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ORIGINAL ARTICLE

In vitro screening of natural product-based compounds for leishmanicidal activity

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Abstract

Leishmaniasis is one of the major parasitic diseases, caused by obligate intracellular protozoa *Leishmania,* having high mortality as well as morbidity rate. As there is no human licensed vaccine available against leishmaniasis, chemotherapy remains the major way of combating this disease. Many disadvantages are known to be associated with the current drug regime including severe side effects and toxicity, long duration and expensive treatment, and the emergence of resistance. An alternative approach is being utilized to search for active molecules using natural sources, rather than relying on synthetic drugs. Many plant-derived secondary metabolites like phenolic compounds, steroids, quinones, etc. are being extensively investigated for their anti-leishmanial potential. One such group of complex phenolic compounds are diarylheptanoids. These compounds have been shown to exhibit anti-inflammatory, anti-parasitic, anti-fungal, and other pharmacological activities. In the present study, a set of sixteen tetrahydropyran derivatives including three natural products were obtained in lyophilized form. These compounds with *trans*-2,6-disubstituted tetrahydropyrans, Diospongin A, Diospongin B (isolated from *Dioscorea spongiosa*) and Centrolobine (*Centrolobium sclerophyllum)* as parent compounds were synthesized by the reaction of 1-phenyl-1-triemthylsiloxyethylene with six-membered cyclic hemiacetals in the presence of iodine as a catalyst. All the sixteen synthesized tetrahydropyran derivatives were used for toxicity analysis against *L. donovani* promastigotes, amastigotes and THP-1-derived human macrophages. IC_{50} values and selectivity index were calculated for all the compounds. Out of these sixteen, five compounds showed the best effect in vitro in terms of both leishmanicidal activity and non-toxicity to human macrophages.

Keywords *Leishmaniasis* · Teterahydropyran derivatives · Phenolic diarylheptanoid · Cytotoxicity

Abbreviations

CL	Cutaneous leishmaniasis
VL	Visceral leishmaniasis
MCL	Mucocutaneous leishmaniasis
PKDL	Post kala-azar dermal leishmaniasis
L. donovani	*Leishmania donovani*
L. major	*Leishmania major*
L. amazonensis	*Leishmania amazonensis*
THP-1	Tohuku Hospital Pediatrics-1
DMSO	Dimethylsulfoxide
FBS	Fetal Bovine Serum
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SDS	Sodium Dodecyl Sulphate
PMA	Phorbol-12-myristate-13-acetate
IC_{50}	Inhibitory concentration 50%
WHO	World Health Organization

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Introduction

Leishmaniasis is one of the many neglected tropical diseases with high rates of mortality and morbidity. It is caused by an obligate intracellular protozoan *Leishmania*, belonging to the family Trypanosomatidae. Transmission of this disease occurs by the bite of the infected female phlebotomine sandflies belonging to the genera *Phlebotomus* (old world) and *Lutzomyia* (new world). Different species of *Leishmania* are the causative agents of the four major clinical forms of the disease namely: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL; renowned in the Indian subcontinent as kala-azar) and post kala-azar dermal leishmaniasis (PKDL), with VL being the most fatal form if left untreated (Alvar et. al. 2012).

Chemotherapeutic agents remain the major means to combat leishmaniasis because of the unavailability of any human licensed vaccines against this disease (Mohapatra 2014). The first line of drugs recommended against *Leishmania* are pentavalent antimonials like sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) but these are now being used restrictively due to the emergence of resistance against them and their severe side effects (Burza et al., 2018). This has led to the administration of a second line of drugs. Amphotericin B or its lipid formulations had been recommended and were found to be effective but due to toxicity and expensive costs, its use in developing countries is restricted. Miltefosine had been registered as the first orally administered drug against leishmaniasis in India in 2002 and has been found to be highly effective with ~98% cure rate but was found to be teratogenic, thus preventing its administration in pregnant women (Maltezou 2010; DNDi 2016). Also, long half-life of miltefosine (150 h) along with long administration duration might result in the emergence of resistance against this drug. Paromomycin which is an aminoglycoside antibiotic has also shown promising anti-leishmanial activity, however in vitro studies have already reported emergence of resistance against this drug along with side effects (Jhingran et al. 2009; den Boer and Davidson, 2006, DNDi 2016).

The above-mentioned factors, namely, increase in the drug resistance, severe toxic side effects, high cost making their use in resource-limited countries restrictive, and the emergence of *Leishmania* coinfections, have led to the search for identification of new drugs and drug targets to eliminate the pathogen (Alvar et al. 2008).

An alternative approach to the search for active molecules apart from synthetic drugs is by using natural sources. Plants have been since long considered a major source of biologically active extracts, essential oils and isolated substances, active against various diseases like microbial and protozoal infections, carcinomas, diabetes, and inflammatory reactions (Harvey 2008). In many resource-limited countries traditional plant-based medicines against parasitic diseases are gaining preference over synthetic drugs due to their accessible nature (Calla-Magarinos et al. 2009).

Plant based products are currently gaining more attention because of their ability to act directly against the pathogens along with acting as immunomodulators (Cragg and Newman 2013). A few examples of the active agent obtained from medicinal plants which are being utilized as chemotheurapetic agents are artemisinin (isolated from *Artemisia annua*) being used for the treatment of malaria, camptothecin (isolated from *Camptotheca acuminata*), vinblastine and vincristine (isolated from *Catharanthus roseus*) and paclitaxel (isolated from *Taxus brevifolia*) being used against cancer.

Many plant-derived secondary metabolites like phenolic compounds, coumarins, alkaloids, etc. are being extensively investigated for their anti-leishmanial potential (Rodrigues et al. 2015; Goncalves de Olievera et al. 2017). Out of these, few alkaloids which have been extracted from various plant species have managed to show significant leishmanicidal activity in vitro conditions. Indole alkaloids (corinanteine, dihydrocorinanteine and corinanteidine), which were isolated from *Corynanthe pachyceras*, were found to be active against *L. major* with an IC_{50} of 30 µM. Coronaridine isolated from *Peschirea australis*, had an IC_{50} of 12 µg/ml against *L. amazonensis*, while other indole alkaloids, like harmane, pleiocarpin and buchtienin, which in turn were obtained from the bark and leaves of *Kopsia griffithii*, were found to be active against the promastigote form of *L. donovani*, with an IC_{50} of 6.25 µg/ml, 25.00 µg/ml and 1.56 µg/ml, respectively (Mishra et al. 2009; Polonio and Efferth 2008; Singh et al. 2014; Delorenzi et al. 2001). The steroidal saponin Racemoside A which was isolated from *Asparagus racemosus* was found to induce apoptosis in *L. donovani* promastigotes and amastigotes with IC_{50} values of 1.31 µg/ml and 0.61 µg/ml, respectively (Polonio and Efferth 2008).

One such group of complex phenolic compounds, known to possess a myriad of pharmacological activities, and are isolated from different parts of plants belonging to Myricaceae, Betulaceae, Zingiberaceae, Aceraceae, Leguminosae and Burseraceae are diarylheptanoids (Per et al. 2002; Kawai et al. 2008; Ibrahim et al. 2017). Their structure consists of two aromatic rings conjugated with seven carbon chains (Brand et al. 2006; Amalraj et al. 2017). Diarylheptanoids isolated from *Alnus glutinosa* were found to protect non-cancerous dividing cells during cancer treatment (Dinić et al. 2015).

