ISOLATION AND CHARACTERIZATION OF IRAQI LEISHMANIAL ISOLATES
عزل وتوصيف عزلاتاللشمانيا العراقية

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Abstract:
Smears, bone marrow and sera were obtained from 25 suspected patients with visceral leishmaniasis (VL) and 25 cutaneous leishmaniasis (CL). Most of patients came from urban and rural areas surrounding Baghdad. Bone marrow cultures were positive in 40% of VL cases, while the serological test was positive in 76% of cases. Parasites were demonstrated in 80% of CL smears against 44% of positive cultures.

The causative organisms in Iraqi patients with leishmaniasis were identified according to the electrophoretic variations of glucose phosphate isomerase (GPI), Glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), malate dehydrogenase (MDH) and hexokinase (HK) enzyme. A total of 63.6% of CL isolates were found to be similar to *Leishmania major* reference strain, while 36.4% were rather similar to *L. tropica* reference strain. In VL isolates, 90% were similar to the Mediterranean strain (*L. donovaniinfantum*), while 10% were similar to the Ethiopian reference strain (*L. donovanidonovani*).

الخلاصة

إشتملت الدراسة الحالية على 25 مريضا باللشمانياالاحشائية، تم التأكد من إصابتهم عن طريق سحب خزع من نخاع العظم لإجراء المسحات وزرعها وإجراء الفحوصات المناعية فضلا عن 25 مريضا باللشمانيا الجلدية تم تشخيص إصابتهم إعتمادا على الأعراض المرضية للقرحة والمسحات والزرع. كان أغلب المرضى من المناطق الحضرية والقروية المحيطة ببغداد. وكان الزرع موجبا بنسبة 40% للشمانياالإحشائية بينما كان الفحص المناعي موجبا بنسبة 76%. اما بالنسبة للشمانيا الجلدية فقد كانت المسحات موجبة بنسبة 80% مقابل 44% للزرع.

شخصت العزلات الموجبة (الجلدية والإحشائية) باعتماد طريقة الترحيل الكهربائي وبإستخدام أنزيمات كلوكوز فوسفيت آيزومريزGPI، كلوكوز-6-فوسفيت ديهايدروجنيزG6PDH، أنزيم الماليك ME، ماليتديهايدروجنيزMDHوهكسوكينيزHK. كانت63.6% من العزلات الجلدية مطابقة لعزلات*Leishmania major* المرجعية و36.4% مماثلة لعزلات*L. tropica*. أما العزلاتالإحشائية فقد كان 90% منها مماثلة لعزلات *L. donovaniinfantum* الشرق أوسطية المرجع و10% مشابهة لعزلة *L. donovanidonovani* الأثيوبية المرجع.

Introduction:
Leishmaniasis is a parasitic disease caused by haemoflagellates belonging to the genus*Leishmania*. There are more than 21 species causing human infection. The infection is transmitted to humans through the bites of female phlebotomine sandflies belonging to 30 species (1). The parasites exist in two forms: an amastogote in the mammalian host and a flagellated promastigote in the insect vector. Leishmaniasis is prevalent in many parts of the world, especially tropical and subtropical countries. Two million new cases of leishmaniasis occur each year. Both VL and CL forms of the disease occur in the Mediterranean basin and their clinical features vary from localized cutaneous affections to the realized life- threatening visceral disease (2).

Leishmaniasis is endemic in Iraq with the visceral form of the disease, caused by the *L.donovani* complex, is mainly endemic in the central part of the country. In contrast, the cutaneous form, caused by *L. tropica* or *L. major*, is more widespread (3). Increased outbreaks of the virulent CL

and VL forms of the disease occurred in Iraq during recent years. VL is more prevalent in Iraq than Afghanistan or Kuwait(4). Cities of Iraq become a breeding ground for the disease (4, 5).

Characterization of *Leishmania* isolates has traditionally been based on biochemical and pathological criteria such as clinical manifestations, geographical location and kinetoplast DNA density. These techniques have not, however, lead to definitive distinction among the various *Leishmania* species. Isoenzyme electrophoresis has proven to be a more reliable and precise method to distinguish at the species and subspecies levels (6). The present investigation was aimed to investigate and characterize Iraqi isolates species from humans by isoenzyme electrophoresis.

