In Vitro Study of The Effect of Some Drugs on *Leishmania Donavani* Replication Rate

دراسة تاثير بعض العقاقير الطبية على معدل نمو طفيلي اللشمانيا الاحشائية خارج الجسم الحي

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Abstract

The effect of some drugs on growth rate of *lishmania donovani* have been estimated. The drugs used have different mechanisms of action, some of them tested on *leishmania donavani* for the first time. The following drugs used like levamisole in a dose (1 mg/ml) , metronidazole(1 mg/ml), bromohexine (0.02 mg/ml), sildenafil (0.5mg/ml) show significant inhibition of *L. donavani* promastigotes proliferation, while paracetamole in a dose of (0.1 mg/ml) enhance the promastigotes growth, meanwhile diclofenac sodium in a dose of (0.5 mg/ml) show little or no effect on growth rate. Also metronidazole and levamisole in a dose of (0.5 mg/ml) has no effect on growth rate. The inhibitory effect of drugs appears from the first day of experiment and fluctuated in second and third days .The effect of paracetamole is continuous throughout the 3 days of experiment. On the other hand, the effect of diclofenac sodium unnoticeable till the third day.

Key words: *leishmania donavani* , levamisole, metronidazole, bromohexine, sildenafil, paracetamole, diclofenac sodium, *in vitro*, replication rates

الخلاصة

تم تقييم تاثير بعض العقاقير على نمو طفيلي اللشمانيا الاحشائية، ان العقاقير المستخدمة تختلف من حيث ميكانيكية التفاعل لاظهار فعاليتها وبعضها يستخدم لاول مرة على الطفيلي وتضم : ليفاميزول بجرعتين (1ملغ / مل) و (0.5 ملغ / مل)، ميترونيدازول (1ملغ / مل)، بروموهيكسين (0.02 ملغ / مل)، سيلدينافيل (0.5 ملغ / مل) وقد لوحظ أن هذه العقاقير قد سببت تثبيطا واضحا وفرقا معنويا في نمو الطفيلي ، بينما اظهر عقار البراسيتامول المستخدم بجرعة (0.1 ملغ / مل) تحفيزا لنمو الطفيلي، بينما لم يظهر عقار ديكلوفيناك صوديوم بجرعة (0.5 ملغ / مل) أي تأثير على نمو الطفيلي (أو تأثيرا طفيفا) وكذلك الميترونيدازول بجرعة (0.5 ملغ / مل) لم تكن مؤثرة. أن العقاقير المثبطة للنمو قد ظهر تأثيرها منذ اليوم الاول للتجربة ومن ثم تذبذب التأثير في اليومين الثاني والثالث. أن تأثير البراسيتامول بقي مستمرا طيلة ايام التجربة الثلاث ومن جهه أخرى بقي تأثير عقار الديكلوفيناك صوديوم غير واضح حتى اليوم الثالث.

Introduction

Leishmaniasis is a disease caused by protozoan parasites that belong to the genus *Leishmania* and is transmitted by the bite of certain species of female sand fly (subfamily phlebotominae)[1]. Most forms of disease are transmissible only from animals, but some can be spread between humans. They are caused by about 20 species of the protozoan *Leishmania*. *Leishmania* has different types of clinical conditions mostly present as cutaneous leishmaniasis, another 2 forms are mucocutaneuos & the most severe fatal form is visceral Leishmaniasis .The treatment of parasitic infections has undergone dramatic changes. The pantavalent antimonials such as glucantime have been the main anti-leishmaniasis drugs for over 60 years but their efficacy is diminishing due to drug resistance [2].

Other antileishmanials such as amphotericin B, paromomycin, pentamidin, and miltefosin are available in cases where antimonials lack efficacy, but their therapeutic windows are limited .thus, the development of a novel & better anti-leishmanials is necessary to meet an urgent medical need on a global scale. Immunotherapy with interferon gamma appears very promising for treatment of visceral Leishmaniasis . In this study we try to use different types of drugs with different mechanisms of action & study their effect on proliferation of *L. donavani* whether enhancing or inhibiting the parasite growth .drugs used are Levamisole which is antihelmenthic drug produce its action by inhancing cell mediated immune response probably by action on macrophage and subsets of T- lymphocyte[3].another drug used in this experiment is Paracetamole which is selective cyclooxygenase -3 inhibitor that is an enzyme abundant in brain and spinal cord ,its inhibition cause relief of pain and fever ,Paracetamole has no anti-inflammatory action [4], [5]. Third drug in our study is Metronidasole which is an antibiotic mainly effective against anaerobic bacteria & is the drug of choice in treatment of Trichomoniasis. It is subsequently proven to be effective for both amebiasis and giardiasis [6], and for cutaneous Leishmaniasis [7].

