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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and biological evaluation of a novel class as antileishmanial agent

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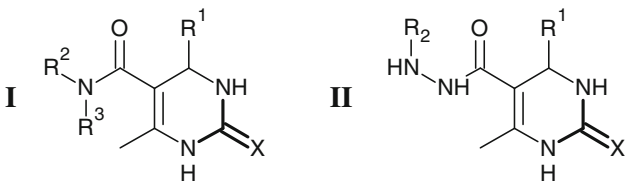
Abstract Leishmaniases are a group of diseases caused by the protozoan parasites of the genus Leishmania. Despite the tremendous progress made in the understanding of the biochemistry and molecular biology of the parasite, the first choice treatment for leishmaniases still relies on pentavalent antimonial developed more than 50 years ago. These drugs are potentially toxic and often ineffective. The spread of drug resistance, combined with other shortcoming of the available antileishmanial drugs, emphasizes the importance of developing new effective, and safe drugs against leishmaniasis. The study reported here was undertaken to examine the antileishmanial activity of novel substituted 1,2,3,4-tetrahydropyrimidine-5-carboxamide (**I**) or cabohydrazide (**II**) analogs both in vivo and in vitro against *Leishmania major*. All tested compounds showed in vitro antileishmanial activity, but only four compounds showed in vivo activity in Swiss strain albino mice model as revealed by clinical cure of the cutaneous lesion, parasitologically by marked reduction in parasitic load and by histopathological changes.



Keywords 1,2,3,4-tetrahydropyrimidine-5-carboxamide or cabohydrazide · Antileishmanial agents · *Leishmania major* · Histopathological study

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Introduction

Leishmaniasis is group of disease caused by various species and strains of *Leishmania.* It is a growing health problem in many parts of the world mainly due to an increasing frequency of drug-resistance and an enhanced risk of co-infection with HIV (Alvard *et al.*, 1997). A wide spectrum of disease in humans, with many different clinical presentations, appearing in three main forms: visceral, cutaneous, and mucocutaneous (Handman, 1999). The severity of the disease is largely dictated by the immunological status of the infected individual and by the species of the *Leishmania* involved (Barret *et al.*, 1999). Approximately, 350 million people in 80 countries are estimated to be threatened by the disease (Desjeux, 2001). Furthermore, as some all types of leishmanial diseases (Barrel *et al.*, 1991). Despite the tremendous progress made in the understanding of the biochemistry and molecular biology of the parasite, the first choice treatment for the several

forms of leishmanisis still relies on pentavalent antimonials, which are the clinical drugs most frequently employed, have been in use since the 1920s. However, antimoniates have a narrow therapeutic window due to their toxicity, and there are additional conditions, which permit the persistence of *Leishmania* in the vertebrate host. When these drugs are ineffective, or cannot be prescribed, treatment with amphotericin B, pentamidine, or paramomycin is indicated (Croft and Coombs, 2003). Miltefosine, an inhibitor of sterol biosynthesis, has been recently approved as the first oral drug for visceral leishmaniosis in India. It yields cure rates of about 98%, has negligible side effects and is used successfully to treat cases resistant to antimonalis (Jha *et al.*, 1999). Recently, miltefosine produced a dramatic clinical and parasitological response in patients with diffuse cutaneous leishmaniasis and improvement continued during drug administration, but relapse occurred after stoppage of the treatment (Zerpa *et al.*, 2007). However, teratogenicity, gastrointestinal upset, potential of resistance development, and a low therapeutic window pose limitations on its use (Croft *et al.*, 2006). A series of 1-[4-(4′-sulfanilyl)phenyl]urea and its derivatives were proved to be efficient in treatment of leishmaniasis (David, 1997). These findings encourage us to synthesize our target compounds as they possess urea or thiourea moieties. Therefore this study was under taken to examine the antileishmanial activity of novel substituted 1,2,3,4-tetrahydropyrimidine-5-carboxamide (**I**) or 5-carbohydrazide (**II**) analogs both in vitro and in in vivo against *leishmania major* disease (Ponte-Sucre, 2003).

Chemistry

The designed target compounds are depicted in Scheme 1. The intermediates **2a** and **2b** were obtained by heating a mixture of ethylacetoacetate and p-aminoacetanilide **1a** or salicylic acid hydrazide **1b** in 6:1 ratio, respectively. On the other hand, compound **2c** was prepared also following the previous procedure, for the synthesis of **2a** and **2b**, starting with diethanolamine **1c** and the further reaction was done on the residue without separation. Whereas, the intermediate **2d** was prepared by two reactions. The first one is hydrazinolysis of crotonylester with excess hydrazine hydrate for 1 h, then evaporation to dryness under vacuum. The residue **1d** was exposed to the second reaction directly using the same procedure described for the synthesis of **2a** and **2b**. The target compounds **3a–f** have been synthesized following the Biginelli reaction (Biginelli, 1893) by heating our intermediate **2a** or **2b** with the appropriate aldehyde with urea or thiouea in ethanol in the presence of hydrochloric acid at reflux temperature. However, compounds **3g** and **3h** have been also prepared by Biginelli reaction but without separating out their intermediate **2d** and **2c**, respectively. Chlorination of compound **3h** using thionyl chloride yielded the dichlorinated product **3i**.

