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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

The cytotoxic effects of diketopiperaizes against *Leishmania donovani* promastigotes and amastigotes

Arindam Maity · Abhijit Hazra · Partha Palit · Shymal Mondal · Sanchaita Lala · Nirup B. Mondal

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Abstract A series of diketopiperazine derivatives (**1**–**12**) were evaluated for their in vitro cytotoxic activity against *Leishmania donovani* promastigotes and amastigotes. Cytotoxicity study revealed that the number and types of the substituents in the phenyl rings have valuable influence on cytotoxic activity. Compounds **1**, **3**, **4**, **11**, and **12** demonstrated appreciable cytotoxic activities with the mean IC_{50} values 1.4, 1.1, 1.02, 1, 0.7 μg/ml on extra cellular promastigotes and 1.6, 1.8, 0.61, 0.53, 1.1 μg/ml on intracellular amastigotes, respectively. The results suggested that diketopiperazine derivatives **4**, **11**, and **12** could be envisaged as new entrants in the domain of antileishmanial agents.

Keywords *Leishmania donovani* · Diketopiperazines · In vitro · In vivo studies

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Introduction

The protozoan parasites of the genus *Leishmania* (Chan-Bacab and Penia-Rodriguez, 2001) are the causative agents of the disease leishmaniasis (WHO, 2010). The disease manifests mainly in three clinical forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). It currently threatens about 350 million people in 88 countries, particularly in subtropical and tropical regions and is estimated to afflict 12 million people worldwide with 2 million new cases affected annually (WHO, 2002, 1999). The co-existence of human immunodeficiency virus (HIV) coupled with leishmaniasis has worsened the incidence more and more and contributed a new profile to the disease (Wolday *et al*., 1999; WHO, 1998).

The treatment of the disease was based on the pentavalent antimonials, pentamidine, and amphotericin-B as first and second line of drugs since their discovery in 1940s (Berman, 1997) and 1950s (Olliaro and Bryceson, 1993). The use of these drugs and their various formulations are associated with various toxic effects like cardiac abnormalities, hypotension, dysglycemia, and renal disfunction (Vande Wa and Tracy, 1993). Miltefosine, an oral alkyl phospholipid has shown promising results in the treatment of VL. But, it cannot be used in children and pregnant women as gastrointestinal toxicity and teratogenicity were evident from the clinical trials carried out in India (Jha *et al*., 1999; Sundar *et al*., 2000).

The other drugs employed for the treatment of leishmaniasis also suffer from various shortcomings, viz. unacceptable adverse effects, poor efficacy, limited accessibility due to high cost, and poor compliance, as they require parenteral administration and long treatment regimens (Nwaka and Hudson, 2006). Moreover, the emergence of drug resistance, particularly towards pentavalent antimonials in the Indian subcontinent (Peters, 1981), and

resurgence of VL with HIV as a co-infection (Savioli *et al*., 2006) has broaden the complexity of the treatment.

The matter of great concern is that the progress in antileishmanial drug discovery has never got momentum as the sufferers are the poorest of the poor, mostly of third world countries, and can not afford the cost for treatment. These afflicted people are being considered as neglected and consequently the disease has been branded as neglected disease by the West. The lack of profitability debars the pharmaceutical houses to give impetus (Pecoul, 2004) to antileishmanial drug discovery program.

These issues emphasize an imperative need to carry out drug discovery programs that would significantly accelerate and facilitate the identification of novel and safer chemotherapeutic agents against leishmaniasis.

In recent years, piperazine (O'Reilly *et al*., 2009, 2010) motifs are prevalent in scientific literature due to high number of positive hits encountered, in biological evaluation with this heterocycle and its congeners (Dinsmore and Beshore, 2002; Doemling, 2005). Among these, diketopiperazine (DKP) derivatives constitute a family of secondary metabolites that are produced by microorganisms (Lautru *et al*., 2002). Innumerable molecules bearing piperazine-2,5-dione-cycle have been isolated from culture and received much attention due to diverse and interesting biological activities (Wang *et al*., 1995; Ikeda *et al*., 1983; Chai *et al*., 1997). In recent years piperazine derivatives (Mayence *et al*., 2004) show good antileishmanial activity but interestingly, no detail studies have yet been appeared in the literature with DKPs. As a part of our continuation searches for antileishmanial agents (Chakrabarti *et al*., 1999; Pal *et al*., 2002; Sahu *et al*., 2002, 2004, Palit *et al*., 2012) the diversified biological activity of piperazine-2,5-dione derivatives intrigued us to explore the possibility of the privileged scaffolds for their antileishmanial activity. It is to be noted that we have reported the chemical synthesis of symmetrically 1,4-disubstituted piperazine-2,5-diones (Fig. 1) in a one-pot sequence (Hazra *et al*., 2007).