Materials and Methods:

Parasite isolations: A total of 25 patients were examined by dermatologists for CL at Baghdad Teaching Hospital during 2004. Their ages ranges from 3 to 45 years. Some information was taken from patients (age, sex, residence, duration of disease and number of ulcers). The samples were aspirated from the edges of the skin lesions. Twenty-five patients with VL were included in the present study. The patients were admitted to the Central Childs Teaching Hospital in Baghdad during 2004. VL patients came from different areas of Iraq. These suspected VL were referred by clinicians for serological tests (IFAT or Dipstick). Data recorded from each patients included age, sex, history of fever, weight loss, nausea…etc. Bone marrow aspirates were taken by sterna or iliac crest puncture for LD bodies. The aspirated material of CL and VL samples was inoculated in semi-solid medium for the primary isolation and all tubes were incubated at 25 C˚. All cultures were incubated and examined for 15-30 days before being considered negatives. Patients were positively diagnosed for CL and VL when actively promastigotes were seen in cultures (7).

Cultivations: The following three media and modifications were used:

Semi-solid medium: This medium was used for the primary isolation of bone marrow aspirates of suspected VL patients and skin lesion aspirates. It was used for the growth maintenance of the promastigotes and recovery of the parasites from infected animals (8).

Biphasic medium: Novy, MacNeal-Nicolle (NNN) medium is made of two phases: solid and liquid phases (Locks solution). This medium was used for sub culturing of parasites and preparation of the antigen (9).

Liquid medium (RBLM): After the achievement of a modification of the semi- solid medium (8), it was used for amplifying the promastigotes.

Preparation of samples for electrophoresis: Promastigotes of CL and VL isolates, growing on semi- solid medium were transferred to liquid medium in order to obtain rich growth and cultures were incubated at 25C˚in an orbital incubator. Active parasites were obtained at log phase and amplified after 3-5 days later (10). The promastigotes were collected by centrifuging the liquid medium overlay at 750g for 15 minutes at 4C˚. The pellets were washed three times (750g for 15 minutes) in normal saline solution, pH 7.2 and stored at -20C˚ until used. Just before application to gel, the frozen pellets were thawed and homogenized with an equal volume of triton X-100 (0.03%) three times for two minutes each time, with a break of 30 seconds between the runs. This was done under cooling conditions. The homogenized promastigotes were centrifuged at 4500g for 20 minutes. The supernatant (crude enzyme) was stored at -20C˚until used. The protein content of the crude enzyme extract and antigen preparation was determined by using bovine serum albumins as a standard (11).

Isoenzyme electrophoresis: Electophoresis was carried out in an LKB 2117 multiphor system, using 0.2M Tris- glycin buffer stock, Ammonium persulfate solution (0.07 M), Acrylamide solution and bromophenol blue solution. The following enzymes were analyzed for strain identification: Glucose phosphate isomerase (GPI), Glucose-6-phosphate dehydrogenase (G6PDH), malic enzyme (ME), malate dehydrogenase (MDH) and hexokinase (HK) enzyme.

Leishmanial reference stocks: The following international reference stocks were used as standards for isoenzyme analysis: MHOM/SA/19884/JisH 252 (5ASKH) *L. major* Ref. from a man with CL in Saudi Arabia. MHOM/SA/Strain- OD (LRC-L39) *L. tropica* Ref. from a man with CL in Kirovabad, Azerbaidzhan SSR, MHOM/TN/80/IPTI; LEM235 (IPTI) *L. donovaniinfantum* from five years old boy with VL in Tunisia and MHOM/ET/67/LV9 (HV3), (LV9 (HV3) *L. donovanidonovani* from a patient with VL in Ethiopia.

Results and Discussion:

The mean ages of CL patients in the present study was 19.6 ± 12.8 years, while in VL patient, it was 2.53 ± 1.8 years .The present results are in agreement with those previously reported for CL and VL in Iraq (12, 13). Number of lesions ranged from 1-5 in 30% of cases with a mean of two lesions. Most of the lesions were found on the exposed areas of the body: face, arms, legs, neck and ears (Fig. 1). These multiple lesions are due to repeated and consecutive bites of sand flies (14). Most patients of CL and VL came from urban and rural areas surrounding Baghdad. This may be associated with vector prevalence.

The percentage of positive CL smears of the present study was 80% with 44% of positive culture (Table 1). The percentage of positive VL smears was 52% while the percentage of positive cultures was 40% (Table 2). This low percentage of positive cultures could be attributed to difficulties in taking samples, especially from children and young patients which may lead to contamination of samples and hence the failure of culturing. From Baghdad, 75% positive smears and 20% positive cultures of CL patients were obtained (12), while from Basrah and ThiQar provinces, 68.2% positive culture of bone marrow aspirates of VL patients were reported (13). The results of the two above studies (12, 13) come in agreement with the present investigation.