The present study deals with the in vitro screening of a set of sixteen natural product-based compounds for

leishmanicidal activity. Both *L. donovani* log-phase promastigotes and amastigotes were cultured in the presence of these compounds to check the effect on their viability. THP-1-derived human macrophages were also cultured with these compounds to determine the toxic effect on the survival of the macrophages. Selectivity index was also calculated for all sixteen inhibitors. Out of sixteen compounds, five compounds showed the best results on the basis of inhibitory effects on *L. donovani* promastigotes and intracellular amastigotes and survival of THP-1-derived macrophages.

Materials and methods

Strains and culture conditions

L. donovani Bob (*Ld*Bob; a derivative of the strain MHOM/SD/62/1SCL2D) promastigotes, originally obtained from Stephen Beverley (Washington University, St. Louis, MO) were cultured at 22 °C in M199 medium (Sigma) and were supplemented with 100 µg/ml of streptomycin, 100 units/ml of penicillin (Sigma-Aldrich, USA) and 10% heat-inactivated fetal bovine serum (FBS; Biowest, UK).

THP-1 cells (Tohuku Hospital Pediatrics-1; acute monocytic leukemia-derived human cell line; 202 TIB) are a human monocytic cell line obtained from ATCC (Rockville, MD). These cells were cultured in RPMI-1640 medium (Sigma-Aldrich, USA) which was also supplemented with 10% heat-inactivated FBS (Biowest, UK), 100 µg/ml of streptomycin and 100 units/ml of penicillin (Sigma-Aldrich, USA), and in turn maintained at 37 °C with 5% CO_2.

Drugs

A set of sixteen compounds (tetrahydropyran derivatives) were obtained from Dr. Debendra K. Mohapatra (CSIR-Indian Institute of Chemical Technology, Hyderabad) in lyophilized form. The compounds were reconstituted by dissolving in dimethylsulfoxide (DMSO, HiMedia) to get the desired concentration (Table 1). Diospongin A, Diospongin B, Centrolobine are naturally isolated from *Dioscorea spongiosa* and *Centrolobium sclerophyllum*, respectively. These three natural compounds served as parent compounds for the synthesis of the tested tetrahydropyrans.

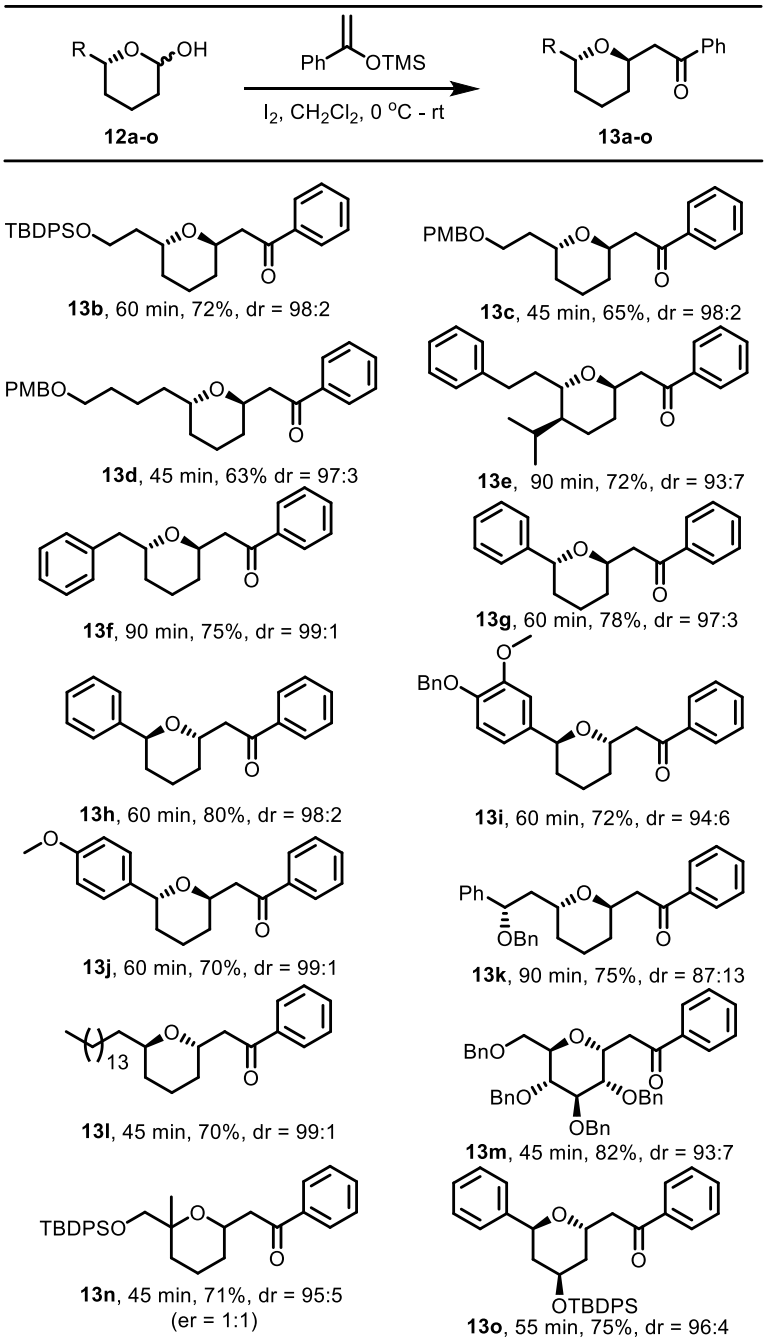
An effective synthetic protocol for the synthesis of *trans*-2,6-disubstituted tetrahydropyrans was developed by following Mukaiyama type aldol reaction through C–C bond formation of a cyclic hemiacetal with trimethyl(1-phenylvinyloxy)silane as a nucleophile via the formation of an oxocarbenium ion intermediate using molecular iodine (Table 2). The protocol offers an efficient, protective group tolerance and practical approach to 2,6-*trans*-disubstituted tetrahydropyrans with good diastereoselectivity. The effectiveness and practicality of the method used was successfully exhibited by the production of diospongin A and B in good yields (Bharath et al. 2019). Centrolobine was also synthesized by following the above protocol.

All sixteen synthesized tetrahydropyrans were tested for toxicity analysis against *L. donovani* promastigotes, intracellular amastigotes and THP-1-derived human macrophages.

