

# Adjunct Effect of Immuno stimulating Tre Haloes Di Mycolate and Pentostam against Experimental Visceral Leishmaniasis

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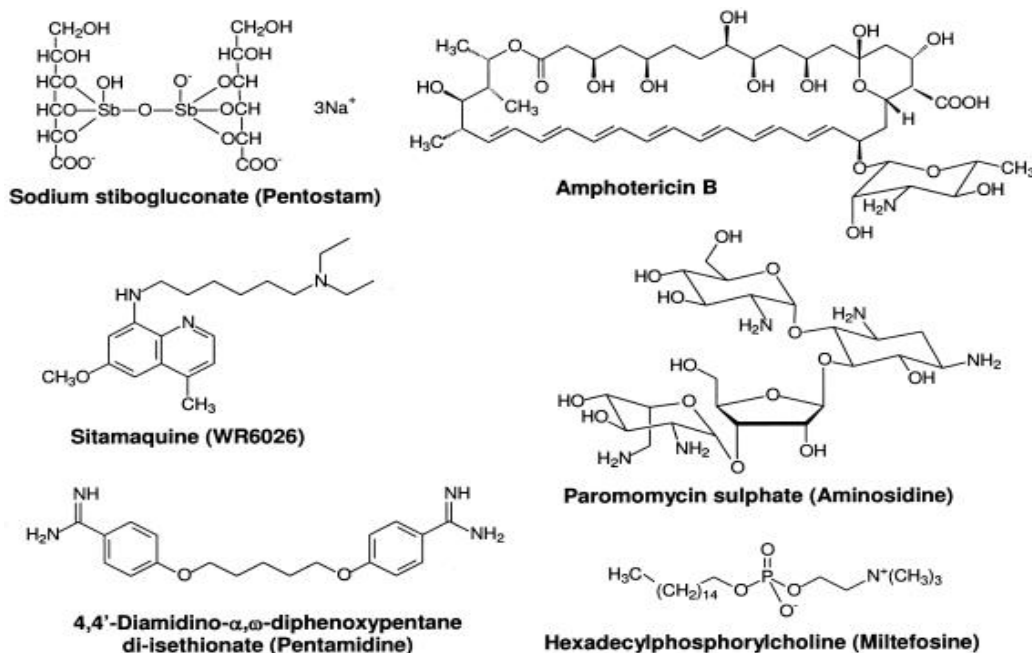
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**ABSTRACT:** Visceral leishmaniasis (VL) is caused by the intracellular protozoan *Leishmania donovani* complex. VL may be asymptomatic or progressive and is characterized by fever, anemia, weight loss and the enlargement of the spleen and liver. The hematological and Biochemical status with VL is a major determinant of the progression, severity and mortality of the disease, as it affects the clinical progression of the disease. Changes in liver enzymes concentration and hematological parameters have major impacts in the host during infection. Our goal in this study was to evaluate the hematological and biochemical alteration in experimental visceral leishmaniasis in golden hamsters in the infected group, treated with high dosage of pentostam alone and with the combined TDM and low dosage of pentostam group. We report the alteration in hematological parameters, liver enzymes, cholesterol and lipid peroxidase which occurred due to experimental leishmaniasis, these altered status was significantly normalized with the treatment with the combinatorial immune-chemotherapeutic approach without any side effects.