The high percentage (76%) of serological tests (IFAT or Dipstick) in VL patients (Table 2) of the present study is due to the fact that these tests are simple to perform, require no specialized equipment and relatively rapid technique, with high sensitivity and specificity. The present results are in agreement with the results from Basrah and ThiQar provinces (13) where 96.5% of positive VL cases by IFAT from patients were obtained.

Of the bone marrow aspirates, 52% showed positive primary cultures and 44% positive culture from CL cases. These results were obtained after 1-2 weeks of incubation. The first subcultures showed good growth as primary cultures. These continued to perform other subcultures later. In the present study, isolation and maintenance of growth of Iraqi CL and VL isolates for many subcultures reached to hundred both *in vitro* and *in vivo.* This finding was obtained here for the first time in Iraq since 1990 as no other researchers until 2003 succeeded in isolation and maintenance of the growth of cultures. Some researchers (12, 15) obtained only primary subcultures which died later. Yet, another study (16) reported that the first subcultures failed to produce a very low recovery rate with no ability to isolate visceralizing stocks. The results of the present study are similar with some previous investigations (17, 18, 19, 20)

In the present study, the isoenzyme GPI, G6PDH, ME, MDH and HK were analyzed for the recovered isolates. The enzymes of four CL isolates were identical with *L. tropica* strains. Most of these isolates were taken from patients in Al-Karkh district in Baghdad, mainly from Al-ShaikhMaaroof region. Other seven isolates were identical with *L. major*, the wet type which is more common in rural areas (Figs. 2&3). The present results are in agreement with some previous studies in Iraq (3, 17, 19) which involved with two species of *Leishmania* in CL infections in Iraq: *L. major*, the causative agent of zoonotic cutaneous leishmaniasis (ZCL) and *L. tropica*. Previously, it was thought that *L. tropica* alone was responsible for CL in Iraq (21, 22). In the present study, *L. tropica* is more dominant than *L. tropica*. The patients came from rural areas of Diyala province.

It was indicated that the most common cases of leishmaniasis acquired by US troops in Iraq were localized CL caused by *L. major* (23) as outbreaks in prevalence of the virulent form of this disease reached 82% in urban and suburban areas especially in Al-Nasiriyah and Baghdad provinces.

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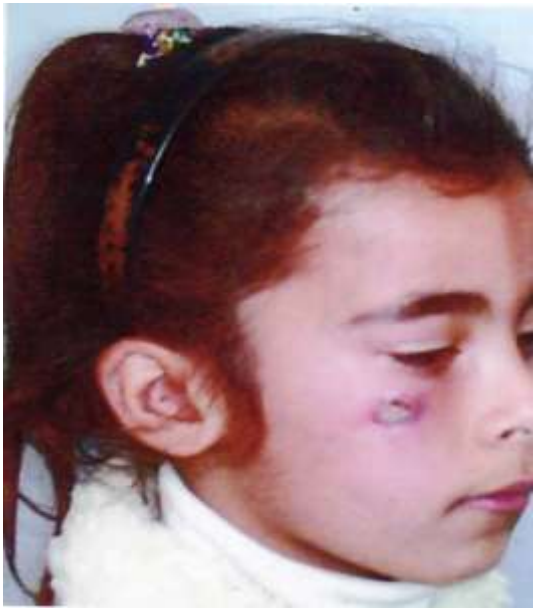

Fig. (3-1): A girl from Wasit province with single lesion of CL.

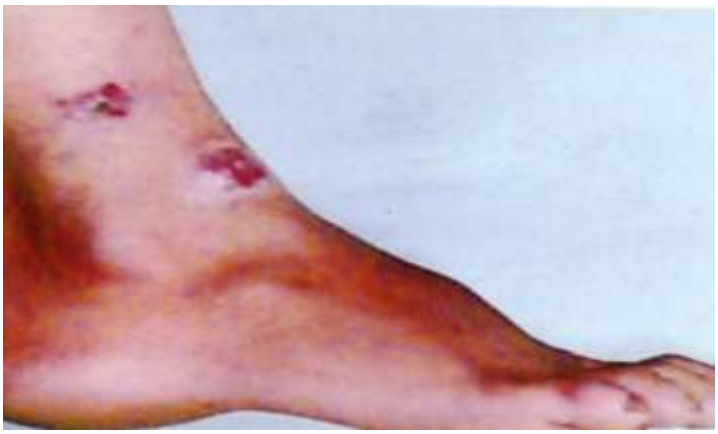

Fig. (3-2): CL lesion on the leg of a young patient from Baghdad.