Bromohexine a derivative of plant adhatoda vasica is a potent bronchodilator and mucolytic ,although it is used for 2000 years in India for treatment of respiratory ailments and abortifacient activities very few recent reports are available for its molecular mechanism of action [8]. It has many other properties as antioxidant , anti-inflammatory , anticestodal and antihelmathic activity [9], [10], [11].

Sildenafil a phosphodiesterase type -5 (PDE5) selective inhibitor that has been successfully deployed as a drug for treatment of male erectile dysfunction and pulmonary hypertention[12]. Diclofenac sodium, the sodium salt of O-(2,6-dichlorophenylamino) –phenylacetic acid (GP45840, voltarin), is a potant inhibitor of prostaglandin synthetase. It is suggested that diclofenac Sodium exerts most of its pharmacological effect via inhibition of prostaglandin synthetase [13].

Aim of study: Find a drug effective against Leishmaniasis so that can be used an alternative in the management of this disease.

Materials and Methods

Equipment and Supply

Six drugs have been used as fellow: levamisole: a syrup 40 mg/5 ml kindly supplied by Kahira Pharm. And Chem. ind. co. Cairo – Egypt. Paracetamole : 500 mg tablet supplied by N.D.I. Iraq. Metronidazole : injection 5 mg/ml J. B. Chemicals & Pharmaceuticals LTD. Ankleshwar – 393 002, India . Bromohexine : tablets 8 mg supplied from SDI. , Iraq. Sildenafil : tablet 100 mg from erica life science ltd. London ,SE6, 4LS, UK. Diclofenac sodium : tablet 50 mg from Troge , Germany. All drugs were dissolved by sterilized distilled water.

Parasite Isolation

A cloned line of L.donovani (MHOM/IQ/2005/MRC10) was obtained from the center of medical research at Al-Nahrayn University in Baghdad; this sample is isolated from the bone marrow of a patient with Kala-azar.

Culture

The promastigotes had been grown on NNN media which is a diphasic media contain; solid phase [14] and Liquid phase which also called lock solution [15], 2 ml of lock solution is added to the solid phase of NNN media.

Growth Studies

Growth rate experiment are conducted by inoculating parasites at a density of 1 x 10^6 cell/ml in NNN media at 24 ◦C after 24 hr. of inoculation. Twenty seven inoculated vials of NNN media has been used for this study with three vials for each concentration of a given drug. When growth is confirmed after 24 hrs of inoculation by counting the base line growth rate, the drugs have been added to groups of Vials each group has three vials and the last group of vials is left as a control media without addition. The growth rate of parasites at different times (within three consecutive days) were counted in a Neubauer hematocytometer under light microscopy by using the following equation:

$$\text{No. of parasites in 1 mm}^3 = \text{No. of parasites in five small squares X 800}$$

Data are analyzed with the analysis of variance test and P values < 0.05 are considered significant.

Results

In the control group, in the second day there is significant difference as compared to the first day, in the third day there is significant difference as compared to the first and to the second days. there is gradual increase in growth rates throughout the three days of experiment. regarding first drug used which is levamisole (Table & Fig 1) , in group of 1 mg/ml concentration in the first day there is significant difference as

compared to the control group . In the second day there is significant difference as compared to the first day .in the third day there is significant difference as compared to the second day & as compared to the control group.

Regarding the other group which use 0.5 mg/ml levamisole , in the first day there is significant difference as compared with control group, and as compared to group of 1 mg/ml levamisole. In the second day the significant difference is found as compared to the control group and group of 1mg/ml levamisole. While in third day of experiment the only significant difference is as compared to the second day.

In respect to paracetamole(Table & Fig. 2) we find that in the first day there is significant difference in comparism to the control group, while in second day there is significant difference as compared to control group & to the first day as well. In the third day also there is significant difference as compared to the control group & to the first day, on the other hand there is no significant difference when compare the second to the third day.

Regarding metronidazole also there are two doses tested on promastigotes (0.5mg/ml) and (1mg/ml) (Table & Fig. 3). in the media of growth where 0.5mg/ml Metronidazole is used, observed that in the first day there is significant difference as compared to the control group & also to group of 1mg/ml metronidazole. In the second day , there is significant difference as compared to the first day, to the control group and to the group of 1mg/ml. in the third day there is significant difference as compared to the second day & to group of 1mg/ml. On the other hand when use 1mg/ml metronidazole, in the first day there is significant difference as compared to the control group, while in the second day there is significant difference as compared to the first day and to the control group, in the third day the significant difference is found in comparism to the first day and the control group.

