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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 extracted (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 anthelminthic 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-Fakhry *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 breed 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 $1x10^6$ 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.

Procedure:	Blank	Standard	Sample
Distilled water	20 µL	……	…….
Protein standard(S)	…….	20 µL	…….
Sample	……	……	20 µL
Reagent(A)	1.0 mL	1.0 mL	1.0 mL

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:

$$\frac{A\ \text{sample}}{A\ \text{standard}} \times C\ \text{standard} = C\ \text{sample}$$

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. Asays 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 10min from OD at 5min (ΔODS). Similarly, calculate the (ΔODNADH) rate for NADH standard (OD5min- OD10 min).

Determine ALT activity using the following equation:

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

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

$$\text{Factor} = 10\ \text{mM NADH} \times \frac{4\ \mu L\ Vol._{NADH}}{210\ \mu L\ Vol._{WR}} \times \frac{200\ \mu L\ Vol._{WR}}{220\ \mu L\ Vol._{Total}} \times \frac{11\ (\text{sample dilution})}{5\ \text{min}}$$

$$= 381\ \mu M/\text{min}$$

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#EASTR-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 rea- gents, 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 subtrac-ting 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:

$$\text{Factor} = 10\ \text{mM NADH} \times \frac{4\ \mu L\ Vol._{NADH}}{206\ \mu L\ Vol._{WR}} \times \frac{200\ \mu L\ Vol._{WR}}{220\ \mu L\ Vol._{Total}} \times \frac{11\ (\text{sample dilution})}{5\ \text{min}}$$

$$= 388\ \mu M/\text{min}$$

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

Collected materials	Control Negative	Control Infected	Experimental		
			T1	T2	T3
A week before infection (analysis)	- ve	- ve	- ve	- ve	- ve
7 days (PI)	- ve	+ ve	+ ve	+ ve	+ ve
15 days (PI)	- ve	+ ve	+ ve	+ ve	+ ve
30 days (PI)	- ve	+ ve	- ve	- ve	- ve
43 days (PI)	- ve	+ ve	- ve	- ve	- ve

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

Variant	CN	CI	T1	T2	T3
Mean	4.24	15.73	9.67	6.52	5.13
SE±	0.23^{b}	0.79^{a}	$0.26^{a,b}$	0.17^{b}	0.17^{b}

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.

Variant	CN	CI	T1	T2	T3
Mean	8.84	4.10	4.9	7.13	9.60
SE±	0.51^{b}	0.22^{a}	$0.08^{a,b}$	0.15^{b}	0.12^{b}

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

Variant	CN	CI	T1	T2	T3
Mean	13.76	7.85	10.40	11.93	13.20
SE±	0.20^{b}	0.20^{a}	0.19^{b}	0.12^{b}	0.18^{b}

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

Variant	CN	CI	T1	T2	T3
Mean	36.21	28.50	30.10	32.41	35.35
SE±	0.22^{b}	0.41^{a}	0.10^{b}	0.19^{b}	0.43^{b}

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

Variant	CN	CI	T1	T2	T3
Mean	466.00	261.00	349.00	406.00	443.00
SE±	4.79^{b}	6.85^{a}	2.76^{b}	3.18^{b}	5.86^{b}

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

Variant	CN	CI	T1	T2	T3
Mean	25.00	83.00	54.00	35.00	28.00
SE±	0.40^{b}	4.60^{a}	0.70^{b}	2.20^{b}	2.00^{b}

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

Variant	CN	CI	T1	T2	T3
Mean	52.00	176.00	107.00	58.00	55.00
SE±	0.60^{b}	9.26^{a}	1.71^{b}	3.92^{b}	0.68^{b}

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

Variant	CN	CI	T1	T2	T3
Mean	3.60	7.70	5.90	5.60	4.10
SE±	0.25^{b}	0.28^{a}	0.10^{b}	0.11^{b}	0.05^{b}

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 *c*rude 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 bdiketone 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 mice. Pentostam, Black seed oil treated mice gave a signi ficant RBCS increase ($P < 0.05$) in mice compared with infected 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 mice. 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 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 decrea sed or impaired production of megacarioc- ytes and platelets as immunomediated process (UIchar *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 antiparasitic 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.

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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 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.