Experimental

Melting points were determined in open glass capillaries and are uncorrected. I.R spectra were recorded for KBr disc using Perkin-Elmer 421 spectrophotometer. ^{1}HNMR spectra were recorded on a Varian EM-390 (400 MHz) spectrophotometer, using TMS as internal standard and DMSO-d_6 as solvent. The MS spectra were determined with GC-MS 1000 Fx Shimadzu mass spectrometer. Microanalyses were performed in the microanalytical unit, University of Assute.

General procedure for synthesis of *N*-(4-acetamidophenyl)-3-oxobutanamide (**2a**) and N_2-acetoacetyl-2 hydroxybenzohydrazide (**2b**)

A mixture of 4-aminoacetanilide (**1a**) or salicylic acid hydrazide (**1b**) (10 mmol) and ethylacetoacetate (7.8 g, 60 mmol) was heated at 150°C for 1 h. After cooling, the reaction mixture was triurated with ethanol (5 ml) and the precipitated material was collected by filtration. The yields and melting points of the synthesized compounds were reported (Gionanni, 1941).

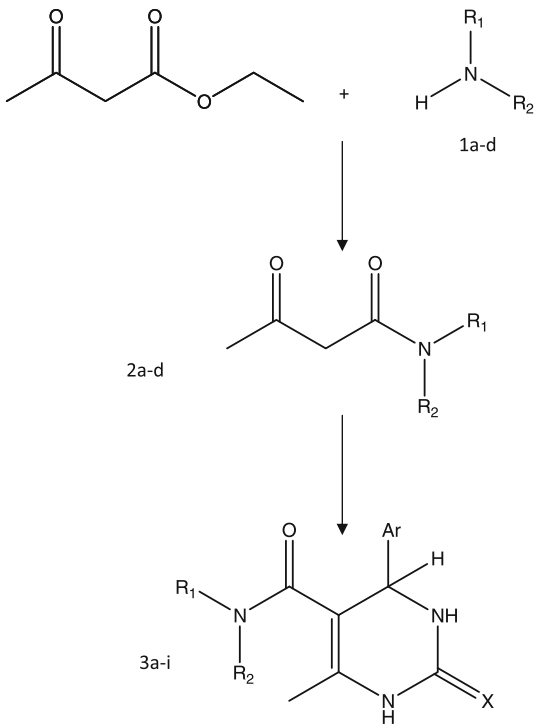
General procedure for synthesis of 4-aryl-6-*N*-substituted-2-oxo or 2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamides (**3a–f**)

To a stirred solution of urea or thiourea (10 mmol), substituted benzaldehyde (10 mmol) and few drops of conc. HCl in ethanol (15 ml), the appropriate ethylacetoacetate derivatives (**2a** or **2b**) (10 mmol) were added. The reaction mixture was heated to reflux for 3–5 h and was then allowed to stand overnight at room temperature. The precipitated products were filtered, washed with ethanol, dried and crystallized from *n*-propanol.

Synthesis of *N*-(but-2-enoyl)-1,2,3,4-tetrahydro-6-methyl-4-(3-nitrophenyl)-2-thiopyrimidine-5-carbohydrazide (**3g**)

Ethylcrotonate (1.1 g,10 mmol) and hydrazine hydrate (2 ml) was refluxed for 1 h.After cooling,the reaction mixture was evaporated to dryness under vaccum.The residue (crotonyl hydrazide) was exposed to the next reaction without separation by heating it with ethylacetoacetate (7.8 g,10 mmol) at 150°C for 1 h.After cooling, the semisolid residue (**2d**) was evaporated to dryness and

Scheme 1



1	R1	R2
a	H	4-(CH_3CONH) C_6H_4
b	NH_2	(2-OH-C_4H_6) CO
c	$(CH_2)_2OH$	$(CH_2)_2OH$
d	NH_2	CH_3-CH=CH-CO

2	R1	R2
a	H	4-(CH_3CONH) C_6H_4
b	H	(2-OH-C_4H_6) CONH
c	$(CH_2)_2OH$	$(CH_2)_2OH$
d	H	CH_3-CH=CH-CONH

3	R1	R2	X	Ar
a	H	4-(CH_3CONH) C_6H_4	O	4-$(CH_3)_2N$-C_6H_4
b	H	(2-OH-C_4H_6) CONH	O	4-$(CH_3)_2N$-C_6H_4
c	H	(2-OH-C_4H_6) CONH	S	4-$(CH_3)_2N$-C_6H_4
d	H	(2-OH-C_4H_6) CONH	O	3-NO_2- C_6H_4
e	H	(2-OH-C_4H_6) CONH	S	3-NO_2- C_6H_4
f	H	(2-OH-C_4H_6) CONH	S	3, 4, 5-$(OCH_3)_3$ C_6H_2
g	H	CH_3-CH=CH-CONH	S	3-NO_2- C_6H_4
h	$(CH_2)_2OH$	$(CH_2)_2OH$	S	3-NO_2- C_6H_4
i	$(CH_2)_2CL$	$(CH_2)_2CL$	S	3-NO_2- C_6H_4

then involved in the second reaction by adding ethanol (15 ml), thiourea (0.76 g,10 mmol),3-nitrobenzaldehyde and few drops of conc. HCl. The reaction mixture was then heated to reflux for 4 h.After cooling, the prepared compound (**3g**) was separated out,filtered and crystallized from n-propanol.

