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Synthesis and systemic toxicity assessment of quinine-triazole scaffold with antiprotozoal potency

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ABSTRACT

A series of hybrid antiprotozoal compounds with quinine-triazolyl scaffold were prepared by copper catalyzed Huisgen 1,3-dipolar cycloaddition via *O*-mesylation with mesyl chloride followed by azide displacement. The synthesized azide derivative was made to react with various aromatic and aliphatic alkynes. The triazolyl-linked quinine scaffolds were synthesized under solvent-free mechanochemical ball milling conditions. Products (6a-s) were screened for *in-vitro* antimalarial and antileishmanial activity. Screening results indicated that out of the synthesized series of 19 products, compounds 6d, 6h, 6l, 6m, and 6n showed significant antimalarial (*P. falciparum*) and antileishmanial activities (*L. donavani*) with IC_{50} values 0.28, 0.28, 0.25, 0.33, 0.76 μM and 8.26, 4.4, 1.78, 3.95, and 4.06 μM, respectively. Further toxicological analysis established the Median lethal dose (LD_{50}), No observed adverse effect level (NOAEL) and human equivalent dose (HED) of the most potent compounds by acute and sub acute toxicity studies performed in rodent animal model. The studies revealed that compounds (6d, 6h, 6l and 6m) did not reveal any toxic manifestation at dose 1000 mg/Kg and from which the corresponding HED was calculated to be 13.84 mg/kg.

1. Introduction

Malaria and leishmaniasis are the leading cause of morbidity and mortality worldwide [1]. Malaria is defined as the infection of the plasmodium species transmitted by the female anopheles mosquitoes [2]. Pathogenesis of leishmaniasis is because of the transmittance of the parasitic protozoan of the genus leishmania by female phlebotomine sandfly [3]. Despite significant therapeutic advancement severe toxicity and increasing incidence of drug resistance have limited the efficacy of the frontline chemotherapy applicable against them [2,4]. Thus, the need of the hour is to develop antiprotozoal therapeutics with a wide therapeutic window and a high threshold for drug resistance.

To this end, we synthesized a series of quinine-1,2,3-triazole hybrids by copper catalyzed click reaction between azide and alkynes. Molecular hybridization is the recognition and unification of pharmacophoric subunits of diverse bioactivities to yield neoactives with pre selected virtues of the original template [5]. We hypothesized that; molecular hybridization of these two diverse scaffolds (quinine and triazolyl) would yield hybrids effective in addressing the issue of drug resistance [6–10].

The quinoline alkaloid rose to fame as the first effective antimalarial agent. It inhibits hemozoin formation causing thereby inducing oxidative death of the malaria parasite. Accumulation of free heme in the acidic vacuoles of the blood schizonts [11]. Furthermore, being weak base the quinolines have the propensity to accumulate in the acidic phagolysosomes of the leishmanial amastigotes. However, drug resistance and high incidence of systemic toxicity have severely compromised the clinical utility of quinoline in the current therapeutic regimen [12–15].

Triazoles on the other hand are relatively safe class of drugs. Studies report LD_{50} of molecule to me > 2000 mg/Kg b.w. in rodents. There potency to damage the pathogenic cell membrane by hampering ergosterol biosynthesis has been widely investigated in context of antileishmanial activity. In the present work we have conjoined the quinine and triazole scaffold to yield hybrids with increased efficacy, reduced toxicity and potency against both the malarial as well as the leishmanial parasites[16].

Studies till date focused on molecular hybridization of quinine and triazole via *o*-alkylation. The said approach was limited because of formation of a considerable number of undesirable by-products. We herein report synthesis and characterization of a novel series of quinine-triazole hybrids via o-mesylation followed by azide displacement under mechanochemical ball milling conditions. The predominant imperative of our scheme was to enhance the yield of the desired compounds and

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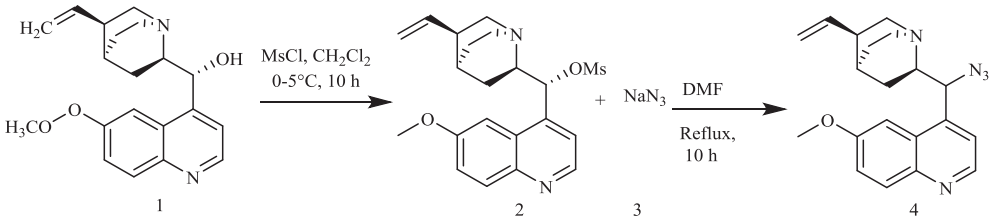


Scheme 1. Scheme for synthesis of triazolyl quinine scaffold.

