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Article

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Synthesis, Structure - Activity Relationships and Biological Studies of Chromenochalcones as Potential Antileishmanial Agents

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ABSTRACT

Antileishmanial activities of a library of synthetic chalcone analogues have been examined. Among them, five compounds (**11**, **14**, **16**, **17**, **22** and **24**) exhibited better activity than the marketed drug miltefosine in *in vitro* studies against intracellular amastigotes form of *Leishmania donovani*. Three promising compounds **16, 17** and **22** were tested in *L. donovani* / hamster model. Oral administration of chalcone **16**, at a concentration of 100 mg/kg of body weight per day for 5 consecutive days resulted in >84% parasite inhibition at day 7 post-treatment and it retained the activity till day 28. The molecular and immunological studies revealed that compound **16** has a dual nature to act as a direct parasite killing agent and as a host immunostimulant. Pharmacokinetics and serum albumin binding studies also suggest that compound **16** has potential to be a candidate for the treatment of non-healing form of leishmaniasis.

INTRODUCTION

Leishmaniasis, an infectious disease caused by protozoan parasites belonging to the genus *Leishmania*, is transmitted to humans through the bite of female phlebotomine sand flies infected with the parasite.[1] It is classified as cutaneous, muco-cutaneous and visceral (kala azar) form depending on the parasite species and cellular immune system of the patient.[1,2] It has been recognized by the World Health Organization (WHO) as an increasing health problem.[3] Many parts of Asia and Africa are vulnerable to leishmaniasis.[4] *Leishmania* parasite has evolved several skills to inactivate protective immune machinery of host to survive inside the cell.[5] The outcome of *Leishmania* infection depends on the proliferation of T-helper 2 (Th2) cell population which is associated with the production of interleukin (IL)-10 and transforming growth factor (TGF)-β.[6] These suppressive molecules distort the healing responses by repression of host-protective microbicidal molecules including cytokines like interferon (IFN)-γ, IL-1, IL-12, and tumor necrosis factor-α (TNF-α), and reactive nitrogen and oxygen intermediates (RNI and ROI).[5-7] One of the major complications for the chemotherapeutic treatment of visceral leishmaniasis (VL) is the depressed immune function exhibited by patients. Therefore, induction of protective immune responses in the infected host by any therapeutic agent / immunomodulator is essential for the successful treatment of VL. The first line treatment options for leishmaniasis are limited and involve the administration of pentavalent antimonials (Sodium stibogluconate (SSG) and Meglumine antimoniate (MA)) and amphotericin B.[8] Second line drugs include, paromomycin and miltefosine, but these drugs have not experienced widespread use due to the severe toxicities, parenteral administration and resistance issues.[8] Pentavalent antimonials, which were developed in 1940s, are presenting high toxicity and low efficacy. Presently, more than 60% VL patients in Bihar (India) are unresponsive to the antimonials.[9] Amphotericin B and its formulations are quite effective for VL, however, these are very expensive, highly toxic and

has a longer half-life.[10] Pentamidine presents several side effects, including renal and hepatic toxicity, pancreatitis, hypotension and cardiac abnormalities.[11] Paromomycin has limited use in the treatment of VL and also present ototoxicity and hepatotoxicity.[10] Miltefosine, an oral drug also suffer from nephrotoxicity, hepatotoxicity and teratogenicity.[10] Up to now, no vaccine approved for human use.[12] Therefore, there is an increasingly urgent need for the development of new, inexpensive, effective and safe drugs for the treatment of leishmaniasis and then, the discovery of new lead compounds for this disease is a pressing concern for global health programs.

Chalcones, or 1,3-diaryl-2-propen-1-ones (Figure 1), are prominent secondary metabolites precursors of flavonoids and isoflavonoids in plants. Chemically, they consist of open-chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system.[13] Natural and synthetic chalcones are described in the literature with different pharmacological profiles, such as antiinflammatory,[14] antibacterial,[15] antiviral,[16] antimalarial,[17] anticancer,[18] antileishmanial,[19] antituberculosis,[20] anti-HIV,[21] and antifungal activities.[22] A thorough assessment of structural requirements for antileishmanial activities of chalcones is vital to develop and designing of novel drug like candidate. Chalcones are natural-like compounds, and as such they can show multi-target profile similar to other compounds described in the literature.[23] Licochalcone A (**II**), is an oxygenated chalcone (Figure 1), isolated from Chinese licorice, efficiently inhibits proliferation of *L. donovani* and *L. major* promastigotes and amastigotes *in vitro* by interfering with the function of the parasite mitochondria. *In vitro* tests have revealed that licochalcone A at lower concentrations inhibits phytohemmagglutinin A-induced proliferation of human lymphocytes.[24] Preliminary studies reveal that changes of the substitution pattern of the chalcones appear to affect the activities against *Leishmania* promastigotes and lymphocytes differently, indicating that it should be possible to prepare chalcones with a high selectivity.

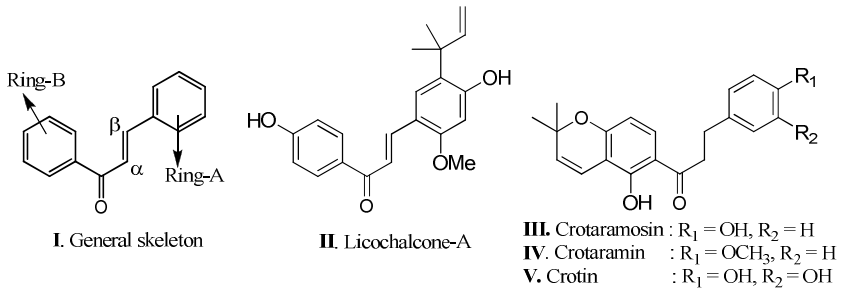


Figure 1. General skeleton and naturally occurring antileishmanial chalcones

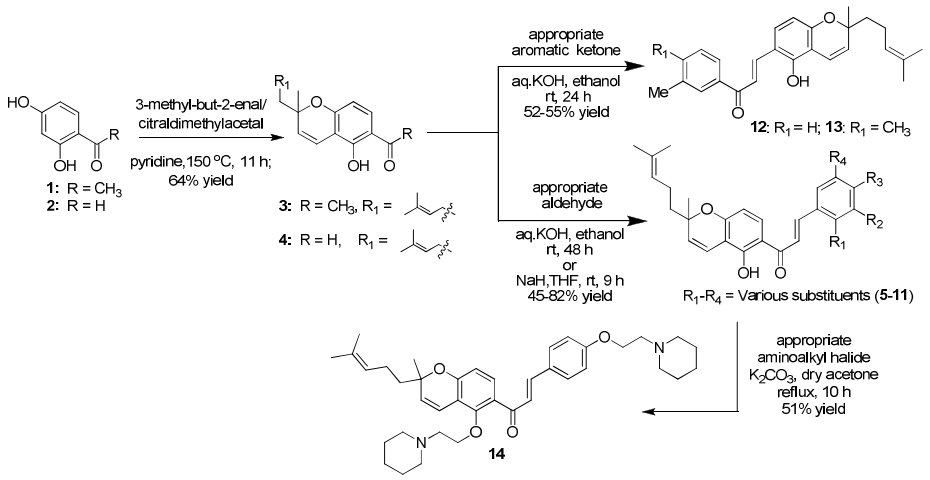
As a part of our drug discovery program on antileishmanial agents from Indian medicinal plants, we recently reported the isolation of three chromenodihydrochalcones, crotaramosmin (**III**), crotaramin (**IV**) and crotin (**V**) (Figure 1) from *Crotalaria ramosissima*,[25]synthesis and *in vitro* antileishmanial activity of **III**–**V** and analogues thereof.[26, 27] In continuation of that program a series of new chalcones with various structural features were synthesized and evaluated for their *in vitro* antileishmanial activity. Several of these compounds were found more active in the *in vitro* screening; few of them were further evaluated for *in vivo* antileishmanial efficacy in a hamster model. Herein, we report a comprehensive assessment of antileishmanial activity, structure−activity relationship (SAR) analyses, pharmacokinetics (PK), and mode of action studies of promising chalcones.

RESULTS AND DISCUSSION

Chemistry

Synthesis of Chromenochalcones. A large number of chromenochalcones were prepared to evaluate their antileishmanial activity. The acetyl/carboxaldehyde chromenes **3** and **4** were synthesized using pyridine-catalyzed condensation between 2,4-dihydroxyacetophenone (**1**) or 2,4-dihydroxy-benzaldehyde (**2**) and citraldimethylacetal.[28] The resultant acetyl chromene **3** and substituted aromatic aldehydes or *p*-hydroxybenzene-1,3-dicarbaldehyde[29] and chromene carboxaldehyde **4** and appropriate acetophenones were subjected to Claisen-Schmidt condensation using either aqueous KOH in ethanol or NaH in dry THF at room

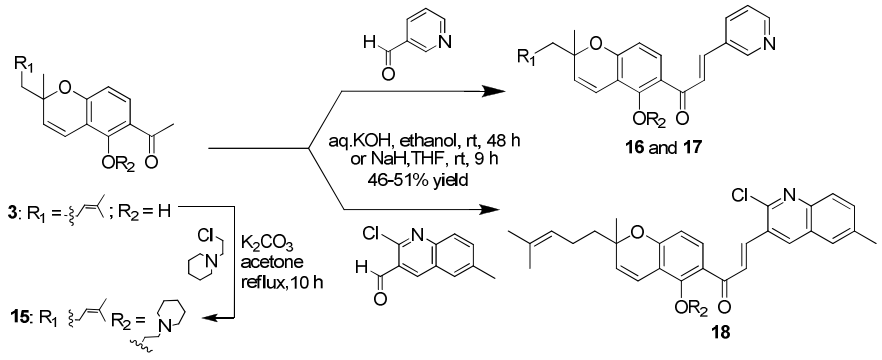
temperature to furnish the corresponding chromenochalcones **5-13** in good yields (Scheme 1, and Table 1).[27] To improve the bioavailability of compounds, the alkylated amine groups were introduced in chromenochalcone core. The synthesis of alkylated amines containing chromenochalcone (**14**) was accomplished by the replacement of the hydrogen of the phenolic hydroxyl groups of the chromenochalcones **7** with the alkyl part of the corresponding amines using K_2CO_3 in dry acetone (Scheme 1).[30]



Scheme 1. Synthesis of Chromenochalcones (**5**–**13**) and Alkylated amine containing-chromenochalcone(**14**)

Synthesis of Chromenochalcones with Hetero Atoms in Ring-A.

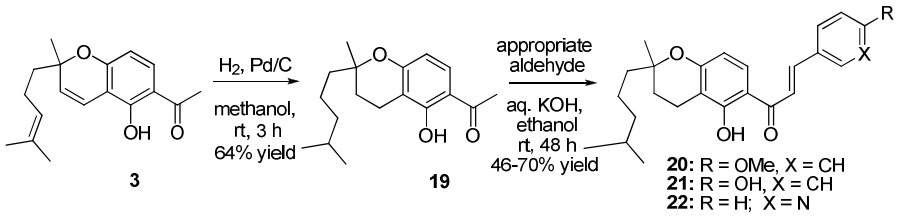
Chromenochalcones **16–18** which have hetero atoms in ring-A (Scheme 2) were synthesized using Claisen-Schmidt condensation from acetyl chromens[28] **3** and **15** and various heteroaryl aldehydes to offer the compounds **16–18** in good yields.