Synthesis of *N*,*N*-bis(2-hydroxyethyl)-1,2,3,4tetrahydro-6-methyl-4-(3-nitrophenyl)-2 thioxopyrimidine-5 carboxamide (**3h**)

A mixture of diethanolamine (**1c**) (1.1 g, 10 mmol) and ethylacetoacetate (7.9 g, 60 mmol) washeated at 150°C for 1 h. After cooling, the reaction mixture was evaporated to dryness under vacuum to give **2c** which was exposed directly to the next reaction by adding ethanol (15 ml), thiourea (0.76 g, 10 mmol), 3-nitrobenzaldehyde and few drops of conc. HCl. The reaction mixture was then heated to reflux for 5 h. After cooling, the prepared compound (**3h**) was separated out, filtered, and crystallized from ethanol.

Synthesis of *N*,*N*-bis(2 chloroethyl)-1,2,3,4-tetrahydro-6-methyl-4-(3-nitrophenyl)-2-thioxopyrimidine-5-carboxamide (**3i**)

A solution of $SOCl_2$ (11.8,100 mmol) in dry benzene (20 ml) was added dropwise, while stirring to a solution of **3h** (3.8 g, 10 mmol) in dry benzene (10 ml).Stirring was continued for 24 h at room temperature. The obtained crystals were filtered, washed with dry benzene, dried and

crystallized from benzene/ethanol. The yields, melting points, and microanalytical data of the prepared compounds were listed in Table 1. IR and ^{1}HNMR spectral data are recorded in Table 2.

Materials and methods

Leishmania strain and its maintenance

The *leishmania* strain used in this study was *L. major* (MHOM/IL/81/FEBNI). It was kindly provided by Prof. Dr. Tamas Lasaky (Professor of immunology, Research Unit, Institute for Medical Microbiology and Hygiene. University of Lübeck, Germany) and further maintained in the laboratory of parasitology Department, Faculty of Medicine, Alexandria University, both in vitro on Tanabe's liquid medium and in vivo by serial passage in Swiss strain Albino mice.

Culture media

(a) Tanabe's medium (Tanabe, 1923) was used for in vitro maintenance of the *L. major* strain.
(b) N.N.N. medium (McCartry-Burke *et al.*, 1991) was used for the in vitro antileishmanial assay of the tested compounds and for mass cultivation of leishmania required for animal inoculation for performance of in vivo study.

Tested compounds

All the newly synthesized compounds (**3a–i**) were screened for their antileishmanial activity both in vitro and in vivo.

Experimental animals

Laboratory bred Swiss albino mice 4- to 8-weeks old were used. For animal inoculation, promastigotes of *L. major* were harvested from N.N.N. medium. The parasites were adjusted to the required concentration of 20×10^6, and inoculated into the left hind footpad.

In vitro antileishmanial assay

The in vitro sensitivity of *Leishmania* promastigote to the tested compounds was performed. The compounds were added at a concentration of 10 μmol to equal volumes of *Leishmania* promastigotes (10^6/ml). 0.5% DMSO and medium alone were used as controls. Parasites were examined microscopically immediately after the addition of the compounds and every 10 min for 1 h, then they were incubated at 23°C in a cooling incubator, parasites were checked for viability and counted daily for 5 days in a

Table 1 The physicochemical properties and elemental analyses of the new compounds

Comp. no.	Yield (%)	MP (°C)	Mol. formula (mol. weight)	Analysis Found/Cald (%)		
				C	H	N
3a	53	>300	$C_{22}H_{25}N_5O_3$ (407.47)	64.85	6.18	17.19
				64.61	6.42	17.32
3b	58	219	$C_{21}H_{23}N_5O_4$ (409.44)	61.60	5.66	17.10
				61.31	5.43	17.06
3c	62	186	$C_{21}H_{23}N_5O_3S$ (425.50)	59.28	5.45	16.40
				58.95	5.13	16.32
3d	68	250	$C_{19}H_{17}N_5O_6$ (411.37)	55.47	4.17	17.02
				55.17	4.42	16.99
3e	56	259	$C_{19}H_{17}N_5O_5S$ (427.43)	53.39	4.01	16.38
				53.62	4.32	16.52
3f	70	131	$C_{22}H_{24}N_4O_6S$ (472.51)	55.92	5.12	11.86
				55.63	5.45	12.01
3g	65	199	$C_{16}H_{17}N_5O_4S$ (375.40)	51.19	4.56	18.66
				51.32	5.82	18.91
3h	75	165	$C_{16}H_{20}N_4O_5S$ (380.42)	50.52	5.30	14.73
				50.41	5.23	14.52
3i	60	123	$C_{16}H_{18}Cl_2N_4O_3S$ (417.31)	46.05	4.35	13.43
				46.35	4.61	13.73