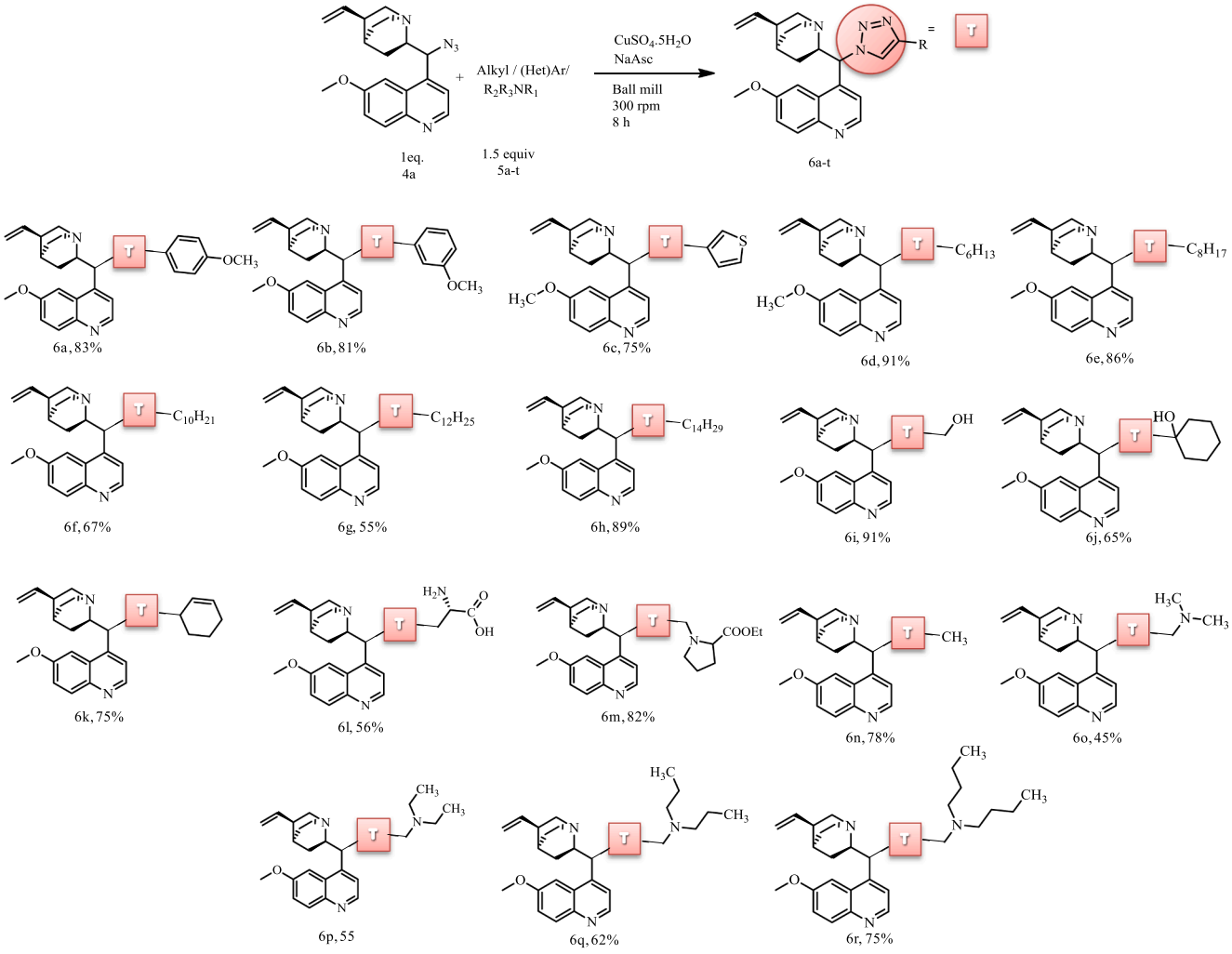
minimize the formation of undesirable by-products.