**KEYWORDS:** visceral leishmaniasis, pentostam, tre haloes di mycolate, *Leishmania donovani*, immunochemotherapy

## I INTRODUCTION

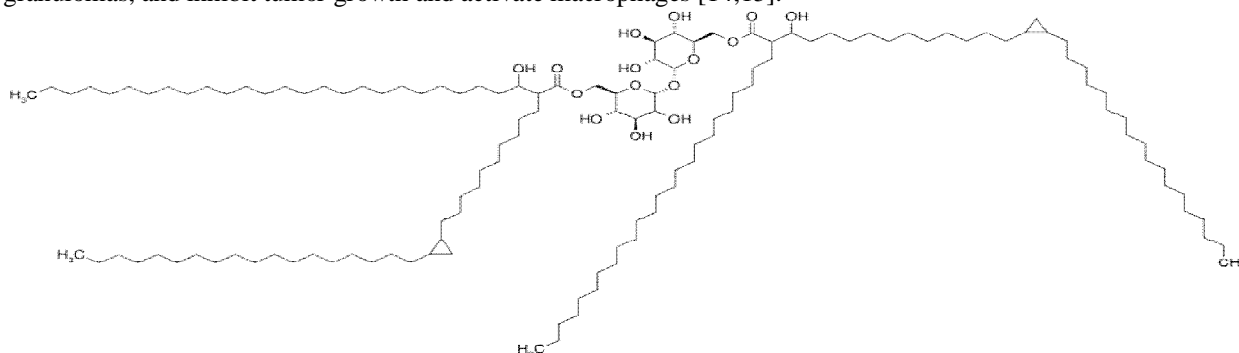
The visceral form of leishmaniasis, commonly known as kala azar, is caused by the parasite *Leishmania donovani*. Approximately 350 million people in 8 countries are estimated to be threatened by the disease. The World Health Organization estimated in 2000 that there were 12 million cases of all forms of leishmaniasis worldwide, with over 500,000 new cases of visceral disease occurring each year [1]. In the disease, the multiplication of the parasite in the reticulo-endothelial system causes prolonged fever, anaemia, hepatosplenomegaly and weight loss. VL is fatal if it is not adequately treated [2]. Despite the tremendous progress that has been made in understanding the biochemistry and molecular biology of *Leishmania* species, chemotherapeutic treatment for visceral leishmaniasis has seen limited progress in recent years. The toxic pentavalent antimonials remain the mainstay of treatment for leishmaniasis. The second-line drugs, pentamidine and amphotericin B, although used clinically, have serious toxic side effects [3]. Miltefosine, an orally active phosphocholine analogue, also appeared to be effective in the treatment of visceral leishmaniasis [4, 5]. commonly use drugs are depicted in Figure(1), despite these advances, improved drug therapy for visceral leishmaniasis still remains desirable. The drugs currently used to treat VL can have severe side effects and the clinical presentation of VL is not sufficiently specific to guide treatment. Highly accurate (both sensitive and specific), cheap and simple rapid diagnostic tests (RDTs) are therefore crucial for case-management of VL. Early case detection followed by adequate treatment is also central to control of VL because, as yet, no vaccine is available and the long-term impact of vector control is unclear [6,7].



**Figure: 1** Drugs currently used in the treatment of *Leishmaniasis*

For drug treatment to be successful, the cooperation of the immune mechanism of the host is often required [8]. Macrophages, the primary target of intracellular *Leishmania* infection, may take on distinct phenotypes in response to parasite signals and inflammatory stimuli within the infected microenvironment. Classically activated macrophages respond to IFN- $\gamma$  and microbial products by generating antimicrobial molecules that effectively kill *Leishmania* and other intracellular pathogens [9, 10]. Central to the killing of intracellular parasites is the production of nitric oxide by the action of inducible nitric oxide synthase 2 (NOS2) on the substrate L-arginine.

One of the strategies is to use a natural immunomodulator that can activate the macrophages so that combinatorial use of low dosage of drugs which is not effective alone can be used effectively to treat the visceral leishmaniasis without any side effects. Cord factor, or trehalose dimycolate, is a glycolipid molecule found in the cell wall of *Mycobacterium tuberculosis* and similar species (Figure 2). It is the primary lipid found on the exterior of *M. tuberculosis* cells [11,12], Cord factor influences the arrangement of *M. tuberculosis* cells into long and slender formations, giving its name [13]. TDM has been observed to influence immune responses, induce the formation of granulomas, and inhibit tumor growth and activate macrophages [14,15].



**Figure2:** Structure of Trehalose dimycolate (TDM)

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In this paper a combined immunochemotherapeutic approach is used to treat the visceral leishmaniasis by using trehalose di mycolate and low dosage of pentostam in experimental leishmaniasis in golden hamsters. Our aim in this studies to evaluate the efficacy of combinatorial immunochemotherapy of TDM and low dosage of pentostam and compared with the standard high dosage treatment which is having toxic effects when use alone. Hematological and biochemical status of hamsters in experimental leishmaniasis was studied to evaluate the efficacy combinatorial immune chemo therapeutic approach.