Regarding bromohexine 0.02mg/ml (Table & Fig. 4) In the first day, there is significant difference as compared to the control group, in the second day there is significant difference as compred to the first day & also a significant difference as compared to the control group, while in the third day there is a significant difference as compared to the control group.

In respect to sildenafil (0.5mg/ml) (Table & Fig. 5) In the first day, there is significant difference as compared to the control group , in the second day there is significant difference as compared to the first day & to the control group . in the third day there is significant difference as compared to the second day & to the control group.

In case of diclofenac sodium(0.5mg/ml) (Table & Fig. 6) In the first day there is no significant difference as compared to the control group , in the second day there is no significant difference as compared to the control group but there is significant difference as compared to the first day ,while in third day there is significant difference as compared to control group .

Table (1): The effects of two concentrations of Levamisole on the *L. donovani* Promastigote growth rates *in vitro*

	Levamisole											
	Count of *L. donovani* promastigotes expressed in mean± standard of error											
	1st day				2nd day				3rd day			
	MEAN		SE		MEAN		SE		MEAN		SE	
CONTROL	5568	±	481.07		11584	±	466.75	*	19872	±	684.58	*a
Levamisol 1mg/ml	2816	±	352.12	b	13120	±	587.88	*	7904	±	406.14	a,b
Levamisol 0.5 mg/ml	7936	±	530.50	b, c	8992	±	564.33	b, c	13248	±	682.08	a

* Significant different at $p<0.05$ as compared with first day values

[a] Significant different at $p<0.05$ as compared with second day values

[b] Significant different at $p<0.05$ as compared with controls values

[c] Significant different at $p<0.05$ as compared with 1mg/ml value

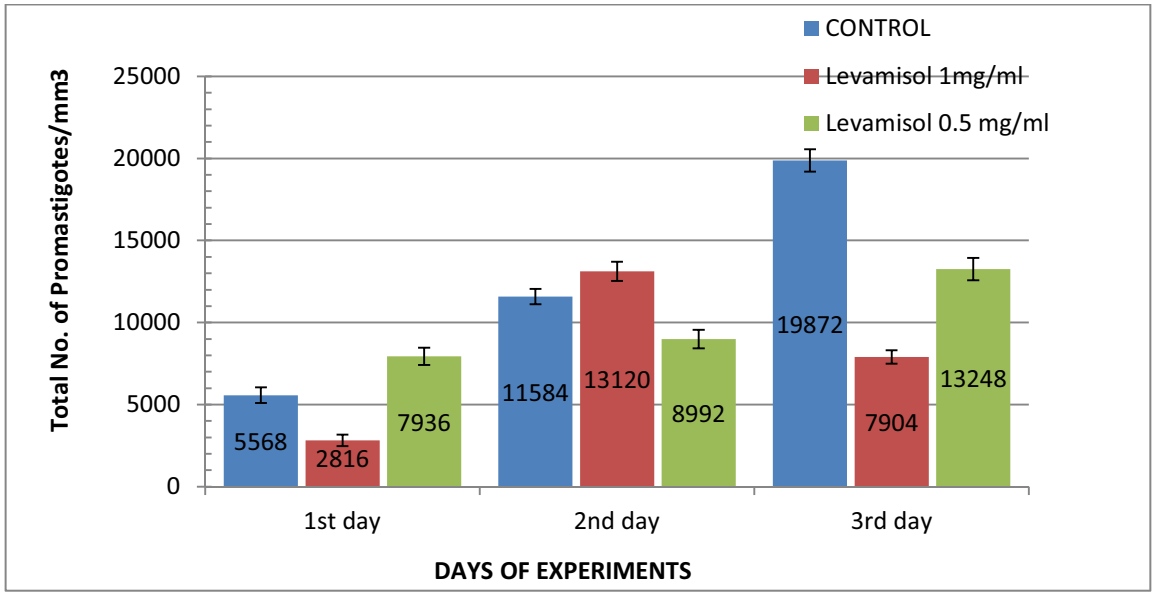

Figure (1): Illustrate the replication rates of *L.donovani* promastigotes treated with two concentration of Levamisol during a period of three days.

Table (2): The effects of one concentrations of Paracetamole on the *L. donovani* Promastigote growth rates *in vitro*

	Paracetamole								
	Count of *L. donovani* promastigotes expressed in mean± standard of error								
	1st day			**2nd day**			**3rd day**		
	MEAN	**SE**		**MEAN**	**SE**		**MEAN**	**SE**	
CONTROL	5568 ±	481.07		11584 ±	466.75	*	19872 ±	684.58	*a
Paracetamol 0.1mg/ml	7808 ±	539.19	♀b	17728 ±	864.94	*,b	17056 ±	679.95	*,b