With the aim of increasing the pharmacological elegance of the synthesized series, we have further introduced aromatic and hetero-aromatic substitutions in the 4th position of the 1,2,3-triazole nucleus. For the purpose of evaluating the onset and expression of toxicity if any, we have reported the toxicological profile of the potent members of the synthesized series as per the norms prescribed by the Organization of Economic Corporation and Development (OECD).

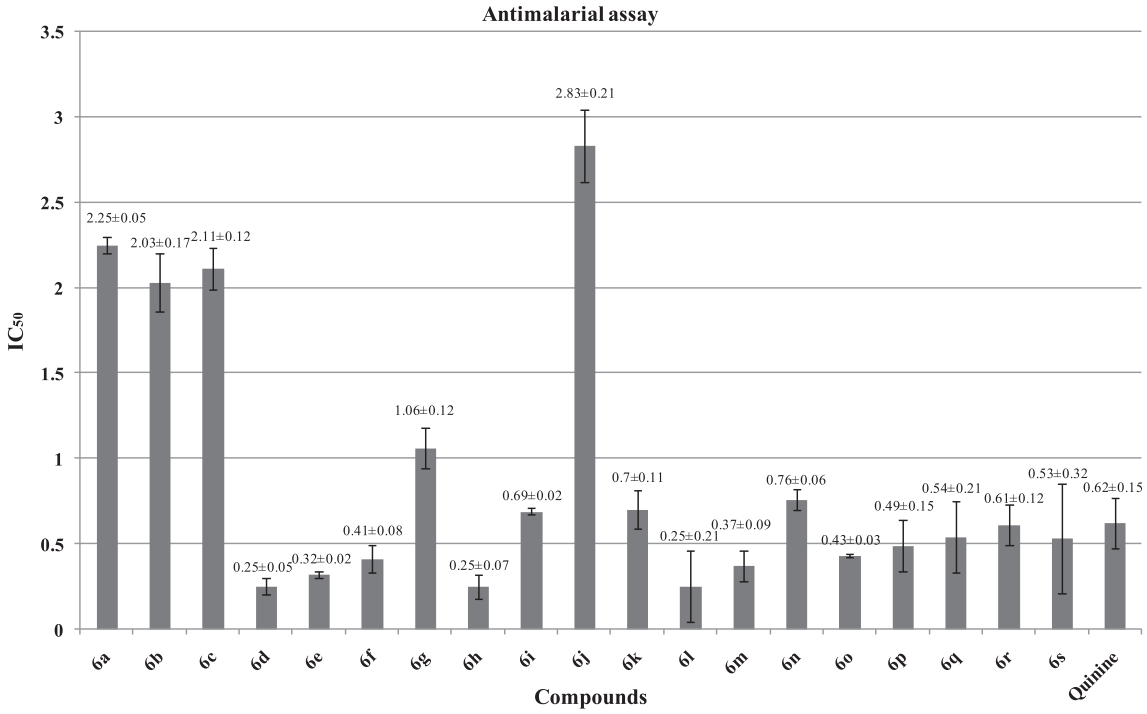
2. Result and discussion

In lieu with the concept of molecular hybridization we designed a quinine-triazole scaffold with the imperative to create hybrid anti-protozoal agent having more than one target of action. The initial concern of the synthetic strategy was to convert hydroxyl quinine into a good leaving group by tethering mesyl group into the quinine moiety. Reacting a solution of quinine in dichloromethane with mesyl chloride as per reported protocol [17] achieved the desired result generating **2** in the process, which was further made to interact with sodium azide under reflux condition [18] to generate azido-dehydroxyquinine **4** (Scheme 1), a key intermediate of the proposed synthesis. After substantial optimization we found that copper-catalyzed cycloaddition between azido-dehydroxyquinine and appropriate alkynes (synthesized or purchased as per reported protocol [19]) yielded the parent triazolyl-quinine scaffold (Scheme 2).