## II RELATED WORK

VL is a systemic infection of the reticuloendothelial system caused by protozoa *Leishmania donovani* (LD) of the genus *Leishmania*. Genus *Leishmania* was created by Ross in 1903. Sir William Leishman discovered the parasite in spleen smears simultaneously with Charles Donovan identifying the same parasite in spleen biopsy [16]. It is observed that the spleen size and duration of illness correlate with some of the main hematological features [17]. Normochromic normocytic anemia is a frequent and clinically significant feature of VL and reported low level of hemoglobin. [18]. Hypersplenism is another primary pathogenetic mechanism [19]. Other mechanisms suggested include increased sensitivity to complement [20], inhibition of erythrocyte and liver enzymes [21-23].

## III MATERIAL AND METHODS

### Laboratory culture

*Leishmania donovani* parasites (NICD-CII strain) were cultured on brain heart infusion agar with inactivated rabbit blood (BHMARB). Dehydrated Brain heart infusion powder and bacto-agar from Difco laboratories; Detroit, Michigan, USA were used for culturing the parasites. Hanks balanced salt solution (HBSS) was prepared according to the prescribed procedure (Figure 3).

### Animals

Healthy male golden hamsters (*Mesocricetus auratus*) each weighing around 60-100 grams were purchased from the animal house facility of the central drug research institute (CDRI), Lucknow, India. Hamsters were routinely used for in vivo maintenance of *Leishmania* parasites and for experimental purposes.

### Immunoadjuvants

An aqueous suspension of trehalose di mycolate (TDM), 1mg/ml prepared from the mycobacterium bovis strain AN% was supplied by the kind courtesy of late Prof. Edgar Lededer, laboratories de Biochimie, Central national de la Recherche Scientifique, 9119 Gif-sur-Yvette France.

### Drug

Sodium stibogluconate (pentostam) equivalent to 0.3 mg/Sbm<sup>-1</sup> was obtained from the welcome foundation, U.K.

### In vitro cultivation of parasites

The promastigotes of *L. donovani* (NICD:CII strain) were maintained on a biphasic medium containing brain heart infusion agar medium with rabbit blood and HBSS as overlay according to the method of Jalees et al [24].

### Animal and Infection

The axenic culture of *L. donovani* was maintained on BHMARB medium containing HBSS as an overlay. The primary inoculum was subculture after 5 days. Promastigotes were harvested at 4°C after successive washing in sterile normal saline containing gentamycin. Hamsters were infected peritoneal using an inoculum of about 1x10<sup>7</sup> promastigotes/0.1 ml per hamster. Two weeks after the inoculation animals were split into six groups. Each group contains 5 animals.

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### Drug and TDM Treatment

Sodium stibogluconate (pentostam) equivalent to 0.3 mg/sb mg<sup>-1</sup> was obtained from welcome foundation, UK on post inoculation day 30, the animals were given pentostam at a dose of 10 mg and 20 mg /kg of body weight for 5 days. An aqueous suspension of TDM (1mg/ml) was taken for inoculation.

### Haematological Studies

Total leukocyte count and Differential count were made from normal, infected and treated animals according to the method described by Dacie and Lewis[25].The hemoglobin was estimated according to th Sahli's method.The gram haemoglobin per 100 ml of blood was converted according to the following formula

Haemoglobin factor = gm% aemoglobin in blood/ average scale of reading

### Biochemical Studies:

#### Tissue collection

Infected and treated animals were sacrificed under light ether anesthesia and the liver tissue were excised and processed according to the experimental design

### Wet and Dry weight of Liver

#### i.Wet weight

The whole liver was taken out from the normal and infected animals at high parasitemia a blotted dry on filter paper and weight quickly.

#### ii.Dry Weight

The liver tissues from the above animals were dried at 50°C for 24 hours. They were dried and weighed.