* Significant different at $p<0.05$ as compared with first day values

[a] Significant different at $p<0.05$ as compared with second day values

[b] Significant different at $p<0.05$ as compared with control values

[c] Significant different at $p<0.05$ as compared with 1mg/ml values

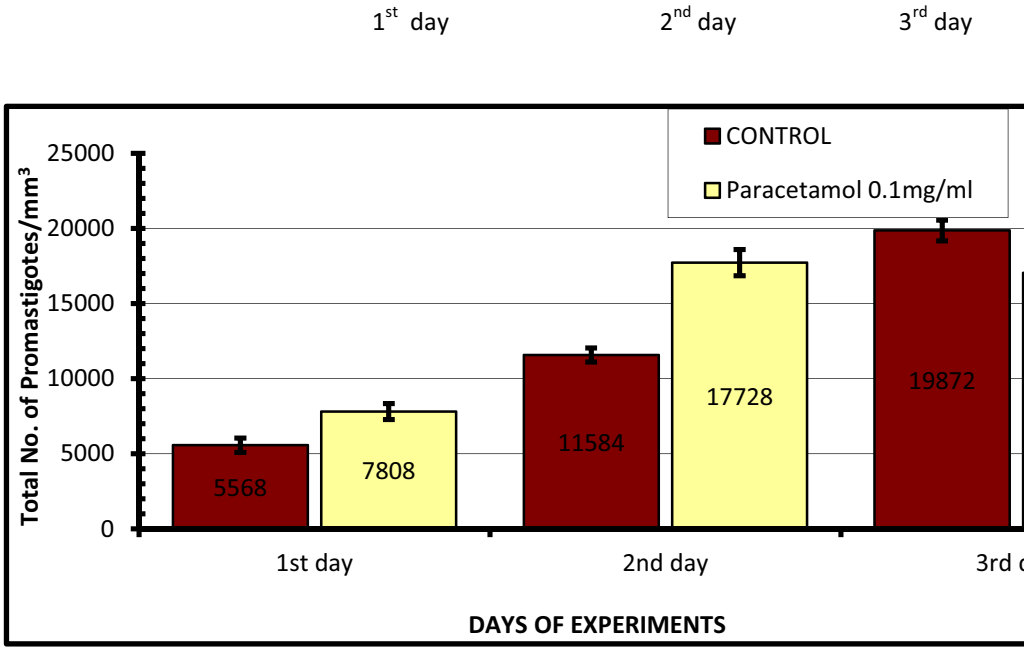

Figure (2): Illustrate the replication rates of *L.donovani* promastigotes treated with (0.1 mg/n Paracetamole during a period of three days.

	Metronidazole								
	Count of *L. donovani* promastigotes expressed in mean± standard of error								
	1st day			2nd day			3rd day		
	MEAN	SE		MEAN	SE		MEAN	SE	
CONTROL	5568 ±	481		11584 ±	467	*	19872 ±	685	*a
Metronidazole 1mg/ml	2656 ±	177	♀b	9632 ±	494	*,b	10688 ±	482	*,b
Metronidazole 0.5 mg/ml	10400 ±	680	b, c	15712 ±	694	*,b, c	19744 ±	617	a,c

Table (3): The effects of two concentrations of Metronidazole on the *L. donovani* Promastigot growth rates *in vitro*

* Significant different at p<0.05 as compared with first day values
[a] Significant different at p<0.05 as compared with second day values
[b] Significant different at p<0.05 as compared with controls values
[c] Significant different at p<0.05 as compared with 1mg/ml values