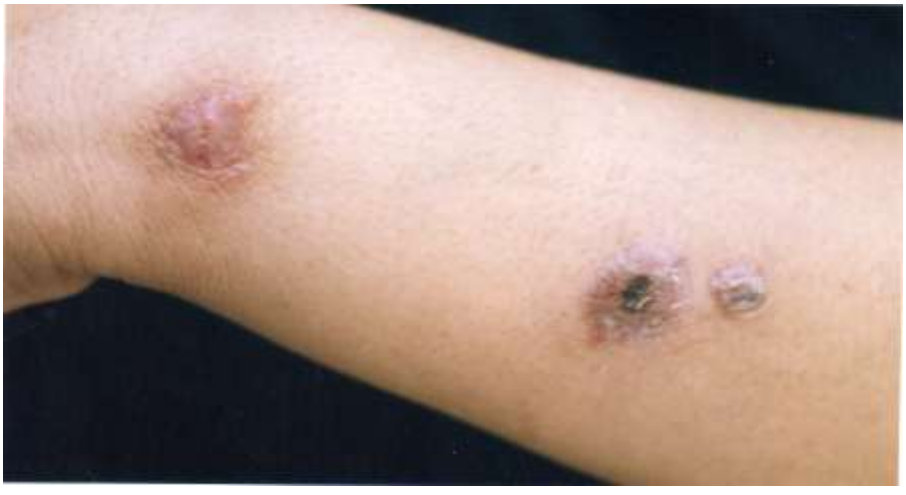

Fig. (3-3): Patient from Diyala province with multiple CL lesions.

