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A natural product inspired hybrid approach towards the synthesis of novel pentamidine based scaffolds as potential anti-parasitic agents

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ABSTRACT

A natural product inspired molecular hybridization approach led us to a series of novel pentamidine based pyrimidine and chalcone scaffolds. All the hybrids were evaluated for their anti-leishmanial potential. Most of the screened compounds have showed significant in vitro anti-leishmanial activity with less cytotoxicity in comparison to the standard drugs (pentamidine, sodium stibogluconate, and miltefosine). Additionally, anti-malarial screening of these compounds was also done and four compounds have shown superior activity against chloroquine resistance strain (K1) of *Plasmodium falciparum.*

Nowadays, neglected tropical disease (NTDs) affect more than one billion people worldwide, causes over 550,000 deaths annually.[1] Research projects aiming to discover new drugs for NTDs have discouraged drug companies from investing due to the low returns of investment.[2] Owing to this, drug discovery pipeline is still almost dry for NTDs. Among NTDs, Chagas disease, sleeping sickness, malaria, and leishmaniasis are the major NTDs with the highest rates of death.[3] In particular, *leishmania* is responsible for cutaneous and visceral infections, endemic in 88 countries in the Horn of Africa, South Asia, and Latin America.[4] Leishmaniasis is a vector born disease caused by different species belonging to the genus *Leishmania*, a protozoa transmitted by the bite of a tiny long (2–3 mm) insect vector, the phlebotomine sandfly.[5] *Leishmania* is manifested in four major clinical forms i.e. cutaneous leishmaniasis, mucocutaneous leishmaniasis, visceral leishmaniasis, and post kala-azar dermal leishmaniasis or PKDL. Among all the four forms, visceral leishmaniasis (VL), caused by the parasite *Leishmania donovani,* is nearly always fatal if not treated.[6]

The first line drugs for the treatment of *leishmania* are pentavalent antimonial compounds, which were discovered almost 70 years ago, and generally require high dose of parental administration. Moreover, this class of drugs need long-term treatment associated with severe side effects including cardiac arrhythmia and pancreatitis.[7] Pentamidine, miltefosine, and amphotericin-B, are the second line drugs for the treatment of VL, which also suffer from moderate to severe side effects.[8] Pentamidine, an aromatic diamidine, is orally inactive and may demonstrate renal, hepatic and pancreatic toxicity beside with hypotension and dysglycemia.[9] Amphotericin-B and its lipid complex is a most useful alternative, however, major draw backs associated with amphotericin-B, such as its high cost leave out of the reach of poor people.[10]

Furthermore, recently introduced first orally active drug Miltefosine, a phosphocholine analogue, has a long half-life (100–200 h) in humans and a low therapeutic ratio, presenting severe gastrointestinal problems and also shows teratogenic effects and cannot be used in the pregnant women.[11] Since the chemotherapy against leishmaniasis is still inefficient, as a result the finding of more effective and safer drug for treating leishmaniasis remains desirable.

The natural product inspired molecular hybridization approach has been emerged as a powerful tool for tackling the problems associated with the standard drugs.[12] Recently, some hybrid molecules of pentamidine with other heterocycles have been synthesized, which showed potent anti-leishmanial activity with low cytotoxicity.[13] Furthermore, following the same approach some pyrimidines and chalcone based hybrids were synthesized and evaluated for their anti-parasitic potential.[14]

Annomontin, a pyrimidine-β-carboline alkaloid, has showed antileishmanial activity 34.8 ± 1.5 against the *Leishmania braziliensis*. On the other hand, licochalcone A, is a well known anti-parasitic

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natural phenol.[15] In the continuation of our ongoing programme to develop new hybrid molecules as potent anti-parasitic agents,[16] and inspired by the antileishmanial and antimalarial activity of pyrimidine and chalcone based natural product annamontine and licochalcone A, we herein report our work on the design, synthesis and anti-protozoal evaluation of novel pentamidine based pyrimidine and chalcone hybrids (Fig. 1).