Under optimized conditions Cu (I) catalyzed cycloaddition of **4** with alkynes **5a-s** (Scheme 2) in ball mill at 300 rpm furnished the desired triazolyl compounds in excellent yield. The compounds thus synthesized were screened by *in-vitro* antileishmanial and antimalarial assays. The most promising compounds were further analyzed in *in-vivo* toxicity assay paradigms. With the purpose of generating a diverse array of quinine-triazole conjugates with maximum efficacy and minimum toxicity, we further coupled azido-dehydroxyquinine **2** with aromatic and aliphatic alkynes using click chemistry in the presence of copper



Scheme 2. Synthesis of triazolyl quinine conjugates.



Data is represented as mean ± SEM. All the experiments were performed in triplicates.

Fig. 1. Effect of the synthesized series on the antimalarial activity. Compounds 6d, 6h, 6l and 6m were found to exert antimalarial activity more potent than that exerted by quinine.

sulfate pentahydrate and L-sodium ascorbate in a Fritsch Ball mill under solvent-free conditions at 300 rpm. The reactions furnished the conjugates **6a-s** in 80–85% yields (Scheme 2).

The synthesized triazolyl quinine conjugates were screened for *in-vitro* antimalarial, antileishmanial and cytotoxicity activity. For antimalarial screening, quinine while for antileishmanial activity amphotericin B was used as a reference standard. Out of the varied substituents lined to the parent quinine-triazole scaffold it was found that the tertiary amines linked compounds were relatively more potent than both the reference standards. On increasing the chain length of the compounds the potency of the compounds also exhibited an increasing trend. This may be attributed to the increased penetrating power rendered to the pharmacophoric lead due to long alkyl chain substitution of the parent scaffold. (Fig. 1)

Intensive *in-vitro* screening for antiprotozoal activity revealed that compounds 6d, 6 h, 6 l, and 6 m exhibited potential cidal action against both *P. falciparum* (Fig. 1) and *L. donavani* (promastigote stage) (Fig. 2). Also in comparison to the reference standard the compounds 6d, 6 h, 6 l, and 6 m exhibited comparatively low *in-vitro* cytotoxicity to human erythrocytes (Fig. 3).

We then sought after the *in-vivo* toxicity profile of the compounds in nullgravida nulliparous wistar albino rats. To establish the LD_{50} (Median Lethal Dose) of the compounds up and down procedure was performed wherein the animals were dosed in stepwise procedure as per OECD TG 425 (Table 1). The starting dose for LD_{50} studies were dtermined by OECD 420 (Fixed dose procedure). LD_{50}, NOAEL, and HED for the most potent members of the series as well as the reference standard quinine and amphotericin B are reported in table 1. From the recorded toxicological data it was observed that the synthesized compounds were found to be having a wide therapeutic window. Furthermore, the reference standard (quinine and amphotericin B) were found to be considerably toxic with LD_{50}.