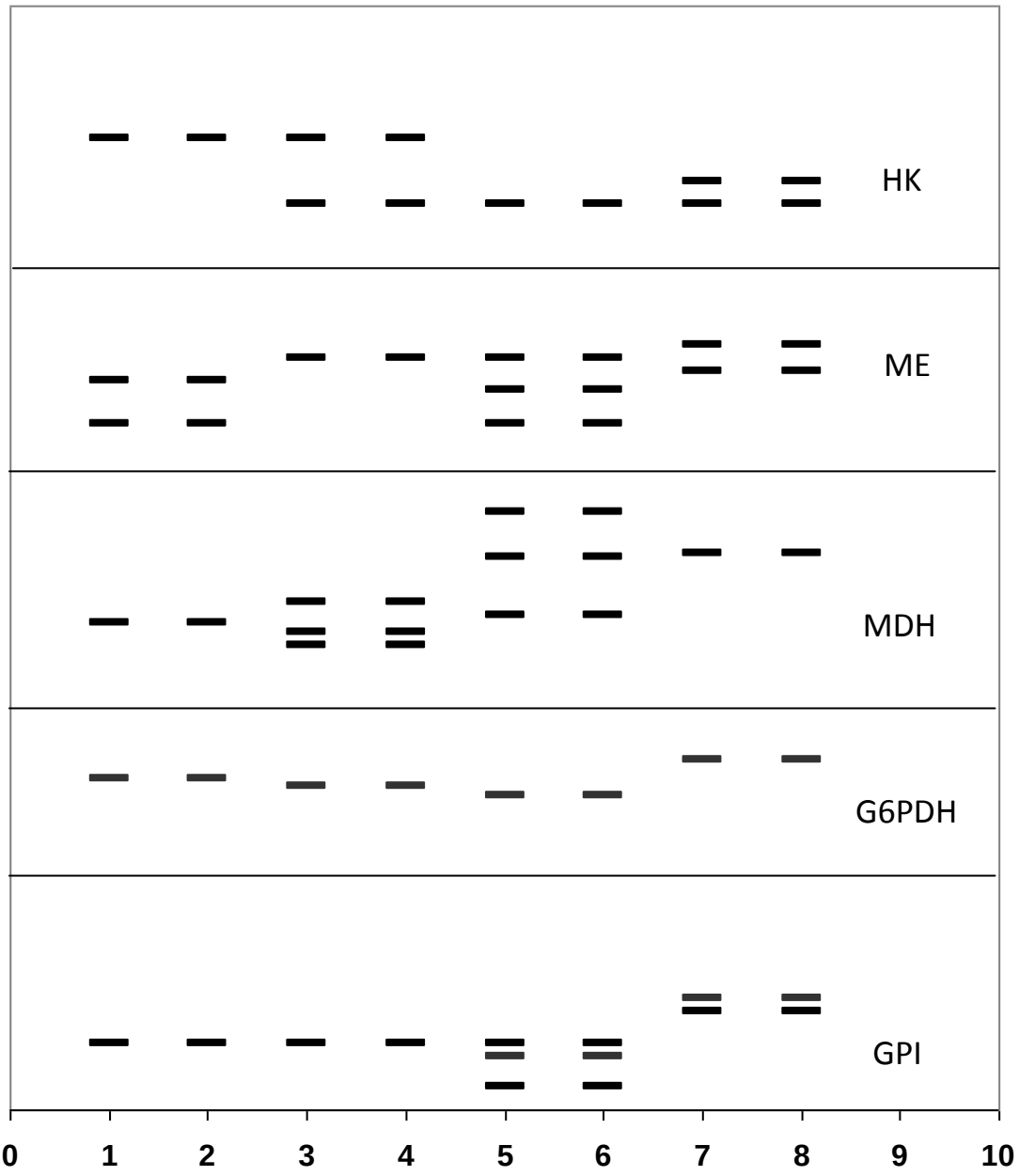

Fig. (3-4): Diagrammatic representation of the electrophoretic enzyme patterns for GPI, G6PDH, MDH, ME and HK of *Leishmania* reference strains and of the species from VL and CL aspirates. 1= 5ASKH (Cutaneous *L. major* reference starin); 2= Sample; 3= LRC-L39 (Cutaneous *L. tropica* reference strain); 4= Sample; 5= IPTI (Visceral *L. donovaniinfantum* reference strain); 6= Sample; 7= LV9 HU3 (Visceral *L. donovanidonovani* reference strain); 8= Sample.

