

RESEARCH ARTICLE

# Biological Activity of Coumarin Derivatives as Anti-Leishmanial Agents

Vineetha Mandlik<sup>1</sup>, Sohan Patil<sup>2</sup>, Ramanamurthy Bopanna<sup>1</sup>, Sudipta Basu<sup>2</sup>, Shailza Singh<sup>1\*</sup>

**1** National Centre for Cell Science, NCCS Complex, SP Pune University Campus, Ganeshkhind, Pune, India, **2** Department of Chemistry, Indian Institute of Science Education and Research (IISER), Pune, Pashan, Pune, India

\* [singhs@nccs.res.in](mailto:singhs@nccs.res.in), [shailza\\_iitd@yahoo.com](mailto:shailza_iitd@yahoo.com)



**OPEN ACCESS**

**Citation:** Mandlik V, Patil S, Bopanna R, Basu S, Singh S (2016) Biological Activity of Coumarin Derivatives as Anti-Leishmanial Agents. PLoS ONE 11(10): e0164585. doi:10.1371/journal.pone.0164585

**Editor:** Imtaiyaz Hassan, Jamia Millia Islamia, INDIA

**Received:** June 22, 2016

**Accepted:** September 27, 2016

**Published:** October 21, 2016

**Copyright:** © 2016 Mandlik et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Data Availability Statement:** All relevant data are within the paper and its Supporting Information files.

**Funding:** Vineetha Mandlik acknowledges the financial support as Senior Research Fellow of Department of Biotechnology, Government of India; and Shailza Singh received the funding BT/PR6037/GBD/27/372/2012, Department of Biotechnology, Government of India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

## Abstract

Cutaneous leishmaniasis affects nearly 0.7 to 1.3 million people annually. Treatment of this disease is difficult due to lack of appropriate medication and the growing problem of drug resistance. Natural compounds such as coumarins serve as complementary therapeutic agents in addition to the current treatment modalities. In this study, we have performed an *in-silico* screening of the coumarin derivatives and their anti-leishmanial properties has been explored both *in-vitro* and *in-vivo*. One of the compounds (compound 2) exhibited leishmanicidal activity and to further study its properties, nanoliposomal formulation of the compound was developed. Treatment of cutaneous lesions in BALB/c mice with compound 2 showed significantly reduced lesion size as compared to the untreated mice ( $p < 0.05$ ) suggesting that compound 2 may possess anti-leishmanial properties.

## Introduction

Leishmaniasis is a neglected tropical disease that is caused by the parasite '*Leishmania*'. Nearly 12 million people worldwide are affected by this disease. There are three forms of leishmaniasis, namely cutaneous, mucocutaneous and visceral forms. Out of these, cutaneous leishmaniasis (CL) is more wide spread and has an higher incidence than the visceral form. CL is more prevalent in parts of Latin America, Middle east and Central Asia. Typical symptoms of CL are the formation of an unpleasant looking skin sores and scars. Treatment of CL is usually done by administration of antimony based compounds (sodium stibogluconate and meglumine antimoniate) in the form of intramuscular injections. However studies reveal that antimonials have their own share of toxicity and side effects. [1] Second line chemotherapy therefore is now focusing on oral anti-fungal compounds, liposomal formulations (Amphotericin B) and topical formulations of paromomycin (Leshcutan). Liposomal amphotericin B (Ambiosome) is also being used for treatment of CL in case of drug resistance to the existing antimonials. Amphotericin B has been shown to be effective against all forms of leishmaniasis, however, the liposomal formulation proves to be expensive, has a lower therapeutic index and is difficult to administer. [2] Paromomycin and Pentamidine are some other compounds that show good efficacy to

**Competing Interests:** The authors have declared that no competing interests exist.

some species but not to all. Moreover, these drugs are usually administered parentally and this limits their use. [3] Miltefosine (hexadecylphosphocholine) is the only oral drug available that has been reported to induce apoptosis in *L. major* though its efficacy in CL is still under consideration. [4,5] With the increasing reports of drug resistance to the available chemotherapeutics, it becomes necessary to identify new compounds that could be used in combination therapy or alone to treat cutaneous leishmaniasis. [6]

Several natural compounds such as alkaloids, phenolic compounds, terpenes and saponins have anti-leishmanial properties. [7,8] Recent studies have also found that coumarins possess anti-leishmanial activity. [9] Two new coumarins i.e. 5-methylcoumarins were isolated from the roots of *Vernonia brachycalyx* and their structures were elucidated using MS and NMR spectroscopy. [10] Coumarin (-) mammae A/BB discovered by Brenzan *et al* shows efficacy over *L. amazonensis* with an IC<sub>50</sub> value < 10 μM. [11] More recently compounds like aurap- tene (LD<sub>50</sub> = 30 μM), osthole (IC<sub>50</sub> = 14.95 μg/ml), coumarin- triazolothiadiazine hybrids (IC<sub>50</sub> = 0.8 μM), triclosan-coumarin hybrids (EC<sub>50</sub> = 9.4; 10.2; 13.5 and 27.5 μg/mL), sesqui- terpene coumarins (IC<sub>50</sub> = 11.5 μg/mL) and indoyl coumarin hybrids (IC<sub>50</sub> = 12.4 μg/mL to 13.4 μg/mL) have been isolated that have been shown to be active against the promastigote form of *Leishmania major*. [12,13, 14, 15, 16, 17] In the present study, we have screened a set of coumarin derivatives. In our computational screening procedure, we have identified a set of five compounds which were screened for their anti-leishmanial properties. For the ease of presentation, these compounds have been designated as C1-C5. The compound 3-(1,3-ben- zodioxol-5-yl)-2-oxo-2H-chromen-6-yl acetate (C2) showed the highest anti-leishmanial properties both *in-vitro* and *in-vivo*. To further enhance the solubility of C2, nano-liposomal formulation was prepared and the efficacy of the compound (C2) was determined.

## Materials and Methods

### *In silico* screening

Using coumarin as a search molecule, its derivatives were obtained from the zinc database. [18] An initial filtering of the compounds was performed based on the “Lipinski rule of five” and the “Vebers rules”. [19, 20]. Further screening included the estimation of the tanimoto combo score using ROCS (Rapid overlay of chemical structures). Molecules with a tanimoto combo score greater than 0.6 were included in further analysis. [21] A total of 30 compounds had a tanimoto combo score greater than 0.6. The 2D structures of the all the 30 compounds were sketched using Chems sketch. [22] For the QSAR analysis [23], a total of 28 molecules with known antifungal and antiprotozoan activity were obtained using literature search, these were included in the training set during QSAR analysis. The test set included the 30 compounds obtained post screening with ROCS. The pIC<sub>50</sub> values of the test compounds was predicted and the top five compounds with the highest pIC<sub>50</sub> value were selected for further experimen- tation. These five compounds were not commercially available and the synthesis of the com- pounds was done by Sigma Aldrich, USA, the schema for the synthesis of the main compound (C2) is shown in S1 Fig. The NMR data and IR data for C2 is shown in S2 and S3 Figs respec- tively. HPLC confirms the C2 to be of 98.5% purity. (Sigma Aldrich,USA)

### *In vitro* screening

**Parasite culture.** *Leishmania major* promastigotes (MHOM/IL/67/JERICHO II) were maintained at 27°C in RPMI-1640 media supplemented with 20% heat inactivated fetal bovine serum (GIBCO BRL, Grand Island, NY) and antibiotics. All experiments were performed with parasite cultures in the logarithmic phase of growth.