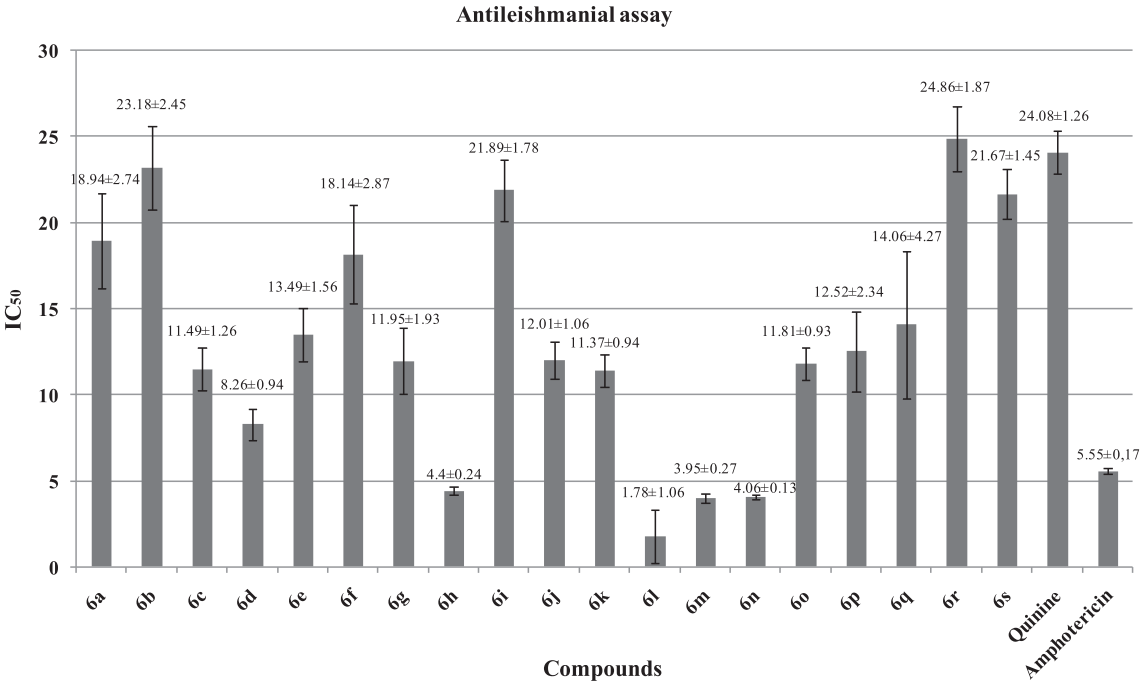
Subsequent sub-acute repeated dose toxicity studies as per OECD TG 407 were performed to determine the NOAEL (No Observed Adverse Effect Level) and HED (Human Equivalent Dose) of the compounds. The studies revealed that compounds (6d, 6 h, 6 l and 6 m) did not reveal any toxic manifestation 1000 mg/Kg and the corresponding HED (Human Equivalent Dose) were calculated to be 13.84 mg/kg respectively.

From the explored biological and toxicological evaluation it was observed that addition of triazole moiety to quinine resulted in reduction of toxicity of the conjugated scaffold. On further increasing the length of the alkyl chain the efficacy of the compound (6d and 6 h), also increased (Fig. 1). Also it was notable that substitution of amino acid proline yielded the most potent compound of the series. While substitution with lysine also produced favorable results.

3. Experimental

3.1. Material and measurements

All reagents were purchased from commercial sources and used without further purification. Quinine and Amphotericin B for microbiological assay was procured from sigma-aldrich, India. Concentration of MTT (4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) solution is 50 μM. The inhibitory potency of the synthesized compounds was measured against the extracellular promastigotes stage of *Leishmania donavani* (DD8) using MTT assay. Absorbance of Purple formazan crystals were measured spectroscopically at 570 nm. Proton nuclear magnetic resonance experiments and carbon-13 nuclear magnetic resonance experiments were recorded on Bruker Advance DX 400 MHz spectrometer. Chemical shifts were reported in parts per million relative to tetramethylsilane as internal standard. The ^1H NMR spectra were referenced with respect to the residual $CHCl_3$ proton of the solvent $CDCl_3$ at 7.27 ppm. Coupling constants were reported in hertz (Hz). ^{13}C NMR spectra were fully decoupled and were referenced to the middle peak of the solvent at 77.00 ppm. Splitting pattern were designated as s, singlet; d, doublet; dd, doublet of doublet; m, multiplet. Mass