### Estimation of Cholesterol and Lipid peroxidation

The cholesterol was calculated by modified Bloor et. al method [26]. 0.5ml of liver extract taken from each group i.e. infected ,treated with low dosage alone ,combined TDM and low dosage of pentostam and finally the infected group of hamsters treated with high dosage alone .The chloroform 4.5 ml was added to each extract and coloring agent consisting of anhydrous and sulfuric acid was added and mixed well. The tubes were kept in dark for 30 minutes at 25°C.The absorbance was read at 660nm against a reagent blank.The amount of malonaldehyde formed after 30 minutes of lipid peroxidation was estimated according to the method described by Utley in all experimental groups of animals [27] .

### Enzymatic Assays in Liver tissues

Liver tissues of normal, infected and treated hamsters were excised and washed in cold KCl 150mM and homogenized in the same medium to give a 10% (w/v) homogenate. The suspension was centrifuged at 15,000xg for 20 minutes and the supernatant was transferred to a clean tube. Freezing and thawing of the supernatant was done a number of times to release acid and alkaline phosphatase before assays.

### Estimation of Liver Transaminases

Aliquots from suitably diluted homogenate were taken from each experimental group separately for glutamic pyruvate transaminases (GPT) and glutamic oxaloacetic transaminase (GOT) determinants. It was calculated according to the method of Reitman and Frankel using sodium pyruvate as a standard[28].

### Estimation of Liver Phosphatases

Acid phosphatase activity was determined according to a slightly modified method of Nelson[29]. A typical reaction mixture in a final volume of 2.0 ml contained 0.5ml homogenate,6.0 moles of ethylene di amine tetra acetic acid,75 micro moles of p-nitrophenyl phosphate(disodium salt)and 150 micro moles of acetate buffer(pH 5.0).

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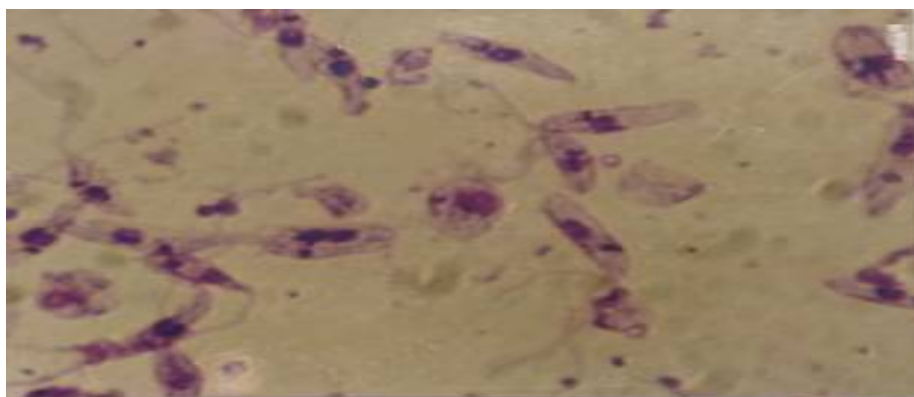
The enzyme concentration of alkaline phosphatase in liver was measured according to the method of Morton .For estimating the concentration of alkaline phosphatase,the reaction mixture in a total volume of 2.0 ml contained 75 moles of p-nitrophenyl (disodium salt),3 micro moles of  $MgCl$ ,3.0 micro moles of  $MgCl_2$  ,150 moles of bicarbonate buffer (pH9.5) and 0.5 ml of a suitably diluted enzyme.The test tubes containing the reaction mixtures were incubated for 30 minutes at 37°C with occasional shaking. The reaction was stopped by the addition of 2.0 ml on NaOH (0.1N).The enzyme in control tubes was supplemented after stopping the reactions. One unit of enzymes was taken as that amount which liberated 1N moles of p-nitrophenyl/minute

### Estimation of serum transaminases

Serum glutamic pyruvictransamine(SGPT) and serum oxaloacetic transaminase (SGOT) in normal and infected serum were estimated according to the procedure of Reitman and Frankel[28].Using sodium pyruvate as a standard. The level of enzyme activity was calculated in terms of micromoles of pyruvate formed per minute per ml of serum.

### Statistical analysis

The data were analyzed by using student't' test. The significant differences between means of experimental and control groups were calculated and 'p' values less than 0.01 were considered as significant.



