HEMATOLOGICAL AND BIOCHEMICAL STUDIES ON LEISHMANIA DONOVANI (ATCC®30030™) IN EXPERIMENTAL INFECTED MICE

By

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Abstract

Leishmaniasis is caused by Leishmania parasites, which are spread by the bite of phlebotomine sand flies. There are several different forms of leishmaniasis in people. The most common forms are cutaneous leishmaniasis, which causes skin sores, and visceral leishmaniasis, which affects several internal organs (usually spleen, liver, and bone marrow). It usually is more common in rural than in urban areas, but also in the outskirts of some cities. Cutaneous leishmaniasis usually heal on their own, even without treatment, but this can take months or even years leaving ugly scars. Mucosal leishmaniasis might not be noticed until years after the original sores healed. Ensuring adequate treatment of the cutaneous infection may help prevent mucosal leishmaniasis. Visceral form If not treated, severe or advanced cases are fatal.

The pentavalent antimonial compounds; Pentostam® is the first line anti-Leishmania treatment. However, its effect on human body was not well evaluated as compared with two plants extract-ed (Black seed oil and Curcumin) with anti-parasitic activities. The effect of the three was studied in experimentally infected Swiss Albino mice.

Key words: Leishmania donovani (Atcc®30030™), Experimental infected mice, Treatment

Introduction

Visceral leishmaniasis is caused by 3 species of L. donovani complex: L. donovani, L. infantum, and L. chagasi. Infections are often asymptomatic, but in others showed severe symptomatic picture by affected several internal organs (Svobodova et al, 2009).

Infantile visceral leishmaniasis was reported in Al-Agamy, Alexandria on 1983 (Mansour et al. (1984), dog reservoir host (Morsy et al, 1983), and detection of Phlebotomus langeroni (Morsy et al, 1989). Again, Madwar et al. (1985) suggested the possibility of IVL in Qalyoubia Governorate. An IVL case was identified in an adult farmer (unusual host) in Qalyoubia with hepatosplenomegaly and never left to Alexandria (Kabil et al, 1988). El Mahdy et al. (1993) in Dakahlia detected Leishmania-positive by IHAT & dot-ELISA in 4/22 hypersplenic patients with Leishmania amastigotes in splenic smears of two patients obtained on splenectomy. Morsy et al. (1993) in Sharkia Governorate reported lymphatic leishmaniasis as an indigenous 30years old male, with cervical lymphadenopathy the only clinical sign without visceral involvement. He was successfully treated with sodium stibogluconate as shown by clinical and parasitological follow-up.

Apart from sand fly bites, VL was transmitted congenitally (Boehme et al, 2006) and by blood transfusion or needle stick injury (Abdel-Motagaly et al, 2017). The spread of L. infantum/HIV co-infection in intravenous drug-users inhabitance in South Europe syringes exchange were reported (Alvar and Jimenez, 1994). Pentostam was the drug of choice (CDC, 2020). Others proved the curative potentiality of plant extracts as anti-protozoal, antifungal, and anthelmintic agents (Gellis et al, 2012).

This study aimed to evaluate two plant products (Nigella sativa) and Curcuma longa as compared to the well documented drug Pentostam® in treatment of experimentally infected Albino mice with L. donovani strain

Materials and Methods

Experimental animals: Clean laboratory bred Swiss Albino mice aged from 3 to 5 weeks, and weighed 25-30gms were used.
They were residing in well aired out cages with holed covers, provided with regular pellet food and water. Bedding was changed daily and allowed to acclimate to the laboratory environment for a week before experimented with (El-Fakhr et al, 1998).

Parasites: *Leishmania donovani* (ATCC® 30030™), strain designation, Khartoum was purchased from the American Type Culture Collection (ATCC), Virginia. Frozen culture was stored at -70°C for approximately one week until use.

Animals: A total of 50 clean laboratory bred male mice were divided into five groups of 10 mice each.

Infection: Each infected mouse was injected with one of therapeutic agent in the hind footpad at a dose of 1×10^6 promastigotes/mouse. Blood were collected from bone marrow of all mice, stained by Giemsa stain and examined by light microscopy.