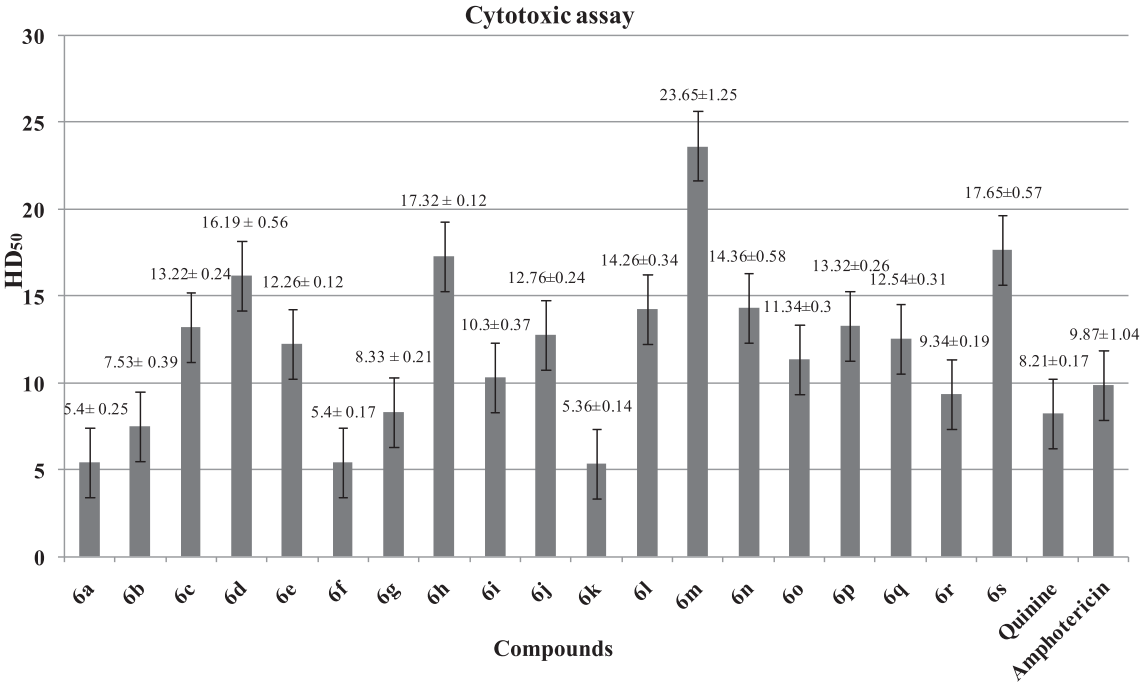


- Data is represented as mean ± SEM. All the experiments were performed in triplicates.

Fig. 2. Effect of the synthesized series on the antileishmanial activity Compounds 6h, 6l and 6m and 6n were found to be exerted antileishmanial activity more potent than that exerted by both quinine and the reference standard Amphotericin.

spectra were obtained on High resolution mass spectroscopy (HRMS) equipment. IR spectra were recorded on a Nicolet FT-IR Impact 410 instrument either as neat or as KBr pellets. Melting points were determined on a melting point apparatus. Compounds were routinely analysed for their purity on the silica gel GF-254 visualized under UV at wavelength 254 nm. Chromatographic purification of synthesized compounds was performed by column chromatography on silica gel (60–120 mesh) eluting with ethyl acetate in hexane.

Representative method for triazole synthesis by planetary ball-milling [20]



- Data is represented as mean ± SEM. All the experiments were performed in triplicates.

Fig. 3. Cytotoxic profile of the synthesized series Median Hemolytic dose (HD_{50})of compounds 6d, 6h, 6l, 6m and 6n were found to be more than that obtained for the both quinine and amphotericin.

Table 1
Toxicity profile of compounds.

Compound	LD50 (mg/kg)	NOAEL (mg/kg)	HED (mg/kg)
6d	< 1000	> 1000	13.84
6h	< 550	> 1000	13.84
6l	< 1000	> 1000	13.84
6m	< 1000	> 1000	13.84
Quinine	300	200	2.76
Amphotericin B	300	200	2.76

Median lethal dose (LD50) of the most potent members of the series (6d, 6h, 6l and 6m) were found to be > 1000 mg/kg, NOAEL < 1000

The azide **4** (1 mmol) and alkynes **5a-s** (1.1 mmol) was taken in a stainless steel (SS) jar (50 mL capacity) containing 10 SS balls (10 mmol) and sodium ascorbate (0.4 mmol) was added to it followed by the addition of $CuSO_4$ (0.2 mmol). The mixture was then ground in a planetary ball mill (Retsch PM-100, Retsch GmbH, Germany) at 300 rpm. After completion of their action the mixture was dissolved in EtOAc and was purified by column chromatography (EtOAc:Hex) to yield analytically pure product. High performance liquid chromatography (HPLC) chromatograph of compound 6m is shown in Fig. 4.

