Anti leishmanial Activity of Methanolic extract of *Juniperus excelsa berries* and *Acacia nilotica*

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Abstract

This study was conducted from December 2012 to May 2013. Samples were taken from the lesions of patients attending Tikrit Teaching Hospital and diagnosed clinically by professional doctors in dermatological department in the hospital clinically identified as *Leishmania sp*. according to lesion characteristics form. Parasites Isolates were harvested at stationary phase of growth injected intradermaly of 40 BALB/c mice for each plant. There were significant differences (P<0.001) in numbers of parasite cells in most concentrations used for methanolic extract of *Juniperus excelsa* berries compared with the control group without agent, and the statistically analysis showed that there were no significant differences (P>0.05) in the numbers of parasite cells in all concentrations used for methanolic extract of *Acacia nilotica* compared with the control group without agent. Also there were significant decrease (P<0.01) in number of parasite cells in control group treated with Pentostam compared with control group without agent.

Keywords: Antileishmania, *Juniperus excelsa* berries, *Acacia nilotica*.

التأثير المضاد للشمانيا للمستخلصات الكحولية لنباتي العرعر juniperus excels berries والصمغ العربي Acacia nilotica

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الخلاصة

اجريت هذه الدراسة للفترة من كانون الاول 2012 – ايار 2013، وتم اخذ العينات من مناطق الافة للمرضى الوافدين لمستشفى تكريت التعليمي وشخصت الافة سريريا" بواسطة الطبيب المختص في قسم الجلدية على انها Leishmania sp حسب خصائص الافة او المنطقة المتضررة . تم عزل الطفيلي عند طور الثبات للنمو وحقن الطفيلي تحت الجلد في 40 فأرا" من نوع BALB السويسرية لكل نبات. لوحظ وجود فروقات معنوية (P<0.001) في اعداد الطفيلي في معظم التراكيز المستخدمة من المستخلص الكحولي لنبات Juniperus excels berries مقارنة مع مجموعة السيطرة المعاملة بالطفيلي فقط ، كما اظهر التحليل الاحصائي عدم وجود فروقات معنوية (p> 0.05)في اعداد الطفيلي جميع التراكيز التي استخدمت للمستخلص الكحولي Acacia nilotica مقارنة مع مجموعة السيطرة المعاملة بالطفيلي فقط. اظهرت الدراسات ايضا" نقصان معنوي (p<0,01) في اعداد الطفيلي المعالج بالعقار بينتوسام Pentosatam مقارنة مع مجموعة السيطرة المعاملة بالطفيلي فقط.