Scheme 2: Synthesis of chromenochalcones with the hetero atoms in Ring A (**16**–**18**)

Synthesis of Chromanochalcones

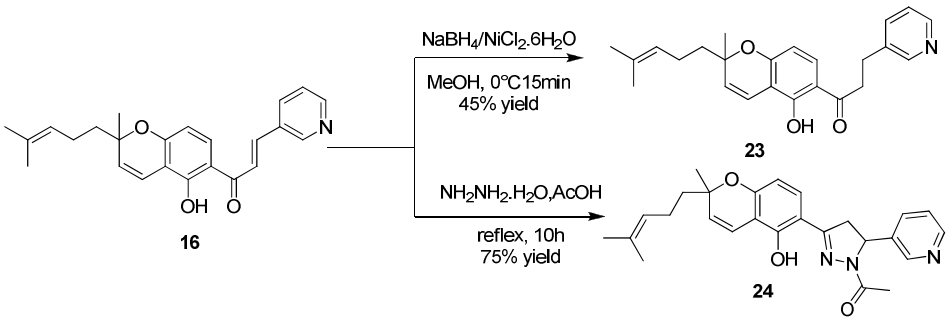
We wanted to study the benzopyran core effect in chromenochalcones; therefore, few chromanochalcones (**20–22**) were synthesized. For synthesis of chromanochalcones **20–22,** intermediate **3** was used as starting material, which smoothly reduced to acetyl chroman **19** with H_2/Pd/C[27] and subsequently subjected to Claisen-Schmidt condensation with aromatic aldehydes to obtain the desired chromanochalcones **20–22** (Scheme 3).



Scheme 3. Synthesis of Chromanochalcones (**20–22**)

Synthesis of Chromenodihydrochalcones and N-acetyl pyrazoline derivative

We wanted to synthesize the chromenodihydrochalcone **23** and N-acetyl pyrazoline **24** to determine the role of the α–β olefinic bond, which connects the ring-A and carbonyl carbon (Figure 1). The chromenodihydrochalcone **23** was prepared from compound **16** by regeoselective hydrogenation with $NaBH_4/NiCl_2.6H_2O$ as shown in Scheme 4. Reaction of the chromenochalcone **16** with hydrazine hydrate in acetic acid under stirring and reflux conditions led to the formation of the N-acetyl pyrazoline derivative **24** (Scheme 4) in good yield.



Scheme 4. Synthesis of chromenodihydrochalcone and N-acetyl pyrazoline derivative (**23** and **24**)

Biological Activity.

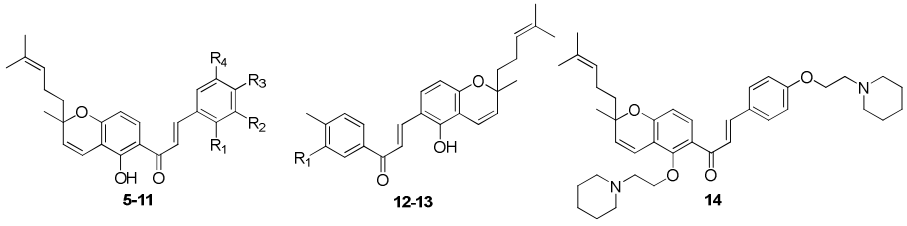
In our efforts to develop new antileishmanial agents, we had earlier reported the *in vitro* antileishmanial activity of few natural and synthetic chromenochalcones.[26,27] This work had shown that the benzopyran core and nature of substitutions on ring A are very crucial for the potent antileishmanial activity. On the basis of these optimistic results, a library of new chalcones was prepared and evaluated their antileishmanial activity against WHO reference strain (MHOM/IN/80/Dd8) of extracellular (62–100% of inhibition at 25 μM) and intracellular (IC_{50} = 1.7 to 20 μM) form (expressing luciferase firefly reporter gene) of *L. donovani*. In parallel, the cytotoxicity of the compounds was also tested using mammalian kidney fibroblast cells (Vero cell line) for the compounds, which have IC_{50} = < 20 μM against amastigotes. Among these, few promising compounds were further tested for their *in vivo* activity against the MHOM/IN/80/Dd8 strain of *L. donovani* in a hamster model. Standard antileishmanial drugs, miltefosine and sodium stibogluconate (SSG) were included in this study as control drugs. The molecular, immunological, pharmacokinetics and serum albumin binding studies were also conducted for most promising compound (see below). (Table 1–4).

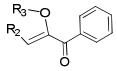
***In Vitro* Antileishmanial Activity of Chromenochalcones**

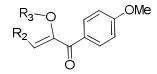
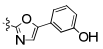
In continuation of our work[27] (Table S1, see in supporting information), we introduced the EWD's and fused hetero cyclic moieties on ring A and maintained the same prenyl unit on benzopyran core as ring B of chromenochalcones **5–11** (Table 1). The introduction of aldehyde group into ring-A as in chalcone **6** effected the *in vitro* activity against amastigotes (**5**: IC_{50} = 16.5 μM vs **6**: IC_{50} > 20 μM, **7**: IC_{50} = 10.7 μM). Since, hydrogen bonding affects membrane transport, as well as the distribution of compound, **6** and **7** exhibited different activity due to presence and/or absence of chelation (between OH and CHO). The presence of aldehyde group in **7** the activity increased, at the same time the selectivity index (SI) was

decreased by two times than **5** (Table 1). It is noteworthy that the insertion of fused hetero cycles to ring A as in chalcones **8–10**, and the interchange of ring A and B as in **12** and **13** led to diminished activity against amastigotes (IC_{50} > 20 μM). Interestingly, chalcone **11**, which contains the oxazolidine moiety on ring A has an improved *in vitro* activity (IC_{50} = 5.8 μM), and toxicological profile (CC_{50} > 400 μM) against amastigotes and vero cells respectively. To improve the bioavailability of chromenochalcone **14**, which has an alkylated amine substituents on both rings was synthesized (Scheme 1) and evaluated for antileishmanial activity as shown in Table 1. This disubstituted analogue **14**, showed 99.87% inhibition of promastigotes at 25 μM concentration with IC_{50} of 6.3 μM and good toxicological profile (SI = 31.9) against amastigotes and vero cells respectively (Table 1). The introduction of alkylated amine substituents on ring A and B (**14:** IC_{50} = 6.30 μM versus **5**; IC_{50} = 16.5 μM against amastigotes) significantly increased the activity profile.

Table 1. *In vitro* antileishmanial activity of chromenochalcones against promastigotes and amastigotes of *L. donovani* and their cytotoxicity



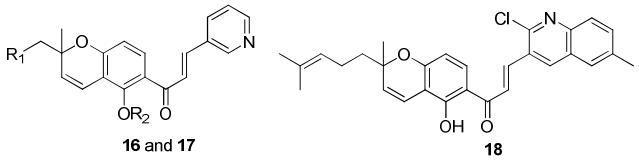
Compd	R_1	R_2	R_3	R_4	*In vitro* activity: Antipromastigote (% inhibition at 25 μM)[a]	*In vitro* activity: Antiamastigote IC_{50} (μM)[b]	Cytotoxicity CC_{50} (μM)[c]	Index (SI)[d]
5	H	H	OH	H	97.5	16.5	189.5	11.5
6	H	CHO	OH	H	94.6	> 20	ND-	-
7	OH	H	H	CHO	91.2	10.7	59.4	5.5
8	H			H	96.5	> 20	ND	-
9	H			H	94.4	> 20	ND	-

10	H			H	92.9	> 20	ND	-
11	H		H	H	99.5	5.8	> 400	> 68.9
12	H	-	-	-	92.7	> 20	ND	-
13	CH_3	-	-	-	93.2	> 20	ND	-
14	-	-	-	-	99.87	6.3	201.2	31.9
SSG	-	-	-	-	No inhibition	49.7	> 400	> 8.0
Miltefosine	-	-	-	-	100	8.4	52.5	6.2

[a] Values are represented as average of at least duplicate measurements (SD ± 2%). [b]IC_{50} and [c]CC_{50} values are represented as average of at least duplicate measurements (SD ± 10%), [d]SI (selectivity index) = CC_{50}/ IC_{50}, ND=not done.

***In Vitro* Antileishmanial Activity of Chromenochalcones with Hetero Atoms on Ring A.** After studying the effect of various substitutions nature and their positions on ring A and benzopyran moiety (Ring B) of chalcones (Table 1), we enthusiastically moved to work on chromenochalcones with hetero atoms on ring A. We synthesized few chromenochalcones **16–18** with hetero atoms in ring A (Scheme 2) and evaluated their *in vitro* antileishmanial activity (Table 2). All the analogues exhibited potent activity against promastigotes (99.5–100% inhibition at 25 μM) (Table 2). On the other hand, compounds **16** and **17**, with exception of **18** (toxic to cells) were very potent (IC_{50} < 5.4 μM) against amastigotes and also have good SI. Alkylated amine substituents on chromanochalcons **17** (IC_{50} = 1.7 μM) increased the activity three fold against amastigotes and SI was increased 6–8 times than reference drugs (Table 2).

Table 2. *In vitro* antileishmanial activity of chromanochalcones with hetero atoms on ring A against promastigotes and amastigotes of *L. donovani* and their cytotoxicity

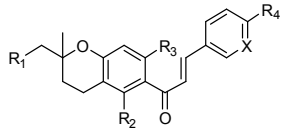


Compd	R_1	R_2	*In vitro* activity		Cytotoxicity CC_{50} (μM)[c]	Index (SI)[d]
			Antipromastigote (% inhibition at 25 μM)[a]	Antiamastigote IC_{50} (μM)[b]		
16		H	99.8	5.4	40.7	7.5
17			99.8	1.7	65.2	38.3
18	-	-	100	Toxic for cells	ND	-
SSG	-	-	No inhibition	49.7	> 400	> 8.0
Miltefosine		-	100	8.4	52.5	6.2

[a]Values are represented as average of at least duplicate measurements (SD ± 2%). [b]IC_{50} and [c]CC_{50} values are represented as average of at least duplicate measurements (SD ± 10%), [d]SI (selectivity index) = CC_{50}/ IC_{50}, ND=Not determined.

***In Vitro* Antileishmanial Activity of Chromanochalcones.** To find out role of the olefinic bond in benzopyran moiety, chromanochalcones **20–22** which contained the chroman ring (dihydrobenzopyran), were prepared (Scheme 3) and screened for their *in vitro* antileishmanial activity (Table 3). Chromanochalcones **20** and **21**, which have the EDG's on ring-A, showed moderated antileishmanial activity against promastigotes (62.3–88.9% inhibition at 25 μM). Chromanochalcones **20** and **21** (IC_{50} > 20 μM) are less potent than the chromanchalcone, which has heteroatom in ring A (**22**: IC_{50} = 5.7 μM versus **20** and **21**: IC_{50} > 20 μM) and selectivity by 3 to 6 times than reference drugs (Table 3). From the above data it is very clear that the *in vitro* activity and toxicological profiles were significantly improved after the incorporation of the hetero atom in ring A in chromenochalcones (Table 2) as well as chromanochalcones (Table 3).

Table 3. *In vitro* antileishmanial activity of chromanochalcones against promastigotes and amastigotes of *L. donovani* and their cytotoxicity



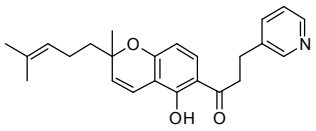
20-22

Compd	R_1	R_2	R_3	R_4	X	*In vitro* activity		Cytotoxicity	Index
						Antipromastigote (% inhibition at 25 μM)[a]	Antiamastigote IC_{50} (μM)[b]	CC_{50} (μM)[c]	(SI)[c]
20		OH	H	OMe	CH	62.3	> 20	ND	-
21		OH	H	OH	CH	88.9	> 20	ND	-
22		OH	H	H	N	99.8	5.7	156.3	27.4
SSG						No inhibition	49.7	> 400	> 8.0
Miltefosine						100 ± 0	8.4	52.5	6.2

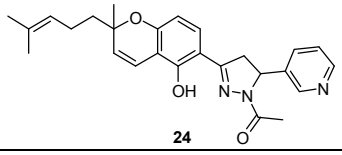
[a]Values are represented as average of at least duplicate measurements (SD ± 2%). [b]IC_{50} and [c]CC_{50} values are represented as average of at least duplicate measurements (SD ± 10%), [d]SI (selectivity index) = CC_{50}/ IC_{50}, ND=not determined.