Table 1 List of sixteen compounds with their chemical formula, molecular weight and concentration of the stock solution obtained after reconstitution with DMSO

S. No	Compound	Chemical formula	Molecular weight	Concentration (mg/ml)
1	DKM-B-BRAT	$C_{27}H_{28}O_4$	416	4
2	DKM-B-VIAT	$C_{23}H_{28}O_4$	368	4
3	DKM-B-DIOB	$C_{19}H_{20}O_3$	296	2
4	DKM-B-INACT	$C_{31}H_{38}O_3Si$	486	6
5	DKM-B-ANAT	$C_{20}H_{22}O_3$	310	6.2
6	DKM-B-CYL	$C_{35}H_{38}O_3Si$	534	10
7	DKM-B-DIOA	$C_{19}H_{20}O_3$	296	2.5
8	DKM-B-EPI	$C_{20}H_{24}O_3$	312	1.5
9	DKM-B-CENT	$C_{20}H_{24}O_3$	312	2
10	DKM-B-LCAT	$C_{28}H_{46}O_2$	414	5.6
11	DKM-B-RBAT	$C_{19}H_{20}O_2$	280	5
12	DKM-B-PHAT	$C_{20}H_{22}O_2$	294	6.8
13	DKM-B-IVAT	$C_{24}H_{30}O_2$	350	6.7
14	DKM-B-PHOBN	$C_{28}H_{30}O_3$	414	10
15	DKM-B-SBAT	$C_{19}H_{20}O_2$	280	9
16	DKM-B-TOBN	$C_{42}H_{42}O_6$	642	7

Table 2 Representing substrate scope[a] towards the generation of tetrahydropyran derivatives



[a]Reaction conditions: Lactol (0.2 mmol), trimethyl(1-phenylvinyloxy)silane (2.0 equiv) and I_2 (10 mol%) in CH_2Cl_2 (2 mL). [b]Isolated yields

In vitro inhibitor susceptibility assay using *L. donovani* promastigotes

In order to establish the susceptibility profile of wild-type promastigotes of *L. donovani* to the above-mentioned inhibitors, MTT assay[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] was performed as described previously (Mosmann1983). Log-phase promastigotes were seeded in a 96-well flat-bottom plate (Nunc) at the cell density of 5×10^4 cells/well, and were in turn, incubated with increasing inhibitor concentrations (5, 10, 20, 40, 80, 160 µg/ml) at 22 °C. Amphotericin B was added as a positive control at the concentration of 0.5 µg/ml, to the promastigotes. After 48 h of incubation, 20 µl of MTT solution (Stock solution—5 mg/ml) was added to each well, at a final concentration of 0.5 mg/ml. This was followed by incubation

of plates at 37 °C for 4 h. The reaction was finally stopped by adding 50 µl of stop solution, which consisted of 50% isopropanol and 20% SDS. This was followed by incubation at 37 °C with gentle shaking for 30 min to 1 h. The absorbance at 570 nm was then, measured in a SpectraMax M2 microplate reader (Molecular Devices). All the experiments were performed in triplicates and the results were expressed as mean ± standard deviation (SD). IC_{50} values were calculated using GraphPad Prism Version 5 software.

In vitro inhibitor susceptibility assay using THP-1 derived human macrophages

In order to establish the susceptibility profile of THP-1 derived human macrophages to the above-mentioned inhibitors, MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] was performed as described previously (Mosmann,1983). THP-1 cells (plated at cell density of 6×10^3 cells/well) were first treated with phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, USA) at the concentration of 50 ng/ml for 48 h to induce their differentiation into macrophage-like cells prior to initiation of drug treatment. These cells were seeded in RPMI-1640 medium containing 10% FBS in a 96-well flat-bottom plate (Nunc). PMA treated cells were then incubated with different inhibitor concentrations (5, 10, 20, 40, 80, 160 µg/ml) at 37 °C in a humidified CO_2 incubator for 48 h. MTT solution was prepared in RPMI-1640 medium (5 mg/ml MTT stock solution diluted appropriately in RPMI-1640 medium to achieve the final dilution of 0.5 mg/ml). After incubation of 48 h, 50 µl of MTT solution was further added to each well. This was followed by incubation of the plates at 37 °C for 2 h. The reaction was finally terminated by adding 100 µl of stop solution (5% formic acid in isopropanol). This was followed by incubation at 37 °C with gentle shaking for 30 min. The absorbance at 570 nm was then measured in a SpectraMax M2 microplate reader (Molecular Devices). All the experiments were performed in triplicates and the results have been expressed as mean ± standard deviation (SD). IC_{50} values were calculated using GraphPad Prism Version 5 software.

In vitro inhibitor susceptibility assay using *L. donovani* amastigotes in infected macrophage model

The susceptibility of wild-type *L. donovani* amastigotes to the compounds was ascertained by microscopic visualization of infected THP-1 derived human macrophages at 48 h after treating these cells with increasing concentrations of the inhibitors and subsequent calculation of the intracellular parasite load by Giemsa staining. THP-1 cells (plated at cell density of 10^6 cells/ml) were first treated with phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, USA) at the concentration of 50 ng/ml for 48 h to induce their differentiation into macrophage-like cells prior to initiation of drug treatment. These cells were grown on cover-slips in 6-well plates in RPMI-1640 medium containing 10% FBS. The matured adherent cells were then infected with late log-phase *L. donovani* promastigotes at a MOI (multiplicity of infection) of 20:1 for 4 h. All the excess non-adherent promastigotes were removed by washing the cells with phosphate-buffered saline (PBS)for 30 s. These cells were subsequently maintained in RPMI-1640 medium containing 10% FBS with 5% CO_2 at the temperature of 37 °C and then incubated with different concentrations of the inhibitors. After 48 h, the cells were first washed with PBS and then fixed with 100% methanol (Merck) for 15 min. To visualize the intracellular parasite load Geimsa staining was performed. The experiments were performed in triplicates and the results have been expressed as mean ± standard deviation (SD). IC_{50} values were calculated using GraphPad Prism Version 5 software.

Results

Cytotoxicity analysis of inhibitors against *L. donovani* promastigotes

L. donovani log phase promastigotes were incubated in the presence of increasing concentrations of these sixteen compounds to test their efficacy. Their growth was assessed by means of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Most of the compounds exhibited inhibition of the growth of promastigotes in a dose-dependent manner (Figs. 1, 2). The effective concentration, which resulted in 50% inhibition of growth (IC_{50}) after 48 h of drug treatment, was found to be as follows,: DKM-B-BRAT—3.689 µg/ml; DKM-B-VIAT—9.887 µg/ml; DKM-B-DIOB—40.16 µg/ml; DKM-B-INACT—147.8 µg/ml; DKM-B-ANAT—6.603 µg/ml; DKM-B-CYL—11.18 µg/ml; DKM-B-DIOA—9.141 µg/ml; DKM-B-EPI—1.558 µg/ml; DKM-B-CENT—1.348 µg/ml; DKM-B-LCAT—78.07 µg/ml; DKM-B-RBAT—12.10 µg/ml; DKM-B-PHAT—14.93 µg/ml; DKM-B-IVAT—13.13 µg/ml; DKM-B-PHOBN—6.388 µg/ml; DKM-B-SBAT—15.21 µg/ml; DKM-B-TOBN—296 µg/ml.