Introduction

Leishmania parasites are the causal agents of leishmaniasis, a group of protozoan diseases transmitted to mammals, including human beings, by phlebotomine sandflies[(1)]. The protozoan *Leishmania* is an obligatory intracellular parasite which exists in two distinctive forms. In man and other hosts it occurs as a non-flagellar amastigote form, while in culture and gut of sandflies the flagellar or the promastigote form is seen. They are neither found in the peripheral blood nor in any visceral organ[(2)]. Cutaneous leishmaniasis caused by *L. tropica* (previously known as anthroponotic or urban anthroponotic cutaneous leishmaniasis) produces painless, frequently multiple, dry ulcers of the skin, which usually heal spontaneously within about 1 year, or sometimes longer, often leading to disfiguring scars. The incubation period is usually 2–8 months[(3)]. Cutaneous leishmaniasis caused by *L. major* (previously known as zoonotic or rural zoonotic cutaneous leishmaniasis) is, like other forms of cutaneous leishmaniasis, painless when the lesions are uncomplicated. The lesions are often severely inflamed and ulcerated and heal within 2–8 months [(4)]. Frequently, they are multiple, especially in non-immune immigrants, becoming confluent and secondarily infected. Such lesions are often slow to heal and may leave large, disfiguring or disabling scars. The incubation period is often less than 4 months[(5)]. The prognosis of the disease varies with the species, the choice of treatment also depends on the causative Leishmania. The species identification by culture and by isoenzymatic examination is fastidious and time consuming (several weeks), and new rapid tools, as genomic amplification by the polymerase chain reaction, are not available widely. This is the reason why clinicians encountering patients with leishmaniasis treat frequently patients without identification of the species[(6)].Pentavalentantimonials (sodium stibogluconate pentostam) become the drug of choice for the treatment of all types of leishmaniasis. The drug can be administered intramuscularly or intravenously, the precise mechanism of action of antimonials by inhibiting parasite glycolysis, fatty acid beta-oxidation and inhibition of ADP phosphorylation , It has also been reported to cause nonspecific blocking of SH groups of amastigote proteins and cause inhibition of DNA topoisomerase I [(7)].Currently, several limitations have decreased the use of antimonials: the variable efficacy against CL and VL, as well as the emergence of significant resistance has been increased . The recommendations have replaced the antimonials by amphotericin B in refractory zones. Second, new generic of Pentostam have been produces with the aim to decrease the high cost of the treatment[(8)].Juniperus (J) is one of the major genera of Cupressaceae family. Juniperus is species are used for the treatment of hyperglycemia, tuberculosis, bronchitis, pneumonia, ulcers, intestinal worms, to heal wounds and cure liver diseases[(9)]. The berry oil from Juniperus species is well reputed for a wide spectrum of pharmacological activities and monographs on it are included in various national pharmacopoeias, while *J. procera* is used in the Southern part of Saudi Arabia for the traditional remedy of tuberculosis and jaundice[(10)]. Earlier investigations on Juniperus leaves and stem bark have yielded several antimicrobial diterpenes, including totarol, ferruginol, 4-epi-abietic acid, 4-epi-abietol, E-communic acid and Z-communic acid, of which totarol and

ferruginol exhibited potentiating activities of INH against four atypical mycobacteria: Examinations of the EtOH extract and n-hexane partitions of the berries from *J. excelsa* showed sufficient antiparasitic activities to warrant further investigation[11]. This led to the identification of previously known abietanestotarol and ferruginol as the principal constituents from these berries,using GC/MS analysis. The EtOH extract of J. excelsa was selected for bioassay-guided fractionation due to its prominent antileishmanial and antimalarial activities[10].

Acacia nilotica, also known as *Mimosa nilotica*, is a member of the family Mimosaceae and is known in the Sudan as Garad. the. *Acacia nilotica* and other Acacia species are used in folk medicine by people in rural areas as a remedy for tuberculosis, leprosy, small pox, dysentery, cough, ophthalmia, toothache, skin ulcers and cancers and as astringents, antispasmodics, aphrodisiac .Phytochemical analysis of the aerial parts of the plant demonstrated the presence of flavonoids and polyphenolic compounds in the flowers, tannins, glycosides, volatile oils, organic acids, coumarins and carbohydrates in the fruits(Application of Acacia niloticasppnilotica Pods (Garad)Powder a s Alternative Vegetable Retanning Materia[12]. Garad tannin is reported to contain chebulinic acid, gallic acid and to have a high sugar content, factors which are common in hydrolysable tanning materials[13]. Garad tannins are therefore mixed tannins i.e. containing condensed tannins as well as hydrolysable tannins containing gallic acid esterified with glucose. When garad pods are crushed, they disintegrate into three parts, the husk with about 12% pure tannins, the seeds with no tannin content and the grain powder with approximately 55% tannins. The seeds and husk form about 63.6% of the weight of the pod, the remainder being the grain powder[14] .

Aim of stud

The aim of this study is to evaluate the efficacy of Methanolic *Juniperus excelsa* berries and *Acacia nilotica* methanolic extractsin vivo and in vitro against leishmanial promastigote (causative agent of Leishmaniasis).

Materials and methods

1-**Sampling:** Samples were taken from the lesions of patients attending Tikrit Teaching Hospital and diagnosed clinically by professional doctors in dermatological department in the hospital clinically identified as *Leishmania a*ccording to lesion characteristics form. All patients underwent lesion aspiration, thoroughly clean the edge of the lesion and surrounding skin with sterile gauze or cotton wool soaked in 70% ethanol or isopropanol, then 0.1-0.2 ml of sterile saline or PBS was injected into the edge of the lesion from 1 ml syringe fitted with a short needle (20gauge), after that the needle was rotated 2-3 times whilst it is in the skin. This cut small pieces of tissue from the edge of the needle wound and applying gentle suction until pink-tinged tissue juice was noted in the hub of the syringe .After the aspirate is obtained, discharge into the Leishmanial culture medium[15,16].