Table 2 IR, ^{1}HNMR and MS spectral data of the newly synthesized compounds

Comp. no.	IR $(cm)^{-1}$	^{1}H-NMR (δ-ppm) (DMSO-d_6)	MS (% relative abundance)
3a	3420 (NH), 1710 (C=O), 1680 (C=O)	2.11 (s, 3H, CH_3–CO–NH–), 2.56 (s, 3H, CH_3, pyrimidine-C_6), 3.11 (s, 6H, $(CH_3)_2N$), 5.14 (d, 1H, pyrimidine-H_4) 6.92–8.39 (m, 9H, 8Ar–H and pyrimidine N_3–H) 9.10(s, 1H, pyrimidine N_1–H, exchangeable with D_2O), 9.94 (s, 1H, –NH–CO–C_5-pyrimidine, exchangeable with D_2O), 10.11 (s, 1H, CH_3–CO–NH-phenyl, exchangeable with D_2O)	
3b	3350–3300(NH, OH), 1715 (C=O), 1580, 1235 (C=S)	2.36 (s, 3H, CH_3, pyrimidine-C_6), 3.23 (s, 6H, $(CH_3)_2N$), 5.21 (d, 1H, pyrimidine-H_4), 7.12–7.98 (m, 9H, 8Ar–H and pyrimidine N_3–H), 8.1 (d, 1H, $j = 6.9$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 9.68 (s, 1H, pyrimidine N_1–H, exchangeable with D_2O), 10.75 (d, 1H, $j = 6.9$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 11.91 (s, 1H, –OH, exchangeable with D_2O)	
3d	3404–3355 (NH, OH), 1720 (C=O), 1685 (C=O)	2.31 (s, 3H, CH_3, pyrimidine-C_6), 5.25 (d, 1H, pyrimidine-H_4), 6.94–8.13 (m, 9H, 8Ar–H and pyrimidine N_3–H), 8.32 (d, 1H, $j = 7.3$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 9.71 (s, 1H, pyrimidine N_1–H, exchangeable with D_2O), 10.81 (d, 1H, $j = 7.3$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 12.01 (s, 1H, –OH, exchangeable with D_2O)	
3e	3410–3355 (NH, OH), 1720 (C=O), 1689 (C=O) 1585,1235 (C=S)	2.29 (s, 3H, CH_3, pyrimidine-C_6), 5.31 (d, 1H, pyrimidine-H_4), 7.12–8.19 (m, 9H, 8Ar–H and pyrimidine N_3–H), 8.45 (d, 1H, $j = 7.4$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 9.71 (s, 1H, pyrimidine N_1–H, exchangeable with D_2O), 10.92 (d, 1H, $j = 7.4$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 12.14 (s, 1H, –OH, exchangeable with D_2O)	M^+, 427 (3.02), 366 (4.23), 302 (574), 267 (5.74), 203 (3.32), 163 (6.65), 121 (81.2), 65 (100)
3f	3390–3365 (NH, OH, 1725 (C=O), 1685 (C=O), 1590, 1230 (C=S)	2.27 (s, 3H, CH_3, pyrimidine-C_6), 3.83 (s, 9H, $3OCH_3$), 5.32 (d, 1H, pyrimidine-H_4) 7.41–8.21 (m, 7H, 6Ar–H and pyrimidine N_3–H), 8.51 (d, 1H, $j = 6.9$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 9.68 (s, 1H, pyrimidine N_1–H, exchangeable with D_2O), 10.89 (d, 1H, $j = 6.9$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 11.99 (s, 1H, –OH, exchangeable with D_2O)	M^+-84, 388 (67.63), 345 (23.60), 299 (5.83), 267 (1.84), 255 (2.90), 193 (33.80), 137 (26.86) 121 (100), 84 (2.08), 66 (58.97)
3g	3350 (NH), 1715 (C=O), 1685 (C=O)	2.12 (s, 1H, CH_3–CH=CH–), 2.39 (s, 3H, CH_3, pyrimidine-C_6), 5.62 (d, 1H, pyrimidine-H_4), 6.11 (d, 1H, $j = 7.4$, –CO–CH=CH– CH_3), 6.31 (d, 1H, $j = 7.4$, –CO–CH=CH– CH_3) 7.56–8.12 (m, 5H, 4Ar–H and pyrimidine N_3–H), 8.91 (d, 1H, $j = 7.3$, –NH–NH–CO-pyrimidine, exchangeable with D_2O), 9.68(s, 1H, pyrimidine N_1–H, exchangeable with D_2O), 10.94 (d, 1H, $j = 7.3$, –NH–NH–CO-pyrimidine, exchangeable with D_2O)	
3h	34104-0 (NH, OH), 1700 (C=O), 1590, 1235 (C=S)	2.31 (s, 3H, CH_3, pyrimidine-C_6), 3.15 (t, 4H, N–(CH_2–CH_2–OH)$_2$, 3.61 (t, 4H, N–(CH_2–CH_2–OH)$_2$, 4.78 (s, 2H, 2OH, exchangeable with D_2O). 5.62 (d, 1H, pyrimidine-H_4), 7.05–7.89 (m, 5H, 4Ar–H and pyrimidine N_3–H), 9.32 (s, 1H, pyrimidine N_1–H, exchangeable with D_2O)	M^+-1, 379 (12.30), 231 (9.09), 194 (11.23), 149 (54.01), 104 (62.03), 91 (19.79), 55(100)
3i	3380- (NH), 1720 (C=O), 1585, 1228 (C=S)	2.34 (s, 3H, CH_3, pyrimidine-C_6), 3.46 (t, 4H, N–(CH_2–CH_2–Cl)$_2$, 3.72 (t, 4H, N–(CH_2–CH_2–Cl)$_2$, 5.67 (d, 1H, pyrimidine-H_4), 7.17–8.10 (m, 5H, 4Ar–H and pyrimidine N_3–H), 9.32 (s,1H, pyrimidine N_1–H, exchangeable with D_2O)	

haemocytometer with a light microscope (LM) and the inhibition was calculated: inhibition (in percent) = 100 − [(number of parasite remaining after treatment)/number of parasites in control] × 100 (Chan and Fong, 1990). Each assay was performed in duplicate and repeated in separate experiments.