***In Vitro* Antileishmanial Activity of Chromenodihydrochalcones and N-acetyl pyrazoline derivative.** To determine the role of the α–β olefinic bond, chromenodihydrochalcone **23** was synthesized from **16** by selective reduction. The chromenodihydrochalcone **23** showed lower order of activity (**23**: IC_{50} = 11.0 μM vs **16**: IC_{50} = 5.4 μM) and better toxicity profile than lead compound **16** (23: CC_{50} = 265.5 μM vs **16**: CC_{50} = 40.7 μM). These results supported the importance of Michael acceptor (α, β-unsaturated ketone) moiety in the inhibition of parasite burden. N-acetyl pyrazoline derivative **24** was prepared to mask the Michael system by reacting **16** with hydrazine. Interestingly, N-acetyl pyrazoline derivative **24** was equipotent to the parent compound **16** against parasites (**24**: IC_{50} = 6.4 μM vs **16**: IC_{50} = 5.4 μM) and its selectivity index increased two fold to parent compound **16** (**24**: SI = 13.8 vs **16**: SI = 7.5), which reiterates the importance of Michael system (Table 4).

Table 4. *In vitro* antileishmanial activity of chromenodihydrohalcone and N-acetyl pyrazoline against promastigotes and amastigotes of *L. donovani* and their cytotoxicity



23



24

Compd	*In vitro* activity		Cytotoxicity	Index
	Antipromastigote (% inhibition at 25 μM)[a]	Antiamastigote IC_{50} (μM)[b]	CC_{50} (μM)[c]	(SI)[d]
23	98.5	11.0	265.5	24.1
24	99.5	6.4	88.5	13.8
SSG	No inhibition	49.7	> 400	> 8.0
Miltefosine	100 ± 0	8.4	52.5	6.2

[a]Values are represented as average of at least duplicate measurements (SD ± 2%). [b]IC_{50} and [c]CC_{50} values are represented as average of at least duplicate measurements (SD ± 10%), [d]SI (selectivity index) = CC_{50}/ IC_{50}

***In Vivo* Efficacy of Chalcones against *L. donovani* / Hamster Model.** Out of six compounds **(11**, **14**, **16**, **17**, **22** and **24)**, which exhibited potent amastigote inhibiting activity, only three compounds (**16**, **17** and **22)** were chosen for further *in vivo* studies after assessing their structural features[31] and tested against the MHOM/IN/80/Dd8 strain of *L. donovani* in a hamster model.[32] The aqueous suspensions of tested compounds were administered for 5 to 10 consecutive days at 50 and/or 100 mg/kg/day either by intraperitoneal (IP) or oral (PO) route. The post treatment splenic biopsies were done on day 7 and day 28 after the last dose administration and amastigote counts were assessed by Giemsa staining. Compounds **22** showed percentage inhibition of 43.92 ± 10.90 on day 7 post treatment (p.t.) at 50 mg/kg/day by oral route (Table 4), and these animals were not survived till day 28 p.t. The chalcone **17** (73.19 ± 10.90 at 50 mg/kg/day), had shown good inhibition on day 7 by oral route, unfortunately, in this case also inhibition was not retained till day 28 and dropped to half (Table 5). Remarkably, compound **16** showed the best dose dependent *in vivo* efficacy among all the tested analogues with an average inhibition of 48.98 ± 12.63%, and 84.74 ± 11.95% on

day 7 at 50, and 100 mg/kg/day by oral administration respectively, and this inhibition was retained till day 28 p.t. and most of the animals were survived after the treatment (Table 5).

Table 5. *In vivo* efficacy of chalcones against *L. donovani* / hamster model

Compd	Dose (mg/kg)	Treated days	Route	% Inhibition ± SD at day 7 (n = 5)	% Inhibition ± SD at day 28
16	50	5	PO	48.98 ± 12.63	55.37 ± 12.15 (n = 5)
16	100	5	PO	84.74 ± 11.95	84.49 ± 10.02 (n = 4)
17	50	5	PO	73.19 ± 10.90	49.98 ± 14.19 (n = 4)
22	50	10	PO	43.92 ± 10.90	NA
SSG	40	5	IP	89.47 ± 4.72	72.51 ± 4.14 (n = 4)
Miltefosine	30	5	PO	98.50 ± 1.10	83.20 ± 3.44 (n = 5)

SD, standard deviation; NA, Not available, IP, intraperitoneal; PO, per oral, SSG, sodium stibogluconate; n=number of animals

Apoptotic-Necrotic Profiling to Determines the Phosphatidylserine Exposure in *Leishmania* Promastigotes by Compound 16. Apoptosis is a physiological phenomenon that induces the cells towards natural death. The induction of apoptosis in a parasite by any therapeutic agent leads to reduced parasitic burden.[33] To ascertain the effect of compound **16** to induce the early apoptosis in *Leishmania* promastigotes, apoptotic-necrotic profiling was carried out. In early apoptotic stage, the membrane phospholipid, phosphatidylserine (PS) is translocated from the inner side of the cell membrane to the cell surface, and exposed to the external cellular environment. Annexin V-FITC which labels PS sites and represent apoptotic cell death was used with propidium iodide (PI). PI was used as a counter stain to differentiate apoptotic cells from necrotic cells. The dot plots of Annexin V- FITC and PI staining have been presented in figure 2. The percentages of promastigotes in early apoptotic phase (lower right quadrant) due to effect of compound **16** at various time points were determined by the comparison of treated promastigotes with that of untreated control. It was observed that in untreated control, 13.70% promastigotes were annexin V positive. The promastigotes treated

with IC_{50} concentration (0.78 μM against promastigotes) of compound **16** for 72 h showed the highest number of early apoptotic cells (> 52%) in comparison to untreated control. At 6, 12, 24 and 48 h time points, compound **16** induces 13.34, 15.23, 17.09 and 23.92% promastigotes, respectively, for early apoptosis when compared with untreated counterpart. It was also observed that this compound triggers maximum PS exposure to annexin V- FITC at late time points (between 48 to 72 h). Interestingly, there was no role of necrosis observed during apoptotic-necrotic profiling. Our results clearly indicate that compound **16** exerts leishmanicidal activity via apoptosis in promastigotes.

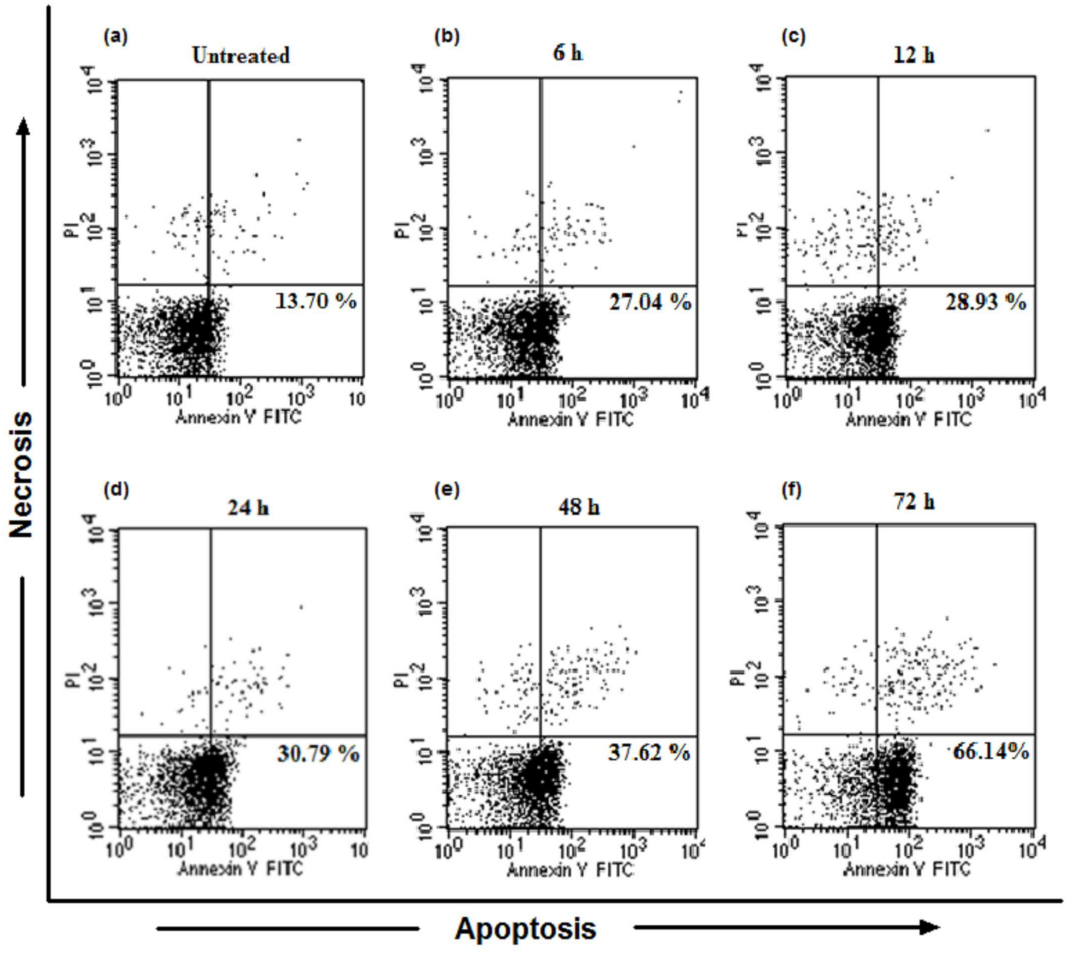


Figure 2: Detection of apoptosis in *Leishmania* promastigotes by annexin V-FITC and propidium iodide (PI) double staining. (a) Untreated promastigotes, (b) 6 h treatment with compound **16**, (c) 12 h treatment with compound **16**, (d) 24 h treatment with compound **16,**

(e) 48 h treatment with compound **16** and (f) 72 h treatment with compound **16**. The lower left quadrant shows unstained living cells (Annexin V^-/ PI^-), the lower right shows early apoptotic cells (Annexin V^+/ PI^-), the upper left shows necrotic cells (Annexin V^-/ PI^+) and the upper right indicates late apoptotic cells (Annexin V^+/ PI^+). Data presented here are the representative of three independent experiments.

Compound 16 Triggers the Drop of Mitochondrial Membrane Potential in *Leishmania* Promastigotes. A mitochondrial enzyme, fumarate reductase (FRD) is a known target of chalcone derivatives.[24] As FRD does not exist in mammalian cells, chalcones selectively inhibited protozoan FRD, which play key role in the respiratory chain of *Leishmania* parasite. The loss in mitochondria membrane potential (MMP) is a distinctive feature of cell death by apoptosis due to the action of drugs. The outcome of our results proved that compound **16** was responsible for loss in membrane potential. The drop in MMP in the treated promastigotes showed that they are undergoing to apoptosis due to loss in MMP, blocks the entry of JC-1 dye in to the mitochondria and fluoresces green. The results are depicted in the figure 3 in the form of dot plots. Our results demonstrated that gradual increase in MMP drop were observed in *Leishmania* parasite, when promastigotes were incubated with compound **16** at IC_{50} concentration (0.78 μM against promastigotes) for 6 to 72 h, respectively. The highest drop (lower quadrant, green florescence) was observed in 72 h post treatment (63.72%) when subtracted with drop in untreated promastigotes (5.87%). The incubation of promastigotes with compound **16** for 6, 12, 24 and 48 h have shown 4.6, 11.59, 26.01 and 55.8% drop in membrane potential, respectively, in comparison with untreated parasite. These data indicate that compound **16** might be target FRD to trigger MMP drop.

