Synthesis of 7, 8 –dihydroxyflavone functionalized gold nanoparticles and its mechanism of action against *Leishmania donovani*

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Research

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Title: Synthesis of 7, 8 –dihydroxyflavone functionalized gold nanoparticles and its mechanism of action against *Leishmania donovani*

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Abstract

Background – The synthesis of gold nanoparticles (GNPs) using drugs, synthetic and natural compounds, proteins, nucleic acids have become beneficial due to improved biological activity coupled with reduced cytotoxicity. In this regard, green synthesis of GNPs using plant extract enriched with flavonoids has shown increased attention due to improved antimicrobial efficacy, greater solubility, and better bioavailability of the flavonoid conjugated with GNPs. We have used 7, 8 dihydroxyflavone (DHF), a flavonoid that is enriched in plants and known for neurotropic and antioxidant activities, for the synthesis of GNP. In this report, we have investigated synthesis, characterization, and biological activity of DHF synthesized GNP against parasite *Leishmania donovani*.

Results – Synthesized DHF functionalized GNPs (DHF-GNPs) are ~35 nm in size with zeta potential values of -34.1 mV, as observed from DLS studies. UV-Visible spectroscopy and FT-IR analysis confirms successful conjugation of DHF over GNP. TEM imaging shows uniform size and spherical distribution of NPs. Against *L. donovani* promastigotes IC₅₀ for DHF and DHF-GNP is ~140 µM and ~40 µM respectively. In *ex vivo* amastigote model, IC₅₀ for DHF and DHF-GNP is ~40 µM and ~22 µM respectively. Even with 1000 µM of DHF-GNP, cytotoxicity is only ~30% on THP1 cells indicating its high biocompatibility. In DHF-GNP treated parasites, activity of arginase decreases in a dose-dependent manner as evident from gene expression and enzyme-based studies. Supplementation of treated cells with ornithine, metabolite of arginase, shows the highest recovery from death. This indicates inhibition of arginase as the main reason for parasite death. Induction of IFN-γ and reduction IL-12 cytokine response shows a possible T_h1/T_h2-mediated cell death. Also, DHF and DHF-GNP are equally effective against sensitive and drug-resistant strains of *L. donovani*.

Conclusion – Low cytotoxicity and high biological activity may provide an alternative but improved delivery of DHF whose solubility increases due to conjugation with GNP. Further efficacy against drug-resistant strains could be beneficial instead of conventional chemotherapy for leishmaniasis.

Keywords: Functionalized gold nanoparticle, DHF, DHF-GNP, arginase inhibition, drug-resistant
Parasite, iNOS activation

Abbreviations: ARG, Arginase; ODC- Ornithine decarboxylase; SpdS-Spermidine synthase; MDR1-
Multidrug Transporter 1; AdoMetDC- S-adenosylmethionine decarboxylase, TryS–Trypanothione
synthase, TryR-Trypanothione reductase, APx- Ascorbate peroxidase , SOD-Superoxide dismutase,
AAP3- Amino acid permease 3; IFN- γ - interferon- γ ; iNOS, inducible nitric oxide synthase; IL-
interleukin 10, RNS- reactive nitrogen species

Background

Gold nanoparticles (GNPs) are the most widely studied metal NPs because of their contribution to various applications [1]. Due to ease of surface modification and biocompatibility, GNPs emerge as an attractive candidate for delivering small molecules [2], antimicrobial agents [3], peptides [4], proteins [5] and nucleic acids [6] to their targets. GNP-based drug delivery is popular due to ease of GNPs functionalization with antimicrobial agents, biological or chemical methods of synthesis, controlled drug delivery, and multiple-targets of biological action with their ability to penetrate biological membranes. Despite several favourable properties such as the ability to self-assemble, encapsulate drugs and stability in biological systems [7], capability to kill drug-resistant microbes [8], widespread usage of NPs is restricted by its toxicity on living organisms as well as the environment [9]. Conventional chemical methods of synthesis render GNPs toxic during delivery in biological systems. Green synthesis apparently provides a solution to this problem [10]. It is regarded as safe, cost-effective, energy-efficient, and environmentally compatible [11]. This technique exploits reducing power of biological systems like plants, algae, fungi and bacteria and natural products, e.g., polysaccharides, phytochemicals, flavonoids, etc. [12]. A number of biological sources have been identified as bio-reductants to generate NPs of desirable size, shape and properties [13]. Different plant extracts have been used for green synthesis of GNP with improved biocompatibility and enhanced biological activity [14,15]. With further advancement in the understanding of green synthesis, raw plant extracts used in green synthesis were gradually replaced with extracted chemicals like alkaloids, flavonoids, tannins, terpenoids, phenolic acids, carotenoids etc [10,16]. High-performance liquid chromatography (HPLC) analysis of NPs synthesized using *Withania somnifera* leaf extract showed the role of flavonoids in green synthesis [17]. A number of studies confirmed that flavonoids might be considered as major contributors and best plant-derived materials for green synthesis [18] of NPs. The presence of hydroxy (–OH) and carbonyl (–CO) groups along with aromatic rings provides a high reducing potential of flavonoids. A large number of flavonoids are sparingly water-soluble with