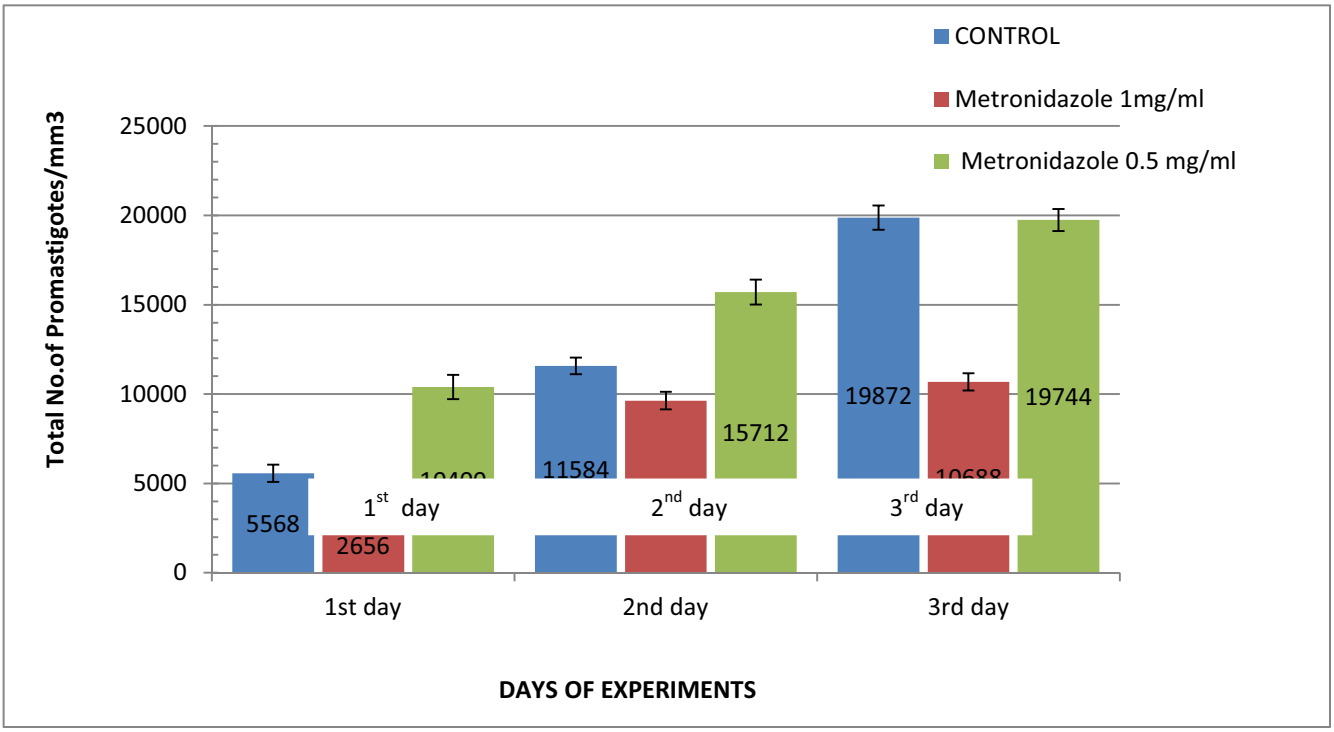

Figure (3) : Illustrate the replication rates of *L.donovani* promastigotes treated with two concentration of Metronidazole during a period of three days

Table (4): The effects of one concentrations of Bromohexin on the *L. donovani* Promastigote *growth vitro*

	1st day			2nd day			3rd day		
	MEAN	SE		MEAN	SE		MEAN	SE	
CONTROL	5568 ±	481		11584 ±	467	*	19872 ±	685	*a
Bromohexin 0.02mg/ml	1024 ±	267	b	2464 ±	206	*,b	2176 ±	157	b