In vivo antileishmanial assay

To examine the therapeutic efficacy of the tested compounds, ♂ Swiss albino mice infected with 20×10^6 promastigotes in the left hind footpad were used. They were allocated into two main groups.

Group I: control group

It was further subdivided into:

- Group Ia: Infected untreated control.
- Group Ib: DSMO control group (infected, received local intralesional injection of 0.5% DSMO)

Group II: experimental group

It was further subdivided equally into nine subgroups each group received one of the nine tested compounds. Treatment was started 21 days post infection. Tested compounds were infiltrated into the lesion at a dose of 20 μmol. Five weeks after local treatment, animals were killed and the effect of the tested compounds on the cutaneous leishmanial lesion was assessed by the following parameters in comparison to infected non-treated control (group I).

(a) Clinical evaluation: Animals of all groups were subjected to examination of the inoculated footpad for the progression of lesion.
(b) Parasitological study: The study includes

(i) Culture was performed to detect the presence of viable parasites in the lesion. Parts of the cutaneous leishmanial lesions from all groups were cultured aseptically on Tanabe's liquid medium and kept at 23–25°C with weekly examination. Culture was recorded as negative if no parasite was observed within 3 weeks.
(ii) Tissue impression smear: Impression smears were made from the cutaneous lesions of all groups, fixed in methanol, and stained with Giemsa stain. The parasite density in the stained smear was examined under oil immersion, and was recorded on empirical scale as follows (Schnur *et al.*, 1973):

+3: More than 150 amastigotes in virtually every field. At least 25 fields were scanned.
+2: About 50 amastigotes in most filed at least 25 fields were scanned.
+1: One to 20 amastigotes in some but not all fields. At least 50 fields were scanned.
±: Very few amastigotes, but at least two were seen. Preparation was scanned for 5–10 min.
–: No amastigotes seen after 10 min search.

(c) Histopathological study: Portions of the cutaneous lesions from all groups were fixed in 10% formalin, embedded in paraffin and cut into sections of 5 μm each. They were stained with haematoxylin and eosin stain.

Results

In vitro antileishmanial assay: DSMO at a conc. of 0.5% didn't have any antileshmanial activity and therefore was used as a control for the in vitro study. All tested compounds that were dissolved in 0.5% DMSO showed antileshmanial effect at a conc. of 10 mmol .Immediate cessation of movement without change in morphology was seen immediately after the addition of the tested compounds, promastigotes exposed to all tested compounds were examined frequently every 10 min under LM for an hour, but no motility was seen and complete death occur.

In vivo antileshmanial assay

Monitoring of the cutaneous lesion of infected control groups (group Ia and Ib) showed redness and swelling of the inoculated footpad. The swelling was visible from the 7th day post infection and progressed gradually with time (Fig. 1) by the 5th week mice developed crust that increased progressively in size by time (Fig. 2a, b), later on gangrene started to develop in the crust and extended to the digits and foot to end by autoamputation of the inoculated footpad by the 8th week post infection (Fig. 3a–c). As regards animals treated with the nine tested compounds by local infiltration into the lesion, only those treated by compounds **3d**, **3e**, **3g**, and **3i** showed cessation of the clinical signs. The swelling and redness started to decrease by time without a need for another injection as clinically the footpad started to return back to the nearly normal appearance by the end of the experiment (Fig. 4).

On the other hand those treated with the rest of the compounds namely **3a**, **3b**, **3c**, **3f**, and **3h** showed progression of the cutaneous lesion but in slower rate than that of the control. At the end of the experiment, while infected

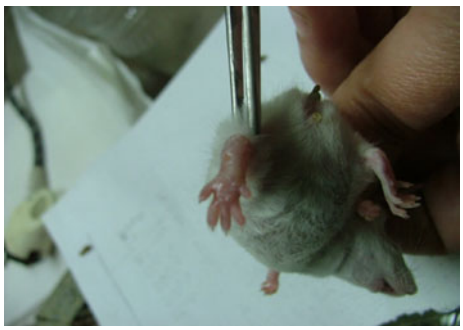


Fig. 1 Inoculated footpad of an infected mouse at the 3rd week post infection showing redness and swelling

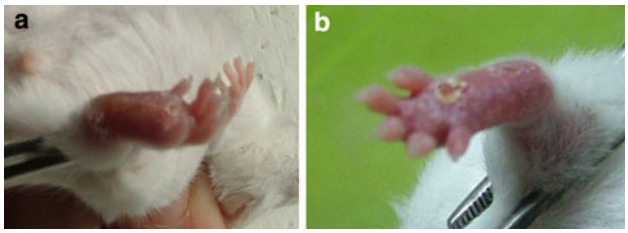


Fig. 2 **a**, **b** Inoculated footpad of infected mouse showing crust formation

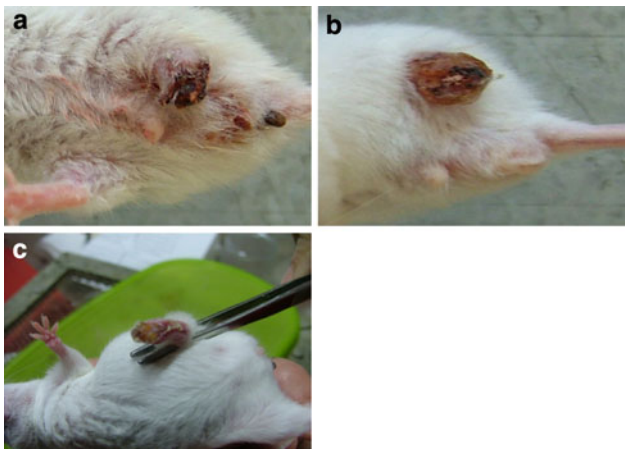


Fig. 3 **a**, **b**, **c** Inoculated footpad of an infected mouse at the 8th week post infection showing autoamputation of the inoculated footpad