**Figure 3: Photomicrograph showing leishman stained *L.donovani* promastigotes from a 6 day old culture in BHAMRB medium(X1000)**

*L. donovani* was cultured in Brain Heat Infusion Agar Medium with rabbit blood.Hank's Balanced salt solution was used as an overlay. The cultures were seeded with  $1.7 \times 10^6$  parasites/ml.The parasitic count were carried out after 24 hours ,maximum count was found at 144hours.

## IV. RESULTS AND DISCUSSION

Visceral leishmaniasis (VL), caused by the intracellular protozoan *Leishmania donovani* or *L. infantum*, is one of the "Neglected Tropical Diseases" that impacts the poor of the world. Active VL is characterized by a relentlessly progressive infection with cachexia, massive splenomegaly, pancytopenia and ultimately death. VL ranks second to malaria in deaths caused by a protozoal pathogen; mortality is reported in up to 10–20% of patients, even with treatment [30]. Till date no effective nontoxic therapy for *L donovani* infection is available. Common side effects during the treatment are vomiting, giddiness, jaundice; hematuria, pneumonia and cardiograph abnormalities and anaphylactic shock which may further complicate the treatment [31]. Furthermore, the above drugs often do not cause complete cure. For drug treatment to be successful, the cooperation of the immune mechanism of the host is often required. In the case of visceral leishmaniasis, the T cell mediated arm of the immune response is of paramount importance in containing and eventually eliminating the parasites from infected macrophages.Our study provided the

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evidence that a combined treatment of lower concentration of drug dose and adjuvant tre –halose-di mycolate (TDM) is equally or more effective than the currently employed standard procedures [32].

TDM is a potent immunomodulator that increases nonspecific resistance to infectious agents and limits tumor growth [33-35]. Direct or indirect attraction of macrophages by TDM to the site of inoculation may be one component of its mechanism of action. Other TDM action would be the priming of newly arrived macrophages at the site of inoculation[36]. In our study the total leukocyte count in normal animals were found to be 4797.6. This count was increased in infected animals (7408.8) and as well as in animals treated with Low dosage of drug . When the animals were treated with a combination of lower drug doses and TDM the count was 5076.6 (Table 1). The changes in the differential count was very significant. The mean polymorph count increased from 32.4 to 60.2 in the infected group and in the treated animals (lower drug dosage alone) the count was 58.8. This count was decreased in the animal group treated with the combination of lower drug and TDM and comparable to those obtained by using higher doses of drug. (Table 2). This hematological change may be due high parasitic infection [37].

The cholesterol level was low and high level of lipid peroxidase infected group that normalized in higher dosage and combined immunotherapy group are in the agreement of previous work of Lal et al [38]. The result showed an important role of free radicals in etiopathogenesis of visceral leishmaniasis which is due to increased production of reactive oxygen species (ROS) as host defense mechanism against invaded parasites [39]. This indicates that hamsters of this group are in oxidative stress which is supported by their decreased level of hemoglobin (Table 3).

**Table 1: Total leukocyte count in Normal, Infected and Treated Animal**

Animal Groups	Mean TLC/mm <sup>+</sup> - SD	Mean Haemoglobin concentration (gm/100ml)
Normal	4796.6 <sub>±</sub> 6.21	17.89 <sub>±</sub> 0.74
Normal +TDM	5054.2 <sub>±</sub> 1.75	16.78 <sub>±</sub> 1.91
Infected	7408.8 <sub>±</sub> 2.00	9.45 <sub>±</sub> 0.36
Infected +TDM	6513.8 <sub>±</sub> 1.86	10.02 <sub>±</sub> 0.25
Infected+Drug(10 mg)	6357 <sub>±</sub> 1.70	10.02 <sub>±</sub> 3.06
Infected + drug(10mg)+TDM	5076.6 <sub>±</sub> 1.60	15.14 <sub>±</sub> 0.50
Infected+Drug(20 mg)	5300 <sub>±</sub> 1.50	15.9 <sub>±</sub> 0.24

\*Each group contained 5 hamsters

\*\*Arithmetic mean of 5 hamsters

Total Leucocyte were found more in numbers in the infected group . There was not effect by using low concentration of drug (pentostam) where as a remarkable difference appeared when combined therapy was used and the result was comparable with the high dosages of drug which has serious toxic side effects . There was marked depletion in the hemoglobin content indite the anemic condition that normalized when combined treatment was done.