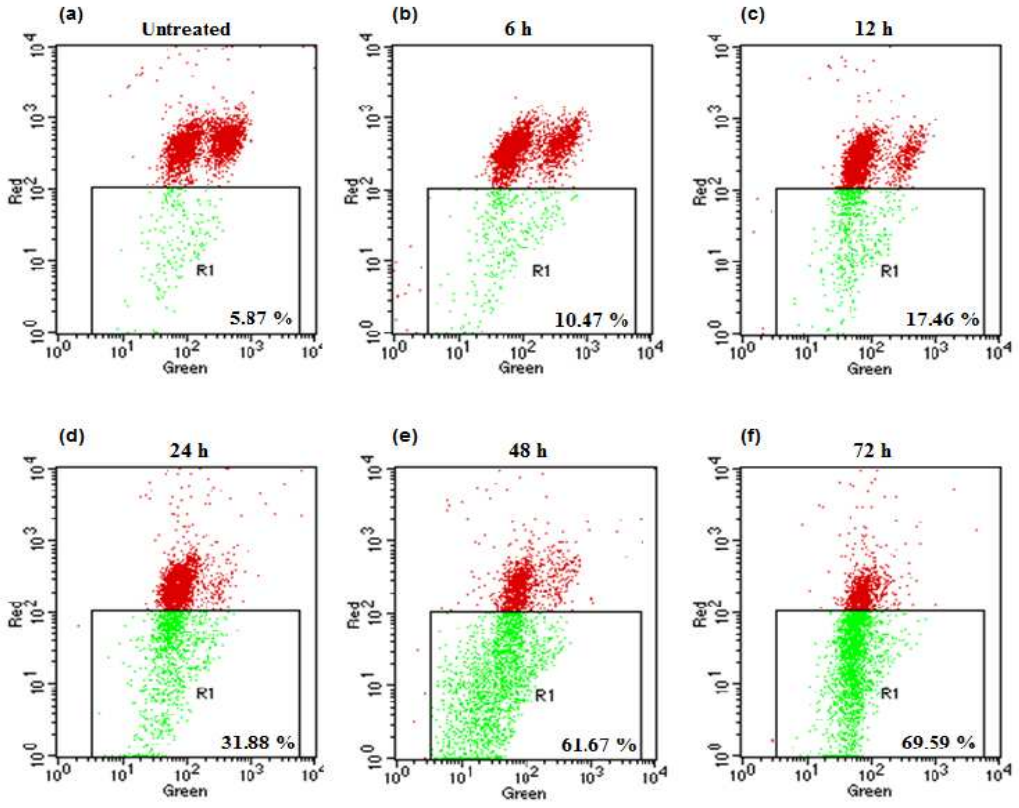


Figure 3: Drop in mitochondrial membrane potential in *Leishmania* promastigotes treated with compound **16** at different time points. (a) Untreated promastigotes, (b) 6 h treatment, (c) 12 h treatment, (d) 24 h treatment, (e) 48 h treatment, and (f) 72 h treatment with compound **16**. Upper quadrant shows red florescent cells (no drop in mitochondrial membrane potential) and lower quadrant indicates drop in mitochondrial membrane potential (green florescent cells). Results are the representative of three independent experiments.

Compound 16 Induces a Host-protective Cytokines Response by *Leishmania*-infected Macrophages. Host immune responses play important role on the effectiveness of any chemotherapeutic agent against leishmaniasis. *Leishmania* infection is classically associated with depression of Th1 type immune cells and expansion of Th2 type cells.[6] Hence, compounds that could boost up the host cell activation by Th1 biased immune responses could be exploited as therapeutic agent for VL. In this context, we have also explored the potential of compound **16** as a host protective immune cells activator. We have chosen

hallmark Th1 (IL-12 and TNF-α) and Th2 (IL-10 and TGF-β) cytokines produced by macrophages which have up- and down-regulated during treatment of *Leishmania* infection. In *Leishmania*-infected mouse macrophages (J-774A.1) treated with compound **16**, we have found 16 fold higher production of IL-12 (figure 4a, $p<0.01$**) and 11.6 fold increases in the production of TNF-α (figure 4b, $p<0.01$**) than infected control. In contrast, among the Th2 type cytokines (figure 4c-d), we have observed considerably ($p<0.01$**) low production of IL-10 (7.11 fold less than infected control) and the level of TGF-β was also down-regulated significantly ($p<0.01$**) in compound **16** treated cells (4.08 fold less production than infected-untreated macrophages). These results suggested that compound **16** has immunostimulatory potential along with direct parasite killing action.

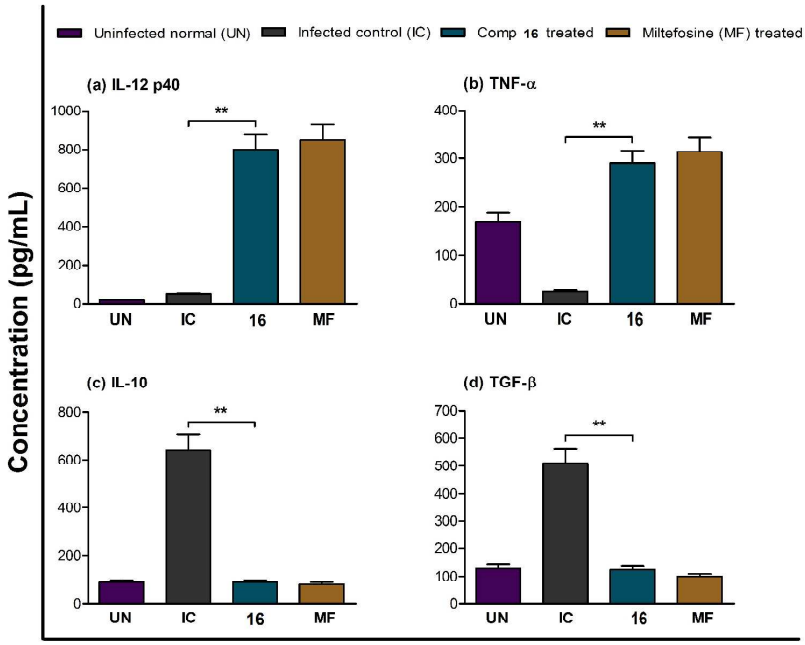


Figure 4: Normal, infected-untreated and infected-treated mouse macrophages (J-774A.1) were analyzed for Th1 cytokines, (a) IL-12 and (b) TNF-α and Th2 cytokines, (c) IL-10 and (d) TGF-β by sandwich ELISA after 24 h post treatment. The levels of cytokine release in the culture supernatant were measured in pg/mL concentration. The significance between infected control versus compound **16** treated group was calculated by student's t test using graph pad Prism (IC versus **16** - $p<0.01$**). Results are the mean ± SD of two independent experiments in duplicates. Miltefosine (MF) was used as a reference drug.

Compound 16 Provides Stimulus to *Leishmania* Infected Mouse Macrophages for Nitric Oxide Production. Nitric oxide (NO) is a potent microbicidal molecule which play critical role in the controlling of *Leishmania* infection.[5] Along with IL-12 and TNF-α, NO is also produced from macrophages to kill the *Leishmania* parasite by oxidative burst.[7] We have estimated the generation of NO by mouse macrophage cells (J-774A.1) in culture supernatant after 24 h post treatment. Our results indicate that *Leishmania*-infected compound **16**-treated macrophages showed 6.3 fold induction in NO generation when compared with corresponding infected control (figure 5, $p<0.01$**). Interestingly, it was also observed that compound **16** induces infected macrophages to production of nitric oxide (26.53 ± 2.6 μM) slight higher than that of standard drug, miltefosine treated macrophages (25.35 ± 2.5 μM). Significantly enhanced production of NO in treated macrophages also suggests that compound **16** activated mouse macrophages for successful elimination of *Leishmania* parasite.

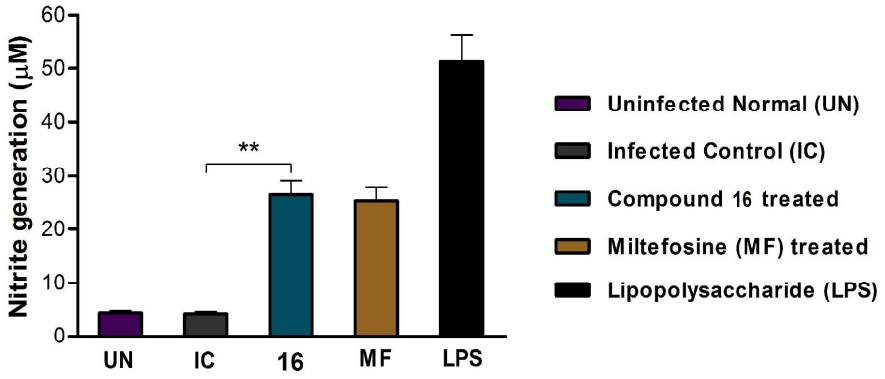


Figure 5: Generation of nitric oxide (NO) in μM concentration form normal, infected-untreated and infected-treated mouse macrophages (J-774A.1) was analyzed by Griess reagent at 24 h post treatment. The absorbance of the reaction product was measured at 540 nm using spectrophotometer. The significance between infected control versus compound **16** treated group was calculated by student's t test using graph pad Prism (IC versus **16** - $p<0.01$**). Results are the mean ± SD of two independent experiments. Miltefosine (MF) and lipopolysaccharide (LPS) was used as a reference drug and as a stimulant, respectively.

***In Vitro* Pharmacokinetic (SGF-SIF and Metabolic Stability) Investigations of Compound 16 in Golden Hamsters.** Compound **16** showed consistent *in vivo* activity at day 7 and day 28 after the last dose administration (Table 5) in hamster model. In order to evaluate stability upon oral dosing; simulated gastric fluid (SGF)/simulated intestinal fluid (SIF) and metabolic stability studies of the drug candidate were performed *in vitro*.

***In Vitro* SGF- SIF Stability Studies**

SGF and SIF stability studies conducted in order to determine compound stability in gastro-intestinal (GI) fluids. Poor ADME properties can occur due to poor pH stability of the drug. Dose absorption from the GI tract will affect if drug has instability in the stomach or intestine. The stability of a drug substance in gastric and intestinal fluids provides support whether drug degradation occur in the GI tract before membrane permeation. Compound **16** found stable in gastric fluids but it shows 30% degradation in the intestinal fluids (Figure 6).

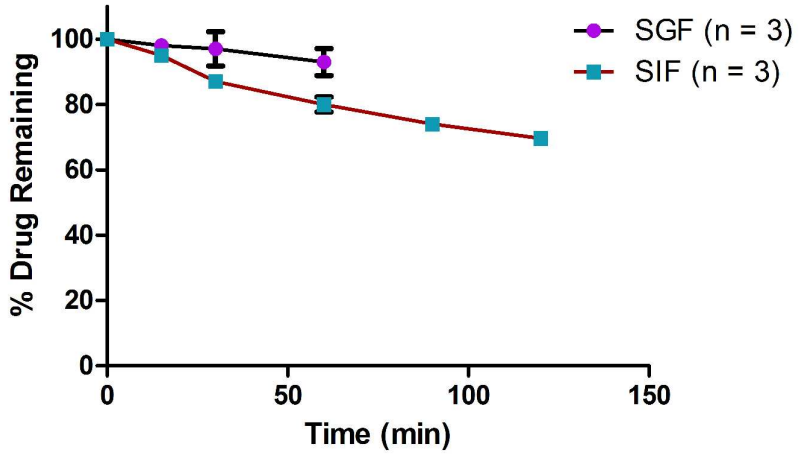


Figure 6: Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) stability of compound **16.** Results are presented as mean ± SEM.