untreated mice showed autoamputation of the inoculated footpad, those treated with compound **3c** showed only swelling and redness. Those treated with compound **3a**, the footpad turned black in color (Fig. 5). Mice treated with compound **3h** showed crust formation whereas compound **3b** and **3f** failed to cessate the lesion development and gangrene and autoamputation of the fingers of the inoculated footpad was observed (Figs. 6, 7). This is evident that these compounds were partially effective as they slower the process of leishmanial lesion development when compared to the infected control.

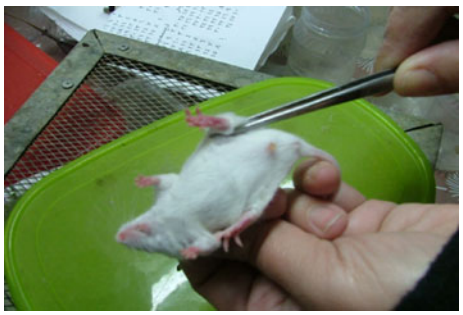


Fig. 4 Inoculated footpad of an infected mouse treated with compound **3g** at the end of the experiment showing almost normal appearance

Fig. 5 Inoculated footpad of an infected mouse treated with compound **3a** showing *black coloration* of the inoculate footpad

Fig. 6 Inoculated footpad of an infected mouse treated with compound **3f** at the end of the experiment showing autoamputation of the fingers

Fig. 7 Inoculated footpad of an infected mouse treated with compound **3f** at the end of the experiment showing autoamputation of the fingers

Parasitological study revealed living parasites in the cutaneous lesion of mice of both infected and infected treated group even those who received compounds **3d**, **3e**, **3g**, and **3i** and showed clinical cure as revealed by positive culture of parts of the footpad on Tanabe's medium and by impression smear. The grading of parasite density in the cutaneous lesion of the infected control group (group Ia and Ib) was +3 (Fig. 8). Compounds **3e**, **3g**, and **3i** succeeded to drop the grade to ± whereas compound **3d** reduces it to grade +1 (Fig. 9). As regards other tested compounds that failed to induce clinical cure the grade was +2 in groups treated with compound **3a** and **3c** grade +3 in groups treated with compound **3b**, **3f**, and **3h**.

Histopathological study of the cutaneous lesion of infected non-treated mice showed focal ulcer on the epidermis, which overlies connective tissue heavily infiltrated by acute and chronic non-specific inflammatory cells, predominately composed of macrophages, lymphocytes, and polymorphs with areas of necrosis (Figs. 10, 11) in the adjacent intact epidermis showed hyperkeratosis,

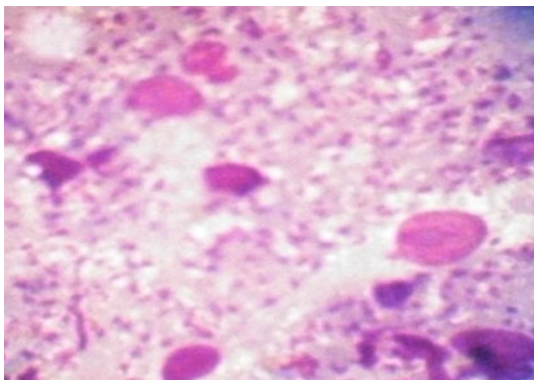


Fig. 8 Impression smear from cutaneous lesion of an infected mouse showing parasite density grade +3 (Giemsa ×1000)

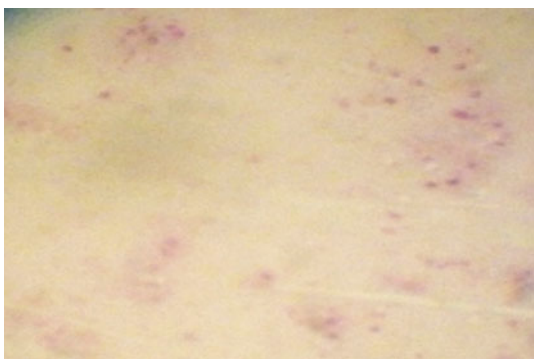


Fig. 9 Impression smear from cutaneous lesion of an infected mouse treated with compound **3d** showing parasite density grade +1 (Giemsa ×1000)

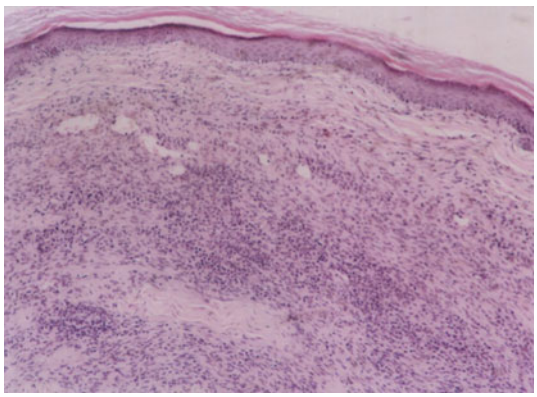


Fig. 10 Section in the skin lesion of infected mouse showing chronic inflammatory cellular infiltrate (H&E, ×100)