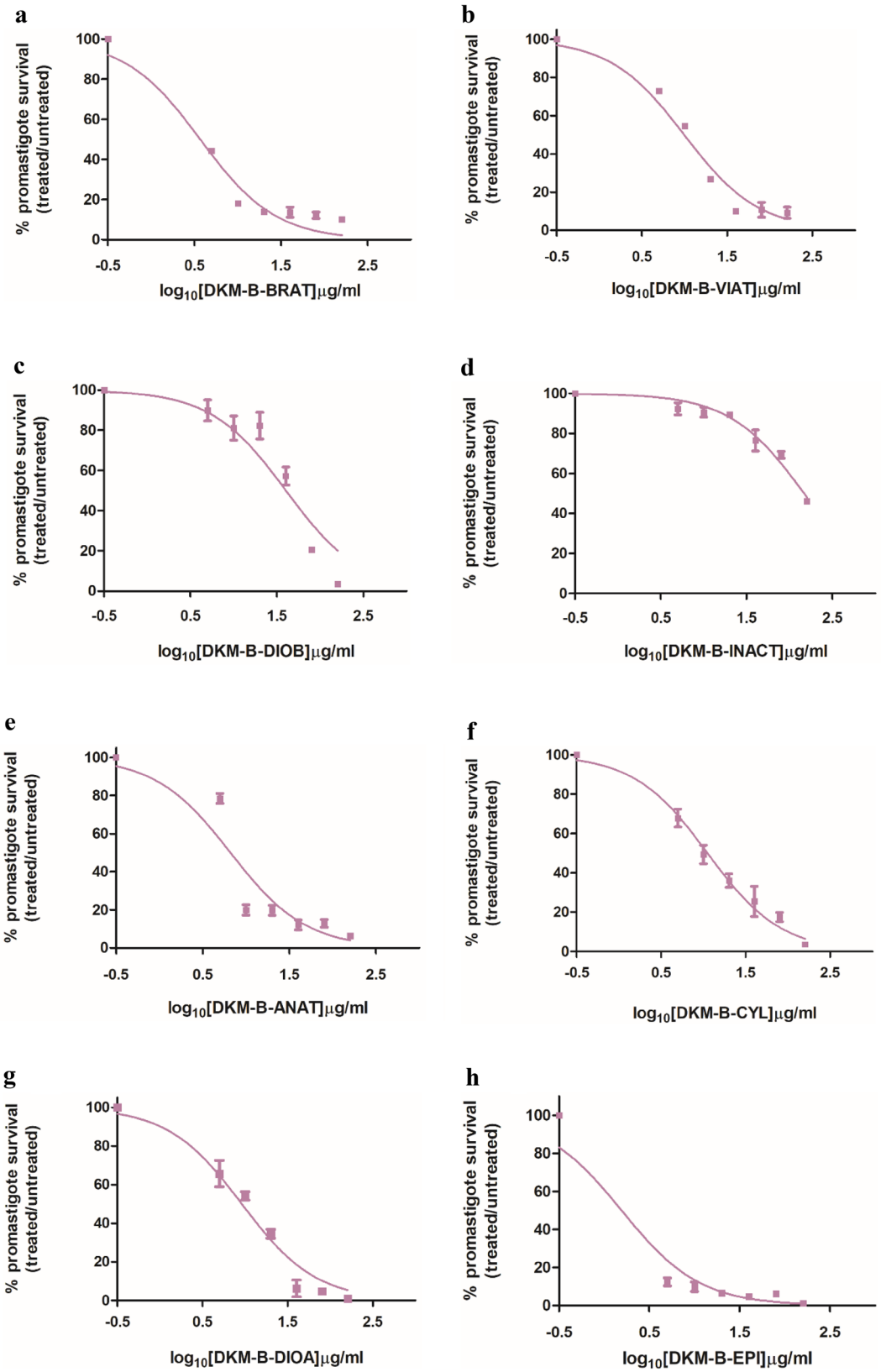


Fig. 1 Effect of inhibitors, DKM-B-BRAT, DKM-B-VIAT, DKM-B-DIOB, DKM-B-INACT, DKM-B-ANAT, DKM-B-CYL, DKM-B-DIOA and DKM-B-EPI on the survival of *L. donovani* promastigotes. Parasite percentage survival was plotted against increasing concentrations of inhibitors

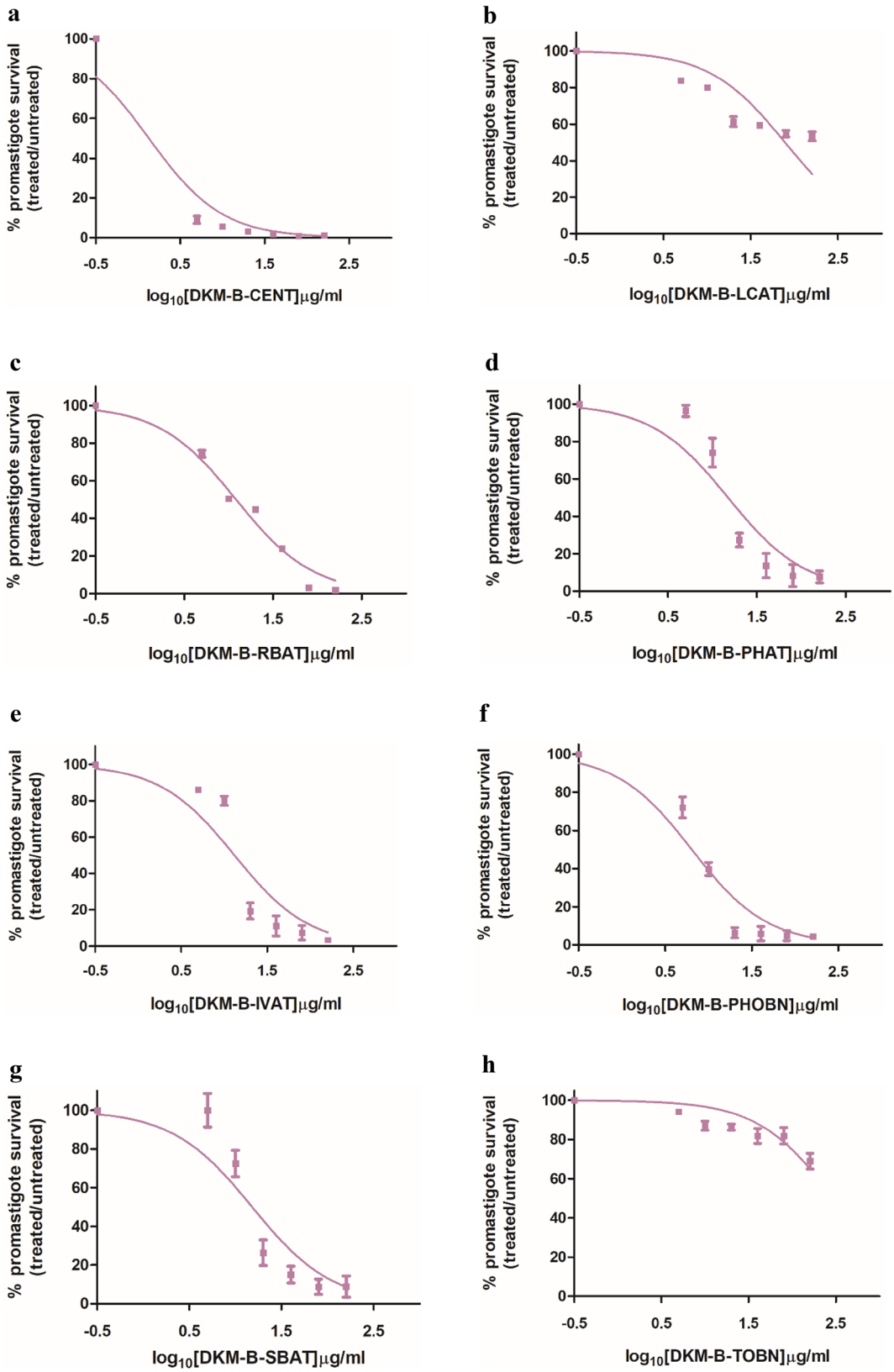


Fig. 2 Effect of inhibitors, DKM-B-CENT, DKM-B-LCAT, DKM-B-RBAT, DKM-B-PHAT, DKM-B-IVAT, DKM-B-PHOBN, DKM-B-SBAT and DKM-B-TOBN on the survival of *L. donovani* promastigotes. Parasite percentage survival was plotted against increasing concentrations of inhibitors