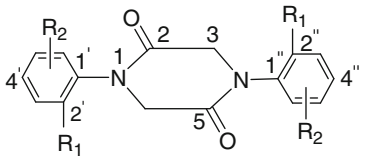
In this investigation, we disclose in vitro and in vivo evaluation of the synthesized products against *Leishmania donovani* promastigotes and amastigotes, the causative agent of leishmaniasis. To the best of our knowledge, this is the first detail report of DKP derivatives as antileishmanial agents.

Materials and methods

Chemistry

General method of preparation

The DKPs were prepared by self-condensation of differently substituted α-chlorophenyl acetamides in a one-pot sequence in presence of sodium hydride under nitrogen atmosphere. The substrate was taken in a three-necked reaction flask fitted with a condenser, a dropping funnel and a stopcock connected with nitrogen cylinder. Sodium hydride was taken in the reaction flask (half of the mole proportion of the substrate) and washed free from mineral oils by dry petroleum ether. Dry dimethylsulphoxide (DMSO) was added to the flask. Heating and stirring continued till the temperature reaches 40–42 °C. The substrate was then added and stirring continued for 1 h at 60 ± 5 °C. The reaction mixture was poured into ice-cold water, extracted with $CHCl_3$, washed free from alkali, dried over anhydrous sodium sulphate, concentrated under reduced pressure and crystallized from CH_3OH (Hazra *et al*., 2007).



1. R_1 = H, R_2 = H
2. R_1 = OMe, R_2 = 5-Cl
3. R_1 = H, R_2 = 4-Me
4. R_1 = Cl, R_2 = H
5. R_1 = Cl, R_2 = 3-Cl
6. R1 = Cl, R2 = 4-Cl
7. R1 = Cl, R2 = 5-Cl
8. R_1 = H, R_2 = 4-OMe
9. R_1 = h, R_2 =3-$COCH_3$
10. R_1 = Cl, R_2 =4,6-Cl
11. R_1 = H, R_2 =3-Cl

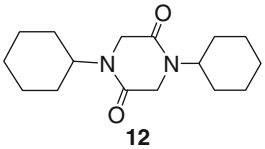


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Fig. 1 Diketopiperazine derivatives

Spectral data of the effective compounds

*1,4-Bis-(2-chlorophenyl)piperazine-2,5-dione (**4**)*

M.p. 134–136 °C. IR: 1694, 1594, 1536, 1445 cm^{-1}. NMR($CDCl_3$): ^1H NMR δ 4.34 (s, 4H, H-3 and 6), 7.09 (t, $J = 8$ Hz, 2H, H-4′, 4″), 7.30 (t, $J = 8$ Hz, 2H, H-5′, 5″), 7.38 (d, $J = 7.8$ Hz, 2H, 6′, 6″), 8.39 (d, $J = 7.8$ Hz, 2H, H-3′, 3″); ^{13}C spectra: ($CDCl_3$) δ 71.9 (t, C-3, 6), 122.0 (d, C-6′, 6″), 123.6 (s, C-2′, 2″), 125.7 (d, C-4′, 4″), 128.2 (d, C-3′, 3″), 129.5 (d, C-5′, 5″), 133.9 (s, C-1′, 1″), 166.4 (s, C-2, 5). MS:(MALDI-TOF, positive ion): *m/z* 373$[M+K]^+$.

*1,4-Bis-(-chlorophenyl)piperazine-2,5-dione (**11**)*

M.p. 234–236 °C. IR: 1671, 1576, 1523 cm^{-1}. NMR ($CDCl_3$): ^1H NMR δ 4.33 (s, 4H, H-3 and 6), 6.99 (d, $J = 7.8$ Hz, 2H, H-6′, 6″), 7.13 (d, $J = 7.8$ Hz, 2H, H-2′, 2″), 7.26 (m, 4H, H-4′, 4″ and 5′, 5″), ^{13}C spectra: ($CDCl_3$) δ 53.2 (t, C-3, 6), 118.7 (d, C-6′, 6″), 120.8 (d, C-2′, 2″), 124.6 (d, C-4′, 4″), 130.2 (d, C-5′, 5″), 134.1 (s, C-3′, 3″),

142.0 (s, C-1′, 1″) 166.3 (s, C-2, 5). MS:(MALDI-TOF, positive ion): *m/z* 373[M+K]$^+$.