The detailed synthetic route to synthesize compounds (**7a–i**) is outlined in scheme 1. The synthesis was achieved by the coupling of substituted pyrimidine with the pentamidine fragment. In this context, pyrimidine derivatives (**2a–i**) were obtained by the condensation of 2,4-dichloro-6-methylpyrimidine with various primary or secondary amines under basic conditions. The pentamidine fragment has been synthesized via a condensation of 1,5-dibromopentane with *N*-(4-hydroxyphenyl)acetamide to furnish the intermediate **5** under basic condition, followed by the deprotection of amine in presence of 20% aq NaOH furnished the pentamidine fragment **6** in 74% yield. Finally, pentamidine fragment **6** was condensed with pyrimidine derivatives (**2a–i**) to afford the targeted pentamidine-pyrimidine hybrids (**7a–i**) in good yields (scheme 1).

In the second phase of our endeavour, we synthesized chalcones-pentamidine hybrid (**10a–f**, scheme 2). In this continuation intermediate **9** has been synthesized by replacing both bromo groups of 1,5-dibromopentane via 4-hydroxy benzaldehyde. Intermediate **9** was condensed with various acetophenones under the reported protocols. The use of 1 equiv of acetophenones leads to chalcone (**10d–f**), while the use of 2 equiv of acetophenones furnished the bis-chalcones **(10a–c)**. All compounds were characterized using ^{1}H NMR, ^{13}C NMR, mass spectrometry and IR spectroscopy (see Supplementary data).[17] The purity of these compounds was ascertained by TLC and spectral analysis.

All the synthesized compounds were evaluated in vitro against transgenic *Leishmania donovani* amastigotes.[18] All the pyrimidine–pentamidine hybrids (**7a–i**) (Table 1) showed very good inhibitory activity with the IC_{50} values in the range of 0.30–1.72 μM and SI