3.2. Biological evaluation

***In-vitro* antimalarial assay:** The *in-vitro* antimalarial assay was carried out in 96 well microtitre plates according to the micro assay protocol of Rieckmann and co-workers with minor modifications [21–22]. The cultures of *P. falciparum* strain were maintained in medium RPMI 1640 supplemented with 25 mM HEPES, 1% D-glucose, 0.23% sodium bicarbonate and 10% heat inactivated human serum. The asynchronous parasites of *P. falciparum* were synchronized after 5% D-sorbitol treatment to obtain only the ring stage parasitized cells. For carrying out the assay, an initial ring stage parasitaemia of 0.8 to 1.5% at 3% haematocrit in a total volume of 200 µl of medium RPMI-1640 was determined by giemsa staining to assess the percent parasitaemia (rings) and uniformly maintained with 50% RBCs (O^+). A stock solution of 5 mg/ml of each of the test samples was prepared in dimethyl sulfoxide DMSO and subsequent dilutions were prepared with culture medium. The diluted samples in 20 µl volume were added to the test wells so as to obtain final concentrations (at five fold dilutions) ranging between 0.4 µg/ml and 100 µg/ml in triplicate well containing parasitized cell preparation. The culture plates were incubated at 37 °C in a candle jar. After 36–40 h incubation, thin blood smears from each well were prepared and stained with giemsa stain. The slides were microscopically observed to record maturation of ring stage parasites into trophozoites and schizonts in presence of different concentrations of the test agents. The test concentration which inhibited the complete maturation into schizonts was recorded as the minimum inhibitory concentrations (MIC). Quinine was used as the reference drug.

3.3. In-vitro antileishmanial assay

The inhibitory potency of the synthesized compounds were measured against the extracellular promastigotes stage of *Leishmania donovani* (DD8) using MTT (4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The promastigotes were cultured in 96-well plate in the presence of graded concentrations of the test samples. Plates were incubated for 48 h at 26 °C. Post incubation MTT solution was incorporated to each well and incubated in dark for 4hrs at 37 °C. Finally the cells were centrifuged at 3000g for 10 min. Purple formazan crystals thus formed were dissolved in DMSO and absorbance was measured spectroscopically at 570 nm. The IC_{50} values of the post-treated viable cells were calculated relative to the untreated control cells and results were expressed as concentration of compounds inhibiting 50% of parasite growth[23].

3.4. Toxicological evaluation

Animal used: For acute toxicological analysis as well as determination of LD_{50} female while for sub-acute toxicological either sex Wistar albino rats were used. All the experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC). The experimental animals were housed at constant temperature (21 ± 2 °C) and RH 55 ± 5% with 12hrs alternate light and dark cycles and free access to food and water. The protocol used in context of the study was pre-approved by the Institutional Animal Ethical Committee. (Reg No: 379/CPCSEA/IAEC-2018/030)

Single dose acute-toxicity studies: Acute toxicity studies of the 6d, 6 h, 6 l and 6 m compounds were performed as per OECD TG 420. Nullgravida, nulliparous animals were dosed in stepwise procedure using fixed dose levels of 5, 50, 200, 300, 2000 mg/Kg b.w.. A total of five animals per dose level were used. Prior to dosing the animals were subjected to overnight fasting. The test substances were administered orally. Sighting studies were performed to establish the starting dose of the study. Based on the presence and absence of signs of toxicity or mortality, further dose levels were analyzed. A period of 24hrs was allowed between administrations of each subsequent dose levels. All the animals were observed for 14 days. Body weight, hematological, biochemical and hematological parameters were analyzed at the end of the study protocol[24] (OECD 420).