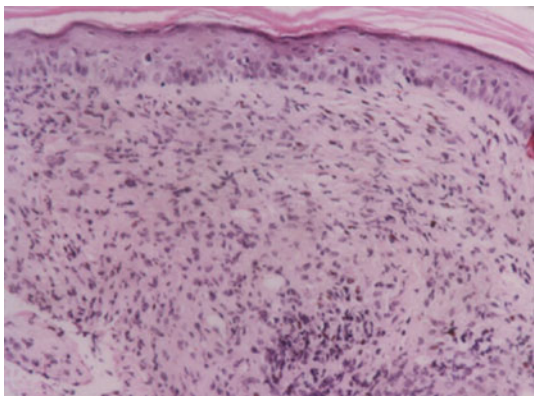


Fig. 11 Higher magnification of Fig. 10 (H&E, ×200)

acanthosis, and parakeratosis (Fig. 12). Parasites were found in large numbers in dermal macrophages, extracellularly and in epidermal cell (langerhans dendritic cells) (Figs. 13, 14). Gangrenous changes were seen. The inflammatory reaction involved the dermis and hypodermis. In animals that received compounds **3d**, **3e**, **3g**, and **3i**, the intensity of inflammatory reaction was remarkably diminished and leishmania parasites could hardly be detected. A striking feature in cutaneous lesions treated with compound **3d** and **3e** is the appearance of nest of epithelial invading the dermis (Figs. 15, 16), indicating sever irritation of the epidermis induced by these compounds. This finding were more apparent in mice treated with compound **3d** than **3e** and this finding raises the possibility of the carcinogenicity of these two compounds.

In compound **3g** and **3i** which induced clinical cure together with marked reduction of intensity of the inflammatory change, the epithelial changes were unremarkable thus these two compounds seems to be promising antileishmanial agents. As regards histopathological finding in groups received compounds **3a**, **3b**, **3c**, **3f**, and **3h** showed the same histopathological findings as the infected control group. Moreover, compound **3a** appeared to be toxic and

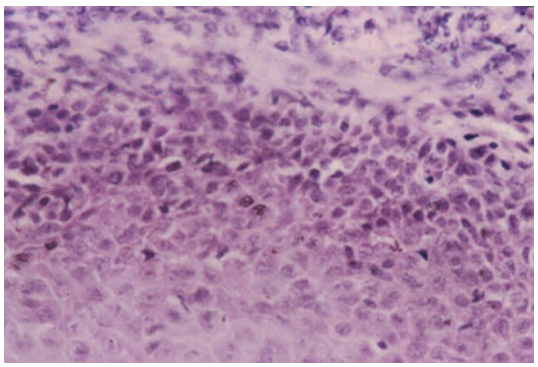


Fig. 12 Section in the skin lesion of infected mouse showing dysplastic changes in basal cell layer (H&E, ×400)

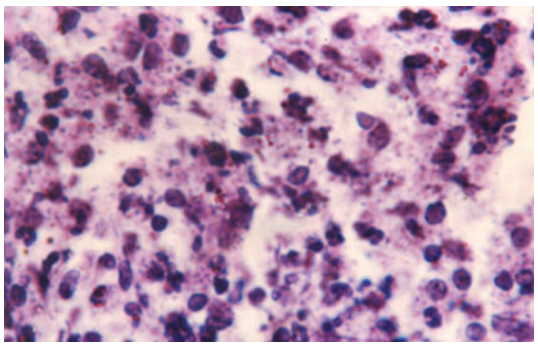


Fig. 13 Section in the skin lesion of infected mouse showing numerous *Leishmania* amastigotes throughout the lesion (H&E, ×1000)

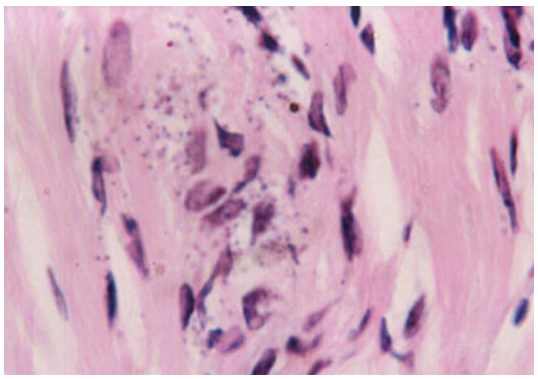


Fig. 14 Section in the skin lesion of infected mouse showing numerous *Leishmania* amastigotes throughout the lesion (H&E, ×1000)

potentially carcinogenic as there was nest of epidermal cells infiltrating the dermis, necrotic areas, subepidermal and intradermal vesicles were seen. In addition, there was vascular thrombosis with collection of haemosidrin in macrophages.