## Anti-leishmanial activity assay

The five compounds (C1-C5) were dissolved in DMSO to prepare a stock of 10mM. The stock was further diluted in the culture media (RPMI-1640) such that the final concentration of DMSO was restricted to 0.7% (v/v). Around  $1 \times 10^6$  *L. major* promastigotes were seeded into each well. Serial dilutions of the drugs were made such that the range of concentrations was from 1–1000  $\mu$ M. The viability of the parasites was assessed using MTT (3-[4, 5-methylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) method. Briefly 20 $\mu$ l of MTT (5mg/ml) was added to each well including the controls. Plates were incubated in dark for a time period of 4 h at 27°C. After the incubation period, centrifugation was performed at 6010Xg for 10min at room temperature. The media was replaced by 100  $\mu$ l of DMSO and absorbance was read spectrophotometrically using a 570nm filter (MultiScan FC, Microplate photometer, Thermofisher).

## Mammalian cell cytotoxicity

To evaluate the cytotoxic effect of the compounds, macrophage cell line (J774) was used. Macrophages were cultured in 96 well microtitre plates ( $1 \times 10^5$  cells/well) in DMEM media (GIBCO BRL, Grand Island, NY) containing heat inactivated 10% FBS and 100  $\mu$ g/ml penicillin-streptomycin. Cells were maintained at 37°C, 5% CO<sub>2</sub> for a period of 24 hours. As soon as the cell confluency was achieved, medium was replaced with fresh medium containing compounds (C2 and C3) at their IC<sub>50</sub> concentrations, incubated for a period of 48h. After incubation, control and treated cells were washed with PBS, pH 7.2. 180 $\mu$ l of PBS and 20 $\mu$ l of MTT solution (5mg/ml) was added to each well. Cells were incubated for 4 h at 37°C, 5% CO<sub>2</sub>. The formazan crystals were dissolved in 100  $\mu$ l of DMSO and the absorbance was read spectrophotometrically using a 570nm filter (MultiScan FC, Microplate photometer, Thermofisher). The percentage of viable cells were calculated as % Cell viability =  $(A_T - A_B / A_C - A_B) \times 100$  where A<sub>T</sub> is the absorbance of treated wells, A<sub>C</sub> the absorbance of control wells (not treated), and A<sub>B</sub> the absorbance of blank wells culture medium and DMSO only). The experiment was carried out in triplicates and a set of two independent experiments were performed. A set of three separate experiments were performed for each drug and the cell viability at IC<sub>50</sub> concentration was determined.

## Development of nanoliposomal formulation of C2

Nanoliposomes of one of the compounds (compound 2) were prepared using the thin film hydration method. Compound 2 (C2), L- $\alpha$ -phosphatidylcholine (PC) (Sigma-Aldrich), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (DSPE-PEG) (Avanti Polar Lipids, Inc.) and cholesterol (Sigma-Aldrich) 1.0: 2.0: 0.1: 0.5 weight ratio were dissolved in 5 mL dichloromethane (DCM) (Sigma-Aldrich). DCM was evaporated using a rotary evaporator to form thin and uniform lipid film. The lipid film was then hydrated with 1.0 mL H<sub>2</sub>O for 1 h at 60°C with intermittent sonication. The resultant solution was passed through the Sephadex<sup>®</sup> G-25 (Sigma-Aldrich), sequentially extruded through 200 nm Whatmann polycarbonate membrane using Avanti<sup>®</sup> mini-extruder (Avanti Polar Lipids, Inc.) The nanoliposomes were stored at 4°C until further use.

**Size and shape of nanoliposomes.** The size, shape and morphology of the nanoliposomes were determined by dynamic light scattering (DLS), field-emission scanning electron microscopy (FESEM) and atomic force microscopy (AFM). Samples were prepared for DLS, FESEM and AFM. Stability of the nanoliposomes was checked as per the procedure mentioned in reference [24]

**Drug loading.** A Calibration curve was plotted in the concentration range of 10 to 30  $\mu$ M by diluting the 1 mM standard stock solution of the C2 in dimethyl sulfoxide (DMSO). The absorbance was measured at 256 nm against the corresponding solvent blank. The linearity

was plotted for absorbance against concentration. For checking drug loading in nanoliposomes, prepared nanoliposomes were dissolved in DMSO in 5%, 10% and 15% dilution. Absorbance was measured at characteristic wave length against the corresponding solvent blank in 200  $\mu\text{L}$  quartz cuvette and from the calibration curve drug loading was measured in triplicates.

**Drug release profile.** Concentrated drug loaded nanoliposomes (200  $\mu\text{l}$ ) were suspended in 200  $\mu\text{L}$  of buffer (pH = 7.4), sealed in a dialysis membrane (MWCO = 500 Dalton) and incubated in 15mL PBS buffer at room temperature with gentle shaking. 200  $\mu\text{L}$  portion of the aliquot was collected from the incubation medium at predetermined time intervals and the released drug was quantified by UV-VIS Spectrophotometer (Chemito Spectrascan UV 2600).

### Cytotoxicity profile of the nanoparticles (C2NL)

To evaluate the cytotoxicity of the nanoliposomes, macrophage cell line (J774) was used. Macrophages ( $1 \times 10^5$  cells/well) were cultured in 96 well microtitre plate as described above. Cells were maintained at 37°C, 5% CO<sub>2</sub> for a period of 24 h. After 24h, the medium was replaced with fresh medium containing different concentrations of liposomes (0–30 $\mu\text{M}$ ) containing C2. After the incubation period of 48h, MTT assay was performed. Absorbance was read spectrophotometrically using a 570nm filter (MultiScan FC, Microplate photometer, Thermofisher) and the percentage of viable cells were calculated. The experiment was carried out in duplicates and a set of two independent experiments were performed. The cytotoxicity profile was obtained and the graph was plotted using GraphPad Prism 5.

### Evaluation of anti-leishmanial activity of the synthesized nanoparticles

Logarithmic phase *L.major* promastigotes ( $1 \times 10^6$  cells/well) were seeded in 96 well plates. To evaluate the inhibitory potential of the nanoliposomes, dilutions of the nanoliposomes were prepared in the culture media (RPMI-1640). The range of concentrations was from 0–30 $\mu\text{M}$ . The plates were incubated for a time period of 48h. Viability of the parasites was assessed using MTT (3-[4, 5-methylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) method. Briefly 20 $\mu\text{l}$  of MTT (5mg/ml) was added to each well including the controls. Plates were incubated in dark for a time period of 4h at 27°C. After the incubation period, centrifugation was performed at 6010Xg for 10min at room temperature. The media was replaced by 100 $\mu\text{l}$  of DMSO and absorbance was read spectrophotometrically using a 570nm filter (MultiScan FC, Microplate photometer, Thermofisher).

### Morphological studies

*L.major* promastigotes were exposed to the compound 2 containing nanoliposomes (C2NL) and the morphological alterations were investigated by optical microscopy. Briefly around  $1 \times 10^6$  *L.major* promastigotes were cultured in 24 well plates and treated with 30 $\mu\text{M}$  of C2NL. The plates were incubated at 27°C for a time period of 48h. Promastigotes were examined using the 40X objective to study the effect of compounds on parasite morphology using EVOS FL Color Imaging System (Life technologies). To estimate the size reduction of the parasites, ImageJ v1.49 software was used. [25]

### SEM analysis of the treated promastigotes

Around  $1 \times 10^6$  *L.major* promastigotes were cultured in 24 well plates and treated with 30 $\mu\text{M}$  of C2NL. The plates were incubated at 27°C for a time period of 48h. Promastigotes were washed twice with PBS pH 7.2, adhered to poly L lysine- coated coverslips. Fixation was done in 2.5% glutaraldehyde solution (pH 7.4) for a period of 3 hours at 27°C. After fixation, the material

was washed twice in PBS and dehydrated in acetone series, coated with platinum using Q150T Turbo- Pumped Sputter Coater. Cells were observed under a Nova NanoSEM field emission scanning electron microscope operating at 15 kV. Images were analyzed using the Gwyddion software. (<http://gwyddion.net/>)

### Mitochondrial potential reduction

To determine the effect of compound on induction of apoptosis and the associated reduction in mitochondrial potential, dual staining of *L.major* promastigotes was performed. Briefly  $1 \times 10^6$  *L.major* promastigotes were treated with  $30 \mu\text{M}$  of **C2NL** for a time period of 48h. Cells were harvested by centrifugation at  $6010 \times g$  and washed with PBS. Cells were incubated with Mitotracker Red CMXRos (Life Technologies) to a final concentration of  $100 \text{ nM}$  for 30 min. Cells were washed with PBS and resuspended in  $100 \mu\text{l}$  of 1X annexin binding buffer. Around  $5 \mu\text{L}$  of Alexa Fluor<sup>®</sup> 488 annexin V was added and cells were incubated in dark at room temperature for 15 min. After the incubation period,  $400 \mu\text{L}$  of 1X annexin-binding buffer was added. Data acquisition was carried out using FACSCalibur (BD Biosciences) and analysis was done using CELLQUEST PRO software. 10,000 events from each sample were acquired. The representative plots are a result of three independent experiments.