*1,4-Dicylohexylpiperazine-2,5-dione (**12**)*

M.p. 224–226 °C. IR: 1636, 1472, 1450, 1324 cm^{-1}. NMR ($CDCl_3$): ^{1}H NMR δ 1.10–1.75 (m, 20H), 3.79–3.83 (m, 2H), 4.99 (s, 4H, H-3 and 6), ^{13}C spectra: ($CDCl_3$) δ 21.9 (t, C-3′, 3″ and 5′, 5″), 27.4 (t, C-4′, 4″), 30.2 (t, C-2′, 2″ and 6′, 6″) 46.2 (d, C-1′, 1″), 50.1 (t, C-3, 6), 169.3 (s, C-2, 5). MS:(MALDI-TOF, positive ion): *m/z* 317[M+K]$^+$.

Parasite culture and growth conditions

Leishmania donovani strain AG 83 was originally obtained from an Indian kala-azar patient (Ghosh *et al*., 1983) and maintained in golden hamsters. Amastigotes were isolated from spleens of *L. donovani* infected golden hamsters as described (Jaffe *et al*., 1984). The spleen was rinsed in ice-cold phosphate-buffered saline (PBS), glucose (55 mM)/EDTA (2 mM), lightly homogenized, macroscopic particles were allowed to settle. The turbid suspension was decanted, centrifuged at 100 g for 10 min at 4 °C. The amastigote-enriched suspension was centrifuged at 800×*g* for 10 min. The pellet was suspended in 45 % Percoll (8.0 ml), and finally 25 % Percoll (4.0 ml) was layered over the amastigote suspension and further centrifuged at 5,000×*g* for 1 h. The band containing amastigotes was taken and washed with PBS (3×) and finally resuspended in Medium-199 (Gibco Laboratories, New York, NY, USA), supplemented with 20 % FBS. Promastigotes were obtained by transforming amastigotes and were maintained in vitro in medium-199 supplemented with 8 % FBS.

In vitro growth of *L. donovani* promastigotes in the presence of diketopiperazines (**1–12**)

Promastigotes (2 × 10^6/ml) were incubated with or without various concentrations of the compounds along with standard antileishmanial drugs in Medium-199 (1.0 ml) supplemented with 8 % FBS at 22 °C for 12 h. Growth of promastigotes was monitored spectrophotometrically at 570 nm using MTT assay. The % of lysis of promastigotes was calculated as described by Sahu *et al*. (2002).

In vitro infection of BALB/c mice peritoneal macrophages and antileishmanial activity of diketopiperazines (**1–12**) on intracellular amastigotes

Thioglycolate-elicited peritoneal exudate was used as the source of macrophages for better recovery and easier isolation. Approximately 2 × 10^6/ml macrophages were allowed to adhere to glass cover slips (20 × 3.25 mm^2) in RPMI-1640 (Gibco Laboratories) supplemented with 10 % FBS and cultured for overnight at 37 °C in 5 % CO_2 before in vitro infection with *L. donovani*. Stationary phase *L. donovani* promastigotes (2 × 10^7/ml) were added to each cover slip and incubated for 6 h at 37 °C in 5 % CO_2. Cover slips were washed (3×) with 10 % FBS-supplemented RPMI-1640 to remove uningested parasites and incubated for 2 days in the presence or the absence of graded concentrations of 1, 3 and 10 μg/ml DKP analogues. Infected macrophage cultures were washed with PBS, fixed with methanol in slides, stained with Giemsa, and examined microscopically under oil immersion. At least 200 target macrophages were examined for each cover slip. Antileishmanial activity was determined by calculating the number of amastigotes per 200 macrophages in comparison to untreated control. All 50 % inhibitory concentrations (IC_{50}s) of these derivatives against promastigotes and amastigotes were determined by linear regression from the percentages of killing compared to untreated vehicle controls (0.5 % DMSO).