125 compromising bioavailability [19] even though they have significant antimicrobial properties.
126 Synthesis of nanoparticles with flavonoids has shown increased bioavailability and aqueous solubility
127 for the flavonoids [14]. Members of flavonoids exhibit anticancer, antimicrobial, antioxidant, anti-
128 inflammatory and neuroprotective properties [20,21]. Flavonoid functionalized GNPs showed
129 significantly improved biological properties in comparison with free flavonoids [18]. Enhancement of
130 biological activity of GNPs in comparison to both flavonoids and NPs alone has been reported [22]
131 [14]. Incorporation of resveratrol, quercetin, baicalein, epigallocatechin gallate (EGCG) on metal ions
132 showed improved antioxidant effect, anti-inflammatory effect, oral bioavailability and reduced
133 toxicity [22,23]. Apart from natural flavonoids, semi-synthetic or synthetic flavonoids have also
134 shown great potential in inhibiting microbes. The synthesis usually includes substitution in hydroxy
135 groups, the addition of halogens or other heteroatomic rings [24].

136 Leishmaniasis is the second-largest protozoal disease after malaria and one of the ‘most neglected’
137 diseases. It is still a major health concern in developing countries like India. An asymptomatic
138 condition like post kala-azar dermal leishmaniasis (PKDL) poses a threat of recurrent disease
139 outbreak. Among all types, the visceral leishmaniasis (VL) caused by *Leishmania donovani* (LD) is
140 fatal and most challenging one to control. Although, there are significant developments of NPs as
141 antileishmanial agents, but its application is severely restricted due to toxicity issues. In recent years,
142 several natural and synthetic flavonoids are considered as lead compounds for developing
143 antiprotozoal agents. Compounds like quercetin, 7, 8- dihydroxyflavone, Luteolin, Apigenin, Fisetin,
144 and Kaempferol had been shown to inhibit LD at very low concentration *in vitro* [25,26]. Quercetin
145 and some of its glycoside derivatives have shown to be active after oral administration on the *L.*
146 *amazonensis* in vivo mice model [27,28]. 7, 8-dihydroxyflavone (DHF) is a naturally occurring
147 flavonoid obtained from plants. It was discovered while searching for molecules that imitate the
148 function of brain-derived neurotrophic factors [29]. DHF rescues cells from damage and death caused
149 by oxidative stress [30]. DHF also reduced the levels of inflammation-causing nitric oxide,

prostaglandin E2 (PGE2), TNF-alpha, and IL-6 in macrophages [31]. DHF treatment failed to protect LD and Trypanosoma brucei infected mice *in vivo* although very high activity was found *in vitro* [27]. It is likely that poor bioavailability of DHF is the cause of failure in mice models. In this study, we synthesized GNP using DHF as a reducing and capping agent. We evaluated the anti-leishmanial potential of DHF-GNP and its mechanism of action in reference to DHF.