### In vivo screening

The experiments were conducted according to the protocols approved by the Institutional Animal Ethical Committee of National Centre for Cell Science (NCCS, Registration number: 7/GO/ReBi/S/99/CPCSEA) under the Project number: EAF/2013/B-197. Mice were bred in the experimental animal facility at NCCS. BALB/c female mice, 8 weeks old with a body weight around 20–25 g were used in the study. *L.major* stationary promastigotes were cultured as per the procedure mentioned above. *L.major* promastigotes ( $2 \times 10^6$  stationary phase promastigotes in 0.04 ml PBS) were injected subcutaneously in the left hind limb footpad of the mice. **C2** was dissolved initially in DMSO and diluted with PBS. The dose for *in vivo* experiments was decided based on the standard dose of miltefosine used for treatment of visceral leishmaniasis ( $2.5 \text{ mg/kg bwt}$ ). Keeping in mind the relatively higher  $\text{IC}_{50}$  value of **C2 and C3**, the compound dose was adjusted to  $5 \text{ mg/kg bwt}$ . Oral administration of **C2 and C3** ( $5 \text{ mg/kg body weight}$ ) was done, treatment was started when the lesion thickness of 3mm was obtained. Mice were administered drug once daily for a period of 8 weeks. Mice treated with PBS were included as a vehicle control. Lesion size was monitored every week post infection using a vernier calliper. Lesion size has been expressed as footpad thickness (mm). Parasite load in the draining lymph nodes was estimated post euthanasia by limiting dilution assay for each of the treated and untreated groups as per the Taswell's method. [26]

### Statistical analysis

A student paired t test has been used for data analysis. Analysis was done using SigmaStat software program (Binary Semantics, Pune, India). Results have been represented as Mean  $\pm$  SEM ( $p < 0.05$  indicating statistical difference between the test and the control)

## Results

### In silico screening of the coumarin derivatives

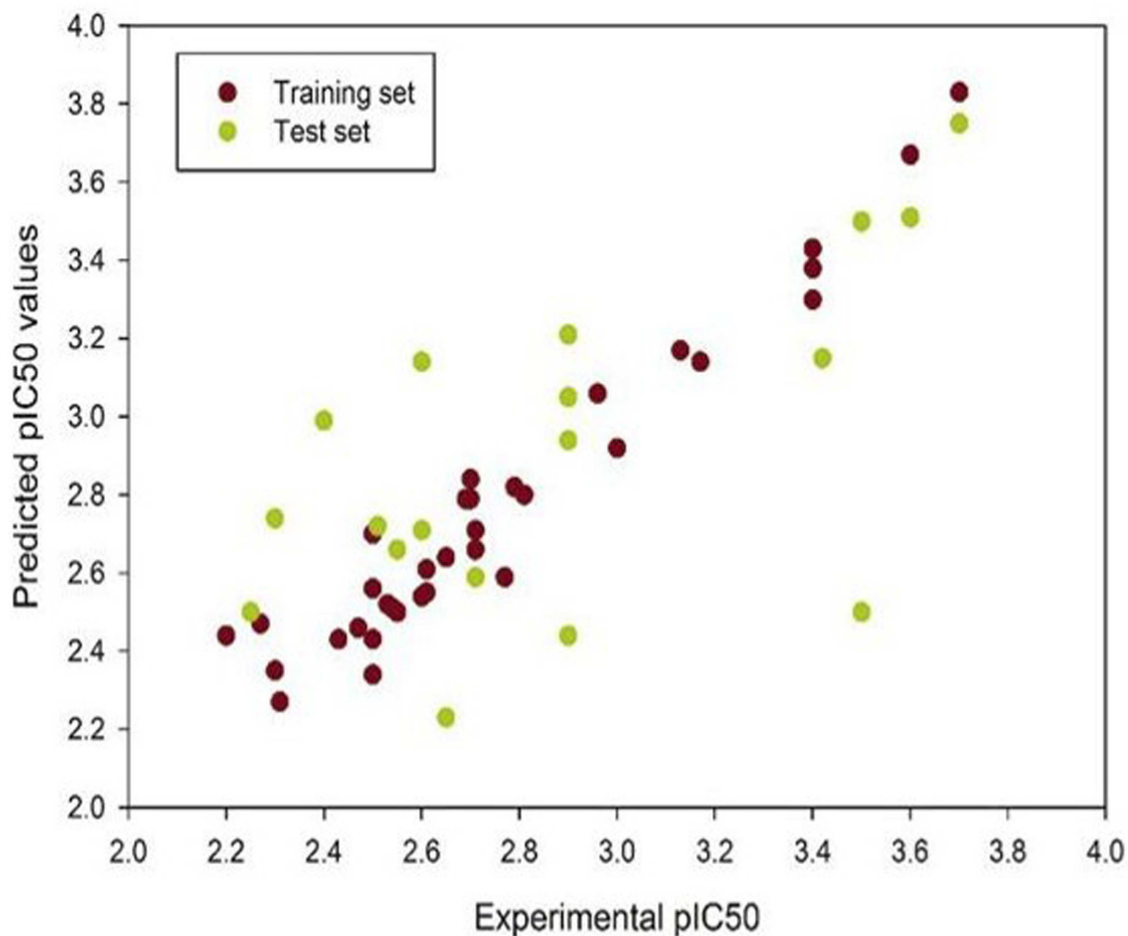
Coumarin derivatives obtained from the ZINC database were further screened based on their compliance with the Lipinski's rule of five and Veber's rules. Shape based screening method was used for screening of the derivatives and only those compounds with a Tanimoto combo

**Table 1. Screening of the compounds using FBQSAR.** Tanimoto combo scores of the top five compounds and their predicted pIC<sub>50</sub> values.