Cytotoxicity analysis of inhibitors against *L. donovani* amastigotes in infected macrophage model

The sensitivity of amastigotes against the inhibitors was also tested by the means of Giemsa staining in the intracellular amastigote-macrophage model. All the compounds were found to affect the growth of *L. donovani* amastigotes in a dose-dependent manner (Figs. 3, 4). The effective concentration which resulted in 50% inhibition of growth (IC_{50}) after 48 h of drug treatment was found to be as follows: DKM-B-BRAT—7.739 µg/ml; DKM-B-VIAT—50.01 µg/ml; DKM-B-DIOB—35.06 µg/ml; DKM-B-INACT—191.7 µg/ml; DKM-B-ANAT—14.37 µg/ml; DKM-B-CYL—36.45 µg/ml; DKM-B-DIOA—21.37 µg/ml; DKM-B-EPI—2.250 µg/ml; DKM-B-CENT—1.466 µg/ml; DKM-B-LCAT—148.6 µg/ml; DKM-B-RBAT—10.64 µg/ml; DKM-B-PHAT—40.53 µg/ml; DKM-B-IVAT—28.64 µg/ml; DKM-B-PHOBN—26.49 µg/ml; DKM-B-SBAT—38.3 µg/ml; DKM-B-TOBN—130.4 µg/ml.

Cytotoxicity analysis of inhibitors against THP-1 derived human macrophages

To test the toxicity of these sixteen compounds on human macrophages, THP-1 derived macrophages were incubated with increasing concentrations of inhibitors. Their survival was estimated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. All the compounds were found to affect the growth of THP-1-derived human macrophages at high concentrations (Figs. 5, 6). The effective concentration which resulted in 50% inhibition of growth (IC_{50}) after 48 h of drug treatment was found to be as follows: DKM-B-BRAT—34 µg/ml; DKM-B-VIAT—61.10 µg/ml; DKM-B-DIOB—59.47 µg/ml; DKM-B-INACT—149.3 µg/ml; DKM-B-ANAT—42.17 µg/ml; DKM-B-CYL—269.9 µg/ml; DKM-B-DIOA—34.71 µg/ml; DKM-B-EPI—5.865 µg/ml; DKM-B-CENT—1.965 µg/ml; DKM-B-LCAT—166.78 µg/ml; DKM-B-RBAT—42.52 µg/ml; DKM-B-PHAT—41.15 µg/ml; DKM-B-IVAT—48.01 µg/ml; DKM-B-PHOBN—111.9 µg/ml; DKM-B-SBAT—91.40 µg/ml; DKM-B-TOBN—135.6 µg/ml.

Discussion

Recently, there has been a renewed interest in traditional plant-based medicines. Current studies are being undertaken for the development of safe and cheaper therapies using medicinal plants for the treatment of infectious and non-infectious diseases. As discussed before, since chemotherapeutics still remain the first choice to treat leishmaniasis, the search for finding novel drugs against this disease remains continuously on. The development of natural products-based chemotherapeutics against leishmaniasis will provide us with a new class of safer, less expensive molecules for the already rapidly exhausting arsenal of anti-leishmanial drugs. WHO is also in support of the development and use of plant extracts as potential leishmanicidal agents in view of increasing cases of resistance against current chemotherapeutic drugs (WHO technical reports 2010). Many plant-based compounds are already being studied for their anti-leishmanial activity. Diospyrin, isolated from *Euclea natalensis*, is a specific inhibitor of the topoisomerase of parasites, and was found to inhibit *L. donovani* promastigotes at an IC_{50} of 0.1 µg/ml (Ray et al. 1998; Lall et al. 2001). Essential oils obtained from *Chenopodium ambroisoides* showed IC_{50} values of 3.7 µg/ml and 4.6 µg/ml against promastigotes and amastigotes of *L. amazonensis* respectively (Monzote et al. 2021). 6,7-dihydroneridienone, a sterol derivative, isolated from a Mexican plant, *Pentalinon andrieuxii*, displayed high leishmanicidal activity with an IC_{50} values of 0.03 µM against *L. mexicana*, with negligible cytotoxicity on healthy murine bone marrow macrophages (Cheuka et al. 2017).

The present study shows the results of the in vitro screening of a set of sixteen natural product-based compounds for leishmanicidal activity. The sixteen tetrahydropyrans were synthesized using centrolobine and diaspongin A and B as parent compounds. Both centrolobine and diaspongin A and B belong to the class of diarylheptanoids. Diarylheptanoids are a class of plant secondary metabolites isolated from various plant sources. These phenolic compounds consist of two phenolic aromatic rings which are linked by a chain of seven carbons. Several studies have shown various health benefits of diarylheptanoids. Curcumin is a known diarylheptanoid compound used as a nutraceutical, and has been studied extensively for its role in protection against many diseases (Kunnumakkara et al. 2017). The compound des-*O*-methylcentrolobine, a phenolic diarylheptanoid derived from *Centrolobium sclerophyllum*, was found to be highly effective against *L. amazonesis* promastigotes (Araujo et al. 1998, 1999). Other diarylheptanoids isolated from *Diascorea spongiosa*, Diospongin B and C have been shown to have anti-osteoporotic activity (Yin et al. 2004). These compounds are able to exhibit a wide spectrum of pharmacological activities as described above and hence, can be used as an alternative source for the development of therapeutics (Ganapathy et al. 2019; Jeong et al. 2010; Beniddir et al. 2012; Lee et al. 2009; Tezuka et al. 2000).

All the sixteen tetrahydropyran inhibitors were incubated with both *L. donovani* promastigotes and amastigotes (infected macrophage model) and the effect of the extracts on the survival of the parasite was studied. Moreover, the