2- **Preparation of media:**Roswell park memorial institute medium (Sigma,St.Louis),L-glutamine (Sigma) medium supplemented with 10% FCS

.Penicillin 1000U/ml(0.00598gm) and 0.3mg/ml streptomycin and nystatin250 U/ml(0.000514gm) were added to avoid contamination[17]. All tubes medium were incubated at 25C° for 21 days and checked every two days. Then the follow up tests were done:

a- Growth rate: Parasites were counted with help of hemocytometer(WBC counting chamber) slide with a light microscopy, calculation done by the following equation:

Total number of promastigotes in ml = the number of promastigotes in 64 small square of haemocytometr ×25×dilution degree×10^3

b-Viability: viability of parasites in cultural media was estimated by mixing 50 µl of growth and 50 µl of methylene blue on clean sterile slide then covered with cover slip and checked with 40 Xobjective of light microscopy[18].

c-Stained smear : a small drop of growth smeared on sterile clean slide and lifted to dry then fixed with methanol for 1 min and dried again, then stained with Giemsa stain for 20-25 min and washed with distilled water and lifted to dry ,Finally examined by oil immersion microscopy 100 X[15].

3-Preparation of Methanolic Extract of *Juniperus Excelsa* Berries

The dried material was ground to fine powder using a mechanical grinder. The berries extract was prepared in analytical grade methanol (100 g in 100 ml of 80% methanol) for 72 hours, Then the methanol was removed and residue was immersed in methanol for further seven days. After that, the methanol was decanted and filtered with Whatman filter paper No 1. The filtrate crude methanolic fruit extract (CME) was kept in 4C° for further use [19].

4-Preparation of Methanolic Extract of *Acacia nilotica*

Samples of plant were dried, coarsely powdered using a mechanical grinder. After that put 100g of sample soaked in 80% methanol over night with continuous shaking at 37C°, Then filtered, and kept at 4C°. According to El-Tahir et al[20] method the counted promastigotes were harvested on day 4-5 of subculture in RPMI 1640 media and used for evaluation of anti- leishmanial activity of methanol extracts of the plant. The stock plant methanol extracts (100mg/ml) were diluted with culture medium for working concentrations (31.25, 62.5, 125, 250, 500, 1000 µg/ml). Furthermore two controls samples were used included, growth medium without agent (100% viability) and growth medium containing studied the anti-Leishmanial drug (Sodium stibogluconate) (Pentostam) (20µg/ml).Then cultured medium enriched with promastigotes (8X 10^6 /ml) were added to all control and test agents. Each concentration was done in triplicate. The parasites were allowed to multiply at 26C°, the promastigotes were counted after 6 ,12,24 & 48 hr. in each well using haemocytometer.The result were expressed as the percentage of growth index GI% which was calculated as follow:

$$GI = \frac{N}{No.} \times 100$$

N = No. of treated promastigote.

No. = No. of untreated (control) promastigote.

5- Inoculation of susceptible mice:. parasites Isolates were harvested at stationary phase of growth injected intradermaly of 40 BALB/c mice for

each plant divided into three groups ,five mice in control group without agent, five mice in control group treated with Pentostam, and thirty mice intreated group of each plant, five mice for each concentration. The mice were examined weekly for appearance of lesion in the injection site up to six to eight weeks. When CL lesion were observed, smears were prepared and stained with Giemsa stain and examined with immersion objective under light microscope .Also small amount of lesion were cultured in RPMI 1640 at 25°C for 14 days[21,22]. Then the plant extract were injected intradermaly in the same area of infection with 0.05ml for treatment.

Results: 1- Growth and viability:

Figure (1):-Shows the Leishmanial promastigote which grown on RPMI1640 medium after 7-14 days of incubation period time. Also the figure shows the rosette shape of the parasite.