In vivo assessment of antileishmanial activity of compounds **1**, **3**, **4**, **11,** and **12**

A group of five BALB/c mice (4–6 weeks) each were injected intravenously with freshly transformed promastigotes of *L. donovani* (2 × 10^7/mouse). After 1-month postinfection, the infected BALB/c mice were treated with **11** at a dose of 4.5 and 10 mg/kg body weight (doses were chosen as 1/22nd and 1/10th, respectively, of LD50 value of compound **11**, data not shown) intraperitoneally two times weekly for 1 month. Each mouse received a total of eight intraperitoneal administrations of compound **11** for 1 month. Mice in the untreated group were received vehicle control (0.2 % Tween 80 in PBS) by the same route. The activity was compared with the standard antileishmanial drug sodium antimony gluconate, which also treated with another group of mice at a dose of 250 mg/kg body weight. All the mice in different groups were sacrificed on 1 month posttreatment. The splenic and liver parasites were determined by impression smear of Giemsa staining. Levels of organ parasite load were determined and expressed as the total parasite burden per organ, using the formula (Stauber *et al*., 1958): (organ weight in mg × the number of amastigotes per cell nucleus × 2 × 10^5).

Serum enzyme assay

The blood sera of normal mice and mice receiving treatment with compounds **1**, **3**, **4**, **11**, and **12** were collected

two times per week for 4 weeks, were subjected to estimate for the enzymes serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) and blood urea, 1-month posttreatment. These enzymes and blood urea were assayed using the kits from Dr. Reddy's Laboratories (Hyderabad, India) following the manufacturer's protocol. Blood urea activity was expressed as mg/dl, whereas SGPT and SGOT activities were expressed as IU/l.

Results and discussion

Antileishmanial activity

All the 12 DKP analogues were tested for cytotoxicity against both extra cellular promastigotes and intracellular amastigotes forms of the parasite *L. donovani* (MHOH/IN/1983/AG83), originally obtained from Indian Kala-azar patient (Ghosh *et al*., 1983) using the MTT assay and giemsa staining counting method. Amphoteracin B was used as the standard.

Effects of diketopiperazine derivatives on the growth of *L. donovani* promastigotes in vitro

The Logarithm phase promastigotes (2×10^6/ml) were incubated with or without various compounds. The compounds were dissolved in 0.2 % DMSO and given to the culture at graded dose and treatment was made for 2 h. The % of lysis was calculated as described by Tim Mosmann (1983). The IC_{50} value of the compounds was evaluated by linear regression analysis method using Graphpad prism 3 software. The results are summarized in Table 1. The IC_{50} values (Table 1) indicated that most of the compounds are more or less cytotoxic to the promastigote forms of the parasite except compound **2**. However, compounds **1**, **3**, **4**, **11**, and **12** showed strong cytotoxic effect on the promastigotes.

Effects of diketopiperazine derivatives in destroying intracellular amastigotes of *L. donovani* in vitro

The protozoan parasites *L. donovani* survive and multiply within mammalian macrophages. It is, therefore, of interest to test the efficacy of the compounds on intracellular amastigotes. Peritoneal macrophages of BALB/c mice were infected with *L. donavani AG 83* promastigotes (Dey *et al*., 2000) in vitro. Infected macrophages after subsequent washings were incubated with different concentrations of the compounds. Similar to their effect on promastigotes, compounds **4**, **11**, and **12**, at a concentration of 10 μg/ml, reduced the parasite burden by 92, 92.19, and 84.66 %, respectively, after 48 h where as, compounds **1**, **3**, **8**, and **9** showed less efficacy, and compound **2** was found to be ineffective in reducing intracellular parasite burden at the same dose, compared with medium or 0.2 % DMSO. The IC_{50} values were calculated by linear regression analysis method using Graphpad prism 3 software. The results are summarized in Table 1.

The results suggested that **4**, **11**, and **12** exhibited promising activity against pathogenic strain *L. donovani* AG 83 promastigotes and amastigotes in a dose dependent manner. The IC_{50} values of **4**, **11**, **12**, and amphotericin-B against intracellular amastigotes were 0.61, 0.53, 1.1, and 0.1 μg/ml, respectively, indicating the compounds could be exploited as antileishmanial chemotherapeutic agents.