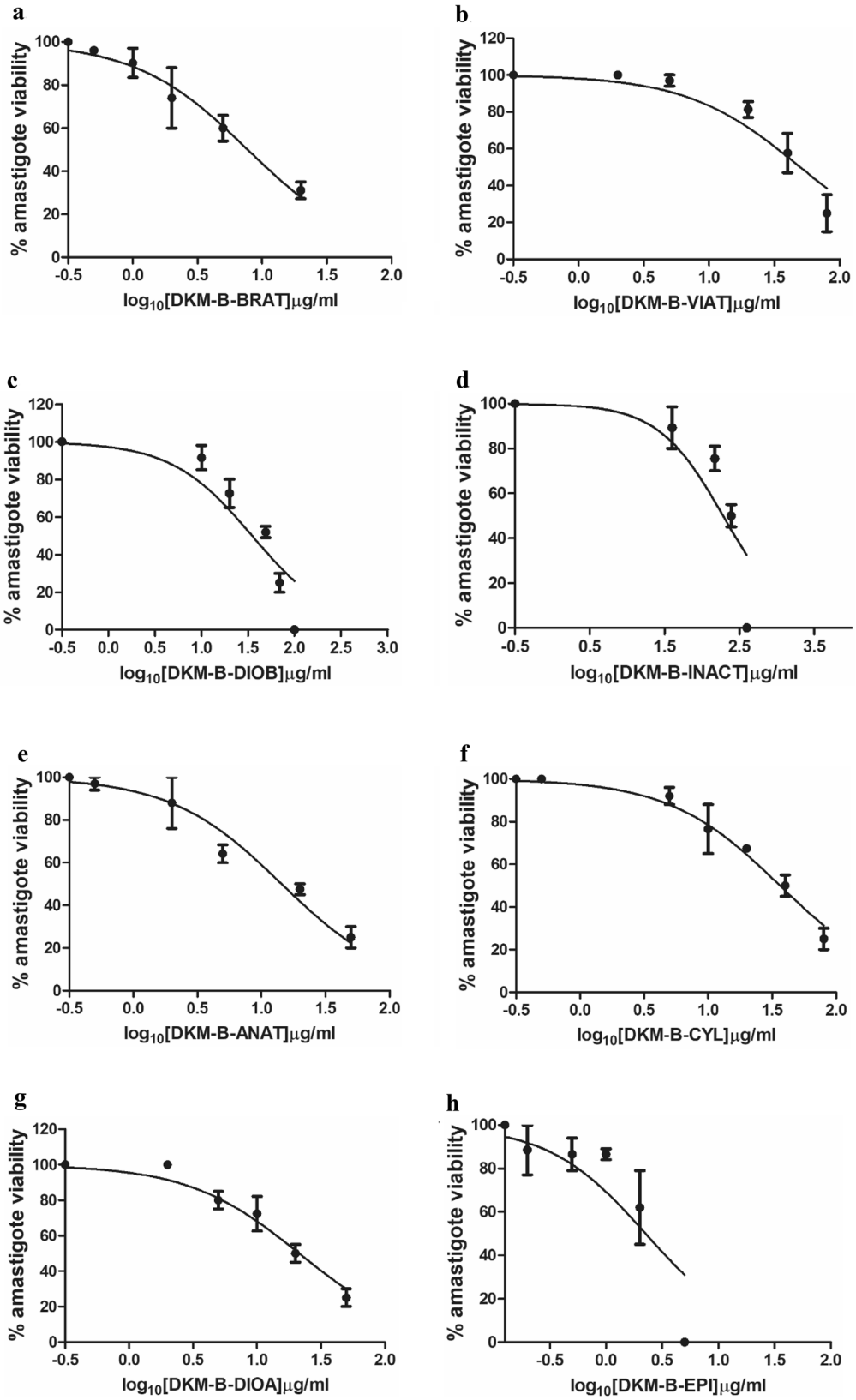


Fig. 3 Effect of DKM-B-BRAT, DKM-B-VIAT, DKM-B-DIOB, DKM-B-INACT, DKM-B-ANAT, DKM-B-CYL, DKM-B-DIOA and DKM-B-EPI on the survival of *L. donovani* amastigotes in the infected macrophages model. The percentage viability of amastigotes was plotted against different concentrations of inhibitors

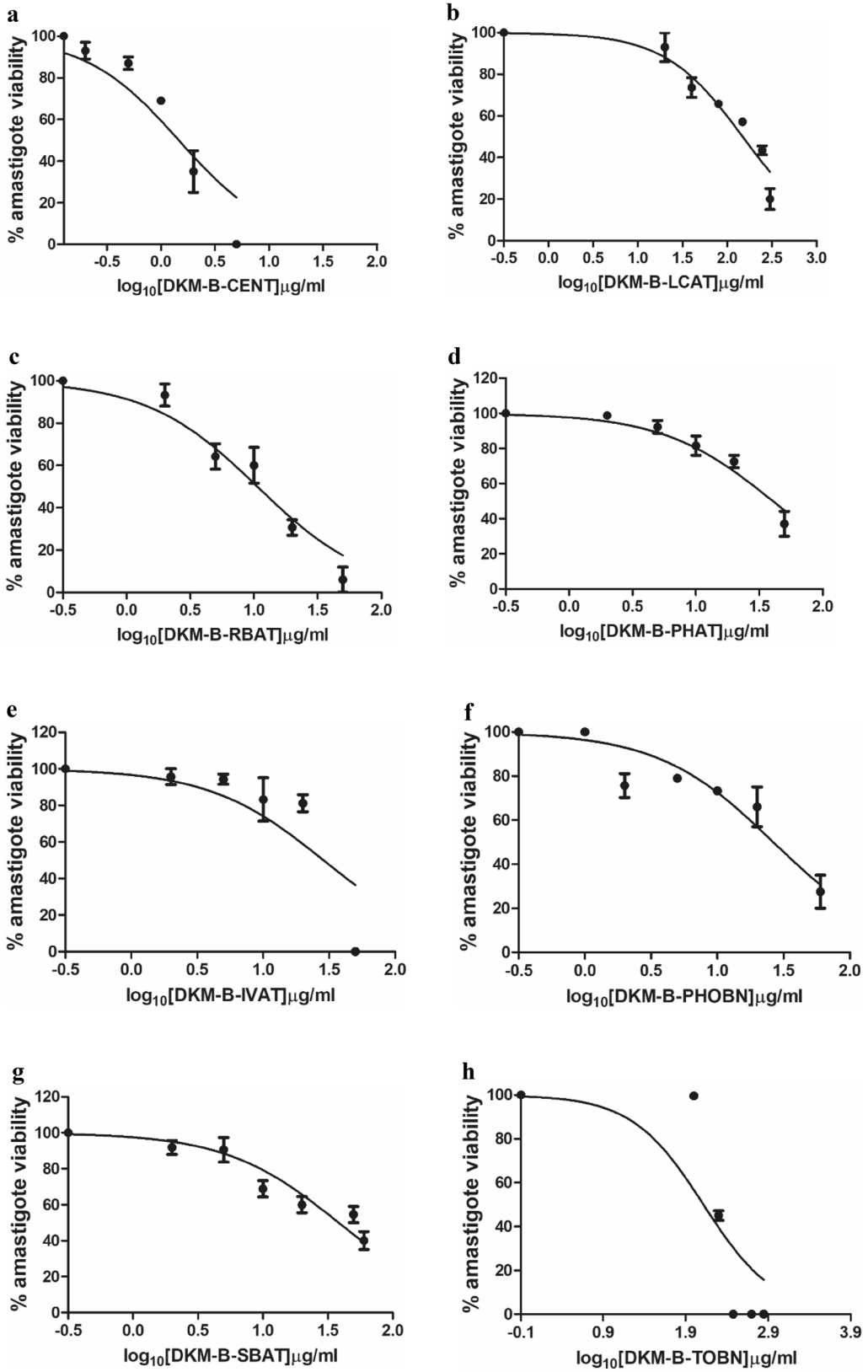


Fig. 4 Effect of DKM-B-CENT, DKM-B-LCAT, DKM-B-RBAT, DKM-B-PHAT, DKM-B-IVAT, DKM-B-PHOBN, DKM-B-SBAT and DKM-B-TOBN on the survival of *L. donovani* amastigotes in the infected macrophages model. The percentage viability of amastigotes was plotted against different concentrations of inhibitors