All treatments started 28 days post infection, using gastric intubation and intravenous injection. The groups were: G1: Control mice (neither infected nor treated). G2: Infected mice were infected in hind footpads with a dose of 1×10^6 stationary phase *L. donovani* promastigotes (infected, not treated), and G3: infected and treated divided as following: SGI: received Curcumin (50mg/kg weight/day) for 28 days. SGII: received Pentostam (20sb/mg/kg weight/day) for 28 days. SGIII: received black seed oil (450mg/kg weight/day) for 28 days. At the experimental end (28 days post infection), animals were sacrificed by cervical dislocation and blood samples were collected on EDTA.

Hematological parameters: Red blood corpuscles (RBCs), white blood cells (WBCs) and platelets, as well as hemoglobin (Hb) content, hematocrit (Ht%), were counted by using hematological analyzer (Sysmex Kx-21), Japan (Dacie and Lewis, 1991).

Biochemical parameters: Total protein concentration was estimated in serum (Henry, 1964), using Biosystems reagents and instruments, Egypt.

Protein in sample reacts with copper (II) ion in alkaline medium forming a colored complex that can be deliberated by spectrophotometry.

2. Mix carefully and let the tubes stand for 10 minutes at room temperature.
3. Read the absorbance (A) at 545 nm against the blank, color is stable for at least 2 hours.

Calculation: Protein concentration was calculated using the following general formula:

\[
A \text{ sample} = \frac{X \text{ C standard}}{A \text{ standard}} \times \text{C sample}
\]

Determination of Alanine Aminotransferase (ALT, GPT) was by EnzyChromTM Alanine Transaminase Assay Kit (Cat# EALT-100). Alanine Transaminase (ALT), which known as serum alanine aminotransferase (ALAT), eases the conversion of alanine and α-ketoglutarate to pyruvate and glutamate. ALT has an effective role in amino acid metabolism and glucon-eogenesis. ALT presented chiefly in liver, and to a lesser extent in pancreas tissues, heart, kidney and muscle. Normal serum values of ALT are low and high serum ALT levels is broadly used as an indicator for liver damage.

Kits: Assay Buffer: 24 mL, LDH: 120µL, Co-substrate: 600µL NADH Reagent: Dried

Procedures: Equilibrate all components to room temperature. Reorganize the NADH Reagent tube with 1000 µL dH2O (final 10 mM). Unused reconstituted NADH reagent is stable for 21 days when stored frozen at -20°C. Mix assay buffer well by forceful
shaking. Keep thawed enzyme on ice. Assays were performed at 37°C or at room temperature. Before assay, bring the working reagents, microplate, and spectrophotometer were brought to the desired temperature. Assay was agreeable with serum or plasma (heparin, EDTA). Samples were clear without particles or precipitates. But, the hemolysis samples were not used.

Calculation: For each Sample, calculate the rate of NADH consumption by subtracting the OD at 10 min from OD at 5 min (ΔODS). Similarly, calculate the (ΔODNADH) rate for NADH standard (OD5min - OD10 min).

Determine ALT activity using the following equation:

\[
ALT = 381 \times \frac{\Delta OD_{STD} - \Delta OD_{NADH}}{OD_{STD} - OD_{BLK}} \quad (U/L)
\]

ODSTD and ODBLK are the OD340 nm values of NADH Standard and Blank at 5 min, respectively. The factor 381 is derived from:

\[
\text{Factor} = 10 \text{ mM NADH} \times \frac{4 \mu L \text{ Vol}_{NADH} x 200 \mu L \text{ Vol}_{WR}}{210 \mu L \text{ Vol}_{WR} x 220 \mu L \text{ Vol}_{total} x 11 \text{ (sample dilution)} / 5 \text{ min}} = 381 \mu M/min
\]

If the ALT activity is higher than 100 U/L, dilute the sample in Assay Buffer and repeat assay again. Multiply the results by dilution factor.

Determination of aspartate aminotransferase (AST, GOP) was by EnzyChromTM Aspartate Transaminase Assay Kit (Cat#EA-STR-100). Aspartate Transaminase (AST), also known as aspartate aminotransferase (ASAT/AAT) or serum glutamic oxaloacetic transaminase (GOT), eases transformation of α-ketoglutarate and aspartate to glutamate and oxaloacetate. There were two isoenzymes in humans: GOT_1 is a cytosolic isoenzyme comes from red blood cells and heart; GOT_2 is the mitochondrial isoenzyme presented chiefly in liver. AST is high in liver and muscle diseases, as a part of prognostic tests for myocardial infarction, acute pancreatitis, liver function, acute severe burns, hemolytic anemia, acute renal disease, and trauma.