In Vitro Metabolic Stability

Major part of drug loss in the body due to metabolism in the liver. Drug metabolism in liver plays significant role in clearance of the drug from the body. Most of the time poor oral bioavailability is due to first pass metabolism in the liver. Testosterone was employed to assess the activation of the hamster liver microsomes. The half life of the testosterone was found as per previously reported literature value. Compound **16** was found stable in the control reaction in absence of cofactor confirmed the chemical stability as well as cofactor dependent degradation. The calculated *in vitro* half-life for compound **16** was 37.78 ± 5.38 min (Figure 7).

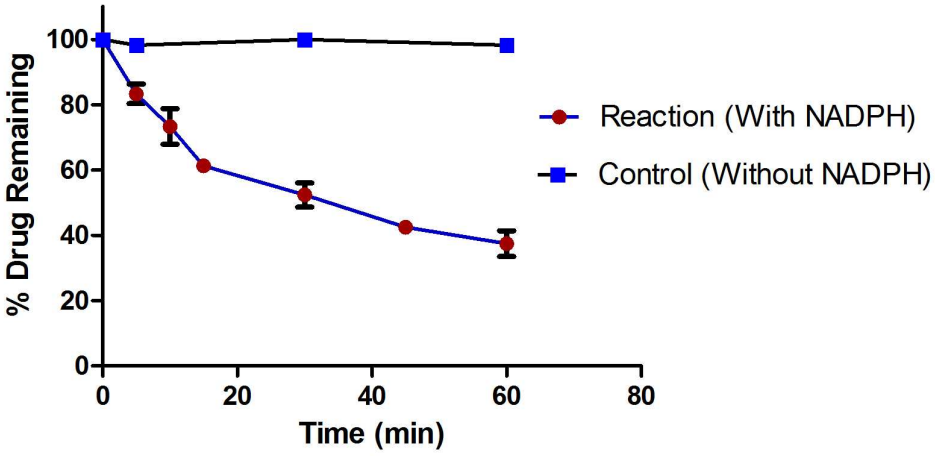


Figure 7: Metabolic Stability of compound **16;** Results are representative of three independent experiments. Data are presented as mean ± SEM.

Oral Pharmacokinetics of Compound 16 in Hamsters. Oral pharmacokinetics studies were performed in hamsters, as it was experimental model for VL. Chalcone **16** was administered as a single oral dose of 100 mg/kg and analysis was done using LC-MS/MS method to get the plasma concentration-time profile. The pharmacokinetics studies showed, no abnormality in the animals as hamsters well tolerated the given dose. Compound **16** was rapidly absorbed by oral route (Figure 8). It showed high Vd/F indicating peripheral distribution (Table 6). The

active site is usually spleen and peripheral tissue, thus compound may have good distribution at target site. The compound **16** showed two T_{max} (0.625 ± 0.3 and 0.78 ± 0.21 h) and two C_{max} (1.47 ± 0.11 and 0.82 ± 0.18 μg/mL) at 100 mg/kg, oral dose. These results indicated that compound **16** has high permeability across the gastrointestinal epithelium. The plasma concentration of **16** showed double peak phenomenon possibly due to enterohepatic recirculation or variability in absorption site (Figure 8). Half life ($t_{1/2}$) of **16** was observed 7.4 h in plasma. Thus the pharmacokinetic behaviour of compound **16** indicated very good oral bioavailability to exhibit antileishmanial activity.

Table 6: Pharmacokinetic estimation of compound **16** in Hamster after single oral 100 mg/kg dose administration

Parameter	Units	Estimates
T_{max1}	h	0.625 ± 0.3
C_{max1}	μg/mL	1.47 ± 0.11
T_{max2}	h	0.78 ± 0.21
C_{max2}	μg/mL	0.82 ± 0.18
$AUC_{0-\infty}$	h*μg/mL	3.7 ± 0.67
Cl/F	L/h/kg	27.84 ± 5.24
Vd/F	L/kg	391.73 ± 79.32
$t_{1/2}$	h	7.4 ± 0.99

T_{max}: time at which maximum concentration achieved in plasma; C_{max}: maximum concentration achieved in plasma; AUC: area under the curve from 0 to ∞ h; V_d: volume of distribution, Cl: clearance; $t_{1/2}$: terminal half life. Each value represents mean ± SD.

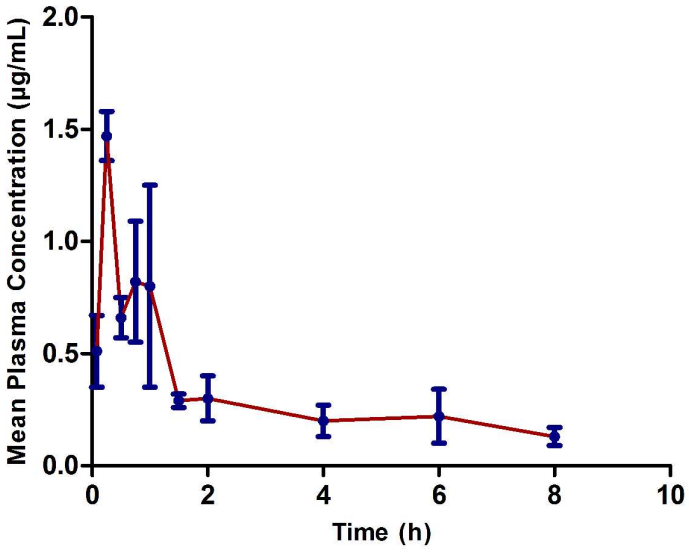


Figure 8: Mean plasma concentration−time profile of compound **16** in Male Syrian Golden hamsters (100 ± 15 gm) after a single oral administration of (100 mg/kg). Results are presented as mean ± SEM.

Binding characteristics of compounds 16 with proteins. By mean of intrinsic fluorescence quenching, we can find out the binding characteristics of small compounds with proteins. Emission spectra of bovine serum albumin (BSA) (excited at 280 nm) showed characteristic peak at 343 nm. Addition of successive amount of compound **16** resulted in to gradual decrease in fluorescence intensity as shown in figure 9a. The decrease in fluorescence intensity is usually described by the Stern-Volmer equation:[34]

$$Fo/F = 1 + Ksv[Q] = 1 + Kq\,\tau o[Q]$$

where F_0 and F are the fluorescence intensities before and after the addition of the compound **16** or quencher, respectively, K_{SV} is the dynamic quenching constant; Kq is the quenching rate constant; [Q] is the concentration of compound added; τ_0 is the average lifetime of the molecule without quencher and its value is considered to be 10^{-8} s.[35] Stern-Volmer plot (Figure 9b) shows good linear relationship within the experimental concentrations.

We observed *Ksv*, 2.7 × 10^5 L mol^{-1} (R, 0.9984) and *Kq*, 2.7 × 10^{13} L mol^{-1} s^{-1} for the compound **16**. In present study, we observed *Kq* value higher than the maximum value of the scattering collision quenching constant (2.0 × 10^{10} L $mol^{-1}s^{-1}$) of biomolecules[36] indicating that probable quenching mechanism of compound **16**-BSA interaction was initiated by complex formation. Also dynamic collisions contribute to the quenching of fluorescence as shown by dynamic quenching constant.

In order to confirm mode of compound **16**-BSA interaction as static complex formation indicated by quenching, the difference absorption spectroscopy was carried out. The absorption spectrum of BSA and the difference absorption spectrum between BSA – compound **16** complex and compound **16** at the same concentration could not be superposed (Figure 10). These results probably indicated that compound **16** form a ground state complex with BSA. BSA absorption spectra showed two peaks, change in peak around 210 nm is a result of change in peptide backbone conformation associated with helix coil transformation, whereas change around 280 nm is explained by change in microenvironment of aromatic amino acid residues (tryptophan and tyrosine).[37] These results showed that compound **16** form ground state complex with BSA associated with alteration in the conformation of BSA.

Binding Constant and Site. Fluorescence data were analyzed by Modified Stern-Volmer[38] equation for the static quenching:

$$Fo/\Delta F = \{1/(faKa[Q])\}+1/fa$$

ΔF is the difference of fluorescence in the absence and presence of compound **16** at concentration [Q], *fa* is the fraction of accessible fluorescence, and *Ka* is the effective quenching constant for the accessible fluorophores, which is similar to the binding constant for the quencher-acceptor systems. The Modified Stern-Volmer plot is shown in figure 9c. The dependence of $F_o/\Delta F$ on the reciprocal value of concentration $[Q]^{-1}$ is linear with the slope equaling to the value of $(faKa)^{-1}$.

The equilibrium in static quenching between free and bound molecules can be given by the equation:[39]

Log *(Fo-F)/F* = log K_A + *n*log*[Q]*

where, *n* is number of binding sites.

Figure 9d shows the double logarithm plot. From above two equations, we can calculate the binding constant (*Ka*) and number of binding sites (*n*). We observed calculated *Ka*, 6.9×10^5 L mol^{-1} (R, 0.9981) and *n*, 0.90 (R, 0.9973). Our results showed that the association constant of compound **16** in the range of 10^5 L mol^{-1} indicated a strong binding with serum albumin and value of the binding sites (*n)* was approximately 1, which showed that the ground state complex formation is seemed to be due to single high affinity binding site on BSA. These studies indicated high binding affinity of compound **16** towards serum protein. Thus, bound fraction of compound **16** might be acting as reservoir from which it is slowly released in the form of unbound fraction. It is also evident from compound **16'**s long half life (7.4 h) in oral pharmacokinetic studies of hamsters (Table 6).

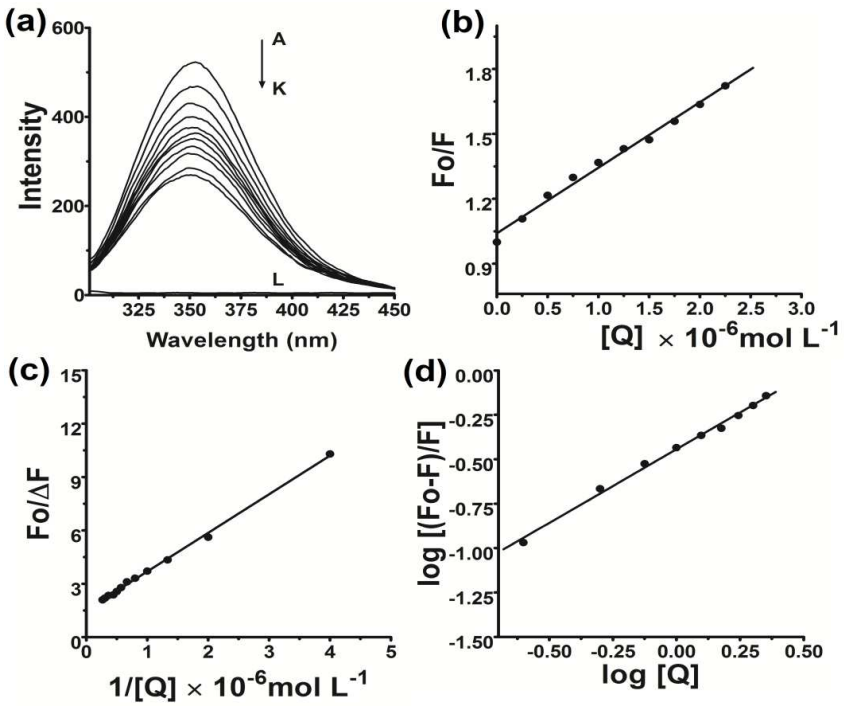


Figure 9: (a) Addition of successive amount of compound **16** resulted in to gradual decrease in fluorescence intensity (b) Stern-Volmer plot (c) Modified Stern-Volmer plots (d) double logarithm plot.