S.No	Identifier	Tanimoto combo score	Predicted pIC <sub>50</sub> value
1.	C1	0.65	3.70
2.	C2	0.84	3.61
3.	C3	0.75	3.46
4.	C4	0.85	3.45
5.	C5	0.74	3.41

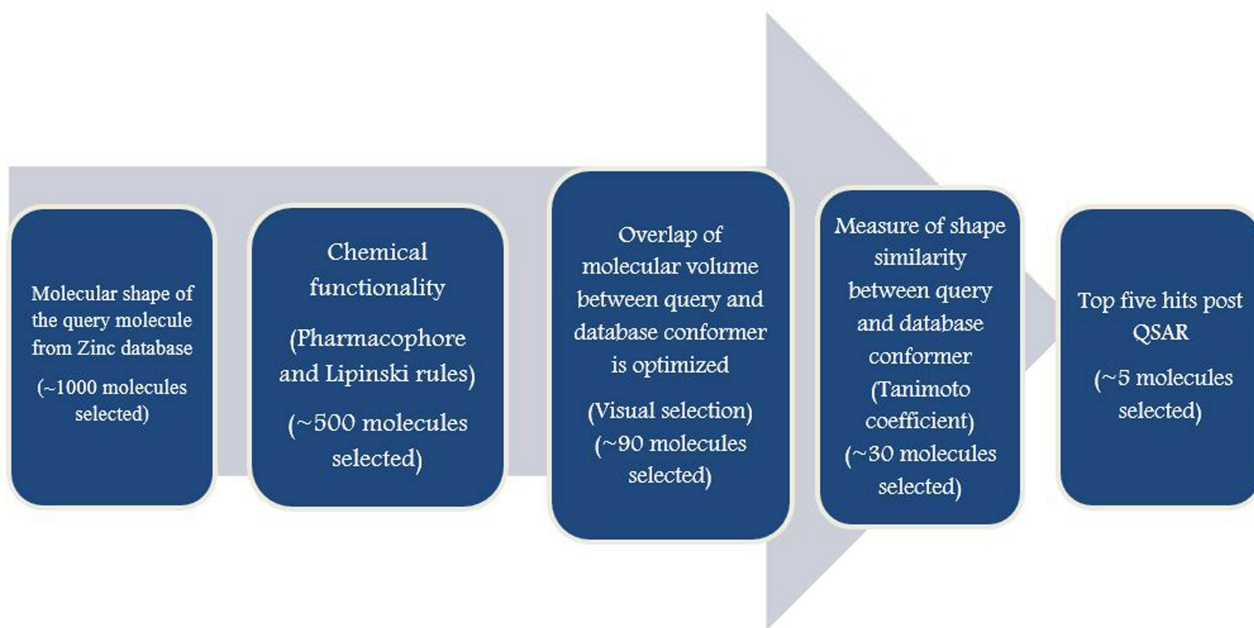
doi:10.1371/journal.pone.0164585.t001

score > 0.6 were considered. (Table 1) Field based QSAR was performed to correlate the physicochemical properties of the compounds with their biological activities. The training set for the QSAR analysis was based on the standard coumarin compounds with known anti-fungal and anti-trypanosomal compounds. [12, 27, 28, 29, 30] The scatter plot showing the test and training set is represented in Fig 1. The model built was reliable as the  $q^2$  value of the model was 0.60 and the  $R^2$  value was 0.83. Based on the FB-QSAR results and the predicted pIC<sub>50</sub> values,

**Fig 1. Screening of the compounds using FBQSAR.** The scatter plot shows the predicted pIC<sub>50</sub> values of the training and test set of the compounds used during the QSAR analysis. The top five compounds with an higher pIC<sub>50</sub> were selected for further analysis.

doi:10.1371/journal.pone.0164585.g001





**Fig 2. *Insilico* screening of the compounds.** This figure depicts the procedure used during the screening of the compounds.

doi:10.1371/journal.pone.0164585.g002

we selected the top five compounds for further *in-vitro* and *in-vivo* experimentation. The screening procedure has been outlined in Fig 2.

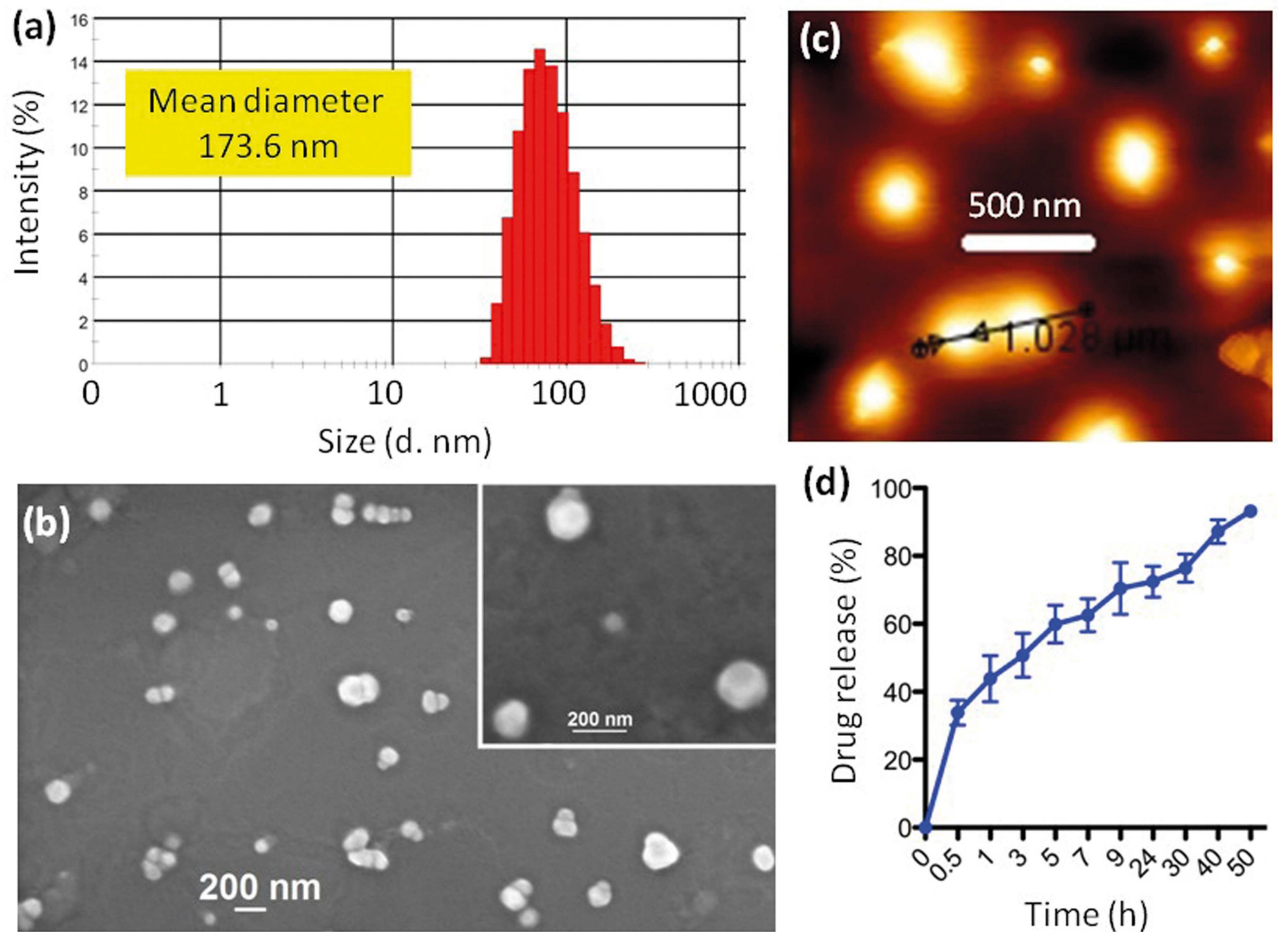
### Anti-leishmanial properties of the compounds

The *in vitro* antileishmanial activity of C1–C5 compounds was determined post 48h treatment of *L. major* promastigotes. The efficacy of the compounds was determined in terms of their IC<sub>50</sub> values. Of the five compounds, compounds (C1, C4 and C5) showed little or no inhibition at all. (IC<sub>50</sub> values > 1mM) while C2 and C3 showed anti-leishmanial properties with an IC<sub>50</sub> value of 524 μM and 223 μM respectively. (Table 2) The percentage viability of the macrophages treated with C2 was around 65%, while that of C3 was around 85%. Thus there was no significant toxicity of macrophages seen with compounds C2 and C3 at their IC<sub>50</sub> values. In the *L. major* promastigotes treated with C3 post 48h of treatment at the IC<sub>50</sub> concentration, we did not observe any morphological changes while promastigotes treated with C2 exhibited size

**Table 2. IC<sub>50</sub> prediction of the compounds in *L. major* promastigotes.** IC<sub>50</sub> values of compounds post 48h of treatment. Compounds giving less than 50% inhibition have been reported to have IC<sub>50</sub> > 1000 μM.