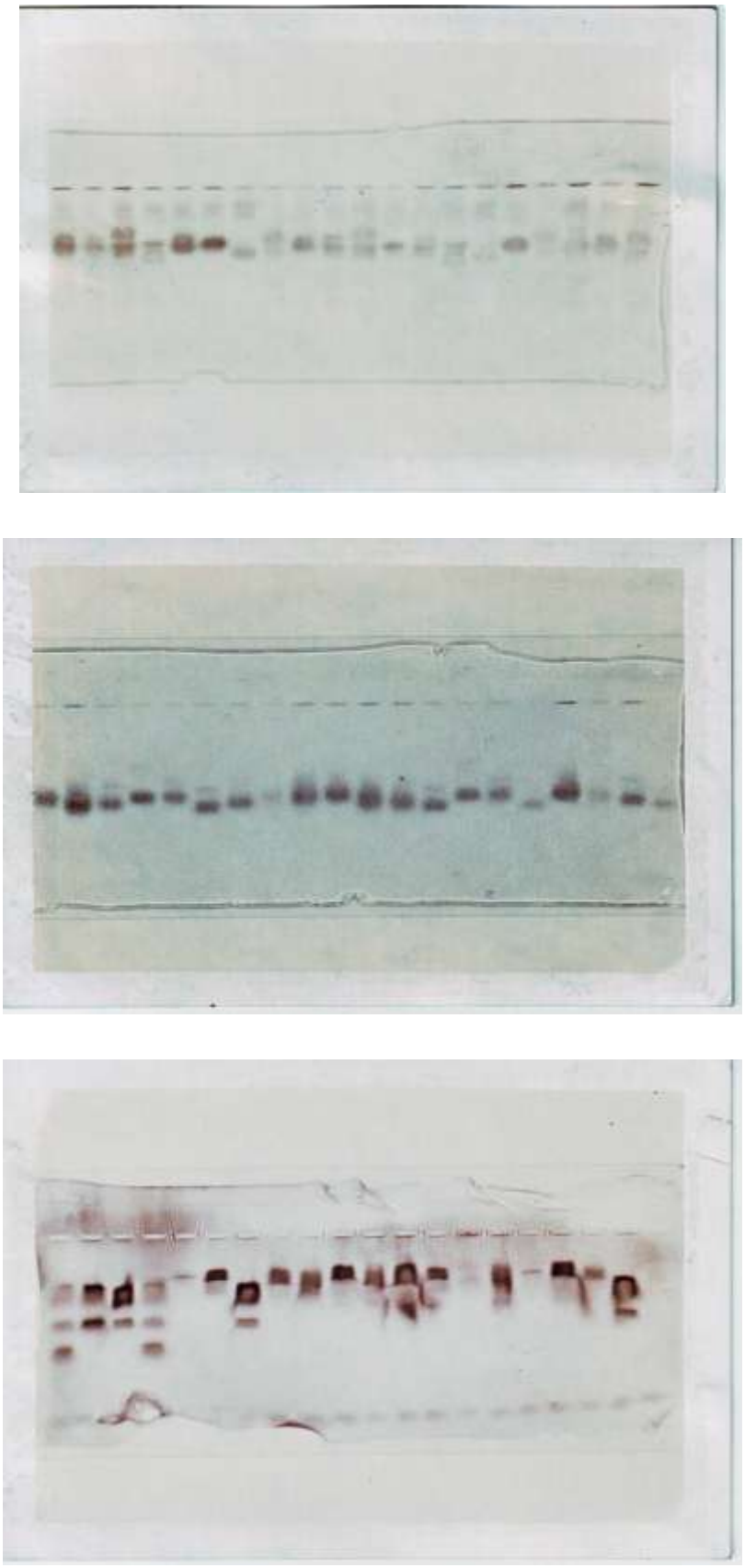

Fig. (3-5a): Photography of polyacrylamide gel showing representatives of the enzyme variant of GPI, G6PDH and MDH of VL and CL including species and reference strains.

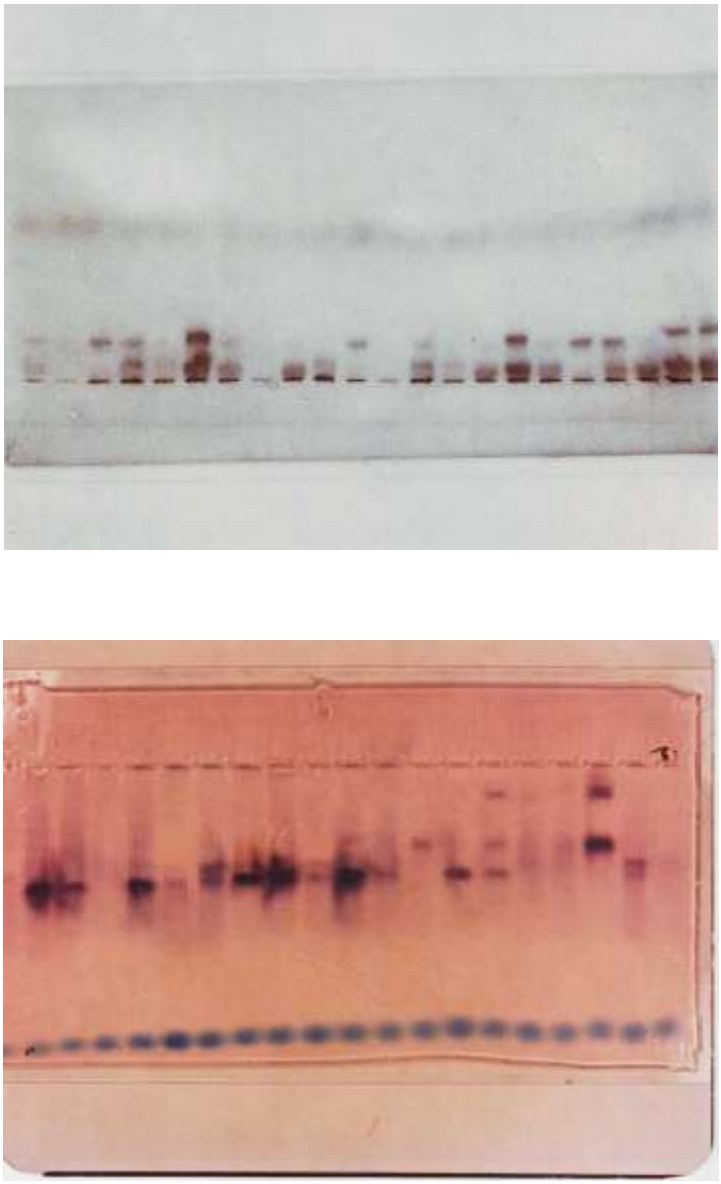

Fig. (3-5b): Photography of polyacrylamide gel showing representatives of the enzyme variant of ME and HK of VL and CL including species and reference strains.