Materials and methods

Medium 199, Fetal Bovine Serum (FBS), RPMI-1640, Penicillin/Streptomycin antibiotic solution, Bradford reagent, DNase I were purchased from ThermoFisher Scientific. Tetrachloroauric (III) acid tri hydrate (HAuCl_4), Trypan blue, Triton X-100, L. arginine, α –isonitrosopropiophenone (ISPF), Nitroblue tetrazolium (NBT), 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH), diphenyleneiodonium chloride (DPI), agarose, ornithine, putrescine, spermidine, 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), Sodium antimony Gluconate (SAG), Miltefosine were obtained from Sigma Aldrich. 7, 8 dihydroxyflavone (DHF) was purchased from Tokyo Chemical Industry (TCI). Kits for RNA isolation, genomic DNA isolation and cDNA synthesis were purchased from Agilent, Qiagen, and BioRad respectively. Other routine chemicals of analytical grade were from Merck, India. Raw 264.1 and THP1 cell lines were procured from National Centre for Cell Science (NCCS) - Pune. All the solvents except ethanol were from HiMedia, India. UV-visible spectra were recorded with a Shimadzu UV-240, Hitachi U-3200 spectrometer with a path length of 1 cm. FT-IR spectra were recorded with a PerkinElmer Inc. (Waltham, MA, USA) Spectrum 400 spectrometer. Zeta potential was analysed using Beckman Coulter (Brea, CA, USA) Delsa nano submicron particle size and zeta potential analyzer. TEM images were taken with a (JEOL-JEM 2010, Tokyo, Japan) transmission electron microscope (TEM) operated at 120 keV.

Cell cultures

LD promastigotes were cultured in medium 199 supplemented with FBS (10%) and penicillin/streptomycin (1%). Mouse macrophage-specific cell line Raw 264.7 cells were cultured in growth medium RPMI-1640 supplemented with FBS (10%) and penicillin/ streptomycin (1%) in the presence of 5% CO_2 at 37 °C. The AmB-resistant clinical isolate of LD was a kind gift from Dr. Syamol Roy, NIPER-Kolkata. A clinical isolate of SAG-Resistant strain was a gift from Dr. Mitali Chatterjee, Institute of Post Graduate Medical Education and Research –Kolkata.

Synthesis and conjugation of GNP with DHF

Reactions were done at room temperature unless mentioned. Chemical synthesis of GNP using sodium-citrate was done as described previously [3]. Synthesis of citrate reduced GNP (c-GNP) was confirmed by measuring absorbance at 530 nm. Green synthesis of GNPs was done by DHF (0.8 mM) solution. Due to the low solubility of DHF in the water of neutral pH, the stock solution of DHF was prepared at pH 9.0. 4 ml DHF solution was added drop wise to 20 mL of HAuCl₄ (0.5 mM) and stirred for 20 min at room temperature followed by heating at 80-90°C. During addition, the color of the reaction mixture changed from pale yellow to wine red. The reaction was continued at room temperature for another 30 min. The resulting mixture was centrifuged at 12000g for 15 min, washed with DDW of pH 9.0 for five times. The pellet was re-suspended in 2ml water of pH 8.0 at 4°C for further use.

Characterization of DHF-GNP nanoparticles

NPs were analyzed by UV-Visible spectrophotometer, DLS, Fourier transform IR (FT-IR) and TEM studies. Hydrodynamic diameter, polydispersity index (PDI) and zeta potential was determined on a Beckman Coulter's Delsa nano submicron particle size and zeta potential analyzer (Brea, CA, USA) as per standard procedures. For FT-IR analysis, purified nanoparticles (DHF-GNP) were scanned over a range of 4000 to 400 cm⁻¹ in the Perkin Elmer Spectrum 400 spectrometer. Furthermore, NPs were processed and analyzed by TEM (JEOL-JEM 2010, Tokyo, Japan) analysis for the determination of their shape using standard procedures [4].

***In vitro* assay against promastigotes**

L. donovani (MHOM/IN/1983/AG83) promastigotes were cultured as described [3]. Log phase cells (1×10⁶/ml) were treated with c-GNP (5-160 μM), DHF (5-320 μM) and DHF-GNP (5-80 μM) for 3-4 days and cell viability was measured by Trypan blue exclusion & 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method after every 24 h using standard procedures.

218 After 48 h, the concentration of drug which showed 50% killing of the parasite was considered IC₅₀
219 for that drug. Drug-treated cells were pre-incubated with N-acetyl L-cysteine (NAC, 1 mM) when
220 required [3].