S. No	Identifier	Compound Name	IC <sub>50</sub> value (μM)
1.	C1	(3-(1,3-Benzodioxol-5-yl)-2oxo-2H-chromen-6-yl-acetate)	>1000
2.	C2	(6-Amino-3-(1,3-benzodioxol-5-yl)-2H-chromen-2-one)	524
3.	C3	3-(1,3-Benzodioxol-5-yl)-6-([(1E)-2-furylmethylene]amino)-2H-chromen-2-one	223
4.	C4	3-(1,3-Benzodioxol-5-yl)-6-([(1E)-1H-pyrrol-2-ylmethylene]amino)-2H-chromene-2-one	>1000
5.	C5	(3-(1,3-Benzodioxol-5-yl)-6-nitro-2H-chromen-2-one)	>1000

doi:10.1371/journal.pone.0164585.t002



**Fig 3. Characteristics of the nanoliposomal formulation.** a) Determination of the hydrodynamic diameter of the nanoparticles using DLS. Particles are sub 200nm in size b) Shape of the nanoparticles as observed by FESEM. Nanoparticles appear monodisperse in nature and are spherical in nature c) AFM characterization of the nanoparticles d) Release profile of compound 2 from the nanoliposomes. The nanoparticles ensure that around 93% drug release occurs over a period of 50h. The designed nanoparticles are capable of releasing the drug in a slow and sustained manner.

doi:10.1371/journal.pone.0164585.g003

reduction and loss of motility. Treated promastigotes were also noninfective to the macrophages (J774 cell line) and lost the ability to proliferate under *in vitro* conditions.

### Nanoliposome development of C2

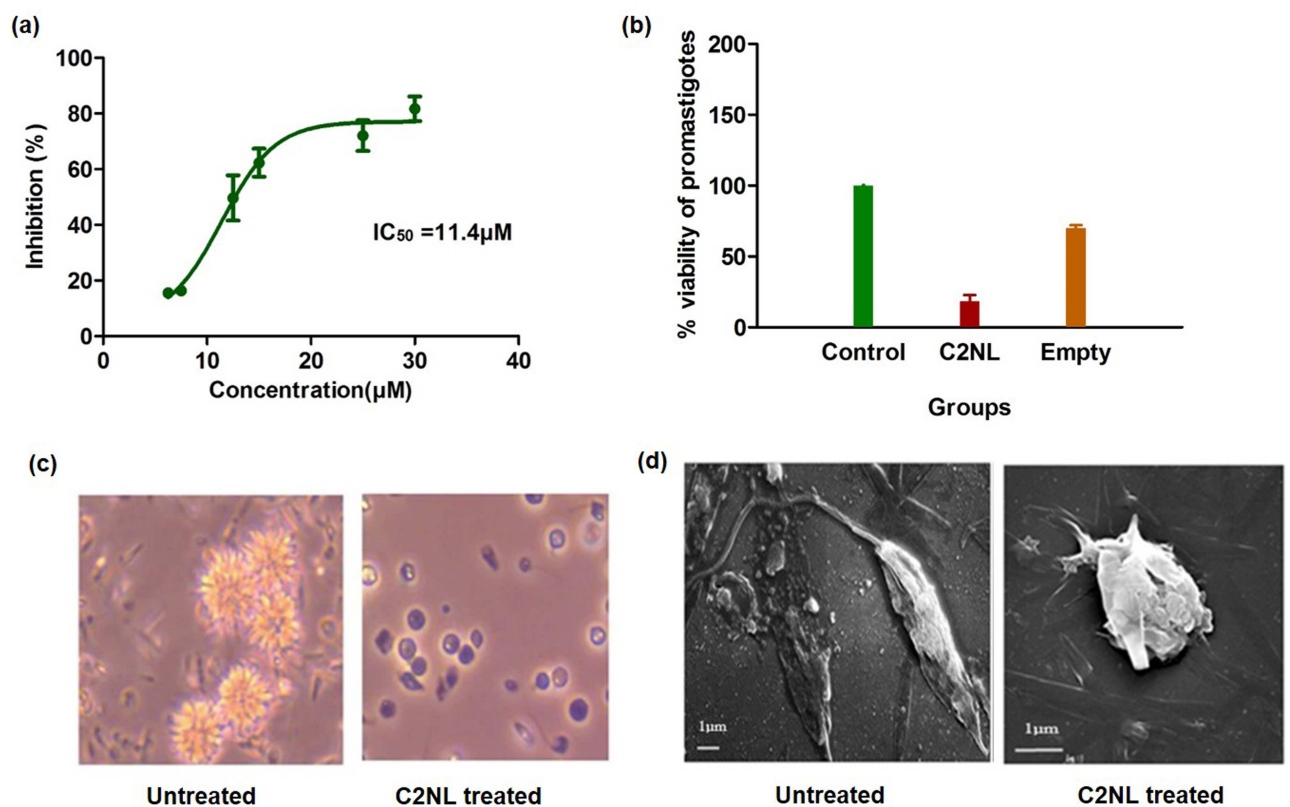
In order to improve the solubility and to study the anti-leishmanial properties of C2, the compound was encapsulated in the form of nanoliposomes. The mean drug loading was found to be 150  $\mu\text{g/ml}$  (loading efficiency = 10%) by UV-Vis spectroscopy through a concentration versus absorbance calibration graph at  $\lambda_{\text{max}} = 256 \text{ nm}$  characteristic of C2. The hydrodynamic diameter of the nanoliposomes was determined to be 173.6 nm through dynamic light scattering (DLS) (Fig 3a). Size, shape and morphology of the drug loaded nanoliposomes were determined by field-emission scanning electron microscopy (FESEM) and atomic force microscopy (AFM) (Fig 3b and 3c). From the FESEM and AFM images, it was clear that the drug loaded nanoliposomes were monodispersed and spherical in shape having sub 200 nm size. The nanoliposomal formulation developed was stable at both 4°C and at 37°C. From the release kinetics



experiment (Fig 3d), it was quantified that almost 93.2% of the compound was released from the nanoliposomes over a period of 50h. Hence, the nanoliposome formulation of C2 (C2NL) ensured the slow and sustained release of the active compound over a long period of time.

### Efficacy of the nanoliposomes

To determine the efficacy of C2 nanoliposomes, we investigated the effects of C2NL on *L. major* promastigotes. Log-phase parasites were cultured in RPMI media and treated with C2NL with the concentrations ranging from 0–30 $\mu$ M. At the fixed points, cells were subjected to MTT assay to estimate the inhibitory potential of the nanoliposomes. It was observed that C2NL treated promastigotes show a dose dependent growth reduction with the IC<sub>50</sub> value of C2NL ranging between 10–15 $\mu$ M. (Fig 4a) At the IC<sub>50</sub> concentration, the viability of the macrophages treated with C2NL was around 80–85% indicating that the nanoliposomal formulation showed lesser toxicity to the host macrophages. (Fig 4b) In order to rule out the possibility of inhibition due to the liposomal mixture, we also performed inhibition assay with empty liposomes. Percentage inhibition of empty liposomes equivalent to 30 $\mu$ M of C2NL over *L. major* promastigotes was around 31% further confirming that the inhibitory effect was due to the presence of C2 rather than the liposomal mixture.