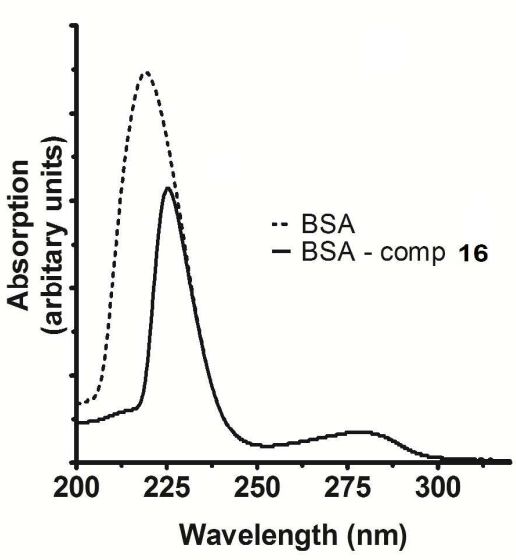


Figure 10: Absorption spectrum between Bovine Serum Albumin (BSA) –compound **16** complex

CONCLUSION

In conclusion, a series of new chalcone analogues with various structural features such as chromenochalcones, chromanochalcones, chromenodihydrochalcone and chromenochalcones with hetero atom in ring-A were prepared based on natural product lead and tested for their antileishmanial activity. Among all, compounds **11, 14, 16, 17, 22** and **24** were found to be significantly more active than the standard antileishmanial drugs, miltefosine and sodium stibogluconate (SSG) in *in vitro* evaluation against *Leishmania* amastigotes. The *in vivo* studies of compounds **16**, **17** and **22** were performed in *L. donovani* / hamster model, in which compound **16** showed consistence activity up to day 28 post treatment (84 % parasite inhibition at 100 mg/kg x 5 days dose by oral route). The molecular and immunological studies showed that compound **16** has dual nature to act as a direct parasite killing agent and as a host immunostimulant. Pharmacokinetics and serum albumin binding studies also

suggest that compound **16** has potential to be a candidate for the treatment of non-healing form of leishmaniasis.

EXPERIMENTAL SECTION

General Methods.

Melting points were recorded on Buchi-530 capillary melting point apparatus and are uncorrected. IR spectra were recorded on Perkin-Elmer AC-1 spectrometer. ^{1}H NMR spectra were recorded on Bruker Avance DPX 200 FT, Bruker Robotics and Bruker DRX 300, spectrometers at 200, 300 MHz (^{1}H) and 50, 75 MHz (^{13}C). Experiments were recorded in $CDCl_3$, CD_3OD, and DMSO-D_6 at 25ºC. Chemical shifts were given in parts per million (ppm) downfield from internal standard Me_4Si (TMS). ESI mass spectra were recorded on JEOL SX 102/DA-6000. Chromatography was executed with silica gel (60–120 or 230–400 mesh) using mixtures of ethyl acetate and hexane as eluants. Reactions, which required the use of anhydrous, inert atmosphere techniques, were carried out under an atmosphere of Nitrogen. 1, 4-Dioxane was distilled over sodium. Commercially available reagents, solvents and starting materials were used without further purification. Elemental analyses were performed on a Vario EL-III C, H, N, S analyzer (Germany), and values were within ±0.5% of the calculated values; therefore, these compounds meet the criteria of >95% purity. Analytical HPLC analyses were performed on a Shimazadu 10ATVP HPLC instrument, Zorbax C18 column (150 × 4.6 mm, 5 μm). A purity of ≥ 95% has been established for compounds, which showed good *in vitro* and *in vivo* activity.

1-(5-Hydroxy-2-methyl-2-(4-methylpent-3-enyl)-2H-chromen-6-yl) ethanone (3). To a magnetically stirred solution of **1** (12.0 g, 79 mmol) in dry pyridine (8.04 mL) was added gradually citraldimethylacetal (15.6 g, 79 mmol) at rt. The whole reaction mixture was refluxed for 4 h at 150^{o}C, additional equivalent of citraldimethylacetal (15.6 g, 79 mmol) was added and refluxed for further 6 h. Excess pyridine, in the reaction mixture was evaporated

by rotary evaporator under reduced pressure. The crude product was subjected to silica gel column chromatography using hexane/ethyl acetate as mobile phase to afford the desired compound **3** (14.45g, 64%). Semi-solid; FT-IR (neat, cm^{-1}): 3436, 1695, 1486, 1430, 1373, 1270, 1086, 758; ^{1}H NMR ($CDCl_3$, 300 MHz) δ: 12.99(s, 1H), 7.54 (d, *J* = 8.8 Hz, 1H), 6.76 (d, *J* = 10.1 Hz, 1H), 6.32 (d, *J* = 8.8 Hz, 1H), 5.54 (d, *J* = 10.1 Hz, 1H), 5.10 (t, *J* = 7.1 Hz, 1H), 2.52 (s, 3H), 2.14 (m, 2H), 1.69–1.80 (m, 2H), 1.67 (s, 3H), 1.59 (s, 3H), 1.40 (s, 3H); MS (ESI): m/z: 287 $(M + H)^+$.

1-[5-Hydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-2H-chromen-6-yl]-3-(4-hydroxy phenyl)-propenone (5). To a stirred solution of 3 (500 mg, 2.2 mmol) in aqueous KOH solution in ethanol (5 mL) was added 4-hydroxybenzaldehyde (311 mg, 2.2 mmol). The whole reaction mixture was stirred for 48 h at rt, and quenched in ice-cold water, acidified with 1 N HCl, extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with water, brine solution, dried over anhydrous Na_2SO_4, and the solvent was evaporated under reduced pressure. The crude product was subjected to silica gel column chromatography using hexane/ethyl acetate as mobile phase to afford the chromenochalcone **5** (346 mg, 45%); semi-solid; FT-IR (Neat, cm^{-1}) 3436, 1635; ^{1}H NMR ($CDCl_3$,200 MHz) δ 13.65 (s, 1H), 7.82 (d, *J* = 15.3 Hz, 1H), 7.70 (d, *J* = 8.8 Hz, 1H), 7.53 (d, *J* = 8.4 Hz, 2H), 7.42 (d, *J* = 15.3 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, 2H), 6.79 (d, *J* = 10.1 Hz, 1H), 6.37 (d, *J* = 8.8 Hz, 1H), 5.53 (d, *J* = 10.1 Hz, 1H), 5.09 (t, *J* = 6.6 Hz, 1H), 2.08 (q, *J* = 6.6 Hz, 2H), 1.73 (t, *J* = 4.5 Hz, 2H), 1.65 (s, 3H), 1.56 (s, 3H), 1.43 (s, 3H); MS (FAB) *m/z:* 391 $(M + H)^+$.

2-Hydroxy-5-{3-[5-hydroxy-2-methyl-2-(4-methyl-pent-3-enyl)-2H-chromen-6-yl]-3-oxo-propenyl}-benzaldehyde (6). To a stirred solution of **3** (575 mg, 2 mmol) in anhydrous THF (5 mL) was added portion wise NaH (120 mg, 5 mmol) and stirred for 20 min at rt under nitrogen. Then *p*-hydroxy-benzene-1, 3-dicarbaldehyde (300 mg, 2 mmol) in 2 mL of THF

was added to the reaction mixture and stirred for 8 h at rt. The mixture was poured into ice-cold water, acidified with 1N HCl. extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water, brine solution, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The crude product was subjected to silica gel column chromatography using hexane/ethyl acetate as mobile phase to afford the chromenochalcone **6** (210 mg, 25%); mp: 105-108^oC; FT-IR (KBr, cm^{-1}) 3430, 1635, 1629,; ^1H NMR ($CDCl_3$, 300 MHz) δ 13.66 (s, 1H), 11.28, (s, 1H), 9.98 (s, 1H), 7.88 (d, *J* = 15.4 Hz, 1H), 7.84 (broad s, 2H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.53 (d, *J* = 15.4 Hz, 1H), 7.07 (d, *J* = 8.8 Hz, 1H), 6.82 (d, *J* = 10.1 Hz, 1H), 6.41 (d, *J* =8.7 Hz, 1H), 5.55 (d, *J* = 10.1 Hz, 1H), 5.11 (t, *J* = 7.0 Hz, 1H), 2.11 (m, 2H), 1.81 (m, 2H), 1.68 (s, 3H), 1.59 (s, 3H), 1.46 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 196.3, 191.4, 163.3, 160.9, 160.3, 142.0, 136.0, 134.4, 131.9, 130.6, 127.1, 127.0, 123.7, 120.6, 119.3, 118.7, 116.3, 113.8, 109.2, 108.2, 80.4, 41.7, 27.2, 25.6, 22.6, 17.6; MS (FAB) *m/z:* 419 $(M + H)^+$, HRMS:419.1843 (MH+).

1-[2-Methyl-2-(4-methyl-pent-3-enyl)-5-(2-piperidin-1-yl-ethoxy)-2H-chromen-6-yl]-3-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-propenone (14). To a stirred solution of chalcone, **5** (250 mg, 1.0 mmol) in dry acetone (20 mL) were added anhydrous K_2CO_3 (2.85 g, 20.8 mmol), 1-(2-Chloro-ethyl)-piperidine hydrochloride (953 mg, 5.2 mmol), and the reaction mixture was refluxed for 5 h. The mixture was filtered off under suction and solvent was evaporated under reduced pressure. The crude product was subjected to silica gel column chromatography using hexane/ethyl acetate as mobile phase to afford the compound **14** Yield: 51%; FT-IR (neat, cm^{-1}) 1652; ^1H NMR ($CDCl_3$, 200 MHz) δ 7.67 (d, *J* = 15.6 Hz, 1H), 7.58 (d, *J* = 8.6 Hz, 1H), 7.53 (d, *J* = 8.5 Hz, 2H), 7.42 (d, *J* = 15.6 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 2H), 6.80 (d, *J* = 10.0 Hz, 1H), 6.61 (d, *J* = 8.6 Hz, 1H), 5.63 (d, *J* = 10.0 Hz, 1H), 5.12 (d, *J* = 6.0 Hz, 1H), 4.14 (t, *J* = 6.2 Hz, 2H), 3.93 (t, *J* = 6.3 Hz, 2H), 2.79 (t, *J* = 6.2 Hz, 2H), 2.62 (t, *J* = 6.3 Hz, 2H), 2.52–2.38 (m, 12H), 1.73–1.25 (m, 21); ^{13}C NMR ($CDCl_3$, 50

MHz) δ 191.4, 161.1, 158.1, 155.6, 143.2, 132.3, 131.6, 130.5 (2C), 129.7, 128.3, 126.4, 124.6, 124.2, 117.8, 115.3 (3C), 112.8, 79.6, 73.9, 59.0, 58.2, 55.4 (2C), 55.2 (2C), 41.8, 27.1, 26.2 (6C), 24.6 (2C), 23.1, 18.0; MS (FAB) *m/z:* 613 $(M + H)^+$.