Kit contents (100 Tests In 96-Well Plates):

- Assay buffer: 24mL Cofactor: 120 µL, & Enzyme mix: 120µL NADH Reagent: Dried
- Procedures: Equilibrate all components to room temperature. Reconstitute NADH reagent tube with 1000µL dH2O (final 10mM). Unused reconstituted NADH reagent was stable for three weeks when stored frozen at -20°C. Mix assay buffer well by vigorous shaking. Keep thawed enzyme on ice. Assays were done at 37°C or at room temperature. Prior to assay reagents, microplate and spectrophotometer were kept to the desired temperature. Assay was co-mpatible with serum or plasma (heparin, EDTA). Samples were clear without particles or precipitates. Hemolyzed samples were not used. NADH consumption was calculated by subtracting OD at 10min. from OD at 5min. (ΔODS). Also, calculate rate (ΔODNADH) for NADH standard (OD5min - OD10min). Determine AST activity by this equation:

\[
AST = 388 \times \frac{\Delta OD_{S} - \Delta OD_{NADH}}{OD_{STD} - OD_{BLK}} \quad (U/L)
\]

ODSTD and ODBLK are the OD340nm values of NADH Standard and Blank at 5 min, respectively. The factor 388 is derived from:

\[
\text{Factor} = 10 \text{ mM NADH} \times \frac{4 \mu L \text{ Vol}_{NADH} x 200 \mu L \text{ Vol}_{WR}}{206 \mu L \text{ Vol}_{WR} x 220 \mu L \text{ Vol}_{total} x 11 \text{ (sample dilution)} / 5 \text{ min}} = 388 \mu M/min
\]
If the AST activity was higher than 100 U/L, dilute sample in Assay Buffer and repeat assay. Multiple results by the dilution factor.

Statistical analysis: Data were assessed by SPSS program using One Way ANOVA test. The data were shown as means ± standard error (SE). P<0.05 were considered significant.

**Results**

The results were shown in tables (1, 2, 3, 4, 5, 6, 7, 8 & 9)

**Table 1: Blood analysis for different mice groups**

<table>
<thead>
<tr>
<th>Collected materials</th>
<th>Control Negative</th>
<th>Control Infected</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1 (CI)</td>
<td>T2 (CI)</td>
<td>T3 (CI)</td>
</tr>
<tr>
<td>A week before infection (analysis)</td>
<td>- ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>7 days (PI)</td>
<td>+ ve</td>
<td>+ ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>15 days (PI)</td>
<td>+ ve</td>
<td>+ ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>30 days (PI)</td>
<td>- ve</td>
<td>+ ve</td>
<td>- ve</td>
</tr>
<tr>
<td>43 days (PI)</td>
<td>- ve</td>
<td>+ ve</td>
<td>- ve</td>
</tr>
</tbody>
</table>

T1= Curcumin, T2= Pentostam, T3= Black seed oil, CN= neither infected nor treated, CI= infected not treated

**Table 2: White blood cells count in treated mice compared with infected and uninfected ones**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.24</td>
<td>15.73</td>
<td>9.67</td>
<td>6.52</td>
<td>5.13</td>
</tr>
<tr>
<td>SE±</td>
<td>0.23b</td>
<td>0.79a</td>
<td>0.26ab</td>
<td>0.17a</td>
<td>0.17b</td>
</tr>
</tbody>
</table>

a = significant with control group (P < 0.05). b = significant with infected mice (P < 0.05). Superscripts with same letters = no significant (P > 0.05).

**Table 3: Red blood cells count in treated mice compared with infected & uninfected ones.**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8.84</td>
<td>4.10</td>
<td>4.9</td>
<td>7.13</td>
<td>9.60</td>
</tr>
<tr>
<td>SE±</td>
<td>0.51b</td>
<td>0.22a</td>
<td>0.08ab</td>
<td>0.15a</td>
<td>0.12b</td>
</tr>
</tbody>
</table>

**Table 4: Hemoglobin concentration in treated mice compared with infected & uninfected ones.**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>13.76</td>
<td>7.85</td>
<td>10.40</td>
<td>11.93</td>
<td>13.20</td>
</tr>
<tr>
<td>SE±</td>
<td>0.20b</td>
<td>0.20b</td>
<td>0.19b</td>
<td>0.12b</td>
<td>0.18b</td>
</tr>
</tbody>
</table>