* Significant different at $p<0.05$ as compared with first day values

[a] Significant different at $p<0.05$ as compared with second day values

[b] Significant different at $p<0.05$ as compared with controls values

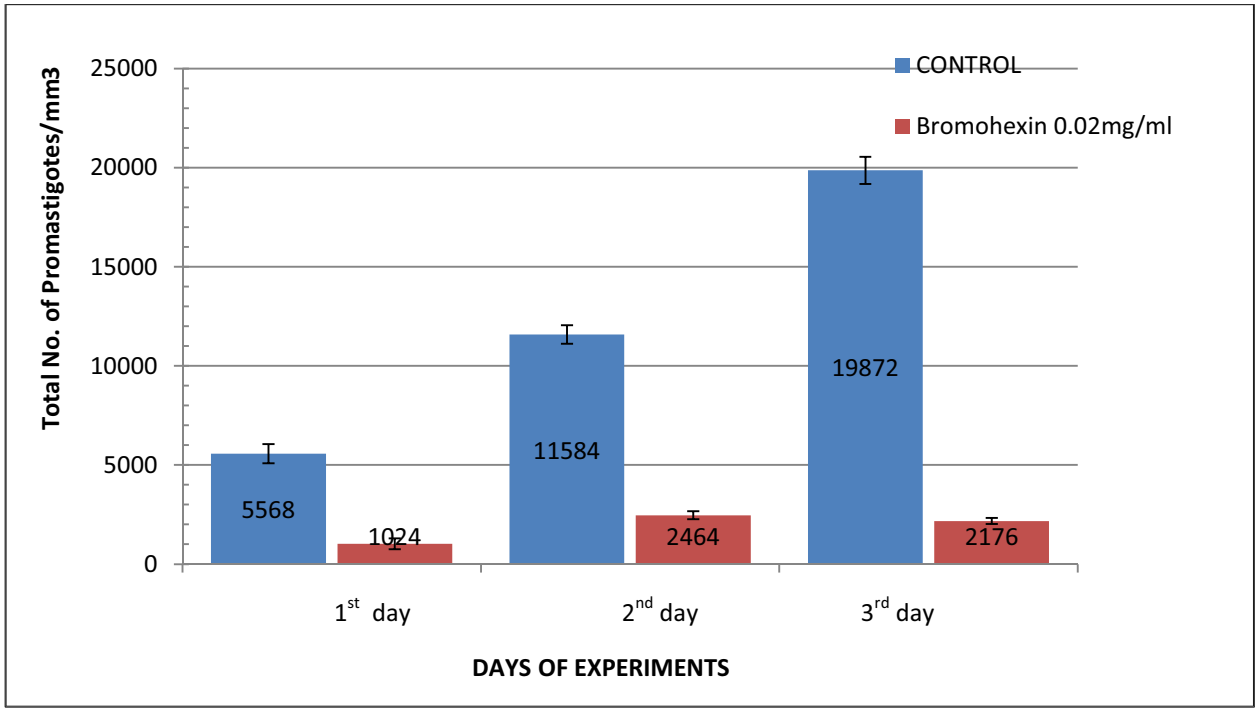

Fig (4): Illustrate the replication rates of *L.donovani* promastigotes treated with one concentration of Bromohexin during a period of three days

	Sildenafil								
	Count of *L. donovani* promastigotes expressed in mean± standard of error								
	1st day			2nd day			3rd day		
	MEAN	SE		MEAN	SE		MEAN	SE	
CONTROL	5568 ±	481		11584 ±	467	*	19872 ±	685	*a
Sildenafil 0.5 mg/ml	1760 ±	253	♀b	7136 ±	369	*,b	5632 ±	290	a,b

* Significant different at $p<0.05$ as compared with first day values

[a] Significant different at $p<0.05$ as compared with second day values

[b] Significant different at $p<0.05$ as compared with controls values

Table (5): The effects of one concentrations of Sildenafil on the *L. donovani* Promastigote growth rates *in vitro*

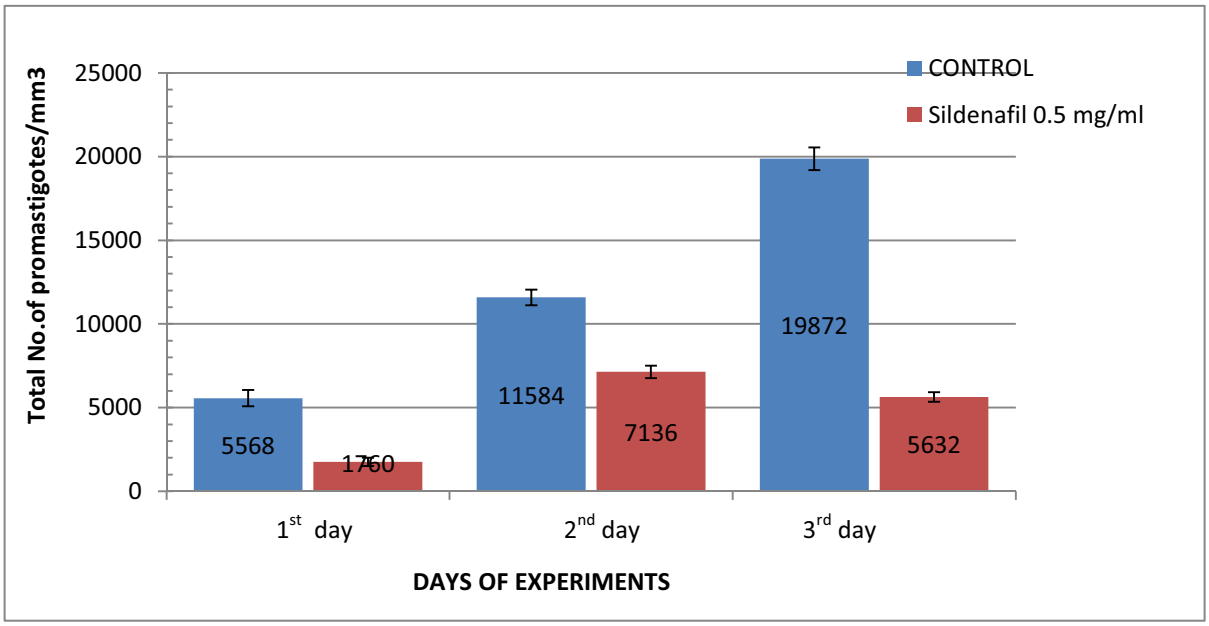

Figure (5): Illustrate the replication rates of *L.donovani* promastigotes treated with one concentration of Sildenafil during a period of three days.