1-(5-Hydroxy-2-methyl-2-(4-methylpentyl) chroman-6-yl) ethanone (19). To a solution of 4 (575 mg, 2 mmol) in methanol (10 mL) was added a catalytic amount of 10% Pd/C. The reaction mixture was shaken in hydrogenation assembly under hydrogen gas at 50 lbs for 2 h. After replacement of air by nitrogen, Pd/C was filtered off and methanol was evaporated under reduced pressure. The crude product was subjected to silica gel column chromatography using hexane/ethyl acetate as mobile phase to afford the title compound 16 (375 mg, 64%); Semi-solid; FT-IR (neat, cm^{-1}) 3430, 1714; ^1H NMR ($CDCl_3$, 200 MHz) δ 7.48 (d, *J* = 8.9 Hz, 1H), 6.33 (d, *J* = 8.9 Hz, 1H), 2.66 (t, *J* = 6.8 Hz, 2H), 2.53 (s, 3H), 1.79 (t, *J* = 6.4 Hz, 2H), 1.35–1.60 (m, 5H), 1.29 (s, 3H), 1.18–1.21 (m, 2H), 0.88 (s, 3H), 0.85 (s, 3H); MS (FAB) (*m*/*z*): 291 $(M + H)^+$.

1-(5-hydroxy-2-methyl-2-(4-methylpent-3-enyl)-2H-chromen-6-yl)-3-(pyridin-3-yl) propan-1-one (23). To a magnetically stirred solution of **16** (100 mg, 0.27 mmol) in methanol (5 mL) was added gradually $NiCl_2.6H_2O$ (64 mg, 0.11 mmol) at rt. The whole reaction mixture was brought to 0^oC and $NaBH_4$ (10 mg, 0.27 mmol) was added portion wise. After addition of $NaBH_4$, the whole solution was stirred for 15 min at 0^0 C. Methanol was removed by vacuum, and then the reaction mixture was dissolved in ethyl acetate and neutralized with 10% HCl solution, the organic layer was washed with water, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. Then the crude product was chromatographed on silica gel to afford the desired compound **24** (60 mg, yield 60%); semi-solid; FT-IR (KBr, cm^{-1}) 3430, 1635; ^1H NMR ($CDCl_3$, 300 MHz) δ 12.85 (s, 1H), 8.53 (s, 1H), 8.45 (broad s, 1H), 7.58 (d, *J* = 7.5 Hz, 1H), 7.48 (d, *J* = 8.8 Hz, 1H), 7.23(m, 1H), 6.74 (d, *J* = 10.1 Hz, 1H), 7.30 (dd, *J* = 78.8Hz, 1H), 5.52 (d, *J* = 10.1 Hz, 1H), 5.01 (t, *J* = 6.6 Hz,

1H),3.22(t, *J* = 7.5 Hz, 2H), 3.06(t, *J* = 7.5 Hz, 2H), 2.05 (m, 2H), 1.74 (m, 2H), 1.65 (s, 3H), 1.56 (s, 3H), 1.41 (s, 3H); MS (FAB) *m/z:* 378 $(M + H)^+$; HRMS: 378.2064 (MH+).

1-(3-(5-hydroxy-2-methyl-2-(4-methylpent-3-enyl)-2H-chromen-6-yl)-5-(pyridin-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)ethanone (24). A mixture of chromenochalcone **16** (100mg, 0.27 mmol), hydrazine hydrate (13.5 mg, 0.27 mmol) and acetic acid (2.0 mL) was heated under reflux for 10 h until complete consumption of the chalcone (TLC control). After cooling, the resulting solution was neutralized with concentrated ammonium hydroxide. Then, the adding of crushed ice to the solution precipitated a solid which was filtered and washed with water. The crude product was subjected to silica gel column chromatography using hexane/ethyl acetate as mobile phase to afford the title compound **23** (86 mg, 75%); semi-solid; FT-IR (Neat, cm^{-1}) 3425, 1648, 1640, 1578; ^1H NMR ($CDCl_3$, 50 MHz) δ 10.53 (s, 1H), 8.55 (m, 2H), 7.55 (d, *J* = 8.2 Hz, 1H), 7.31 (broad s, 1H), 6.41 (d, *J* = 8.7 Hz, 1H), 6.81(d, *J* = 10.1 Hz, 1H), 6.41 (d, *J* = 8.7 Hz, 1H), 5.62 (d, *J* = 10.1 Hz, 1H), 5.10 (t, *J* = 6.5 Hz, 1H), 3.87(m, 2H), 3.25(m, 2H), 2.31 (s, 3H), 2.12. (m, 2H), 1.78 (m, 2H), 1.67(s, 3H), 1.57 (s, 3H), 1.43 (s, 3H); MS (FAB) *m/z:* 432 $(M + H)^+$. HRMS: 432.2267 (MH+).

Experimental Section for Biology

Parasite, Cell culture and Animals

The WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) was maintained as promastigote *in vitro* in medium 199 supplemented with 10% heat inactivated fetal bovine serum (HIFBS) at 24 ± 2ºC incubator and as amastigotes in golden hamsters (*Mesocricetus auratus*). Adherent mouse macrophage cell line (J774-A.1) were maintained in RPMI -1640 medium with 10% HIFBS at 37ºC in a 5% CO_2 incubator.[40] Healthy, inbred hamsters (initial weight 40-45 gm) of both sexes were used in *in vivo* studies. For oral pharmacokinetic study, male Syrian Golden hamsters (weight 100 ± 15 gm) were used. Throughout the study, the

animals were housed in climate-controlled (23 ± 2°C; Relative humidity: 60%) and photoperiod-controlled (12 h light-dark cycles) animal quarters. Animals were fed standard rodent pellet and had free access to drinking water. All the *in vivo* studies were performed in compliance with the Institutional Animal Ethics Committee (IAEC) guidelines for use and handling of animals.

Promastigote Growth Inhibition Assay

The antileishmanial activity of these compounds on the extracellular promastigote form of *L. donovani* was assessed as described earlier.[40] The late log phase of promastigotes (expressing firefly luciferase gene) were seeded with complete M-199 medium at 5 x 10^5/mL/100µL/well in 96-well plates and incubated with tested compounds in a 24ºC incubator for 96 h. Miltefosine was used as a standard drug. After 96 h of incubation, 50 μL of promastigote suspension was pipette out from each well in to another 96-well plate and mixed with an equal volume of Steady Glo® reagent (Promega) and luminescence was measured by using a luminometer. The values were expressed as relative luminescence unit (RLU). The inhibition of parasitic multiplication is determined by comparison of the luciferase activity of compound treated parasites with that of untreated control.

Reporter Gene - based Antiamastigote Assay

Mouse macrophage cell line (J-774A.1) infected with promastigotes (expressing luciferase firefly reporter gene) was used for the assessment of the activity of compounds against the amastigote form of *Leishmania* parasite. J-774A.1 cells were seeded in a 96-well plate (4 x 10^4/mL/100μL/well) in RPMI-1640 medium containing 10% HIFBS and the plates were incubated at 37ºC in a CO_2 incubator (5% CO_2- 95% air mixture). After 24 h, the medium was replaced with fresh medium containing stationary phase promastigotes (4 x 10^5/mL/100μL/well). Promastigotes were phagocytized by the macrophages and inside the

phagolysosomes, they were transformed into amastigotes form. Each well of the plate was washed with plain RPMI medium after 24 h of incubation to remove the un-internalized promastigotes. The test compounds were added at dilutions up to 7 points starting from 40μM concentration in complete RPMI medium and the plates were incubated at 37ºC in a CO_2 incubator for 72 h. After incubation, the drug containing medium was aspirated and 50 μL Phosphate buffer saline (PBS) was added in each well and mixed with an equal volume of Steady Glo® reagent. After gentle shaking for 1-2 min, the reading was taken in a luminometer.[40] The values are expressed as RLU and data were transformed into a graphic program (Excel). IC_{50} value of each compound was calculated by nonlinear regression analysis of the concentration response curve using the four parameter Hill equations.

Cytotoxicity Assessment Assay

The cytotoxicity of the compounds was determined by following the method of Mosmann[41] with some modifications. As described previously,[40] mammalian kidney fibroblast cells (vero cell line) (1 x 10^5/mL/100μL/well) were incubated with test compounds (in 7 concentrations starting from 400μM) at 37ºC in a CO_2 incubator. After 72 h of incubation, 25 μL of MTT (3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent (5 mg/mL) in PBS medium was added to each well and incubated at 37°C for 2 h. At the end of the incubation period, the supernatant were removed and 150 μL of pure DMSO was added to each well for solubilizing the formazan crystal. After 15 min of shaking, the readings were recorded as absorbance at 544 nm on a micro plate reader. Fifty percent cytotoxic concentration (CC_{50}) values were estimated as described by Huber & Koella.[42] The selectivity index (SI) for each compound was calculated as ratio between, cytotoxicity (CC_{50}) in vero cells and activity (IC_{50}) against *Leishmania* amastigotes.

In Vivo Evaluation in *L. donovani* / Hamster Model

The *in vivo* antileishmanial activity was determined in golden hamsters infected with MHOM/IN/80/Dd8 strain of *L. donovani*. The method as described by Gupta *et al*[32] was used for *in vivo* evaluation. Golden hamsters of either sex were infected intracardiacally with 1 x 10^7 amastigotes per animal. After establishment of infection in 15-20 days, pre-treatment spleen biopsy was performed to assess the degree of infection in all the animals. The animals with +1 grade infection (5-10 amastigotes / 100 spleen cell nuclei) were included in the chemotherapeutic trials. Five to six infected animals were used for each test compound. Drug treatment in different dose regimen by intraperitoneal (IP) or per oral (PO) route was initiated after 2 days of pre- treatment biopsy and continued for 5-10 consecutive days. Miltefosine and SSG were used as a reference drugs. Post-treatment biopsies were done on day 7 and day 28 of the last dose administration and amastigote counts are assessed by Giemsa staining. Intensity of infection in both, treated and untreated animals, and also the initial count in treated animals was compared and the efficacy was expressed in terms of percentage inhibition (PI) using the following formula:-

PI = 100- [(ANAT x 100) / (INAT x TIUC)]

Where PI is Percent Inhibition of amastigotes multiplication, ANAT is Actual Number of Amastigotes in Treated animals, INAT is Initial Number of Amastigotes in Treated animals and TIUC is Time Increase of parasites in Untreated Control animals.