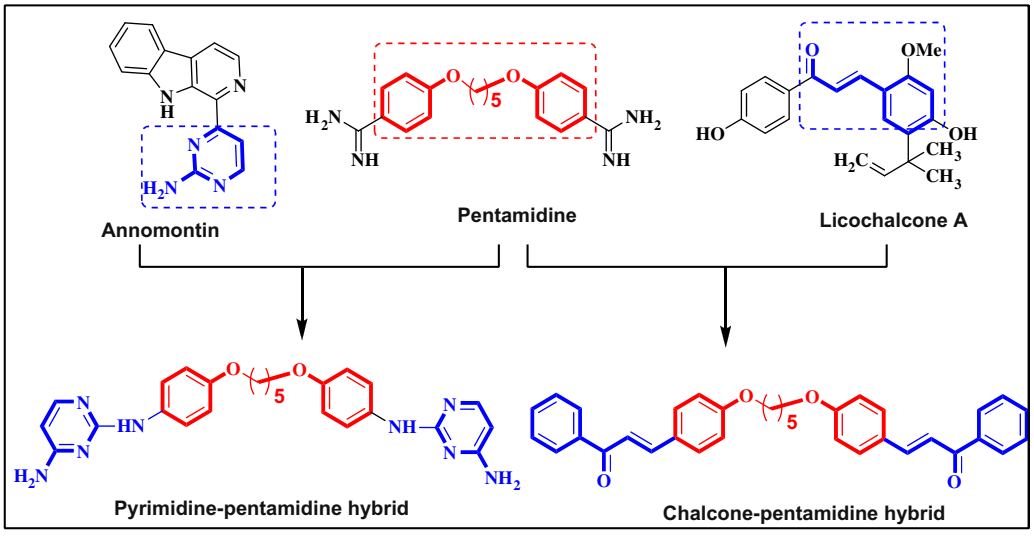
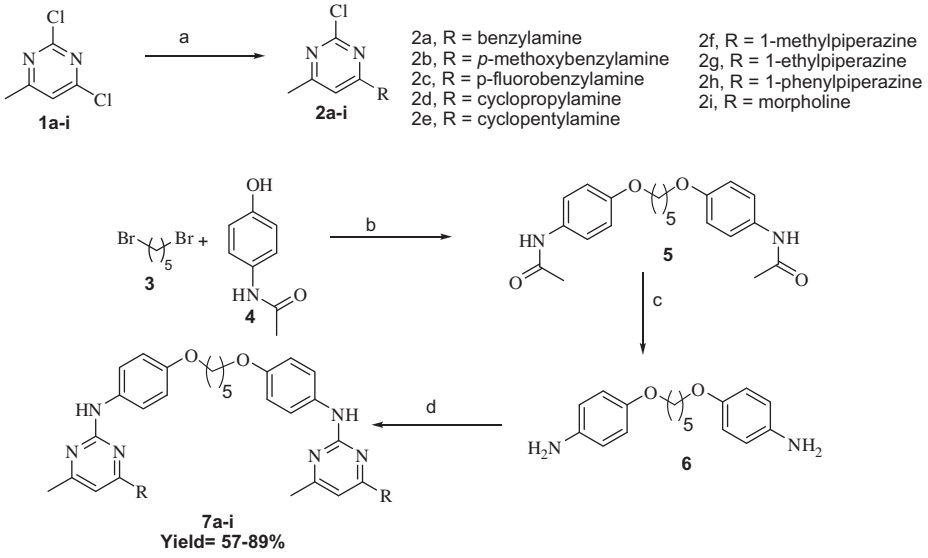
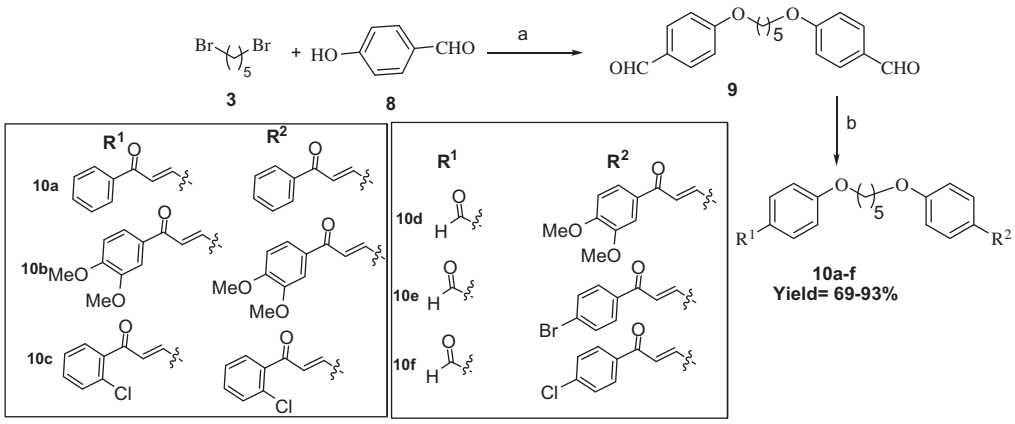


Figure 1. A natural product inspired hybrid approach to the synthesis of anti-parasitic agent.

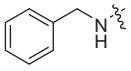
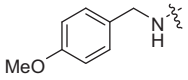
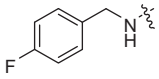
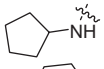
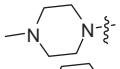
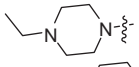
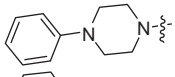


Scheme 1. Synthesis of pyrimidine–pentamidine hybrids (**7a–i**). Reagents and conditions: (a) amines, DIPEA, EtOH, rt, 2 h (b) K_2CO_3, acetone, reflux, 10 h (c) 20% aq NaOH, H_2O:EtOH (1:1), 90 °C, 5 h (d) **2a–i**, DIPEA, DMF, MW, 200 °C, 30 min.