Table (6): The effects of one concentrations of Diclofenac sodium on the *L. donovani* Promastigote growth rates *in vitro*

	Diclofenac								
	Count of *L. donovani* promastigotes expressed in mean± standard of error								
	1st day			2nd day			3rd day		
	MEAN	SE		MEAN	SE		MEAN	SE	
CONTROL	5568 ±	481		11584 ±	467	*	19872 ±	685	*a
Diclofenac 0.5 mg/ml	5152 ±	343	♀	10496 ±	782	*	10432 ±	517	b

* Significant different at $p<0.05$ as compared with first day values

[a] Significant different at $p<0.05$ as compared with second day values

[b] Significant different at $p<0.05$ as compared with controls values

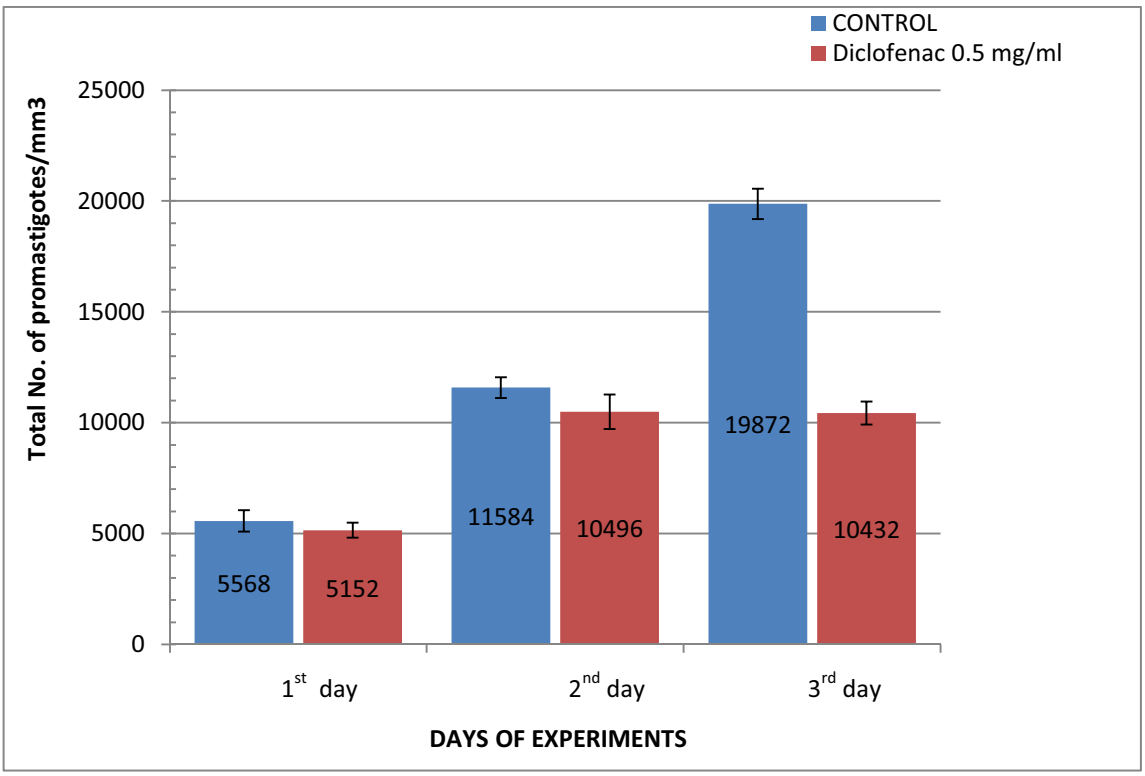

Figure (6): Illustrate the replication rates of *L.donovani* promastigotes treated with one concentration of Diclofenac sodium during a period of three days.

Discussion

In the control group there is a gradual increase in growth rate of parasites which suggest that the media of growth was efficient & well equipped with nutritional materials so that the large increase in number of parasites not lead to crowding and competition for survival.

In the present study most types of drugs used have significant difference between the growth rates of parasites and control group in the first day which mean that they have killing effect on promastigotes in away or another according to their mechanisms of action[16]. In the second day most of groups that have been treated with drugs show an increase in growth rates with a significant difference as compared to the first day which can be attributed to diminished actin of drugs after 24 hours (there is no addition) and also to the development of drug resistance[17].Third reason for the increase in growth rate is that there is a sufficient nutrient & suitable environment in the media which cause the parasites to replicate again[18].

