parasite, this can be a specialized primer can distinguish *L. donovani* from other leishmaniasis as *L. major* and *L. tropica* [11]. The aimed of the research is the detection the infected mice with the visceral leishmaniasis which treated with *viscum* extract and compare thus with negative and positive control .

Materials and Methods

A- Location and date performing of the research

The study presented in the University of Tikrit , laboratories of the Biological Department ,Collage of Education for Pure Sciences and in Al- Balsam medical laboratory for the period from 1/3/2016 to 1/12/2016.

B- *Leishmania* used and Culture media :

MHOM/IQ/2005/MRU15 *Leishmania donovani* stock culture used. the culture is obtained from the College of Medicine AL-Nahreen University . The obtained culture is cultivated in RPMI 1640 medium which developed by Moore, *et al.* (1976) [12] and added fetus serum calf according to Sundar, *et al.* (2001) [13] and added gentamycin to prevent the growth of bacteria as following :

9 ml from RPMI 1640 +1ml from fetal calf +3 μ Gentamycin. Putting 9.9 ml of culture media to a test tube and taking 0.1 ml From the stuck culture contain on 2 x 10³ cells / cm incubate for 4 days at a temperature of 23-25°C to develop the parasite. determination the number of parasites every day by taking 0.02 cm and adding them to 0.4 cm normal saline containing of 1 % formalin to kill and fix the parasites, the number of parasites was calculated by using the Neubauer hemocytometer chamber [14].

Injected the Balb/c Male mice intravenously with 0.3 ml of media that contain about 6.3 – 8.1 × 10³ parasite in promastigote phase .

C- *Viscum album* alcoholic extraction

Fruit of *V. album* collected from local markets in Tikrit city, then alcoholic extract according to Taskin, *et al.* (2005) [15] , the fruit were milled into coarse powder, then treated with petroleum ether on magnetic stirrer for 72 h. and filtrated. taking the un filtrated materials and adding absolute ethanolic alcoholic on magnetic stirrer for 72h. too. then filtrated and removed the un filtrated particles. Put the filtrated liquid in rotary evaporator to obtained the final extract .

D- Treatment the infected mice :

The infected male mice were divided into three groups, each group contain 5 mice , one of which was treated with pentostam (20mg/kg), the other two

groups, one was treated with the 0.92 mg/kg Of *viscum* extract by inter muscular injection, the other was treated orally with 0.92 mg/kg Of *viscum* extract.

E- Collect blood samples from mice: Blood samples from experimentally infected mice were withdrawn at different intervals after 5, 10, 15, 20, 25, and 30 days , in additional to positive and negative control groups, blood samples were placed in glass tubes containing an EDTA (blood anticoagulant) and kept in freezing at minus 8 ° C until use.

F- DNA Extraction : All the genomic DNA of the parasite has been isolated initially from the promastigotes which grow in culture media , and then extracted the DNA from the blood mice which kept in the EDTA-containing tubes . As well as need a small amount of blood ranging from 250 - 350 micro-liters. The concentration of genomic DNA ranged from 200 to 500 ng / μ l, and Its purity ranged from 1.5 to 1.8. This concentration and purity are ideal for PCR reactions. Some modifications have been made to the reaction program temperatures to obtain clear results that could be easily read on the agarose gel.

The concentration and purity of DNA extracted from blood samples by Bartlett and Stirling method was estimated in 2003 [16] using the Nano drop device by taking 1 μ of the sample DNA samples after the mixture was discarded Microfuge device for 3-5 seconds to complete the mixing of sample components, then loaded onto the device and took the reading focused and purity that appeared on the computer screen. The molecular size of the DNA was estimated by conducting the electrolyte transferred on the agarose gel, using the well-known molecular weight (L λ). The agarose gel was presented at a concentration of 1.5%. The interactions were based on Salotra, *et al.* (2001) [11] in DNA samples extracted from mice using the LdI primer and equipped with promega, USA. Two special KDNA kinetic primers were used with LdF and LdR.

Primer	Sequence	Expected size (bp)	Ann. Tem. (°C)
LdF	AAATCGGCTCCGAGGCGGAAAC	700	55
LdR	GGTAACTCTATCAGTAGCAC		

3- Procedure :

1- Two random samples were selected from each group.

2- Determined of the concentration of DNA in the studied samples at a concentration of 50 ng / μ l by Nano drop.

3- Prepared the reaction tubes with a capacity of 0.2 ml and added 12.5 microliters of the reaction mixture to each tube and put in the snow until the completion of the additions shown in the table below note that the final size of the reaction 25 microliters.