221 ***Ex vivo* assay against amastigotes**

222 RAW 264.1 cells were maintained in RPMI-1640 medium and infected with promastigotes as
223 described [3]. Parasite-infected macrophages, grown for another 24 h, were treated with varying
224 concentrations of DHF (5-160 µM), DHF-GNP (5-160 µM) and c-GNP (5-80 µM) and chamber slides
225 were washed and supplemented with fresh medium and kept in a CO₂ incubator for another 12 h.
226 Untreated parasite-infected macrophages were used as control. Amastigotes from 100 macrophage
227 nuclei per well were counted, at least, under the oil immersion objective of a light microscope (Nikon
228 Eclipse TS100, Japan) after methanol fixation and Giemsa staining of the slides. IC₅₀ for the drug was
229 determined by standard protocol after 48 hours.

230 **Cytotoxicity assays**

231 Cytotoxicity was measured by MTT assay, against human THP-1 cell lines. Briefly, THP-1 cells
232 were cultured with RPMI-1640 medium in 6 well plates (2×10⁶/well) and treated with c-GNP, DHF-
233 GNP and DHF at 5-1000 µM concentration for 48 h and cell viability was measured as described [3].

234 **Determination of ROS in promastigotes**

235 The generation of reactive oxygen species upon drug treatment was measured by H₂DCFDA mediated
236 fluorimetric assay [32]. The Promastigotes (1×10⁶ cells in each assay) were treated with 5 µM c-GNP,
237 40-280 µM DHF and 40-280 µM of DHF-GNP for 12 h and accumulated ROS was measured by
238 H₂DCFDA with respect to untreated cells [33].

239 **Determination of RNS in amastigotes**

240 Reactive nitrogen species in amastigotes were measured with Griess after treatment with different
241 doses of DHF (40-160 μ M), DHF-GNP (20-80 μ M) and c-GNP (2.5-10 μ M) as described earlier.
242 Macrophages were pre-treated with DPI (0.1 mM) when required [33].

243 **DPPH assay**

244 DPPH assay was used to evaluate antioxidant activity using a standard protocol [34]. Due to a strong
245 absorption band at 520 nm, the DPPH radical has a deep violet color in solution, and it becomes
246 colourless or pale yellow when neutralized. This change in color can be detected and measured by
247 spectrophotometry. The use of the DPPH assay provides a way to evaluate antioxidants. 120 μ l of
248 0.1mM DPPH solution was added to 1.5 ml of methanol (98%). 5-80 μ M each of DHF and DHF-GNP
249 was incubated with 100 μ l of prepared stock of DPPH solution. The mixtures were shaken and
250 allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm
251 using a UV-VIS spectrophotometer.

252 **Arginase assay**

253 To evaluate the effect of DHF and NPs on LD arginase, arginase assay was performed as described
254 previously [35]. Promastigotes were treated with IC₅₀ doses of c-GNP, DHF and DHF-GNP and cell
255 lysates were prepared by resuspending harvested cells in lysis buffer with 0.1% of Triton X-100 [36].
256 25 μ l of cell lysates were solubilized with 25 μ l of lysis buffer containing: 0.1% Triton x-100, 10 mM
257 MnCl₂ and 50 mM Tris-HCl (pH 7.5). Arginase was activated by heating for 7 min at 56°C. L-
258 arginine hydrolysis was done by incubating the activated lysates with 50 μ l of L-arginine (pH 9.7) at
259 37°C for 60 min. The reaction was stopped by the addition of 400 μ l acid solution (H₂SO₄ (96%)/
260 H₃PO₄ (85%)/H₂O (1:3:7, v/v/v). Then 20 μ l of 9% ISPF (α -isonitrosopropiophenone) (dissolved in
261 100% ethanol) was added, heated at 100°C for 45 min and spectrophotometric reading was taken at
262 540 nm [37]. Assay without L-arginine was used as a negative control.

263 **Supplementation assay**

264 To evaluate the involvement of enzymes of polyamine biosynthesis pathway in parasite death and
265 survival, culture medium was preincubated with 1 mM arginine, ornithine, putrescine and spermidine
266 before treatment with 140 μ M of DHF and 40 μ M of DHF-GNP [38]. Cells were treated with various
267 drug concentrations and cell viability was estimated after every 24 hours for 3 days by both trypan
268 blue exclusion assay and MTT assay. The percentage of survival was calculated using drug-treated
269 cells as control.

270 **Superoxide dismutase (SOD) assay**

271 Promastigotes (5×10^6 cells/ml) were treated with DHF (120-240 μ M), DHF-GNP (40-80 μ M) and c-
272 GNP (20 μ M) for 12 hours and then harvested. Cells were lysed and the SOD enzyme activity assay
273 was performed as described [3]. AmB (0.2 μ M) –treated parasites are used as a positive control.