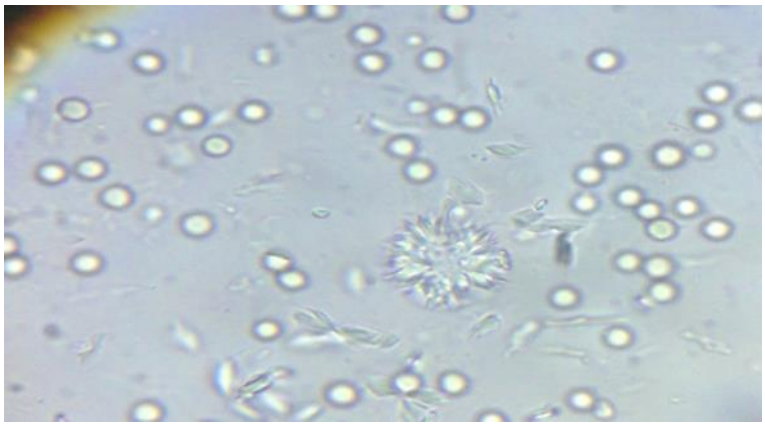

Fig.(1):- Promastigote of *Leishmania* in direct smear from cultural medium RPMI 1640, with rosette shape of the parasite.

2- Effect of plants extract *in vitro:*

The morphology of promastigote as seen by light microscope showed that the treated promastigote became smaller and rounded in size, slow and loss of motility as compared to the normal spindale shaped flagellated promastigote. These changes were more evident at higher concentration of extract. In Table (1) showed an increase in the number of parasite cells of untreated control group (control without agent) and decrease in number of treated group for Methanolic Extract of *Juniperus excelsa* berries, during 48 hours of plant extract exposure, and the statistically analysis show that there were significant differences in the in umber of parasite cells in all concentrations used except 31.25µg/ml compared with the control group without agent ($P<0.001$). Table (2)showed that there were increase in the number of parasite cells of untreated control group(control without agent) and treated group for Methanolic Extract of *Acacia nilotica*, during 48 hours of plant extract exposure ,this means that there were no effect of

alcoholic extract of *A.nilotica* of the growth of promastigote of Leishmanial parasites, and the statistically analysis show that there were no significant differences in the numbers of parasite cells in all concentrations used compared with the control group without agent ($P>0.05$), Also from table (1 &2) it is clear that there were decrease in number of parasite cells in control group treated with Pentostam compared with control group without agent, and the statistically analysis show that there were significant differences in the in number of parasite cells between Pentostam and control group without agent ($P<0.01$).

3-Effect of plants extracts *in vivo* (infected mice):

Clinical examination: The infection started on mice at the site of inoculation as swelling, thickening and redness which noticed and observed at fifth weeks post infection on infected control group (control without agent) , while the lesion in infected treated group for methanolic extract of *Juniperus excelsa* Berries characterized by more decrease in size, cellular infiltration, swelling and redness. At the first week of infection, the Juniperus extract was given at that time. It was observed that the lesion appeared as ulcer and swelling started to decrease gradually after the onset of treatment regimen. The swelling continued to decrease, two weeks after the treatment onset (figures 2,3,4, respectively). The topical treatment with methanolic extract of *Acacia nilotica*, lesions usually showed a weak response to these extracts and treatment with different concentrations of plant extract as shown in figure (5). The lesion in control group treated with Pentostam was more decrease in size, cellular infiltration, swelling and redness.