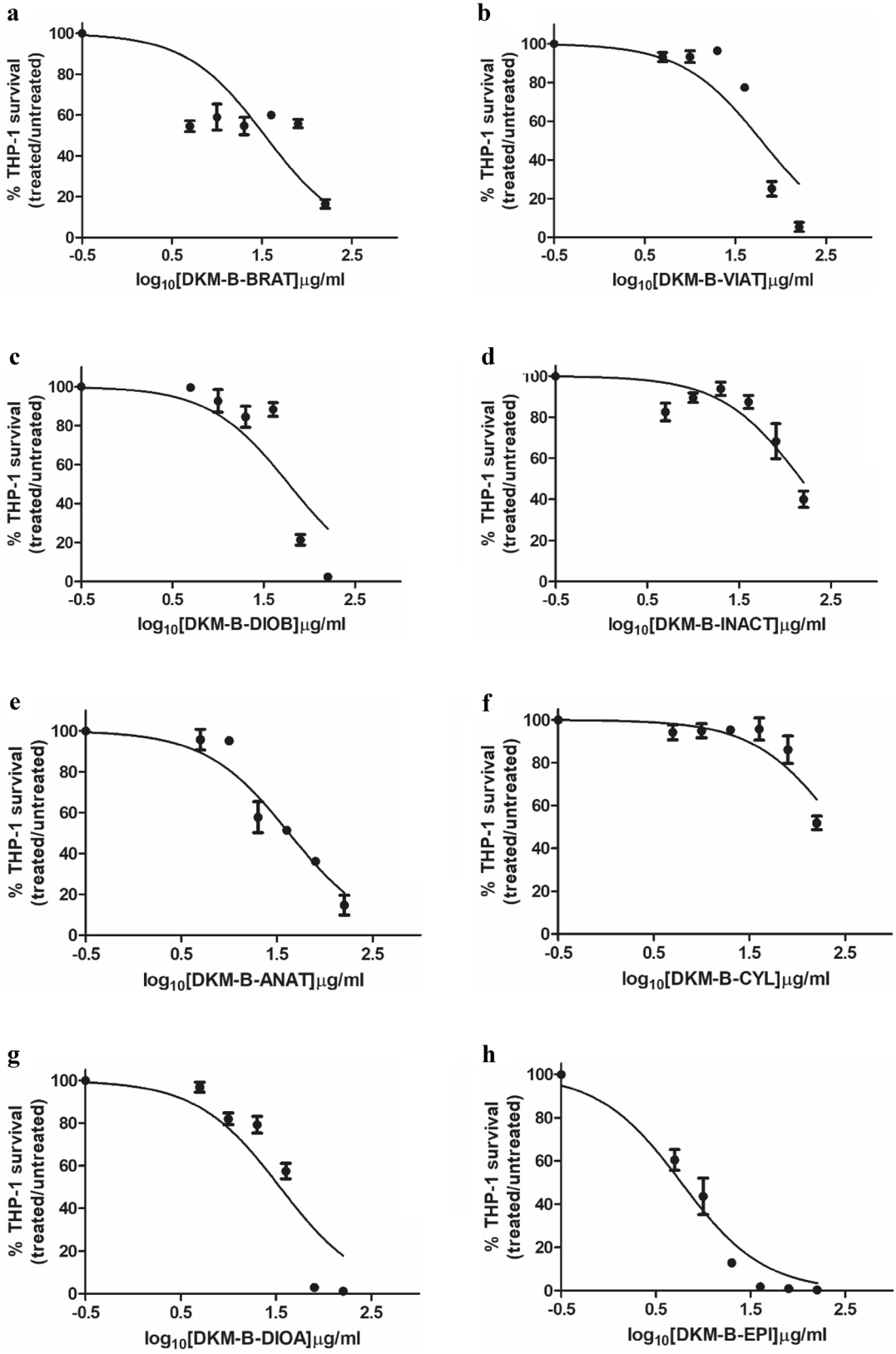


Fig. 5 Effect of DKM-B-BRAT, DKM-B-VIAT, DKM-B-DIOB, DKM-B-INACT, DKM-B-ANAT, DKM-B-CYL, DKM-B-DIOA and DKM-B-EPI on the survival of THP-1 derived macrophages. The percentage survival of macrophages was plotted against different concentrations of inhibitors