274 **Ascorbate peroxidase (APx) assay**

275 Log phase promastigotes were treated with 120-240 μ M DHF, 40-80 μ M of DHF-GNP and 20 μ M of
276 c-GNP and allowed to incubate for 12 hours. After incubation the cells were harvested and washed
277 with 1X PBS. A cell lysate was prepared by resuspending harvested cells in lysis buffer with 0.1% of
278 Triton X-100. An equal volume of cell lysate from each treated sample was used for APx assay. The
279 assay was started by adding 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1
280 mM EDTA, 0.1 mM H_2O_2 , cell lysate and adjusting the volume upto 2 ml with double distilled water.
281 The reaction was allowed to run for 5 min at 25 °C. APx (Ascorbate peroxidase) activity was assayed
282 by recording the decrease in optical density at 290 nm. The activity of the enzyme was determined in
283 terms of μ mol per min [39]. 1 μ M miltefosine-treated cells were used as a positive control.

284 **Isolation of total RNA**

285 To study the role of genes in DHF and DHF-GNP treated parasites, total RNA was isolated after drug
286 treatment followed by semi-quantitative PCR. For isolation of RNA parasites (5×10^6 cells/well) were

treated with 10 μ M c-GNP, 140 μ M DHF, and 40 μ M DHF-GNP for 6 hours. Treated cells were centrifuged at 4200g for 10 minutes, washed thrice with PBS, and then total RNA was isolated by the addition of TRIZOL solution. Pellet of RNA was air-dried, re-suspended in 100 μ L of RNase-free water, and treated with DNase I (1U/ μ l) at 37°C for 30 minutes. Digested RNA was loaded on RNeasy Mini Kit columns, and RNA was eluted in 30 μ l of RNase-free water. RNA quality was checked by gel electrophoresis and quantified by Nanodrop spectrophotometer (Thermo scientific, Nanodrop 2000, USA). For isolation of RNA from macrophage-infected parasites, RAW 264.1 cells (1 $\times$ 10⁶/well) were grown in a 6 well plate and infected with 1 $\times$ 10⁷ parasites for 12 hours. Non-phagocytic cells were washed out; fresh medium was added and then incubated further for 12 hours. Parasite-infected macrophages were treated with c-GNP (20 μ M), DHF (40 μ M), and DHF-GNP (22 μ M) for 6 hours, and then RNA was isolated from the cell pellet as described above. The isolated RNA was converted into cDNA with the help of BioRad cDNA synthesis kit following the manufacturer's protocol.

Semi-quantitative PCR for gene expression studies

Reverse transcription was performed using 1 μ g of total RNA by cDNA synthesis kit (Roche, USA) according to the manufacturer's instruction. The synthesized cDNAs (from RNA of promastigotes) were amplified by PCR (primer detail is mentioned in Supplementary Table 1) for specific genes viz. arginase, ornithine decarboxylase (ODC), trypanothione reductase (TryR), spermidine synthase (SPD), S-adenosylmethionine decarboxylase (AdoMetDC), trypanothione synthase (TryS), multi drug transporter 1 (MDR1), amino acid permease 3 (AAP3), glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and α -tubulin. The synthesized cDNAs (from RNA of amastigote infected macrophages) were amplified by PCR for genes viz. IFN- γ , inducible nitric oxide synthase (iNOS), IL-10. GAPDH was used as a loading control for both promastigotes and macrophage models. For promastigote model α -tubulin is also used as additional loading control. The PCR mixture (25 μ l) contains 0.6-0.8 μ M of forward and reverse primer, 0.5 mM of each dNTP, 2 mM MgCl₂, 0.5 μ g of synthesized cDNA

and 1 µl Taq polymerase. The sequence of PCR primers, annealing temperature, and the size of PCR products were shown in supplementary Table1. The PCR was done for 25 cycles, where each cycle had denaturation at 95°C for 45 seconds, annealing (ranging from 55-62°C) for 30 seconds, and extension at 72°C for 45 seconds. Samples were preheated at 95°C for 3 minutes before PCR. The products were run on 1.5% agarose gel, stained with ethidium bromide (0.5 µg/ml), and finally documented and quantified using the gel documentation system and associated Gene-tool software (Syngene, USA).