Discussion

The results of the present study showed that *Juniperus excelsa* berries extracts had antileishmanial activity agents Leishmanial promastigote at various concentration in vitro, and *in vivo*. It was found that alcoholic extract had a strong effect on the growth and cell division of the parasite. The results demonstrated a decrease of density of promastigotes with the increase of the concentration of the extracts. There is a general lack of affectivity and in expensive chemotherapeutic agents for the treatment of leishmaniasis. Although trivalent antimonial like potassium antimonyl tartrate and pentavalent antimonial drugs are the first-line treatment for this disease, with amphotericin B and pentamidine being used as alternative drugs, all of these have serious side effects and resistance has become a challenge problem. Therefore, new drugs are urgently required, natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans[14] . The essential oils, leaves, and berries of Juniperus species have been used for cosmetic and medicinal purposes for several centuries. The oils obtained by either steam distillation of berries and wood or by dry distillation of heartwood (cade oil, juniper tar oil) are applied for treatment of many diseases, from leprosy and typhoid to tape worm. *J.uniper* berries and leaves are used for diuretic, antiseptic, carminative, stomachic, antirheumatic, and antifungal purposes and as a disinfectant in many countries [9]. The investigation trials in BALB/c mice of the present study demonstrated that Juniperus extract has great effect against amastigote, there is significant decrease in the thickness and ulceration of infected treated mice when

compared with control mice. Signs of ulcer healing and clinical response were more clear in infected treated mice than control group (without agent), few number of amastigote were detected by cutaneous smear. After each successive increase in concentration the parasiticidal effect is increased to reach a mean (3.11×10^6) promastigote/ml in a concentration 125µg/ml. The parasiticidal effect more increased when adding (250,500,1000µg/ml) to reach a mean (2.90,2.21 and 1.12 $\times10^6$) promastigote/ml respectively. These observations are in consistence with the study of Sajid .*et.al*, who showed that Juniperus extract has significant Antileishmanial activity at 14.4 µg/ml[21] these antileishmanial effects could be due to the presence of Alkaloids, Flavonoids, Phenols, Saponins and Diterpenes in the Juniperus plant. In another study Samoylenko, *et. Al* (2008) determined the bioassay-guided fractionation of *Juniperus berries* and found that the plant yielded antiparasitic, nematicidal and antifouling constituents, including a wide range of known abietane, pimarane and labdanediterpenes. Among these, abieta-7,13-diene demonstrated in vitro antimalarial activity against *Plasmodium falciparum* D6 and W2 strains , while totarol , ferruginol and 7β-hydroxyabieta-8,13-diene- 11,12-dione inhibited *Leishmania Donovani* promastigote with concentration of 3.5–4.6 µg/mL. In addition, totarol demonstrated nematicidal and antifouling activities against *Caenorhabditise legans* and *Artemiasalina* at a concentration of 80 µg/mL and 1µg/mL, respectively[10].The extract of *A. nilotica* showed a weak antileishmanial activity. Although earlier reports, showed that the ethyl acetate extract of *A. nilotica* had showed potent antiparasitic activity against *Plasmodium falciparum* on the other hand Eltayeb[23]study which concluded that *A. indica* leaves lack anti leishmanial activity . In another previous study, it was reported that the methanol extract of *A. indica* leaves exhibited a moderate antileishmanial activity[14]. In fact, *A. indica* preparations are used locally in treatment of many parasitic diseases. Oil extracted from the seeds is used in both human and animals as an antihelmintic, and in scabies, abdominal ulcers, rheumatism and muscular pain. *A. nilotica* methanol extract had LC50equal 89.5 µg/ml. on *L. major* promastigote and this result showed low activity of *A. nilotica*, while Garad ethyl acetate extract possessed high activity (LC50 1.5 µg/ml) against *Plasmodium falciparum*. *A. nilotica* is widely used in folk medicine to treat a variety of diseases, where the gum stem bark, leaves and fruits are used for treatment of colds, bronchitis, pneumonia, ophthalmia, diarrhea and hemorrhage[24]. In a preliminary phytochemical screening of the ethyl acetate extract of *A. nilotica* husk, the presence of tannins and phenolic compounds was confirmed when a blue-black color was observed when ferric chloride reagent was added to a solution of the extract. Several studies stated that many plant species have immunomodulatory action. The ethyl acetate extract of *A. nilotica* husk, at low concentrations revealed an increase of human lymphocytes cells count. This may be an indication of the

ability of this extract to enhance human immunity. A potent antileishmanial activity was observed when *L. donovani* was treated with *A .maritima* extracts (IC50 <5 µg/ml). Further in vivo studies are needed to confirm the bioactivity of this plant.