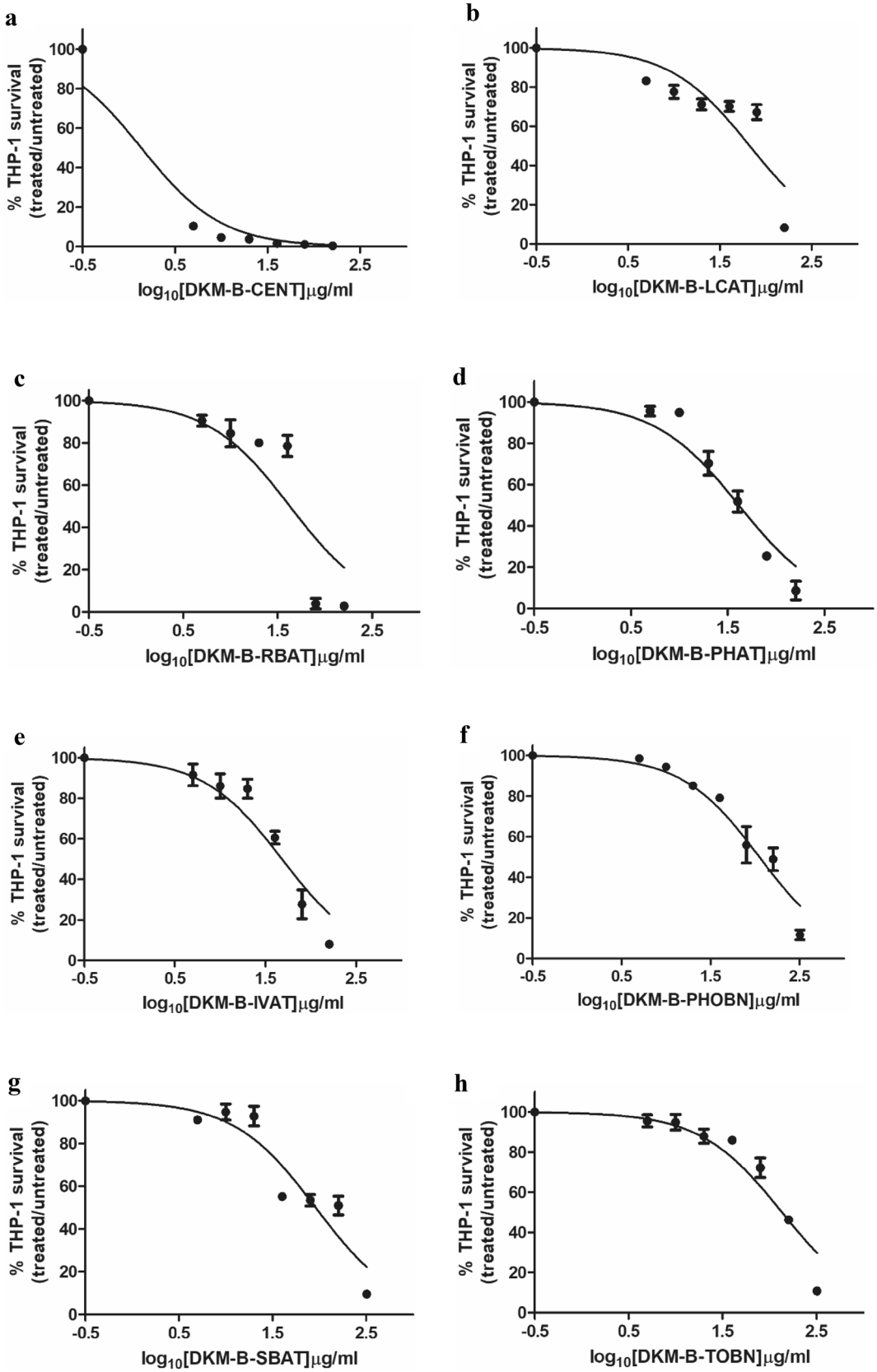


Fig. 6 Effect of DKM-B-CENT, DKM-B-LCAT, DKM-B-RBAT, DKM-B-PHAT, DKM-B-IVAT, DKM-B-PHOBN, DKM-B-SBAT and DKM-B-TOBN on the survival of THP-1 derived macrophages. The percentage survival of macrophages was plotted against different concentrations of inhibitors

toxicity of the extracts on THP-1-derived human macrophages was also studied. For each case, IC_{50} values and selectivity index were also calculated for all the sixteen inhibitors (Table 3).

Cytotoxicity analysis of inhibitors against *L. donovani* promastigotes revealed that at higher concentrations all the compounds except three, DKM-B-LCAT, DKM-B-INACT and DKM-B-TOBN (IC_{50}–78.07 µg/ml,147.8 µg/ml and 296 µg/ml respectively), were lethal for the promastigotes. All the other compounds showed toxicity even at lower concentrations. DKM-B-BRAT, DKM-B-ANAT, DKM-B-CYL, DKM-B-RBAT and DKM-B-PHOBN (IC_{50}—3.689 µg/ml, 6.603 µg/ml, 11.18 µg/ml, 12.10 µg/ml and 6.388 µg/ml respectively) showed 80% inhibition of the promastigotes at lower concentrations between 10 and 20 µg/ml.

Similarly, cytotoxicity analysis of these inhibitors against *L. donovani* amastigotes in infected macrophage model revealed that, all the compounds at higher concentrations were lethal for the amastigotes in the infected macrophage model. DKM-B-BRAT, DKM-B-ANAT, DKM-B-RBAT and DKM-B-PHOBN (IC_{50}—7.739 µg/ml, 14.37 µg/ml, 10.64 µg/ml and 26.49 µg/ml respectively) showed 80% inhibition of the amastigotes at concentrations between 20 and 60 µg/ml.

Cytotoxicity analysis of these inhibitors against THP-1 derived human macrophages revealed that most of the compounds were in fact lethal for human macrophages at higher concentrations (80–160 µg/ml). Two compounds, DKM-B-EPI and DKM-B-CENT, had lethal effect on THP-1 derived human macrophages at very low concentrations (2–5 µg/ml) deeming them unfit for consideration even though they had lethal effect on *L. donovani* promastigotes at very low concentrations (Table 3). Out of the sixteen, only six, DKM-B-INACT, DKM-B-CYL, DKM-B-LCAT, DKM-B-PHOBN, DKM-B-SBAT and DKM-B-TOBN showed an IC_{50} of ≥ 100 µg/mL (Table 3) for THP-1 derived human macrophages, indicating low toxicity towards human macrophages.

The selectivity index can be defined as the ratio of 50% cytotoxic concentration of the drug against macrophages to the 50% inhibitory concentration of the drug against *Leishmania* spp. amastigotes (CC50/IC50). A compound with an SI value greater than 1 is considered to be more selective against *Leishmania* spp. parasites and is regarded as a promising potential agent in the treatment of leishmaniasis (Koutsoni et al. 2019). Out of sixteen inhibitors, only one, DKM-B-INACT, displayed a selectivity index less than one (0.77) rest all had a selectivity index greater or equal to one (Table 3). DKM-B-CYL had the highest selectivity index of 7.40, with a high IC_{50} value for THP-1 macrophages (269.9 µg/ml) and low IC_{50} values for both promastigotes and amastigotes (11.18 µg/ml and 36.45 µg/ml respectively), indicating comparatively higher selectivity of the drug towards the parasite than the host cells. DKM-B-BRAT, also showed a high selectivity index of 4.39, with low IC_{50} values for both promastigotes and amastigotes (3.689 µg/ml and 7.739 µg/ml respectively) but a comparatively higher IC_{50} value for THP-1 macrophages (34 µg/ml). A similar pattern was also observed in the case of DKM-B-PHOBN, DKM-B-RBAT, and DKM-B-ANAT, while the rest of the compounds had comparatively lower values of selectivity index (Table 3).

Table 3 List of sixteen compounds with their inhibitory concentrations 50% (IC_{50}) at 48 h for promastigotes, intracellular amastigotes and THP-1 derived macrophages and selectivity indexes

S. No	Compound	Promastigotes IC_{50} (µg/ml)	Amastigotes IC_{50} (µg/ml)	THP-1 derived macrophages IC_{50} (µg/ml)	Selectivity Index
1	DKM-B-BRAT	3.689	7.739	34	4.39
2	DKM-B-VIAT	9.887	50.01	61.10	1.221
3	DKM-B-DIOB	40.16	35.06	59.47	1.69
4	DKM-B-INACT	147.8	191.7	149.3	0.77
5	DKM-B-ANAT	6.603	14.37	42.17	2.93
6	DKM-B-CYL	11.18	36.45	269.9	7.40
7	DKM-B-DIOA	9.141	21.37	34.71	1.62
8	DKM-B-EPI	1.558	2.250	5.865	2.60
9	DKM-B-CENT	1.348	1.466	1.965	1.34
10	DKM-B-LCAT	78.07	148.6	166.78	1.122
11	DKM-B-RBAT	12.10	10.64	42.52	3.99
12	DKM-B-PHAT	14.93	40.53	41.15	1.015
13	DKM-B-IVAT	13.13	28.64	48.01	1.67
14	DKM-B-PHOBN	6.388	26.49	111.9	4.22
15	DKM-B-SBAT	15.21	38.3	91.40	2.38
16	DKM-B-TOBN	296	130.4	135.6	1.03

Conclusion

From the present study, it can be concluded that out of the given set of sixteen compounds, five compounds, DKM-B-CYL, DKM-B-PHOBN, DKM-B-BRAT, DKM-B-RBAT and DKM-B-ANAT showed the best effect in vitro in terms of both leishmanicidal activity and human macrophage survival (Table 3). All these compounds also had a high selectivity index (Table 3), indicating higher selectivity of the compounds towards *Leishmania* amastigotes than the human macrophage cells. Further studies will be required to decipher the exact *modus operandi* of these inhibitors in *Leishmania*, followed by checking their efficacy in vivo in a murine model. If required, further development of better compounds, in terms of leishmanicidal activity and human macrophage survival, using these compounds as scaffolds can be done, as there is a desperate need for the discovery of new lead compounds, which can be used to combat leishmaniasis, amidst the fear of emerging resistance against the existing drug arsenal.

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Declarations

Conflict of interest None to declare.

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