Scheme 2. Synthesis of chalcone-pentamidine hybrids (**10a–f**). Reagents and conditions: (a) K_2CO_3, acetone, reflux, 10 h (b) 10% aq KOH, MeOH, rt, 2 h

Table 1
In vitro anti-leishmanial activity against intracellular amastigotes of pyrimidine–pentamidine hybrids

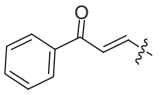
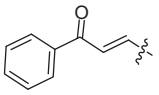
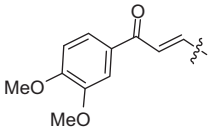
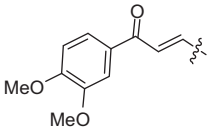
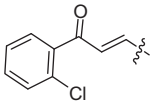
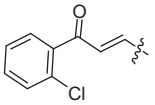
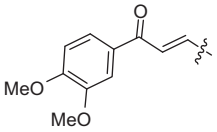
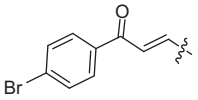
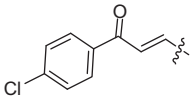
Compd no.	R	Antiamastigote activity IC_{50} (μM)	Cytotoxicity CC_{50} (μM)	Selectivity[a] index (SI)
7a		1.67	26.04	15.60
7b		0.57	2.11	3.70
7c		1.41	8.68	6.16
7d		0.42	2.68	6.38
7e		1.53	1.69	1.11
7f		0.81	2.93	3.62
7g		0.30	3.65	12.17
7h		1.72	>400	>232
7i		1.18	5.51	4.67
Pentamidine		20.43	52.70	2.58
SSG		71.90	397.60	5.53
Miltefosine		12.5	3.2	0.25

SSG = sodium-stibo-gluconate (IC_{50} & CC_{50} in μg/mL)
[a] Selectivity index (SI) defined by the ratio CC_{50} (in KB cell lines)/IC_{50} (Intracellular *leishmania* amastigotes).

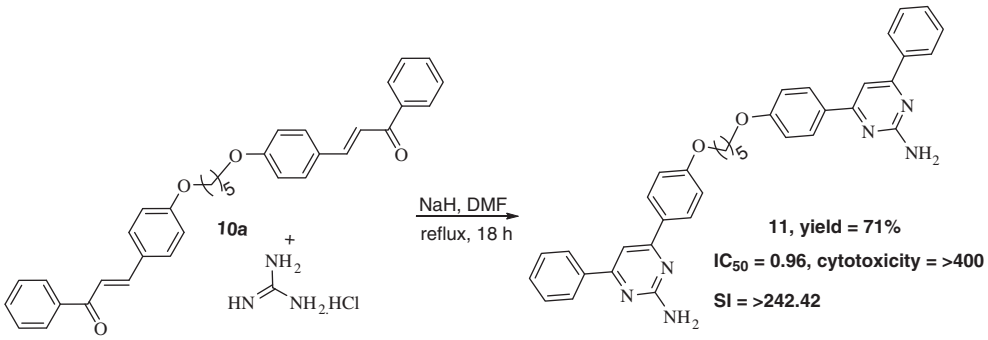
values in the range of 1.11–>232, when compared to the standard drugs like Pentamidine (IC_{50} = 20.43 μM, SI = 2.58), SSG (IC_{50} = 71.90 μg/mL, SI = 5.53) and miltefosine (IC_{50} = 12.5 μM, SI = 0.25). Among these pyrimidine-pentamidine hybrids, compound **7b** having *p*-methoxy benzyl amine functionality showed more activity with IC_{50} = 0.57 μM than the compounds **7c** having *p*-fluoro benzyl amine functionality (IC_{50} = 1.41 μM) whereas compound **7c** showed less toxicity (SI = 6.16) than the **7b** (SI = 3.70). Moreover, in case compounds **7d** and **7e** having cyclopropyl and cyclopently amine functionalities showed IC_{50} = 0.42, 1.53 μM and SI = 6.38, 1.11, respectively. Next, we introduced the aliphatic cyclic heterocyclic amines (**7f–7i**), the most active compound found to be **7g** having ethyl piperazine substitution showed high inhibitory activity against *L. donovani* with IC_{50} = 0.30 μM and selectivity index (SI) = 12.17, but compound **7h** having phenyl piperazine substitution proved to be the most promising compound of the series IC_{50} = 1.72 μM and SI >232 which was manifold times more active and less toxic than the standard drugs. Encouraging by the in vitro activity of pentamidine–pyrimidine hybrids, we have also screened chalcones-pentamidine hybrids against *L. donovani*, but only some of these showed good inhibition Table 2. It was observed that compound **10a** is the most active with IC_{50} = 1.65 μM, and SI = 242.42 than the compound **10d** with IC_{50} = 28.4 μM. Furthermore, the most active bis-chalcone-pentamidine hybrid **10a** has been utilised for the synthesis of pyrimidine–pentamidine hybrid **11** by the reaction of guanidine hydrochloride in the presence of NaH (scheme 3). To our delight, this pyrimidine–pentamidine hybrid showed potential activity with IC_{50} = 0.96 μM and SI = 242.42 against the *L. donovani*, which was manifold times better active and less toxic than the used standards.