Conclusions

The present study found that there were effect of *Juniperus excelsa* berries extracts on Leishmanial promastigote at various concentration *in vitro*, and *in vivo*, and no positive results for the treatment by *Acacia Nilotica* extracts.

Table (1):- The effects of various concentration of alcoholic extract of *Juniperus excelsa* berries on the *Leishmania* promastigote *in vitro*.

Hours after plant extract Exposure	Total No. of parasite cells / ml (x 10^6) Plant extract concentrations (µg/ml)							
	31.25	62.5	125	250	500	1000	Control with (Pentostam)	Control Without agent
6	6.11	5.22	4.43	4	3.32	4.41	3.18	5
12	7.23	4.52	3.37	3	2.61	3.34	2.21	5.22
24	9.34	3.82	3.11	2.90	2.21	1.12	1.56	5.50
48	12.54	3.33	3	2.4	2.1	Zero	Zero	6
Mean of Concentration ± SD	8.805 ± 5.9	4.22 ± 0.	3.48 ±	3.08 ± 0.336	2.56 ± 0.	2.217 ±1.	1.7375 ± 0.583	5.43 ± 0.1397

Table (2):- The effects of various concentration of alcoholic extract of *Acacia nilotica* on the *Leishmania* promastigote *in vitro.*

Hours after plant extract Exposure	Total No. of parasite cells / ml (x 10^6) Plant extract concentrations (µg/ml)							
	31.25	62.5	125	250	500	1000	Control with (Pentostam)	Control Without agent
6	16	14.9	15.7	9.1	8.5	8	7.5	8
12	14.5	14	13.3	13	12.9	11.3	6.1	13
24	28	26	24	22.3	221	19.5	4.9	24
48	37	34	32	30.2	29	28.1	Zero	36

Mean of Concentration ± SD	**23.875± 9.208**	**22.225 ± 8.279**	**21.25 ± 7.36**	**18.65± 8.213**	**17.85 ± 7.847**	**16.725 ± 7.788**	**4.625 ± 2.824**	**20.25 ± 10.75**

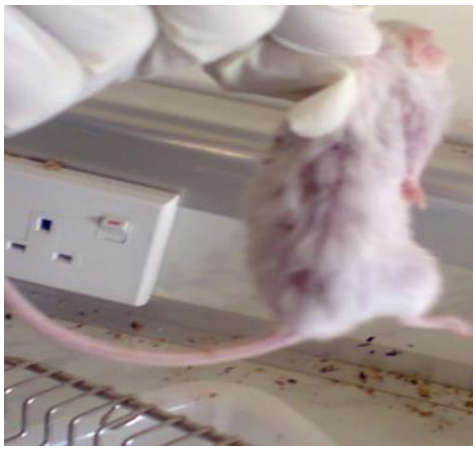

Figure (2)

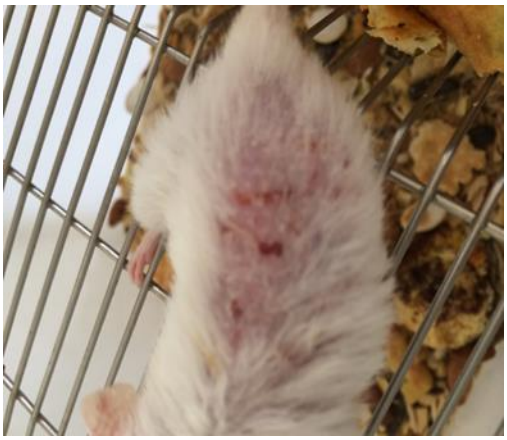

Figure (3)

Fig.(2):- The multiple lesions of cutaneous leishmaniasis on infected sites in mice after five weeks of injection with Leishmanial promastigotes

Fig. (3):- Starting of treatment with alcoholic Juniperus excelsa berries extract ,(the size, and ulceration of lesion decrease after one week of treatment)

Fig.(4):- The healing of ulcerated lesion after 10 days of treatment.

Fig. (5):- The left mice infected treated mice with extract, right mice represent infected treated mice with pentostam.

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