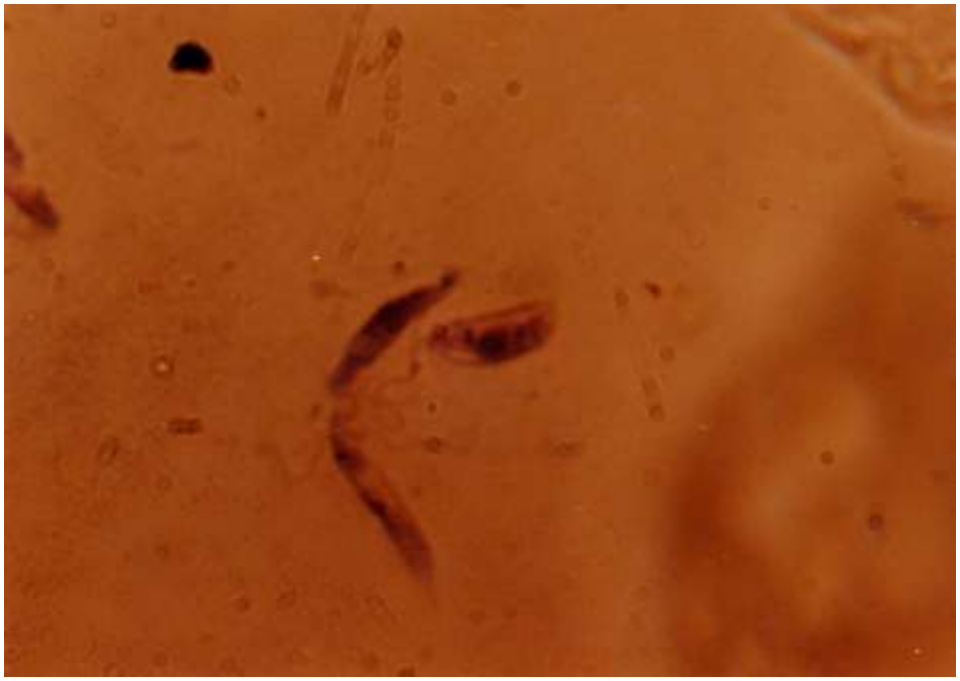

Fig. (3-6): *Leishmania* spp. promastigotes from *in vitro* culture at 26Cº.

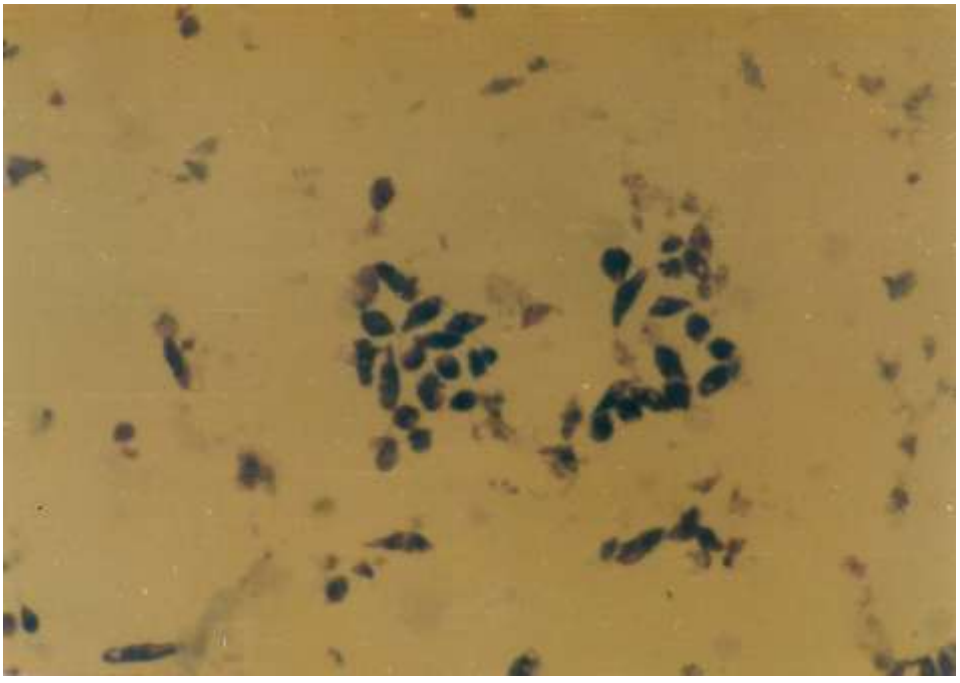

Fig. (3-7): *Leishmania*spp. promastigotes from *in vitro* culture at 26Cº after treatment with either *G. glabra*or *C. roseus*.