Additionally, the anti-malarial activity of pentamidine was already reported over many years ago, but presence of other suitable drugs stuck its anti-malarial development.[13] Due to the, growing

Table 2
Anti-leishmanial in vitro activity against intracellular amastigotes of chalcone-pentamidine hybrids

Compd no.	R^1	R^2	Antiamastigote activity IC_{50} (μM)	Cytotoxicity CC_{50} (μM)	Selectivity index [a](SI)
10a			1.65	>400	>242.42
10b			NI	ND	ND
10c			–	–	–
10d			28.39	13.86	0.49
10e			NI	ND	ND
10f			–	–	–
Pentamidine			20.43	52.70	2.58
SSG			71.90	397.60	5.53
Miltefosine			12.5	3.2	0.25

SSG = sodium stibo-gluconate (IC_{50} & CC_{50} in μg/mL).
[a] Selectivity index (SI) defined by the ratio CC_{50} (in KB cell lines)/IC_{50} (Intracellular *leishmania* amastigotes). NI = no inhibition, ND = not determine, NA = not available.



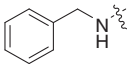
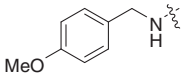
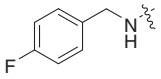
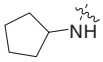
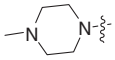
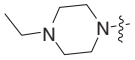
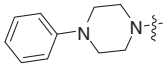
Scheme 3. Synthesis of pyrimidine-pentamidine hybrid **11** using chalcone-pentamidine hybrid **10a** as precursor.

drug resistance of malaria, there are urgent needs for novel classes of compounds that are effective against sensitive as well as resistant *Plasmodium falciparum* strains.

In previous studies, pyrimidine and chalcone have also been showed a therapeutic effect on *Plasmodium falciparum* strains.[14] Prompted by the anti-malarial activity of pyrimidines, chalcones, and pentamidine, the synthesized hybrid compounds were also screened against *Plasmodium falciparum*.[18] Among the pyrimidine–pentamidine hybrids, some of these showed very good in vitro activity against the 3D7 (chloroquine-sensitive strain) and K1 (chloroquine-resistant strain), while the chalcone based hybrids of pentamidine were found to be inactive against *Plasmodium falciparaum*. Moreover, compounds **7d** having cyclopropyl amine functionalities showed very good activity against the 3D7 (IC_{50} = 6.25 ng/mL) and K1 (IC_{50} = 11.26 ng/mL) (Table 3). Compounds **7b**, **7g** and **7h** having *p*-methoxy benzyl amine, *N*-ethyl piperazine, and *N*-phenyl piperazine units respectively showed good activity against the K1 cell line (Table 3).