Table 1 In vitro antileishmanial studies of the diketopiperazine analogues (**1**–**12**) and the reference drugs pentamidine and amphotericin-B; through MTT assay on promastigotes; and on intracellular amastigotes within murine peritoneal macrophages

Compounds	IC_{50} μg/ml[a] (promastigotes) through MTT assay	IC_{50} μg/ml[a] (amastigotes)	M.T (μg/ml)[b] (amastigotes)
1	1.4	1.6	>30
2	NA	NA	>100
3	1.1	1.8	>40
4	1.02	0.61	>25
5	2.2	4.3	>50
6	2.9	6.4	>35
7	2.6	6.6	>60
8	21.8	2.2	>60
9	3.4	2	>50
10	9.4	>10	>70
11	1.0	0.53	>25
12	0.7	1.1	>50
Pentamidine	2.1	2.8	>90
Amphotericin-B	0.16	0.1	>4

MTT assay for *Leishmania donovani* promastigotes (MHOH/IN/1983/AG83) lysis by different compounds; % of lysis on promastigotes was evaluated using the MTT assay with comparison to untreated 0.2 % DMSO control. All assays were carried out in triplicate. Thioglycolate-elicited peritoneal macrophages of BALB/c mice were infected in vitro with *L. donovani* promastigotes and after conversion into amastigotes, the macrophage culture was incubated with graded concentrations of the compounds (dissolved in 0.2 %DMSO) for 2 days at 37 °C for the determination of anti-leishmanial activity on intracellular amastigotes

NA Not active

[a] IC_{50} are the sample concentrations that kill 50 % cells compared to solvent controls

[b] MT are the sample concentrations that destroy the host macrophage, indicates the toxic dose

In vivo antilishmanial activity in established visceral leishmaniasis on BALB/C mice model

The in vitro studies results of compounds **1**, **3**, **4**, **11**, and **12** prompted us to investigate the antileishmanial activity in established VL on BALB/c mice model through intraperitoneal administration. BALB/c mice were infected with *L. donovani* as described (Sahu *et al*., 2002). After 1 month, groups of four mice were treated with phosphate-buffered saline (PBS), sodium antimony gluconate (SAG) or compounds **1**, **3**, **4**, **11**, and **12** (intraperitoneally). The parasite load in the spleen and in the liver was determined as described (Chakrabarti *et al*., 1999). The treatment led to significant reduction of parasite burden in spleen 91.35 % ($P < 0.0001$) and 67.97 % ($P < 0.0001$) and in liver 94.75 % ($P < 0.0001$) and 69.73 % ($P < 0.0001$), compared to untreated controls, 30 days post intraperitoneal treatment with **11** at a dose of 10 and 4.5 mg/kg body weight, respectively. Compounds **4** and **12** had also rendered appreciable results by reducing 71.81 and 80.95 % splenic and 80.37 and 81.74 % liver parasite load, respectively, in experimental mice. Dose of 250-mg/kg body weight of SAG was able to reduce the parasite burden from spleen and liver only by 60.16 and 57.73 %, respectively.

Hence, both the doses of compounds **4**, **11**, and **12** were much more effective compared to the standard drug sodium antimony gluconate and other DKP analogues in eradicating the parasite load from the spleen and liver. Moreover, treatment with **4**, **11**, and **12** showed significant decrease in weights of the spleen and liver too, compared to untreated controls (Table 2).

To check the liver and kidney function, the specific serum enzyme and blood urea levels of normal mice receiving treatment with the active compounds were analyzed and the results are shown in Fig. 2.

The levels of both SGPT and SGOT in mice upon the active compounds (Table 3) approach more or less normal values with respect to untreated control. Blood urea level after treatment with different active compounds is within the normal acceptable range. It was evident that the values of serum enzyme levels of treated mice are almost lower than the untreated normal mice. The results revealed that compound **11** is nontoxic to kidney and liver up to a dose of 10 mg/kg body weight intraperitoneally.

The structure–activity relationship of the diketo piperazines as revealed from the studies indicate that the cytotoxicity was strongly influenced by the nature of the substituent on the phenyl rings. The presence of halogen in the aromatic rings has a great impact on cytotoxicity in both forms of parasites. But surprisingly, the presence of more than one halogen in aromatic rings (**5**, **6**, **7**, and **10**) has decreased the efficacy. However, the reason of remarkable activity of compound **12**, which lacks of aromaticity and also of any substituent effect, is obscure to us.

Table 2 Effect of compounds **1**, **3**, **4**, **11**, **12** on weight of spleen and liver after 1 month intraperitoneal treatment

Compounds	Dose (mg/kg body wt)	Spleen wt (mg) after treatment (±SE)	Liver wt (mg) after treatment (±SE)
Untreated infected control	_	509 ± 25	1,746 ± 56
1	10	166 ± 12.7	999 ± 25.9
3	10	184 ± 19.5	1,127 ± 30.4
4	10	106 ± 9.5	976 ± 19.7
11	10	87 ± 8.5	89 ± 32.6
11	4	155 ± 10.5	1,134 ± 47.6
12	10	139 ± 24.6	1,076 ± 46.05
Sodium antimony gluconate	250	303 ± 14.67	1,317 ± 45.1