**Table 2: Differential Leukocyte Count in Normal, Infected and Treated Animals**

Animal Group	Mean** DLC percent <sub>±</sub> SD			
	P	L	M	E
Normal	32.4 <sub>±</sub> 2.07	63.0 <sub>±</sub> 2.12	2.4 <sub>±</sub> 0.55	1.4 <sub>±</sub> 0.55
Normal+TDM	31.2 <sub>±</sub> 2.59	63.0 <sub>±</sub> 1.87	2.0 <sub>±</sub> 0.71	1.4 <sub>±</sub> 0.55
Infected	60.2 <sub>±</sub> 1.48	75.6 <sub>±</sub> 2.19	3.6 <sub>±</sub> 0.89	4.2 <sub>±</sub> 1.73
Infected +TDM	56.8 <sub>±</sub> 2.38	62.4 <sub>±</sub> 2.79	2.8 <sub>±</sub> 0.84	4.0 <sub>±</sub> 0.45
Infected+Drug(10mg)	58.8 <sub>±</sub> 1.48	72.4 <sub>±</sub> 1.14	3.0 <sub>±</sub> 0.89	3.8 <sub>±</sub> 1.3
Infected+Drug(10mg)+TDM	33.5 <sub>±</sub> 3.53	65.0 <sub>±</sub> 3.83	2.4 <sub>±</sub> 0.55	1.5 <sub>±</sub> 0.55
Infected+Drug(20mg)	57. <sub>±</sub> 1.92	6.4 <sub>±</sub> 3.78	3.0 <sub>±</sub> 0.71	1.6 <sub>±</sub> 0.89

\*Each group contained 5 hamsters

\*\*Arithmetic mean of 5 hamsters

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P=Polymorph,L=lymphocyte,M=Monocyte,E=Eosinophil

Polymorph increased in infected group as compared to the normal. There was slight variation in lymphocytes and monocytes in infected group but marked increase found in eosinophil that become normal when combined immunotherapy was given.

The alteration in hematological parameters showed the incidence of parasitic infection and effective treatment of immunotherapeutic approach with TDM and low dosage of pentostam.

**Table 3: Changes in Cholesterol and Lipid peroxidation contents in the Liver of Normal, Infected and Treated Animals**

Animal Groups	Biochemical Assays			
	Cholesterol	+ SD 'p' value	Lipid peroxidation	'p' value
Normal	121.64+-1.66	-	172.35+-3.50	-
Normal +TDM	120.42+-1.131	-	168.59+-3.50	-
Infected	67.86+-4.32	p<0.01	746.64+-48.13	p<0.001
Infected +TDM	69.82+-2.75	P<0.10	789.93+-42.08	p<0.10
Infected+Drug(10mg)	80.29+-216	P<0.05	634.2+-47.75	p<0.05
Infected +Drug(10mg+TDM)	111.66+-2.1	P<0.001	186.15+-3.60	p<0.001
Infected +Drug (20 mg)	91.21+-2.04	P<0.01	188.45+-2.25	p<0.001

\*Each value is the Arithmetic mean of 5 values +-SD

\*All values of p<0.01 are significant

\*Significance was calculated by student 't' test

The cholesterol level was decreased in the infected group and low dosage treatment alone was not effected significantly but in immunotherapeutic group treatment results in normalizing the cholesterol level. Lipid peroxidation was higher in infected groups of animals and values obtained after immune-chemotherapeutic group and high dosage treatment were comparable

**Table 4: Changes in Total Carbohydrate, Glycogen contents of Liver of normal, infected and Treated Animals**

Animal Groups	Biochemical Assays			
	Total Carbohydrates (mg/g liver) +- SD	'p' value	Glycogen (mg/g liver) +- SD	'p' value
Normal	76.70+_0.96	-	51.45+_0.26	-
Normal+TDM	75.10+_0.96	-	51.51+_0.26	-
Infected	25.35+_0.44	p<0.1	23.28+_0.37	p<0.001
Infected +TDM	25.89+_0.89	p<0.001	25.32+_0.15	p<0.1
Infected +Drug(10 mg)	27.77+_1.95	p<0.01	23.29+_0.20	p<0.05
Infected +Drug(10mg)+TDM	75.35+_1.66	p<0.001	51.13+_0.17	p<0.001
Infected + Drug(20mg)	75.19+_1.0	p<0.001	50.52+_0.44	p<0.01