Determination of LD_{50}: To determine the LD_{50} of the synthesized conjugates OECD test guideline 425 (up and down procedure) was followed with slight modification. As per the described study design animals were segregated into control and treatment groups and fasted overnight prior to the study. The test compounds were orally administered in a single odered test progression (175, 550, 1000 and 2000 mg/Kg), one at a time at 48 h interval. Subsequent animals were administered a lower or higher dose on the basis of appearance of either morbidity or mortality. Dosing was discontinued if three consequtive animals survived at the upper bound dose. Following which an estimate of LD_{50} was calculated using the maximum likelyhood method (Table 1) [25].

3.5. Repeated dose sub-acute toxicity study

Sub-acute toxicity study was performed using 28 days repeated dose protocol prescribed by OECD TG 407 with slight modification. The compounds 6d, 6 h, 6 l and 6 m were daily administered orally at a dose of 1000 mg/kg b.w. for a period of 28 days. Animal were routinely checked for signs for morbidity and mortality. From the data obtained (No Observed Adverse Effect Level) NOAEL and Human Equivalent Dose (HED) was calculated, considering the standard human weight as 60Kg. The obtained HED was further divided by a factor value 10 to yield the starting dose for human studies[26–28].

$$\mathbf{HED(mg/kg) = Animal\ NOAEL(mg/kg) * (AnimalWeight[kg])/HumanWeight[kg])^{(0.33)}}$$

Statistical Analysis The *in-vitro* assays were performed in triplicates. The results of *in-vivo* studies are presented as mean ± standard error of the mean (SEM). Difference in mean values was analyzed by one way ANOVA followed by Dunnett's test using software GraphPad Insat 3 software.

4. Conclusions

Malaria and leishmaniasis are the two most predominant parasitic menaces plaguing the developing world. Mounting incidence of drug resistance and excessive peril of systemic toxicity has limited the therapeutic efficacy of the frontline chemotherapeutic agents. To this end, we synthesized a series of quinine-triazole molecular hybrids via click reaction. From systematic execution of synthetic strategies and

SHIMADZU LabSolutions Analysis Report

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Sample ID	: Cal5		
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Batch Filename	: amit.lcb		
Vial #	: 1-12	Sample Type	: Standard
Injection Volume	: 2 uL	Level	: 5
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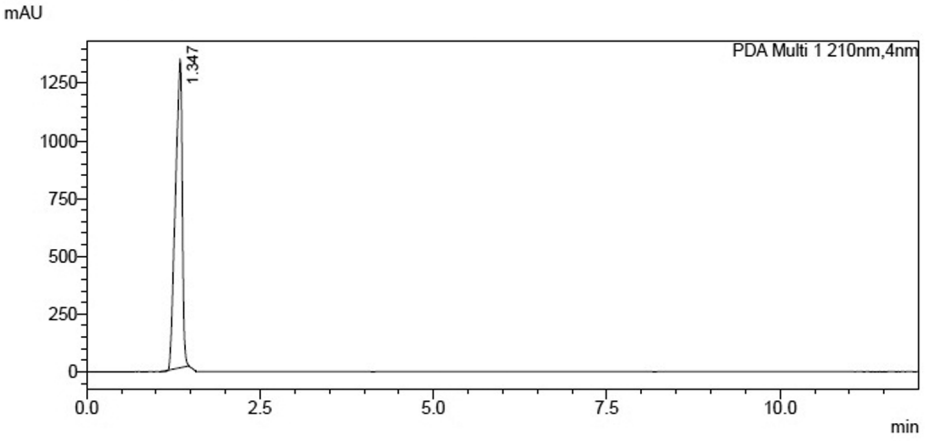

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PDA Ch1 210nm

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Total		9078680			

Fig. 4. High performance liquid chromatography (HPLC) chromatograph of compound 6m.

careful evaluation of biological corollary we were able to engender antiprotozoal compounds which were not only at par with the current antimalarial and antileishmanial agents, but were also devoid of the systemic toxicity associated with the established regimen.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.102939.

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