Statistical significance compared to untreated mice is indicated as follows: *** $P < 0.001$ significant versus untreated controls (analyzed by unpaired student t test). Values are mean ± standard error of results for mice

Table 3 Serum clinical chemistry evaluations

	Dose (mg/kg)	SGPT (IU/liter)	SGOT (IU/liter)	Blood urea (mg/dl)
Vehicle		28 (5)	71 (4)	29 (6)
1	0	34 (2)	74 (3)	32 (5)
3	10	33 (6)	75 (2)	34 (4)
4	10	28 (5)	72 (5)	30 (7)
11	10	28 (4)	67 (5)	29 (3)
11	4	29 (7)	69 (6)	28 (1)
12	10	30 (3)	73 (4)	31 (5)

Values are means (standard deviations) of four animals

SGPT serum glutamate pyruvate transaminase, *SGOT* serum glutamate oxaloacetate transaminase

Conclusion

In conclusion, DKPs are new additions in the domain of antileishmanial chemotherapeutic agents. The antileishmanial activity on intracellular amastigotes of compounds **4**, **11**, and **12** was quite promising in a dose-dependent manner. The indicated effective dose of the above compounds was proved to be nontoxic by MTT assays. The DKP nuclei could serve as a lead molecule for further modification to obtain a clinically useful novel class of antileishmanial agents.

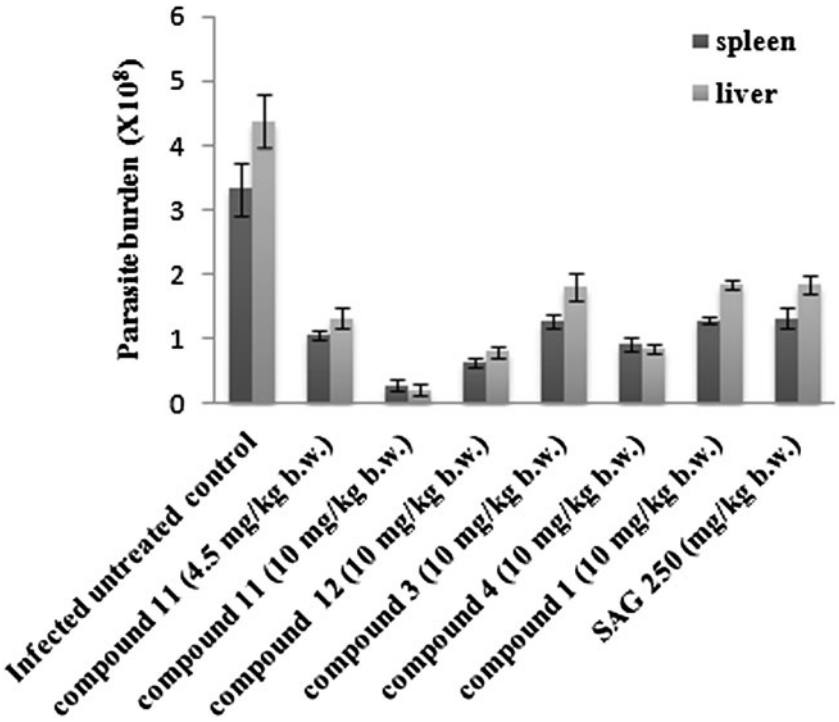

Fig. 2 Antileishmanial activity of compounds **1**, **3**, **4**, **11**, and **12** in vivo against established VL infection by *L. donovani* AG83 in BALB/c mice. One-month postinfection, infected mice were treated with compound **11**, intraperitoneally at a dose of 4.5 and 10 mg/kg body weight and compounds **1**, **3**,**4**, and **12** at 10 mg/kg body weight in 0.2 % Tween 80 in PBS (0.02 M), two times weekly for 1 month. Another group of mice were treated with standard antileishmanial drug sodium antimony gluconate at a dose of 250 mg/kg body weight intraperitoneally two times weekly for 1 month. Control untreated group received only 0.2 % Tween 80 in PBS (0.02 M). Mice were sacrificed 4 weeks posttreatment. Levels of parasite burden in spleen and liver were counted from impression smears after Giemsa staining and expressed as the total parasite load per organ, using the formula (organ weight in mg $\times$ the number of amastigotes per cell nucleus $\times$ 2 $\times$ 10^5). Values represent the mean $\pm$ SE of five animals

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