**Fig 4. Testing the efficacy and cytotoxicity of the nanoliposomal formulation.** a) IC<sub>50</sub> prediction of C2NL. *L. major* promastigotes were treated for 48 hours with 30 $\mu$ M of the C2 containing nanoliposomes. Bar graph indicates the percentage viability of parasites. A comparison has also been made with that of empty liposomes. Inhibitory effect was evaluated using MTT assay. b) Cytotoxicity of the nanoliposomes over J774 macrophage cell line. c) Microscopic analysis of the treated parasites. Parasites treated with C2NL show abnormal morphology and reduced motility. d) SEM images of the treated and untreated promastigotes. Treated promastigotes tend to round up and shrink in size. They also lose their flagella and become less motile. Bar = 1  $\mu$ m.

doi:10.1371/journal.pone.0164585.g004

## Morphological changes

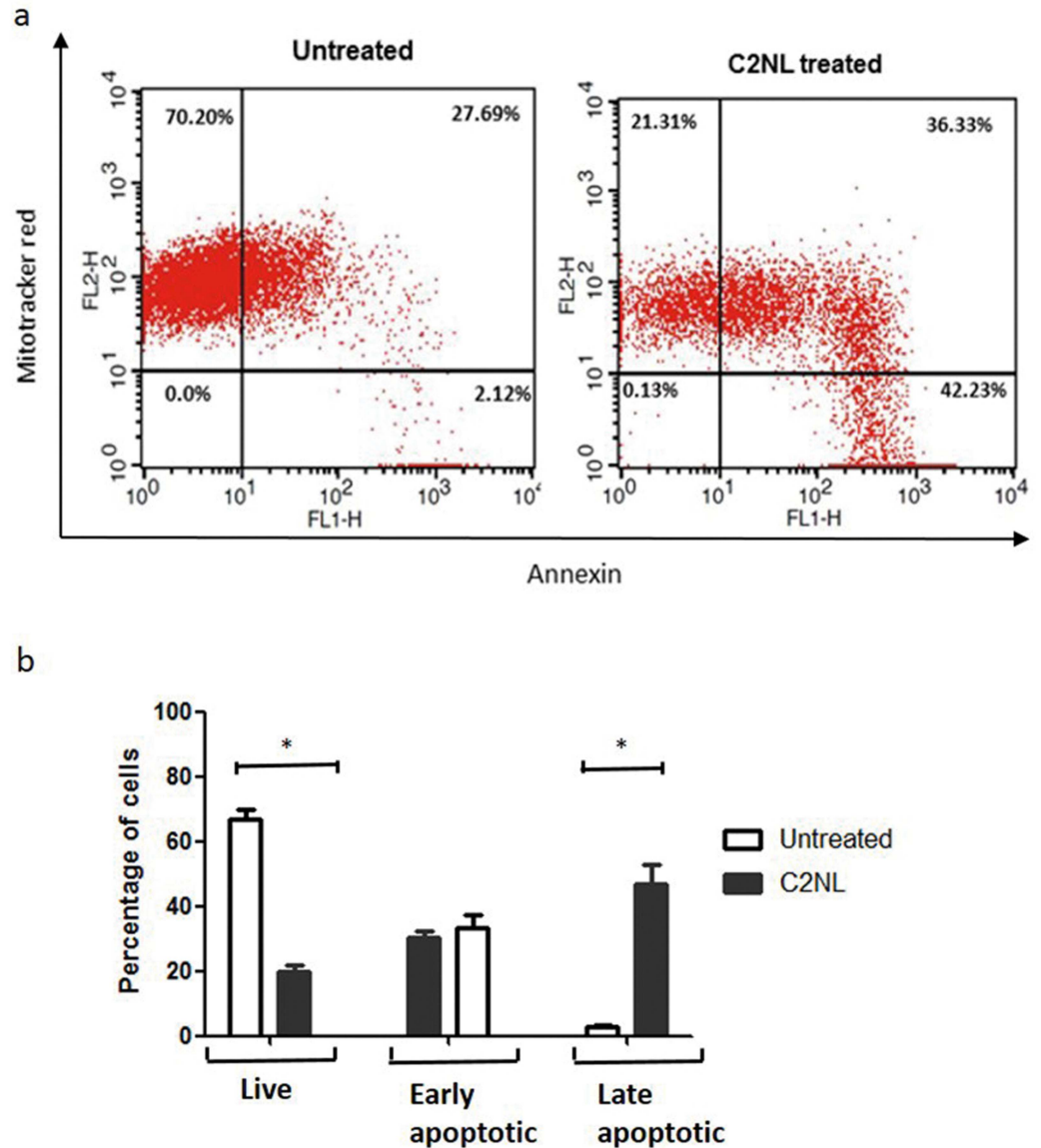
At a concentration of 30 $\mu$ M post 48h treatment of *L.major* promastigotes with C2NL, we also observed morphological changes in the parasites like reduction in size and motility (Fig 4c). To further confirm this observation, the percentage area reduction of the C2NL treated parasites was calculated which was around  $48.1 \pm 13\%$  as compared to the untreated parasites. We also performed SEM analysis of the treated parasites which indicated that C2NL treated promastigotes appear to lose their flagella and exhibited size reduction in comparison to the control. This indicates that C2NL is capable of reducing the motility of the *L.major* promastigotes. (Fig 4d)

## C2 induces mitochondrial membrane potential reduction in *L.major* promastigotes

Mitochondrial membrane potential reduction is a characteristic feature observed in the cells undergoing programmed cell death.[31] C2NL treated *Leishmania* promastigotes were investigated for a change in the  $\Delta\Psi_m$ . In addition, we also tried to check if C2NL induced apoptosis in *Leishmania*. Mitotracker dyes get aggregated within the normal mitochondria, which retain the  $\Delta\Psi_m$  giving a bright red fluorescence. Externalization of phosphatidyl serine is one of the common feature of apoptotic cells. Cells undergoing apoptosis can be detected using Annexin V as it has the affinity to bind to the exposed phosphatidyl serine. [32] In the C2NL treated *L.major* promastigotes, the reduction in mitochondrial membrane potential and phosphatidyl serine externalization was studied by performing a dual staining with the mitochondrial potential specific Mitotracker Red CMXRos dye and the apoptosis specific Annexin dye. Cells were analyzed using flow cytometry and classified as early or late apoptotic based on the ability to retain  $\Delta\Psi_m$  coupled with the annexin exposure of cells. The *L.major* promastigotes were treated with 30 $\mu$ M of C2NL for a period of 48h before performing the staining. We observed a loss of the red fluorescence signifying reduction of the  $\Delta\Psi_m$  in the C2NL treated promastigotes. (Fig 5a) In the untreated cells (promastigotes without treatment), there was a higher Mitotracker red staining. In the treated promastigotes, a larger population of promastigotes had lower Mitotracker red staining indicating that one of the properties of C2 is to lower mitochondrial membrane potential in *L.major* promastigotes. We also observed that the percentage of late apoptotic cells was higher in the treated sample. In the 48h treated sample, 36.3% of the cells were under early apoptotic stage while 42.3% of the cells were late apoptotic while in the untreated sample, 27.6% of the cells were under early apoptosis and only 2% of the cells were late apoptotic. This clearly indicates that C2NL was capable of inducing mitochondria mediated apoptosis in *Leishmania major* promastigotes. (Fig 5b)

## Efficacy of C2 in *L.major* infected mice

Based on the in-vitro experiment the effect of C2 and C3 was tested in BALB/c mice infected with *L.major*. Oral administration of the compounds at a dose of 5mg/kg/bwt orally was done for a period of 8 weeks. The progression of the infection was assessed based on the footpad thickness. The footpad thickness of the mice treated with C2 was significantly lower as compared to the untreated mice. (Fig 6a) No reduction in footpad thickness was seen in C3 treated mice and it was seen that this compound was ineffective (Fig 6b). The parasite loads in the draining lymph nodes of the mice treated with C2 was lower as compared to the untreated mice (Fig 6c).