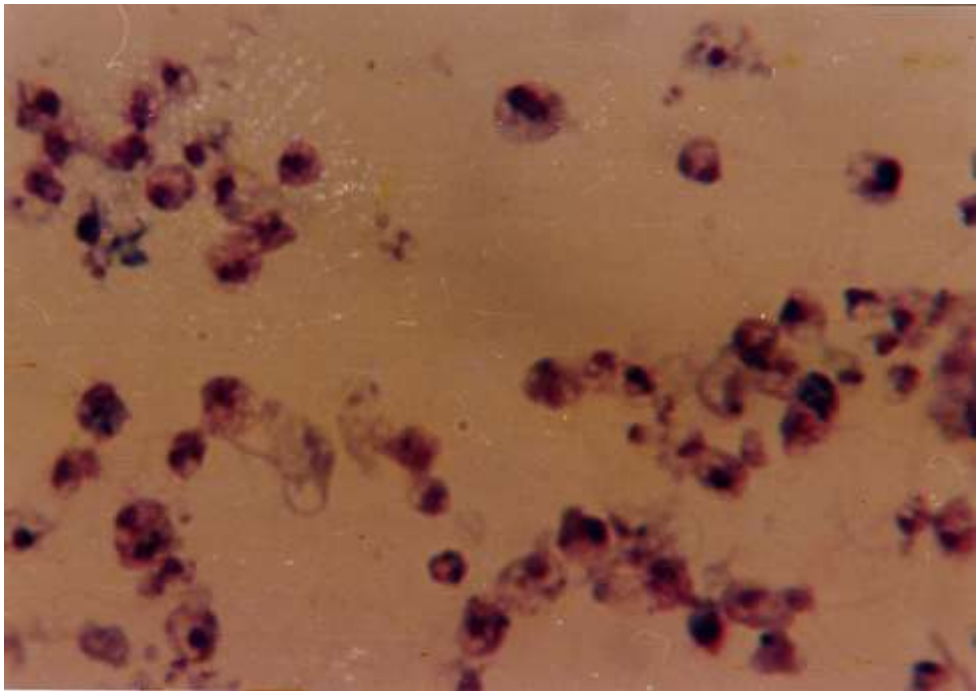

Fig. (3-8): *Leishmania* spp. amastigotes from *in vitro* culture at 37Cº.

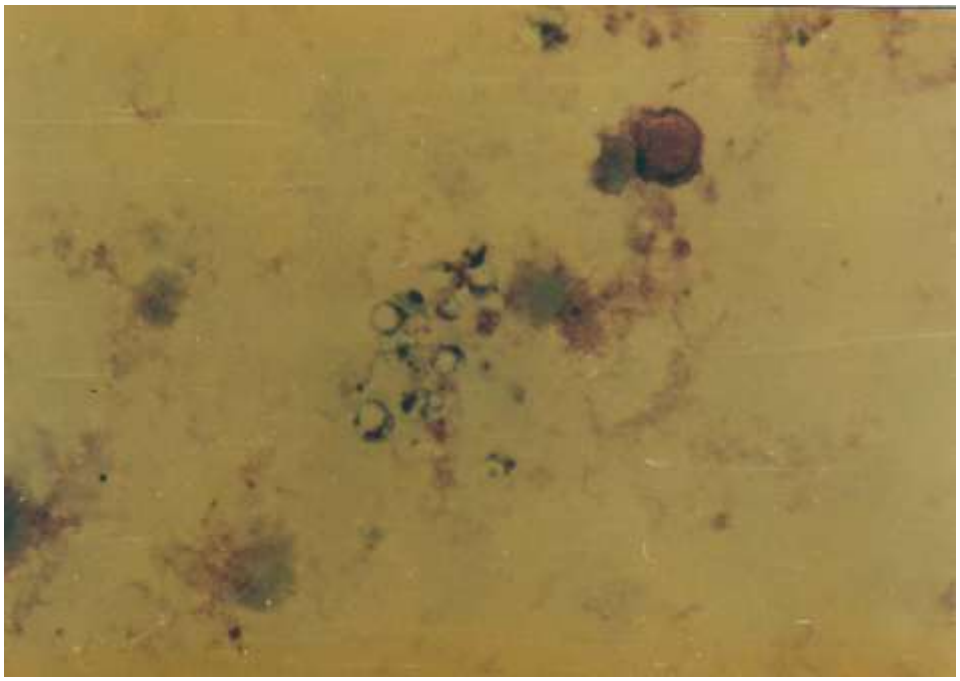

Fig. (3-9): *Leishmania* spp. amastigotes from *in vitro* culture at 37Cº after treatment with either *G. glabra* or *C. roseus*.