In conclusion, a series of highly potent and less toxic hybrids of pentamidine as anti-parasitic agents were designed and synthesized. The synthesized hybrid molecules exhibited promising in vitro antileishmanial activity, whereas several compounds showed remarkable antimalarial potency in vitro, especially against CQ-R *Pf* (K1) strain. Due to the better in vitro antileishmanial activity and less toxicity than the standard drug (pentamidine, SSG, and miltefosine), these compounds can be very valuable for further optimization work in anti-leishmanial chemotherapy.

Table 3
Anti-malarial in vitro activity of pyrimidine–pentamidine hybrids against the sensitive 3D7 and resistance K1 cell lines of *Plasmodium falciparum*

Compd no.	R	Anti-malarial activity 3D7 IC_{50} [b](ng/mL)	Anti-malarial activity K1 IC_{50} (ng/mL)	Cytotoxicity CC_{50} (ng/mL)	Selectivity index [c](SI)
7a		51.45	382.59	22080	429.15
7b		11.69	23.61	3370	288.28
7c		39.41	358.43	41440	1051.51
7d		6.25	11.26	560	89.6
7e		313.29	ND[d]	18080	57.71
7f		245.0	ND	10800	43.97
7g		45.15	22.13	7130	157.9
7h		59.24	53.06	100000	1688
7i		263.69	ND	2060	7.82
[a]CQ		2.45 ± 1.03	141.52 ± 25.6	75000	30612

[a] CQ = Chloroquine, 3D7 (CQ-sensitive strain) and K1 (CQ-resistant strain).
[b] IC_{50} (ng/mL): concentration corresponding to 50% growth inhibition of the parasite.
[c] Selectivity index(SI) defined by the ratio: CC_{50} (in Viro cell lines) /IC_{50} values of antimalarial activity against 3D7 cell line).
[d] ND = not done.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.10.101.

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17. (a) *Spectroscopic data for* ***7h***: yellow solid, yield = 79%, mp = 107–110 °C, FT-IR (KBr) ν (cm^{-1}): 3420, 2918, 1591, 1507, 1443, 1229, 923, 826, 758; ^{1}H NMR (300 MHz, $CDCl_3$):δ 7.50 (d, J = 8.7 Hz, 4H), 7.34 (d, J = 8.1 Hz, 4H), 6.99–6.88 (m, 12H), 5.94 (s, 2H), 4.03 (t, J = 6.3 Hz, 4H), 3.79 (br s, 8H), 3.27 (br s, 8H), 2.30 (s, 6H), 1.91–1.86 (m, 4H), 1.70–1.68 (m, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.8, 163.4, 159.6, 153.5, 134.7, 120.6, 114.7, 93.3, 68.0, 66.3, 29.0, 24.1, 22.8 ppm, ESI-MS (*m/z*): 790.4 $(M+H)^+$; Anal. Calcd for $C_{47}H_{54}N_{10}O_2$: C, 71.37; H, 6.88, N, 17.71. Found: C, 71.29; H, 6.86, N, 17.68.
(b) *Spectroscopic data for* ***10a***: white solid, yield = 81%, mp = 130–134 °C, FT-IR (KBr) ν (cm^{-1}): 3447, 1654, 1598, 1510, 1291, 1216, 1018, 764, 670, 571; ^{1}H NMR (300 MHz, $CDCl_3$):δ 8.04 (d, J = 7.2 Hz, 3H), 7.86–7.78 (m, 3H), 7.63–7.40 (m, 9H), 7.10 (s, 2H), 7.03–6.94 (m, 5H), 4.13–4.04 (m, 4H), 1.92 (br s, 4H), 1.73–1.66 (m, 2H) ppm, ^{13}C NMR (75 MHz, $CDCl_3$): δ 191.2, 188.9, 163.5, 160.8, 144.0, 137.8, 132.9, 131.8, 130.8, 129.4, 128.6, 128.4, 127.1, 119.3, 114.8, 67.9,

67.6, 28.2, 22.0 ppm, ESI-MS (*m*/*z*): 516.2 $(M+H)^+$; Anal. Calcd for $C_{35}H_{32}O_4$: C, 81.37; H, 6.24. Found: C, 81.32; H, 6.22.