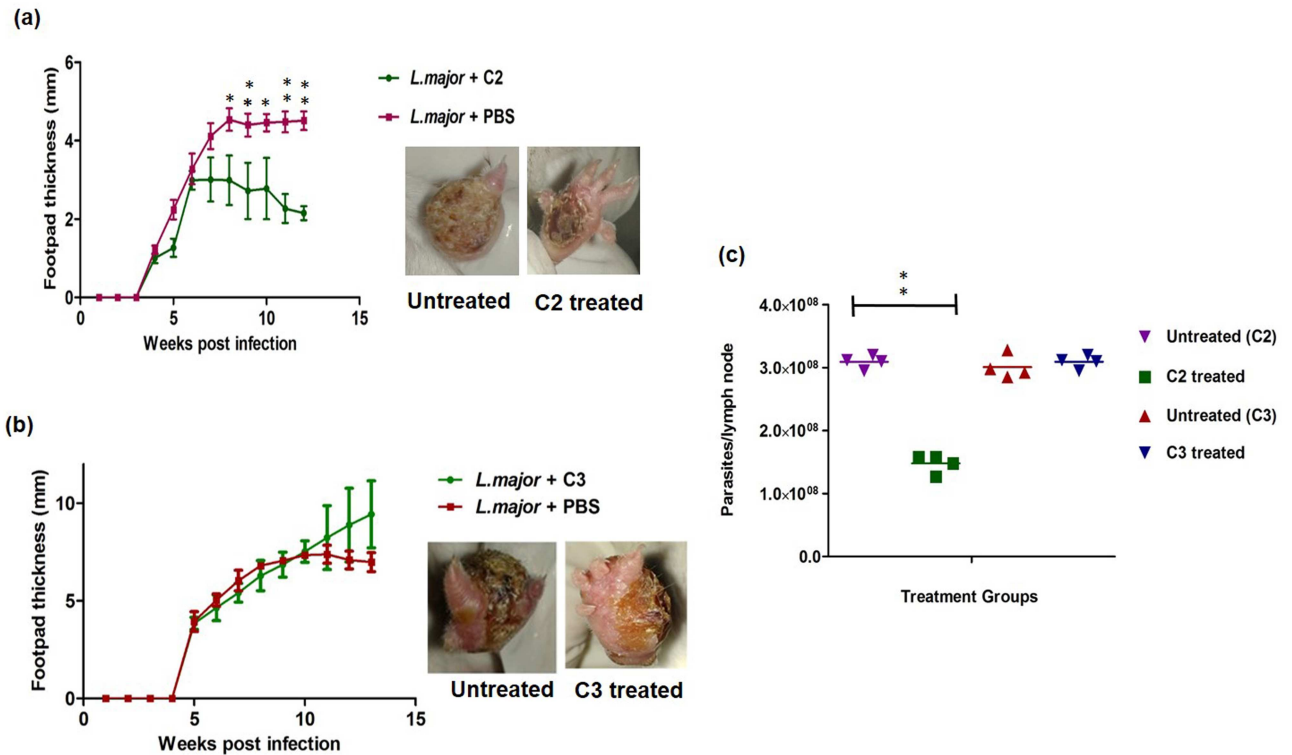


**Fig 5. C2 induces mitochondrial mediated apoptosis in *L. major* promastigotes.** a) FACS analysis showing the reduction of mitochondrial potential and the induction of apoptosis in the C2NL treated *L. major* promastigotes. Promastigotes were treated with 30µM of C2NL for a period of 48hours. The image shown is a representative image of three independent experiments b) Percentage of cells showing an increase in the number of cells in the late apoptotic phase in the treated samples as well reduction in the number of live cells. Experiment was performed in triplicates. Significance is indicated in the figures as \*p<0.01. Error bars represent the standard deviation of the mean.

doi:10.1371/journal.pone.0164585.g005

## Discussion

Coumarins are potent anti-fungal compounds and a few studies have also reported that coumarins have anti-leishmanial properties. Though their exact mechanism of action has not yet been deciphered, their ability to enhance the phagocytotic activity of macrophages is well known. [33] We identified a set of coumarin derivatives and tested their efficacy on *Leishmania major*. Shape based matching of coumarin derivatives with their parent compound was done,



**Fig 6. Course of *L. major* infection in mice treated with C2.** a) Footpad swelling progression during drug treatment. Weekly recordings of footpad swelling are shown for the untreated and the mice treated with compound 2. Footpad swelling is expressed as the increase of the infected over the noninfected footpad. Data shown summarizes the (Mean±SEM) from 4 animals each. \*p < 0.05, \*\*p < 0.01. Photographs of the footpad at the end of the treatment indicate the progression of the ulceration. b) Footpad swelling progression of the miltefosine treated mice. c) Footpad swelling progression of the compound 3 treated mice d) Parasite load assay of the treated groups. Significance is indicated in the figure as \*\*p < 0.01. Every dot indicates one mice.

doi:10.1371/journal.pone.0164585.g006

which excluded the compounds having lower similarity. 3D-QSAR which exploits the 3D properties of the ligands to predict their biological activities was used for further screening. We further tested these compounds over *L. major* promastigotes to identify the compounds with anti-leishmanial properties. It was seen that treatment with compound 2 leads to a dose-dependent inhibition of proliferation. To further improve the solubility of the compound and to explore the anti-leishmanial properties of this compound, nanoliposomal formulation was prepared. Parasites treated with C2 showed drastic reduction in motility and reduction in size. They also lose their ability to infect the macrophages (J774 cell line). Dual staining with annexin-mitotracker red indicated that the nanoliposomes developed were able to initiate mitochondrial mediated apoptosis in the treated parasites. Based on the *in vitro* screening, we tested the efficacy of C2 and C3 in *L. major* infected BALB/c mice at a dose of 5mg/kg/bwt. Our observations indicate that there was a healing effect in the footpads of mice treated with C2. Together, our study indicates that C2 serves as an initial hit which could be further modified and developed for realizing its potential as a potent anti-leishmanial compound.

### Conclusion

There is an urgent need to design and develop novel anti-leishmanial compounds due to various problems associated with the current chemotherapeutic. We have designed coumarin derivatives using *in silico* approaches. Five of the inhibitors were selected for further *in vitro*

experiments, their cytotoxicity and efficacy against *L. major* has been tested. Parasites treated with compound 2 and its nanoliposomes showed significant morphological changes. Thus the possibility of using coumarin derivatives could thus be exploited towards treatment of cutaneous leishmaniasis.

## Supporting Information

**S1 Fig. Schema for the synthesis of C2.**

(TIF)

**S2 Fig. NMR Spectra of C2.**

(TIF)

**S3 Fig. IR Spectra of C2.**

(TIF)

## Acknowledgments

Vineetha Mandlik acknowledges the financial support as Senior Research Fellow of Department of Biotechnology, Government of India; and Shailza Singh received the funding BT/PR6037/GBD/27/372/2012, Department of Biotechnology, Government of India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Authors would like to thank the Central Instrument facility at SP Pune University, Pune for carrying out the SEM imaging. Authors also acknowledge BHPCF (Bioinformatics & High Performance Computing Facility), FACS and Animal house facilities of NCCS.