Apoptotic- Necrotic Profiling with Annexin V and Propidium Iodide (PI)

To assess the apoptotic-necrotic profiling of untreated and drug treated *L. donovani* promastigotes, apoptosis detection kit (Sigma Aldrich, USA) was used as per manufacturer's instructions. Exposure of phosphatidylserine due to apoptosis was studied using annexin V-

FITC (Fluoroscein isothiocynate) in untreated and treated promastigotes and cell impermeable dye, propidium iodide (PI) was also used to label the cellular DNA in necrotic cells to differentiate cell death via apoptosis or necrosis. Briefly, promastigotes of log phase (1 x 10^6/ mL / well) were seeded in 24 wells plate. These promastigotes (except control) were treated with IC_{50} concentration of test compound and incubated at 24 ± 2°C for different time points (6, 12, 24, 48 and 72 h). After incubation, treated and untreated promastigotes were washed with PBS by centrifugation followed by addition of 5 μL annexin V- FITC and 10 μL PI to each tube. Samples were incubated for 30 minutes at room temperature. Fluorescence of cells immediately determined with Cell Quest FACS Calibur (Becton Dickinson). At least 10,000 cells were analyzed for each sample. Cells stained with annexin V-FITC and PI were analysed by flow cytometry using excitation wavelength, 488 nm and 536 nm and emission wavelength, 530 nm and 617 nm on FL1 and FL2 channel, respectively.[43]

Measurement of Mitochondrial Membrane Potential Drop

To assess the change in mitochondrial membrane potential (ΔΨm), a cell-permeable, cationic and lipophilic dye, JC-1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Invitrogen, USA), was used following the manufacturer's instruction. JC-1 dye aggregates in mitochondria and give red florescence at higher membrane potential and at lower membrane potential, JC-1 cannot bind with mitochondria and remains as monomer form in cytoplasm and give green florescence. Briefly, *Leishmania* promastigotes in log phase were centrifuged and suspended in complete M-199 medium. A population of 1 x 10^6 promastigotes / mL / well were seeded in 24 wells plate. Test compound in IC_{50} concentration was added in each well (except control well) and incubated at 24 ± 2ºC for different time points (6, 12, 24, 48 and 72 h). After incubation, cells were washed and resuspended in 1 mL PBS and incubated with 10 μL of 200 μM JC-1 (2 μM final concentration) for 15 min at 37ºC and analysed by flow cytometry using 488 nm excitation wavelength and 530 nm emission

wavelength on FL1 channel. Data acquisition was carried out using a FACS calibur and analysed using Cell Quest Pro software.[44]

Estimation of Th1 / Th2 Cytokines

Culture supernatant from treated and untreated mouse macrophages (J-774A.1 cell line) were analyzed for production of various cytokines (IL-12, TNF-α, IL-10 and TGF-β) by using an OptEIA set ELISA kit (BD Biosciences, USA) according to manufacturer's instructions. Briefly, 1 x 10^6 mouse macrophages (J-774A.1) infected with *Leishmania* parasite (1:10 ratio) were incubated with tested compounds at IC_{50} concentration in 6-wells plate at 37ºC in CO_2 incubator. Culture supernatant of normal, infected-untreated and infected-treated cells from each well collected after 24 h incubation for estimation of the cytokines level by ELISA as described previously.[45] At the end of the experiment, the absorbance was measured at 450 nm in a microplate reader (BioTek instruments, USA).

Quantification of Nitric Oxide (NO)

Accumulation of nitrite in culture supernatant of normal, infected-untreated and infected-treated macrophages was detected by the Griess reaction for the quantification of NO generation.[46] Briefly, *Leishmania* infected macrophage cells (1×10^6 / mL / well) were incubated with tested compounds for 24 h before nitrite assay. LPS (10 μg/mL) was used as a stimulant. After 24 h of incubation, cell supernatant (500 μL) were collected from each well and mixed with an equal volume of Griess reagent (Sigma Aldrich, USA) at room temperature. The absorbance of the test samples was measured at 540 nm in a spectrophotometer (molecular devices, USA). The concentration of NO in macrophage supernatants was quantified by a standard curve generated with sodium nitrite.

In Vitro SGF-SIF Stability

Simulated gastric fluid (SGF) / simulated intestinal fluid (SIF) mimic GI tract in terms of acidity/basicity and molarity and are a perfect media to determine the stability of the drug candidate *in vitro*. The simulated gastro-intestinal fluids were prepared according to USP specifications.[47] Briefly, 10 μM of compound was incubated in SGF and SIF at 0, 15, 30 and 60 min time points for the gastric stability experiment and at 0, 30, 60, 90, 120 min for the intestinal stability experiment. Organic content in reaction mixture was kept less than 1%. At each time point, 200 μL of sample was taken and quenched with 200 μL of ice cold methanol. Samples were processed using liquid-liquid extraction (LLE) and quantify using validated HPLC-UV method. Stability results were expressed in % remaining vs time graph. Calculation was done by % loss with respect to zero minute time point. The percentage of parent compound remaining at each time point relative to the 0 min sample is calculated from peak area ratios.

In Vitro Metabolic Stability

In vitro metabolic stability studies were performed in early drug discovery to choose compounds with favorable pharmacokinetics based on half life in liver fractions. Most commonly used liver fraction is S9 consists of both soluble and membrane bound enzymes, and contains a wide variety of both Phase I and Phase II enzymes. Incubations were performed in triplicate in test tube at 37°C in a bench-top Lab-Line shaker (Julabo sw 23) for 60 min. The incubation was performed at final 10 μM concentration. Incubation reaction mixture contains 2 mg/mL protein hamster liver S9, 50 mM tris buffer, 20 mM $MgCl_2$ and 2 mM NADPH. Briefly, mixture of Tris buffer, $MgCl_2$, Protein and NADPH were pre-incubated for 10 min and reaction was initiated with tested compound. Control reaction without NADPH was also performed to reveal any chemical instability or non-cofactor

dependent enzymatic degradation. A final organic content was kept 1% in all the reaction mixtures. Testosterone was used as positive control to check the activity of liver fraction. The reaction sample was taken at 0, 5, 10, 15, 20, 30, 45 and 60 min time points. Control reaction samples were taken at 0, 15, 30 and 60 min. Samples (200 μL) were taken at each time points and quenched with 200 μL ice cold methanol. Samples were processed using LLE.[48] The S9 fraction stability data is expressed as % parent remaining at different time points relative to the parent at 0 minute (100% parent). The data will be fitted to the one phase exponential decay equation ($A = A_o e^{-kt}$) using GraphPad Prism software. *In vitro* half life ($t_{1/2}$) generated by software is reported.

Oral Pharmacokinetics Studies in Golden Hamsters

Oral pharmacokinetic study was performed in Male Syrian Golden hamsters. Compound was administrated to male hamsters orally by gavage (100 mg/kg). Oral formulation was prepared in aqueous suspension. The Blood samples were collected from retro-orbital plexus of hamster under light ether anesthesia into microfuge tubes containing heparin as an anti-coagulant. The samples were taken at 0.08, 0.25, 0.5, 0.62, 1, 1.5, 2, 4, 6, 8, 12 and 24 h post-dose. The plasma was separated and stored at −70°C until analysis. The amount of compound was determined in hamster plasma by HPLC-UV method. The plasma concentration–time profile of compound was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, USA).

Spectroscopic Analysis of BSA-Compound Interaction

Intrinsic fluorescence was measured the on a Varian fluorescence spectrophotometer in a quartz cell with 5 nm excitation / emission slits. Change in BSA emission was measured in a 2.5 mL solution having 1.00×10^{-5} mol L^{-1} BSA (in 0.10 M Tris-HCl buffer, pH 7.4) with successive additions of 1.00×10^{-3} mol L^{-1} compound. Titrations were done manually by

micro-injector. The excitation wavelength was 280 nm and fluorescence emission spectra were recorded between 300 and 450 nm. The UV–vis absorption spectra were recorded in a double beam spectrophotometer (Shimadzu UV 2450) with 1.0 cm path length matched strain free cells. BSA in buffer was taken in sample cell and same aliquots of compound were added in both reference and sample cell. Finally difference spectra of reference and sample cells were recorded between 200-350 nm wavelengths. Similarly, absorption spectra of compound with increasing concentration of BSA were recorded.[40]

ASSOCIATED CONTENT

Supporting Information

Final compounds characterization data and NMR spectra of selected chalcones, *in vitro* activity of previously reported chromenochalcones and quantification of compound **16** by HPLC-UV method are associated with this article. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

BSA, bovine serum albumin; FITC, Fluoroscein isothiocynate; FRD, fumarate reductase; IC_{50}, half maximal inhibitory concentration; iNOS, inducible nitric oxide synthase; IP, intraperitoneally; MMP, mitochondrial membrane potential; NO, nitric oxide; PI, propidium iodide; PO, per oral; SAR, structure–activity relationship; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SSG, sodium stibogluconate; p.t., post treatment; VL, visceral leishmaniasis.

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31. (a) The compound **16** (IC_{50} = 5.4 μM) can be synthesized with two steps, whereas **11** (IC_{50} = 5.8 μM) need four steps with low overall yield. (b) Compound **14** is an alkylamine derivative of **5**. In general, alkylamine derivatives increases the bioavailability as a result the activity enhances, which is true, if we compare the *in vitro* activity of parent compound **5** (IC_{50} = 16.5 μM) with compound **14** (IC_{50} = 6.3 μM). Similar scenario exists with the compound **16** (IC_{50} = 5.4 μM) and its alkylamine derivative **17** (IC_{50} = 1.7 μM), however, in *in vivo* studies compound **17** has failed to show consistence activity on day 28. Hence, compound **14** was not taken up for *in vivo* studies. Compound **24** was hydrazine derivative prepared to mask the Michael system of **16**.

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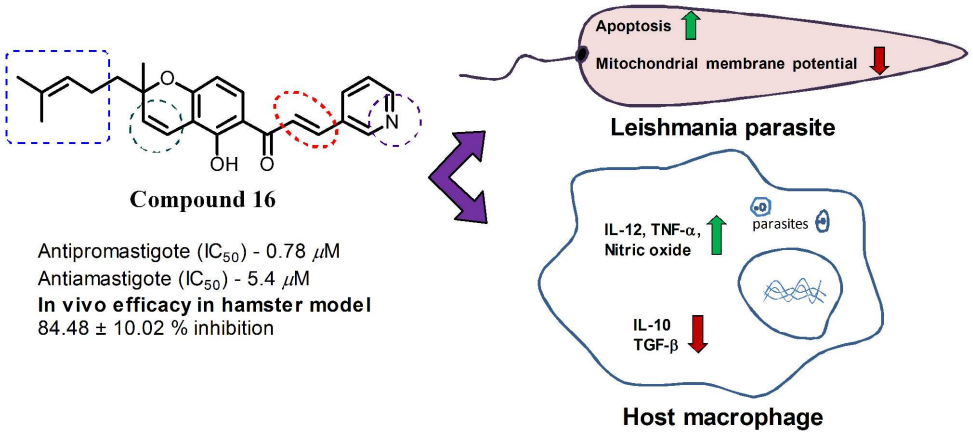

Compound 16

Antipromastigote (IC_{50}) - 0.78 μM
Antiamastigote (IC_{50}) - 5.4 μM
In vivo efficacy in hamster model
84.48 ± 10.02 % inhibition

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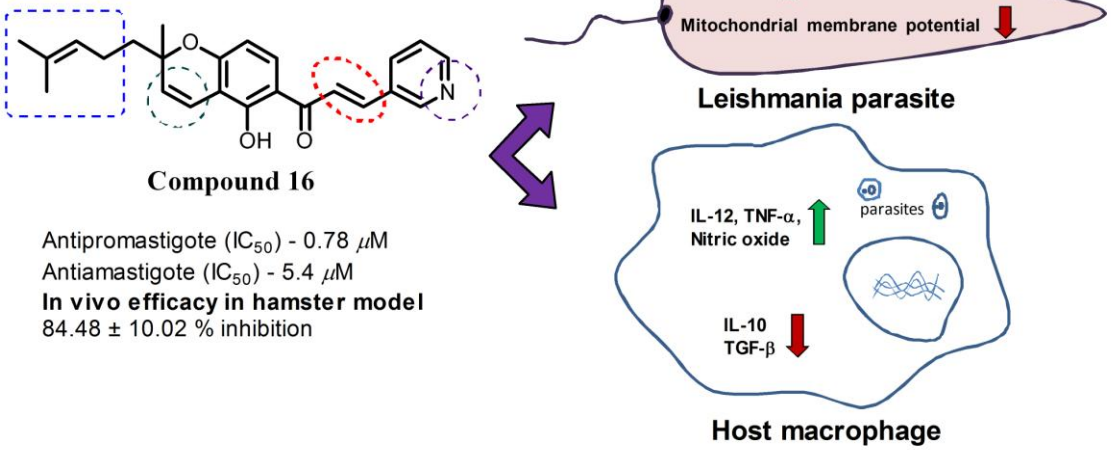

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