18. *Bioevaluation methods In vitro antileishmanial assay*: The anti-leishmanial evaluation of these hybrids was performed on luciferase-transfected *L. donovani* promastigotes maintained in our laboratory (Sunduru et al, 2009). In order to assess the activity of compounds against the amastigote stage of the parasite, the mouse macrophage cell line (J-774A.1), infected with promastigotes was used. Cells were seeded in a 96 well plate at a density of 4×10^3 cells/100μL/well in RPMI-1640 containing 10% fetal calf serum and the plates were incubated at 37 °C in a CO_2 incubator. After 24 h, the medium was replaced with fresh medium containing stationary phase promastigotes (4×10^4/100μL/well). Promastigotes were phagocytised by the macrophage and transformed into amastigotes. The test compounds were added in up to 7 concentrations in fresh complete medium starting from 40 μM concentration, and the plate were incubated at 37 °C in a CO_2 incubator for 72 h. After incubation, the drug containing medium was decanted and 50 μL PBS (Phosphate Buffer Saline) 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 (Porwal et al, 2009). The values are expressed as relative luminescence units (RLU). Data were transformed into graphical program (Excel) and the inhibition of parasitic growth is determined by comparison of the luciferase activity of drug treated parasites with that of untreated controls. The 50% inhibitory concentration (IC_{50}) for anti-leishmanial activity was calculated by logit regression analysis. (Sunduru, N.; Nishi; Palne, S.; Chauhan, P. M. S.; Gupta, S. *Eur. J. Med. Chem.* **2009**, *44*, 2473). *In vitro antimalarial assay*: The compounds were evaluated for antimalarial activity against both 3D7 (CQ- sensitive) and K1 (CQ-resistant) strains of *Plasmodium falciparum* using Malaria SYBR Green I nucleic acid staining dye based fluorescence (MSF) assay as mentioned by Singh et al (2011)[1]. The stock (5 mg/ml) solution was prepared in DMSO and test dilutions were prepared in culture medium (RPMI-1640-FBS). Chloroquine was used as reference drug. The compounds were tested in 96well plate (in duplicate wells). 1.0% parasitized cell suspension containing 0.8% parasitaemia was used. The plates were incubated at 37 °C in CO_2 incubator in an atmosphere of 5% CO_2 and air mixture. After 72 hours 100 μl of lysis buffer containing 2× concentration of SYBR Green-I (Invitrogen) was added to each well and incubated for another one hour at 37 °C. The plates were examined at 485 ± 20 nm of excitation and 530 ± 20 nm of emission for relative fluorescence units (RFUs) per well using the fluorescence plate reader (FLUO star, BMG lab technologies). The IC_{50} values were obtained by Logit regression analysis using pre-programmed Excel spreadsheet (S. Singh, R. K. Srivastava, M. Srivastava, S. K. Puri, K. Srivastava, *Experimental Parasitology*., **2011**, *127*, 318).[1] Cytotoxicity of the compounds was carried out using KB cell line (oral carcinoma cell line) and Vero cell line (C1008; Monkey kidney fibroblast) following the method of Mosmann (1983) with certain modifications.[2] The cells were incubated with compound dilutions for 72 h and MTT was used as reagent for the detection of cytotoxicity. 50% cytotoxic concentration (CC_{50}) was determined using nonlinear regression analysis using pre-programmed Excel spreadsheet. Selectivity Index was calculated as $SI = CC_{50}/IC_{50}$. (T.J. Mosmann, *Immunol. Methods*., **1983**, *65*, 55). [2]