**Table 5: Hematocrit percentage in treated mice compared with infected & uninfected ones.**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>36.21</td>
<td>28.50</td>
<td>30.10</td>
<td>32.41</td>
<td>35.35</td>
</tr>
<tr>
<td>SE±</td>
<td>0.22a</td>
<td>0.41a</td>
<td>0.10b</td>
<td>0.19b</td>
<td>0.43b</td>
</tr>
</tbody>
</table>

**Table 6: Platelets count in treated mice compared with infected & uninfected ones.**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>466.00</td>
<td>261.00</td>
<td>349.00</td>
<td>406.00</td>
<td>443.00</td>
</tr>
<tr>
<td>SE±</td>
<td>4.79a</td>
<td>6.85a</td>
<td>2.76b</td>
<td>3.18b</td>
<td>5.86b</td>
</tr>
</tbody>
</table>

**Table 7: ALT concentration in sera of treated mice compared with infected & uninfected ones.**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>25.00</td>
<td>83.00</td>
<td>54.00</td>
<td>35.00</td>
<td>28.00</td>
</tr>
<tr>
<td>SE±</td>
<td>0.40b</td>
<td>4.60a</td>
<td>0.70b</td>
<td>2.20b</td>
<td>2.00b</td>
</tr>
</tbody>
</table>

**Table 8: AST in sera of treated mice compared with infected & uninfected ones.**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>52.00</td>
<td>176.00</td>
<td>107.00</td>
<td>58.00</td>
<td>55.00</td>
</tr>
<tr>
<td>SE±</td>
<td>0.60b</td>
<td>9.26a</td>
<td>1.71b</td>
<td>3.92b</td>
<td>0.68b</td>
</tr>
</tbody>
</table>

**Table 9: Total protein in sera of treated mice compared with infected & uninfected ones.**

<table>
<thead>
<tr>
<th>Variant</th>
<th>CN</th>
<th>CI</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>3.60</td>
<td>7.70</td>
<td>5.90</td>
<td>5.60</td>
<td>4.10</td>
</tr>
<tr>
<td>SE±</td>
<td>0.25a</td>
<td>0.28a</td>
<td>0.10b</td>
<td>0.11b</td>
<td>0.05b</td>
</tr>
</tbody>
</table>

**Discussion**

Generally, *Nigella sativa*: (N. sativa, family Ranunculaceae) or black seed is rising as an astounding herb with a wealthy historical and religious culture with a wide spectrum of pharmacological potentialities (Ali and Blunden, 2003). Its crude extracts and essen-
tial oil have many pharmacological properties as anti-oxidant (Burits and Bucar, 2000), anti-diabetic (Rchid et al, 2004), anti-inflammatory (Salem, 2005), anti-tumor (Ivankovic et al, 2006), anti-fungus (Inouye et al, 2006), anti-parasitic (El Wakil, 2007), antibacterial (Abu-Al-Basal, 2009), and protective against nephrotoxicity (Shokri, 2016).

The turmeric is a spice groomed from rhizome of Curcuma longa plant used in diet, cosmetically as skin facemask, healing wound, to treat liver disorders, diabetes and inflammatory circumstances such as rheumatism, sinusitis and arthritis (Joe et al, 2004). Its polyphenol architecture with the two ferulic acids associated with a methylene bridge at the C- atoms of carboxyl groups, double bonds in alkene part of the molecule, hydroxyl groups of benzene ring and central b-diketone grant it to hunt nitric oxide radical (Bansal et al, 2005). Pentostam® is well accepted for treating all forms of leishmaniasis several years ago (Sebai et al, 1975).

In the present study, there was a significant increase (P < 0.05) in WBCs count in infected mice as compared with normal value in control uninfected untreated ones. Curcumin, Pentostam and Black seed oil treated mice gave a significant WBCs count decrease (P < 0.05) in mice compared with infected untreated mice. This agreed with Bansal et al. (2005) who found that mice controlled infection with WBCs response increased to allergic reaction and parasite.

In the present study, there was significant decrease (P < 0.05) in RBCs count in infected mice as compared with normal value in control uninfected untreated ones. Pentostam, Black seed oil treated mice gave a significant RBCs count increase (P < 0.05) in mice compared with infected untreated ones. In Curcumin treated mice there was a significant RBCs decrease in mice as compared to uninfected untreated mice. In Curcumin treated mice, there was a significant RBCs decrease in mice as compared to uninfected untreated mice. The RBCs value indicated mild to severe anemia due to decreased erythropoiesis or increased hemolysis. This agreed with Zulmira and Batista (2008) who found that anemia severity in L. donovani depend on bone marrow affected degree, with complex normocytic hypochromic, which decrease due to their damage by the parasite (de Abreu et al, 2011), or from bone marrow dysfunction (Momo et al, 2014).