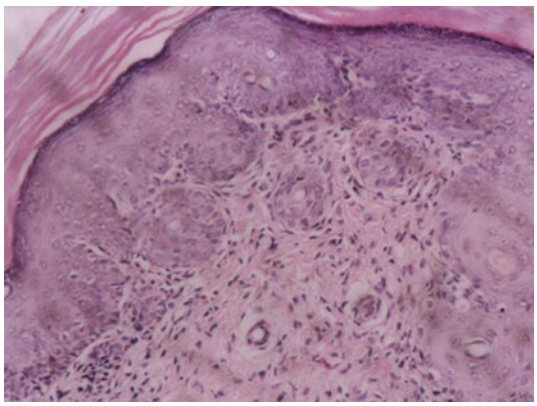


Fig. 15 Section in the skin lesion of infected mouse treated with compound **3d** showing downward growth of squamous epidermis into dermis (H&E, ×200)

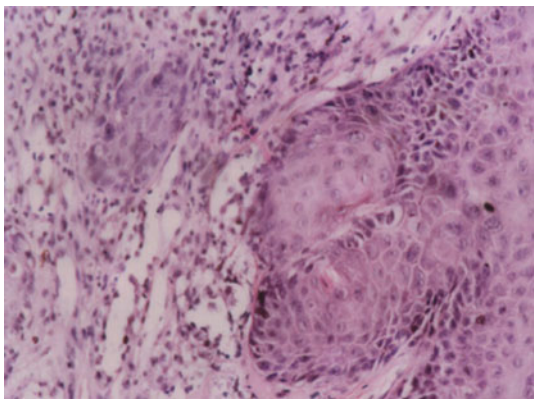


Fig. 16 Section in the skin lesion of infected mouse treated with compound **3d** showing downward extension of epidermal nests into the dermis with an intact basement membrane (H&E, ×400)

Discussion

To date, there are no vaccines against leishmaniasis, and chemotherapy remains the mainstay for its control. The drugs of choice used were significantly toxic, expensive, and with a growing frequency of refractory infections. Dramatic increase in the rates of infections, drug resistance, and the non-availability of safe vaccines has highlighted the need for identification for novel and inexpensive antileishmanial agents (Dutta *et al.*, 2007).

Efforts to find new chemotherapeutics for leishmaniasis have been ongoing for decades, and numerous potentially new drug candidates and/or putative drug targets have indeed been proposed (Croft and Yardley, 2002).

In this study, the antileishmanial activity of the novel compounds (**3a–i**) was evaluated both in vitro and in vivo against *L. major*. In both the assays, DMSO was used a solvent in a concentration of 0.5%. This concentration did not show any antileishmanial activity, similarly, to what was previously reported by Ma *et al.*, (2004) and Tiuman *et al.*, (2005).

In vitro results revealed that all tested compounds showed a dramatic and amazing antileishmanial activity with immediate cessation of parasite movement with no apparent morphological changes by LM. Elucidations of mechanism(s) by which these compounds act on *L. major*, and ultrastructural changes which could occur are points which require further investigations.

Numerous studies reported potent in vitro antileishmanial activity of several agents such as a hypocrellin A, two synthesized sterol families, crude ethanolic extract, lignoid fraction, and the purified compound yangambin (Ma *et al.*, 2004; Bazin *et al.*, 2006; Monte *et al.*, 2007). Such an activity justifies further in vivo antileishmanial evaluation. This is because not all compounds that show in vitro effect should show the same in vivo. Therefore, in this study, all the tested compounds that showed potent antileishmanial activity in vitro were subjected to in vivo evaluation in experimental animals and evaluated clinically, parasitological, and pathologically.

Clinical examination of infected untreated animals showed that in the inoculated footpad, leishmanial lesion developed and ends by autoamputation of the footpad. In contrast, animals that received compounds **3d**, **3e**, **3g**, and **3i** could achieve clinical cure. On the other hand, compounds **3a**, **3b**, **3c**, **3f**, and **3h** were partially effective, they showed different degrees of clinical presentation starting from swelling and redness in animals treated with compound **3c** to autoamputation in animals received compound **3b**.

Positive culture of parts of the footpad in both infected and all infected treated groups even those treated with **3d**, **3e**, **3g**, and **3i** showed clinical cure that was demonstrated. This finding coincide with that obtained by Hepburn *et al.* (1994) where it was possible that *Leishmania* present in clinically treated lesion, hence a post treatment culture is useful. This finding has an applicable medical importance, that patient treated with one of antileishmanial drug and showed marked clinical response, should be carefully examined for fear of the presence of viable parasite which may have important consequence if the patient subsequently becomes immunosuppressed.

In the in vitro study, inspite that all tested compound showed potent antileishmanial activity, four of them showed potent in vivo activity and reduced activity of the remaining five compounds. It has been suggested that reduced responsiveness inside the host is due to either drug bioavailability or host factors (Ismail *et al.*, 1992; Ismail *et al.*, 1999). It is also well known that the cutaneous lesion is insensitive even to the known standard drug as sodium stiboglucan (Evans *et al.*, 1989).

As regards the histopathological findings, H&E stained sections of cutaneous lesion of mice treated with compounds **3d**, **3e**, **3g**, and **3i** were markedly diminished. Compounds **3d** and **3e** showed epidermal changes indicating potential carcinogenicity of these compounds which need further investigations. On the other hand, compounds **3a**, **3b**, **3c**, **3f**, and **3h** showed the same histopathological findings of control group. Furthermore, compound **3a** appeared to be toxic as vascular thrombosis with collection of haemosidrin in macrophages was seen beside epidermal changes as what observed with compound **3d** and **3e** which raise its possibility of being also carcinogenic.

Thus it can be concluded that, in spite that all these compounds showed in vitro antileishmanial activity, in vivo activity was apparent only in compounds **3d**, **3e**, **3g**, and **3i**. Compounds **3d** and **3e** appeared to be potentially carcinogenic; however, compounds **3g** and **3i** appeared to be safe. Thus, further studies and restricted toxicological analysis should be performed on the promising compounds **3g** and **3i** for the possibility of their development as new potent antileshimanial agents.

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