The size of one sample is microliter	Final concentration	Ingredients
9.5	-	Distilled water
1 Microliter of F primer	10 micromall	The primer
1 Microliter of R prime		
1	50 ng / microliter	DNA template
12.5	-	PCR Master Mix

4 – Thermal cycler using the following conditions: 94°C for 5 min and 40 cycles of 94°C for 30 s, 55°C for 1 minute , and 72°C for 1.30 minute followed by 72°C for 10 min. [17] .

Number of courses	Time required in minutes	Temperature	Stage
1	5	94	Primary denaturation
40	1	94	Normal denaturation
	1	55	Annealing
	1.5	72	Extension
1	10	72	Final Extension

5- The PCR products from amplification of primer by thermo cycler were then electrophoresis on an ethidium bromide-stained 1.5% agarose gel. 100v, 20A for 25-30 minutes. The presence of specific bands was indicative of the infections (positive). The bands visualized under UV light and Gel documentation .

Results and discussion

The genomic DNA in the current study was extracted as Bartlett and Stirling method [16], it is good quality in terms of being inexpensive, and their materials are available and do not need sophisticated devices and is conducted in a relatively short time does not exceed two hours. The LdI primer was used in the current study, which amplifies the 700bp bands of *L. donovani* parasite according to Salotra, *et al.* (2001) ,These primer is a specialist in the detection of leishmaniasis caused by *L. donovani* infection alone. [11].

The results of the present PCR study are high-resolution results that differ from Other serological, chemical and clinical diagnostic methods; for accuracy in terms of specificity and sensitivity. The

using of this technique in this study was intended to be accurate to obtain convincing and accurate results, through which can detect parasites in the blood, even if only one parasite [18].

After electrophoresis the DNA to the agarose gel , significant results were found in the ability of the alcoholic extracted to inhibitory the parasitic growth in infected mice . Negative results were shown by the absence of the 700bp bands for the group of pentostam treated animals after 20 days of treatment, the same result that appeared in the group of animals treated intramuscularly with the extract of *Viscum* (image 1 and 2). While the group of animals which treated orally With *Viscum* extract showed negative results after 25 days of treatment, Table 4. That the appearance of the specialized band of 700bp on the agarose gel is a definite indication of the presence of the parasite and the absence it that referred to absence of the parasite in the sample.

The present study showed that there is a slight difference between the muscular injection group with the extract of alcohol and with those treated orally by it . This may be due to the fact that treatment through the digestive system goes through complex stages starting from digestion, absorption, metabolism and then the arrival of active substances to the target sites where the parasite is present, and may be affected by the treatment of juices and digestive enzymes and acids such as acid HCL contagious, trypsin and Chymotrypsin and other enzymes and this It takes longer to inject the substances into the muscles or to enter them into the bloodstream directly.

The ability of the *Viscum* extract therapeutic may return to the contents of alkaloids and other compounds or other important elements.

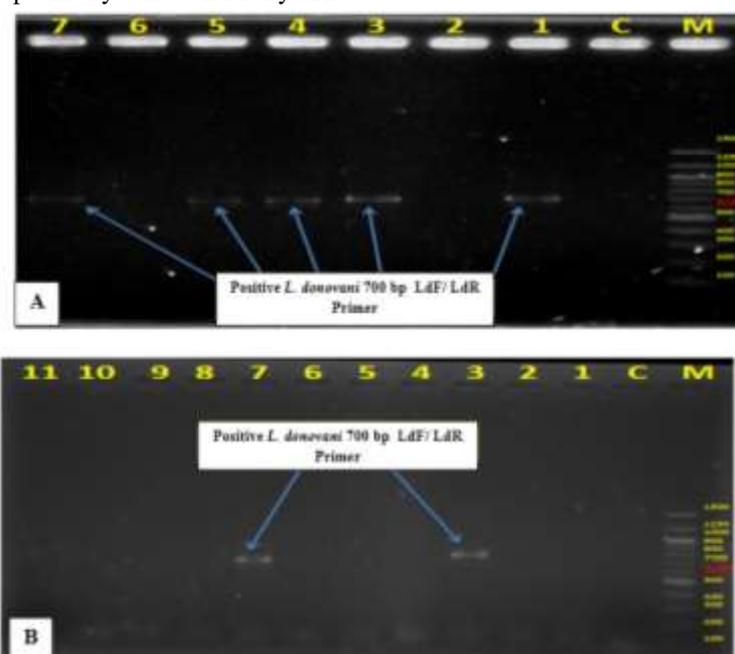


Fig. 1: A and B, the Broad band of *leishmania* (Promastigote) at 700bp and electrophoresis on 1.5% agarose are shown

Table 1 shows the results of the present study with polymerase chain reaction technology (PCR), where the (+) symbol indicates the appearance of the

parasite packet while the symbol (-) indicates that it is not present.