In the present study, there was a decrease (P < 0.05) in hematocrit percent in infected mice as compared with the normal value in control uninfected untreated mice. In contrast, in Pentostam, and Black seed oil treated mice, there was a significant Hemoglobin (Hb) increase (P < 0.05) in mice compared with the infected untreated mice. In Curcumin treated mice, there was a significant Hb decrease in mice compared with uninfected untreated mice. The hematocrit (HCT) is a marker of organism hydration degree and decreases due to the hemodilution, caused by decreased erythropoiesis and/or increased hemolysis or polydipsia, caused by the high blood urea level (Nicolato et al, 2013).

In the present study, there was a significant Hb conc. decrease (P < 0.05) in infected mice compared with control uninfected untreated ones. But, in Pentostam, and Black seed oil treated mice, there was a significant Hb increase (P < 0.05) in mice compared with infected untreated mice. In Curcumin treated mice, there was a significant Hb decrease in mice compared to uninfected untreated mice. Decrease in Hb was attributed to bone marrow dysfunction, high parasite load, or decreased in secreted erythropoietin due to the chronic renal failure (Nicolato et al, 2013).

In the present study, there was a significant platelets count decrease (P < 0.05) in infected mice compared with normal control uninfected untreated ones. In contrast, in Pentostam and Black seed oil treated mice, there was a significant platelets count increase (P < 0.05) in mice compared with infected untreated mice. In Curcumin treated mice, there was a significant platelets count decrease in mice compared to uninfected untreated mice. The thrombocytopenia is a common hematological finding in VL mice (Solano-Gallego et al, 2009), and the platelet seques-
tration or consumption during clotting process decreased or impaired production of megacaryocytes and platelets as immunemediated process (Uchar et al, 2015).

No doubt, ALT& AST predominate in liver, lesser quantities in kidneys, heart and skeletal muscle. So, liver has a role in metabolic conversion and enzymes elimination (Jonker et al, 2009), and ALT & AST determine liver function (Jennifer, 2017).

In the present study, L. donovani infected mice showed increase in ALT (IU/L) &AST (IU/L) compared with control and treated ones. This agreed with Agarwal et al. (2006) and Rahim and Ashkan (2007), who reported AST increased to 441 (IU/L) and ALT to 221 (IU/L). But, moderately elevated AST & ALT were reported in human visceral leishmaniasis (Yazici et al, 2008). Generally, visceral leishmaniasis showed increase of liver enzymes with different rates. The fall of AST and ALT levels as key liver enzymes correlated to treatment with an anti-parasitic immune response. ALT & AST increase due to parasitic effect on liver caused hepatomegaly with affected function tests (Bhattacharyya, and Hati, 2004).

In the present study, there was a significant increase (P < 0.05) in total protein concentration in infected mice as compared with the normal value in control uninfected untreated ones. In Pentostam, and Black seed oil treated mice, there was a significant decrease (P < 0.05) in total protein concentration in serum of mice compared with infected untreated mice. In Curcumin treated mice, there was a significant increase in total protein concentration in serum as compared to uninfected mice, which agreed with Solano-Gallego et al. (2001).

**Conclusion**

The outcome of experimentally L. donovani infected mice showed that the treatment changed the biochemical frame and hematological status of them, more or less returned to the normal levels. The best result was achieved with Black seed oil treated mice, followed by Pentostam® and the least effective one was Curcumin.

**References**


Bhattacharyya, J, Hati, A, 2004: Leishmaniasis, IDRC 21:4-9


Explanation of figures

Fig. 1: Giemsa stained *L. donovani* amastigotes in blood of infected mice (x1000).
Fig. 2: Mean levels of White blood cells count in blood of treated groups.
Fig. 3: Mean levels of Red blood cells count in blood of treated groups.
Fig. 4: Mean levels of Hemoglobin concentration in treated groups.
Fig. 5: Mean levels of Hematocrit percentage in treated groups.
Fig. 6: Mean levels of Platelets count in treated groups.
Fig. 7: Mean levels of ALT concentration in serum of treated groups.
Fig. 8: Mean levels of AST concentration in serum of treated groups.
Fig. 9: Mean levels of total protein concentration in serum of treated groups.