Regarding Levamisole, 2 doses have been used in the experiment separately (0.5 and 1 mg/ml) 0.5 mg/ml is suboptimal and have no effect on the parasite growth. The growth rate is larger than that of the control group in the first day and increasing in the second day and more in the third day. While using 1mg/ml Levamisole leads to decrease in growth rate in first day with significant difference when compare to control group. The increase in growth rate in the second day could be explained by limited duration of action of Levamisole and overgrowth of remaining parasites might be due to abundant nutrients after large decrease in their numbers in the first day was also suspected [19].The effect of Levamisole is more apparent in the third day where it cause large decrease in growth rate and significant difference with control group. The effect of Levamisole on *L.donavani* promastigotes is attributed to Levamisole enhancing effect on cell mediated immunity [3]. Our results goes with a study on guinea pigs infected with *L. donavani*, which demonstrate that Levamisole-treated guinea pigs either did not develop an ulcerative cutaneous lesion or developed a much smaller lesion than untreated animals[20],the difference from our results is that their findings studied *in vivo*.

Regarding Paracetamole , from the data collected we observed that there is a gradual increase in growth rates in the first & second days more than what had been calculated in the control group which indicate that the Paracetamole may have stimulating effect on growth of
L. donavani , the little decrease in growth rate in the third day can be explained by competition between the parasites on nutritional materials and crowding,our data could not be explained by the mechanism of action of paracetamole[4].

In case of Metronidazole we observed that the use of small dose 0.5mg/ml leads to increase in growth rate of parasites even more than the control group in the first & second day and approximately similar rate of growth between the control group & group of 0.5mg/ml Metronidazole , this can be explained by development of drug resistance of parasite when treated by suboptimal dose[17]. While in the other group where the dose of Metronidazole used is 1mg/ml ,it show significant decrease in growth rate of parasite which may be an optimal dose.The killing effect of

Metronidazole appears in the first day more obviously while in second & third day the growth rate has been increased in comparism to the first day but is less than that of control group so its effect on parasites may be diminished after 24 hours which may indicate the necessity of repeating the dose daily . The use of Metronidazole in treatment of cutaneous Leishmaniasis is proved its efficacy in another study where the patients lesion had shrunk to half its size by the tenth day of treatment[21].

In regard to Bromohexine the results shows a strongly potent inhibitory action of Bromohexine on the growth of promasitigotes from the first day & continue to inhibit growth in the second &third day , There is no significant difference between second & third days in growth rates but still very low rates, the most accepted explanation for the present data is that the decrease in growth rates of promasigotes might be related to Bromohexine inhibiting action on cAMP phosphodiesterase enzymes which is important for promastigotes proliferation [22].in a study in India prove that peganine hydrochloride isolated from peganum harmala seeds in dehydrated form is shown to have exhibited *in vitro* activity against both extracellular promastigotes as well as intracellular amastigotes residing within murine macrophage in *L. donavani.* Furthermore it also exhibit in-vivo antileishmanial activity[23].

In present study Sildenafil in a dose 0.5mg/ml leads to a significant decrease in growth rates of promastigotes throughout the three days of experiment, this is because sildenafil cause inhibition of phosphodiesterase enzyme of *L.donavani* which shown to play important roles in cell proliferation & apoptosis of the parasites by controlling cellular concentration of cAMP and cGMP that are key regulators of many physiological processes in leishmania[24]. Early studies show that three human phosphodiesterase enzyme inhibitors (Etazolate, Dipyridamole, and Trequinsin) inhibit the proliferation of *L. major* promastigotes and *L. infantum* amastigotes with IC50 values in the range of 30 – 100 μM [25].

The Diclofenac sodium in the experiment show a significant difference in the second day as compared to the first day but in increasing the Growth not decreasing & as there is no significant difference as compared to the control group In the second day so this could be explained as that the Diclofenac sodium has no effect on growth rate in the second day which mean that the parasite nearly has the same conditions of Environment as the control group. The only significant difference in decreasing growth rate was at the third day of experiment when compared to the control group which mean a delayed action of Diclofenac as mentioned above but still there is a very little decrease in growth rate from second to third day which may be mean that the more likely cause of death of parasites is decrease in nutritional supply in addition to Diclofenac sodium effect and not only to the effect of diclofenac sodium .as the mechanism of action of Diclofenac sodium is inhibition of prostaglandin E2. It has been shown that an increased production of PGE2, PGF2α ,LTC, TXB2, andPGD2 occur during the course of murine infection with *L.donavani* [26]. In a contradictory report on role of prostaglandin in immunology mention that Prostaglandin of E Series increase cAMP and by this mechanism it inhibits the Leishmanicidal activity of macrophage So decrease production of PGs by Diclofenac sodium will cause increase in the killing of *Leishmania* by macrophage [27].

Conclusion

Selective inhibitors of phosphodiesterase may potentially represent a novel class of drugs for the treatment of Leishmaniasis .Limited trials of Levamisole on cutaneous Leishmaniasis may give promising results. Paracetamole can be used in media for stimulation of Leishmanial growth. being antileishmanial, Bromohexine can further help to prevent the disease. When use Metronidazole in treatment of Leishmaniasis, need to give daily dose.

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