\*Each value is the Arithmetic mean of 5 values +-SD

\*All values of p<0.01 are significant

\*Significance was calculated by student 't' test

Total carbohydrates and glycogens were found to be depleted in the in treatment infected group and significantly recovered after the combined therapy of TDM and low dosages of pentostam and comparable with the high dosages of pentostam alone treatment that has toxic side effects

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**Table5: Changes in Glutamic Pyruvate Transaminase and Glutamic transaminase, Acid phosphatase and Alkaline Phosphatase in Normal, Infected and Treated Hamsters**

Animal Group	Biochemical Assays							
	Liver glutamic pyruvate Transaminase (unit/mg/tissue protein) + SD	'p' value	Liver Oxaloacetic Transaminase (unit/mg/tissue protein)+_SD	'p' value	Liver Alkaline phosphatase (unit/mg/tissue protein)+_SD	'p' value	Liver Acid phosphatase (unit/mg/tissue protein)+_SD	'p' value
Normal	170.19+_4.45	-	45.77+_1.18	-	0.064+_0.007	-	0.16+_0.003	-
Normal +TDM	169.12+_4.10	-	42.35+_1.10	-	0.052+_0.006	-	0.14+_0.005	-
Infected	118.16+_4.92	p<0.001	21.98+_0.80	p<0.001	0.104+_0.01	-	0.496+_0.32	p<0.001
Infected + TDM	117.07+_3.53	p<0.001	21.8+_0.75	p<0.001	0.107+_0.01	-	0.40+_0.06	-
Infected+Drug(10 mg)	133.87+_3.09	p<0.05	27.21+_1.17	p<0.001	0.089+_0.004	p<0.05	0.72+_0.08	p<0.05
Infected+Drug(10 mg)+TDM	168.02+_5.75	p<0.001	44.86+_1.48	p<0.001	0.061+_0.008	p<0.01	0.15+_0.012	p<0.01
Infected+Drug(20 mg)	166.94+_3.64	p<0.001	43.78+_1.87	p<0.001	0.007+_0.007	p<0.001	0.14+_0.01	p<0.001

\*Each value is the Arithmetic mean of 5 values +\_SD

\*All values of Pp<0.01 are significant

\*Significance was calculated by student't'test

Liver glutamic pyruvate transaminases and Liver oxaloacetic transaminases showed marked depletion in the specific activity in the infected group whereas marked increased in the liver alkaline phosphatase and acid phosphatase was observed. The enzymes altered levels were normalized after the treatment with combined therapy of TDM and the low drug dosages

There was significant decrease in the total carbohydrate and glycogen in the infected group from 76.7mg/gram of liver to 25.35 mg/gram of liver and in the treated with TDM and low dosage of drug was 75.35mg/gram of liver for total carbohydrates (Table 4) .The decrease in the total carbohydrates and glycogen in the L. donovani infection may be due to the fact that amastigotes residing in macrophages drain glycogen from the hepatic cells or deriving energy. It has been observed that liver GPT and GOT were decreased significantly and are in agreement with the earlier observation made by Lal and Hussain[40].In the SGPT and SGOT levels in the serum of infected hamsters, SGPT and SGOT levels are increased during leishmaniasis. Similar findings were also reported by Khanna et al in Malarial infection [41].

### V. CONCLUSION

Based on the present study, we conclude that experimental animals in visceral leishmaniasis are affected by oxidative stress, which may contribute to the progression of the disease. These results clearly show that the high level of liver enzymes together with the decrease in hemoglobin and increase in DLC could be a clue for diagnosis of VL besides the clinical symptoms and other laboratory features. This study is the first of its type in which combinatorial approach of



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tre haloes di mycolate as an immnomodulator and low dosage of pentostam is used to treat the visceral leishmaniasis without any toxic side effects.

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