## Author Contributions

**Conceptualization:** SS.

**Data curation:** VM SS.

**Formal analysis:** VM SP RB SB SS.

**Funding acquisition:** SS.

**Investigation:** VM SP.

**Methodology:** SS SB.

**Project administration:** SS.

**Resources:** SS RB SB.

**Software:** SS VM.

**Supervision:** SB SS.

**Validation:** VM SP SB SS.

**Visualization:** VM SB SS.

**Writing – original draft:** SS SB VM.

**Writing – review & editing:** SS SP SB VM.



## References

1. Haldar AK, Sen P, Roy S. Use of antimony in the treatment of leishmaniasis: current status and future directions. *Molecular biology international*. 2011.
2. Yardley V, Croft SL. Activity of liposomal amphotericin B against experimental cutaneous leishmaniasis. *Antimicrob Agents Chemother*. 1997; 41: 752–756. PMID: [9087483](#)
3. Sundar S, Chakravarty J. Paromomycin in the treatment of leishmaniasis. *Expert opinion on investigational drugs*. 2008; 17: 787–94. doi: [10.1517/13543784.17.5.787](#) PMID: [18447603](#)
4. Khademvatan S, Gharavi MJ, Rahim F, Saki J. Miltefosine-induced apoptotic cell death on *Leishmania major* and *L. tropica* strains. *The Korean journal of parasitology*. 2011; 49: 17–23. doi: [10.3347/kjp.2011.49.1.17](#) PMID: [21461264](#)
5. Paris C, Loiseau PM, Borjes C, Bréard J. Miltefosine induces apoptosis-like death in *Leishmania donovani* promastigotes. *Antimicrobial agents and chemotherapy*. 2004; 48:852–9. doi: [10.1128/AAC.48.3.852-859.2004](#) PMID: [14982775](#)
6. Croft SL, Barrett MP, Urbina JA. Chemotherapy of trypanosomiasis and leishmaniasis. *Trends in parasitology*. 2005; 21:508–12. doi: [10.1016/j.pt.2005.08.026](#) PMID: [16150644](#)
7. Fournet A, Muñoz V. Natural products as trypanocidal, antileishmanial and antimalarial drugs. *Current Topics in medicinal chemistry*. 2002; 2:1215–37. PMID: [12171582](#)
8. Mishra BB, Kale RR, Singh RK, Tiwari VK. Alkaloids: Future prospective to combat leishmaniasis. *Fito-terapia*. 2009; 80:81–90. doi: [10.1016/j.fitote.2008.10.009](#) PMID: [19015012](#)
9. Sauvain M, Gimenez A, Muñoz V, Callapa J, Le Men-Olivier L, Massiot G et al. Bioactive phenolic glycosides from *Amburana cearensis*. *Phytochemistry*. 1999; 50:71–4.
10. Oketch-Rabah HA, Lemmich E, Dossaji SF, Theander TG, Olsen CE, Cornett C et al. Two new antiprotozoal 5-methylcoumarins from *Vernonia brachycalyx*. *Journal of Natural Products*. 1997; 60:458–61. doi: [10.1021/np970030o](#) PMID: [9170288](#)
11. Brenzan MA, Nakamura CV, Dias Filho BP, Ueda-Nakamura T, Young MC, Côrrea AG et al. Structure–activity relationship of (–) mammea A/BB derivatives against *Leishmania amazonensis*. *Biomedicine & Pharmacotherapy*. 2008; 62:651–8.
12. Napolitano HB, Silva M, Ellena J, Rodrigues BD, Almeida AL, Vieira PC et al. Aurapten, a coumarin with growth inhibition against *Leishmania major* promastigotes. *Brazilian journal of medical and biological research*. 2004; 37:1847–52. PMID: [15558191](#)
13. Ibrar A, Zaib S, Jabeen F, Iqbal J, Saeed A. Unraveling the Alkaline Phosphatase Inhibition, Anticancer, and Antileishmanial Potential of Coumarin–Triazolothiadiazine Hybrids: Design, Synthesis, and Molecular Docking Analysis. *Archiv der Pharmazie*. 2016; 349: 553–565 doi: [10.1002/ardp.201500392](#) PMID: [27214743](#)
14. Otero E, Vergara S, Robledo SM, Cardona W, Carda M, Vélez ID et al. Synthesis, leishmanicidal and cytotoxic activity of triclosan-chalcone, triclosan-chromone and triclosan-coumarin hybrids. *Molecules*. 2014; 19:13251–66. doi: [10.3390/molecules190913251](#) PMID: [25170948](#)
15. Kermani EK, Sajjadi SE, Hejazi SH, Arjmand R, Saberi S, Eskandarian AA. Anti-*Leishmania* activity of osthole. *Pharmacognosy research*. 2016; 8: S1. doi: [10.4103/0974-8490.178650](#) PMID: [27114685](#)
16. Sangshetti JN, Khan FA, Kulkarni AA, Patil RH, Pachpinde AM, Lohar KS et al. Antileishmanial activity of novel indolyl–coumarin hybrids: Design, synthesis, biological evaluation, molecular docking study and in silico ADME prediction. *Bioorganic & medicinal chemistry letters*. 2016; 26: 829–35.
17. Bashir S, Alam M, Adhikari A, Shrestha RL, Yousuf S, Ahmad B et al. New antileishmanial sesquiterpene coumarins from *Ferula narthex* Boiss. *Phytochemistry Letters*. 2014; 9:46–50.
18. Irwin JJ, Shoichet BK. ZINC—a free database of commercially available compounds for virtual screening. *Journal of chemical information and modeling*. 2005 Jan 24; 45(1):177–82. doi: [10.1021/ci049714](#) PMID: [15667143](#)
19. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced drug delivery reviews*. 2012; 64:4–17.
20. Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD. Molecular properties that influence the oral bioavailability of drug candidates. *Journal of medicinal chemistry*. 2002; 45:2615–23. PMID: [12036371](#)
21. ROCS; OpenEye Scientific Software: Santa Fe, NM
22. ACD/Chemsketch, version 5.12, Advanced Chemistry Development, Inc., Toronto, ON, Canada, Available: <http://www.acdlabs.com>
23. Field-Based QSAR, version 2014–3, Schrödinger, LLC, New York, NY, (2014).

24. Mallick A, More P, Ghosh S, Chippalkatti R, Chopade BA, Lahiri M et al. Dual drug conjugated nano-particle for simultaneous targeting of mitochondria and nucleus in cancer cells. *ACS applied materials & interfaces*. 2015; 7:7584–98.
25. Rasband WS. Image J, version 1.41. Maryland, Bethesda: National Institutes of Health
26. Taswell C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *The Journal of Immunology*. 1981; 126:1614–9. PMID: [7009746](#)
27. Iranshahi M, Arfa P, Ramezani M, Jaafari MR, Sadeghian H, Bassarello C et al. Sesquiterpene coumarins from *Ferula szowitsiana* and in vitro antileishmanial activity of 7-prenyloxycoumarins against promastigotes. *Phytochemistry*. 2007; 68:554–61. doi: [10.1016/j.phytochem.2006.11.002](#) PMID: [17196626](#)
28. Mogana R, Adhikari A, Debnath S, Hazra S, Hazra B, Teng-Jin K et al. The antiacetylcholinesterase and antileishmanial activities of *Canarium patentinervium* Miq. *BioMed research international*. 2014.
29. Sardari S, Mori Y, Horita K, Micetich RG, Nishibe S, Daneshtalab M. Synthesis and antifungal activity of coumarins and angular furanocoumarins. *Bioorganic & medicinal chemistry*. 1999; 7:1933–40.
30. Montagner C, de Souza SM, Groposo C, Delle Monache F, Smânia EF, Smânia A Jr. Antifungal activity of coumarins. *Zeitschrift für Naturforschung C*. 2008 Feb 1; 63(1–2):21–8.
31. Saudagar P, Saha P, Saikia AK, Dubey VK. Molecular mechanism underlying antileishmanial effect of oxabicyclo [3.3. 1] nonanones: inhibition of key redox enzymes of the pathogen. *European Journal of Pharmaceutics and Biopharmaceutics*. 2013 Nov 30; 85(3):569–77. doi: [10.1016/j.ejpb.2013.08.014](#) PMID: [24002022](#)
32. Shukla AK, Patra S, Dubey VK. Iridoid glucosides from *Nyctanthes arbortristis* result in increased reactive oxygen species and cellular redox homeostasis imbalance in *Leishmania* parasite. *European journal of medicinal chemistry*. 2012 Aug 31; 54:49–58. doi: [10.1016/j.ejmech.2012.04.034](#) PMID: [22608855](#)
33. Piller NB. A morphological assessment of the stimulatory effect of coumarin on macrophages. *British journal of experimental pathology*. 1978, 59:93. PMID: [638035](#)