Table 1 shows the results of the PCR gene study during the treatment period

Days	Oral Dosage group	Intramuscular injection group	Pentostam group	Positive control group	Negative control group
Before treatment	+	+	+	+++	*
After 5 days	+	+	+	+	
After 10 days	+	+	+	+	
After 15 days	+	+	+	+	
After 20 days	+			+	
After 25 days				+	
After 30 days				+	

* Not showing 700bp band ** The emergence of the band that indicates the presence of the parasite.

The current study was agreed with those of Salotra *et al.* (2001) [11] who used the same specific primer LdI to diagnose leishmaniasis infection in 107 blood samples of people with leishmaniasis, Where their study showed that 102 positive sample 95.3% and sensitivity rate up to 96% using blood samples instead of bone marrow or fluid of the spleen .

They also managed to diagnose the infection in the blood of 45 out of 48 people suffering from post kala azar dermal leishmaniasis (PKDL), at the same time, they suffered from other infections such as mycobacterium tuberculosis, Mycobacterium leprae and Plasmodium, up to 93.8%. This ratio is much higher than that reported by Osman , *et al.* (1998) in Sudan [19] which indicated that the sensitivity of this technique to 82.7% in 32 patients with post kala-azar dermal leishmaniasis PKDL. Also the study of Ranasinghe, *et al.*(2015)[20] in Sri Lanka confirmed that they were able to diagnose kala azar in 27 people out of 38 (71%) using the same primer. And agreed with the findings of Yaseen and Ali in 2016 [21] in

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Baghdad, who used the same primer specialized in the diagnosis of leishmaniasis. also Osman, *et al.* (1997) [22] reported differences in the degree of PCR sensitivity in the diagnosis of *L. donovani* infection for previous studies ranging from 45% to 94%. This variance may be due to varying sample sizes in these studies and to primers In which used.

Present study concludes that *V. album* alcoholic extract show a promising *in vivo* antileishmanial activity and can be considered as new drug in treatment of leishmaniasis. The use of polymerase chain reaction technology has given clear evidence of the efficacy of treatment with *V. album* extract.

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تشخيص اللشمانيا الأحشائية باستخدام تقنية التفاعل السلسلي لأنزيم البلمرة في الفئران المخمجة

تجريبياً والمعالجة بمستخلص *Viscum album* و البنتوستام

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الملخص

داء اللشمانيا الإحشائية أو الكلا أزار هو مرض يصيب الجهاز الشبكي البطاني، وترتبط الوفيات الناجمة عنه بإصابة الأحشاء الداخلية. طفيل *Leishmania donovani* هو المسبب لهذا الداء. في الدراسة الحالية تمت إصابة ذكور الفئران البيض من سلالة Balb/C تجريبياً بطفيل اللشمانيا الإحشائية في الطور أمامي السوط، ومن ثم تمت معالجتها بمستخلص الدبق الكحولي ومقارنة النتائج مع وبالينتوستام. الهدف من هذه الدراسة هو تقييم حساسية وخصوصية التفاعل السلسلي لأنزيم البلمرة (PCR) في الكشف عن الحمض النووي اللشمانيا في عينات دم الفئران المصابة، وكذلك، تقييم قدرة مستخلص الدبق لعلاج داء اللشمانيا الإحشائية، وقد أعطت الدراسة الجينية نتائج ممتازة في تشخيص الخمج طوال فترة العلاج مقارنة مع مجموعة السيطرة الموجبة والسالبة. باستخدام البادئ المتخصص LdI، حيث تم تشخيص الحزمة bp700 العائدة للطفيل، والتي شوهدت في عينات السيطرة الموجبة وفي المجموعة المعالجة بالبنتوستام ومجموعة الحقن العضلي بمستخلص الدبق في الأيام الـ 15 الأولى من المعالجة، وإيضاً في مجموعة الفئران المجرعة فمويًا بالمستخلص في الـ 20 يوماً الأولى من بدء المعالجة. في حين أن هذه الحزمة لم تظهر فيما سواها. وقد أظهرت الدراسة الحالية نتائج واعدة في استخدام مستخلص الدبق في علاج داء اللشمانيات الحشوية وأيضاً أعطت نتائج مثيرة للإعجاب في تشخيص الخمج باستخدام تقنية التفاعل السلسلي لأنزيم البلمرة.