Statistical analysis

The statistical analysis was done by one-way and two-way ANOVA using Graphpad Prism software (version 5.00; Graphpad Software Inc., La Jolla, CA, USA). The results were measured as mean±SD of at least three independent experiments. The results were shown as approximate mean values. Differences between group data were considered statistically significant and highly significant when $P<0.05$ and $P<0.001$, respectively.

333

334 **Results and Discussion**

335 Leishmaniasis affects around 12 million people in 98 countries of mostly the developing world. It is
336 transmitted by sand flies (*Phlebotomus* spp.), small biting mosquito, which breeds in moist soil, forest
337 areas or caves and feed from infected animal reservoir hosts or humans [40]. It comprises of a
338 complex group of diseases ranging from self-healing cutaneous lesions (CL) to severe and systemic
339 visceral leishmaniasis (VL). VL (also known as kala-azar) is a parasitic disease affecting an estimated
340 500,000 new cases per year mostly in the Indian subcontinent and East Africa [41]. Conventional
341 chemotherapy which primarily uses Amphotericin B (AmB) as deoxycholate formulation (fungizone)
342 was effective but came with severe nephrotoxicity [42]. Application of liposomal AmB reduces the
343 problem of toxicity [43] but comes with a high cost in treatment [37,38]. Different natural compounds
344 from plants such as Flavonoids, Polyphenols, Chalcones, Lignins, etc. were reported as antiparasitic
345 agents with the possible mechanism of action [44,45]. Although flavonoids/ polyphenols are less
346 cytotoxic than standard chemotherapeutic agents their use was severely restricted due to poor water
347 solubility and less bioavailability [46]. Very high first-pass metabolism involving phase II conjugation
348 (i.e., glucuronidation and/or sulfonation) of polyphenols is the leading cause of their poor
349 bioavailabilities [47]. Flavonoid functionalized NPs were reported to increase the bioavailability of
350 flavonoids with a consequent increase in antileishmanial efficacy than standard chemotherapeutic
351 agents against sensitive and drug-resistant strains [48]. GNPs synthesized from Flavonoids were
352 found to be non-cytotoxic with improved macrophage delivery and antileishmanial efficacy [49]. In
353 this work, we have used DHF as a reducing and capping agent for the green synthesis of GNP. DHF
354 was earlier reported in an animal model of VL but was found to be ineffective [27]. Till then, no
355 further study was reported for DHF against parasitic diseases although DHF was extensively used in
356 various neurological diseases with safety and efficacy [29,50]. Due to poor solubility, DHF was
357 solubilised in water of pH 9.0 and then used for the synthesis of GNP. The characteristic peak at ~530

358 nm in UV-Visible spectroscopy, along with a change from colourless to a red wine colour confirms
 359 the reduction and synthesis of GNP. Further, a separate peak at 350 nm, which comes from DHF,
 360 confirms the association of DHF in DHF-GNP (Figure 1A). Measurement of concentration in UV-
 361 visible spectroscopy for unbound and washed DHF showed conjugation efficiency of ~8-10% DHF in
 362 DHF-GNP. Therefore, 40 μ M gold is associated with 1.33 μ M DHF in DHF-GNP. From here on, the
 363 given concentration of DHF-GNP in each assay will indicate the concentration of GNP in it. The
 364 synthesized GNP is stable at room temperature for 6 months with no change in biological activity. In
 365 FT-IR analysis, a broadened O-H stretch at $\sim 3000\text{ cm}^{-1}$ was observed due to H-bonding, which is
 366 reduced in DHF-GNP and shifted towards $\sim 3400\text{ cm}^{-1}$. This is, possibly, due to the association of -OH
 367 group of catechol ring of DHF in DHF-GNP (Figure 1B). Further, numerous bending vibrations of
 368 DHF, which were seen in the fingerprint region of $600\text{--}1400\text{ cm}^{-1}$, are reduced in DHF-GNP due to
 369 non-covalent attachment in GNP (Figure 1B). DLS studies revealed the diameter of DHF-GNP as
 370 $35\pm 7.4\text{ nm}$ with PDI 0.19 ± 0.01 , which indicates well-nigh uniformity in the particle size. The notable
 371 high value of the zeta potential of DHF-GNP (-34.1 mV) indicates the excellent stability of our
 372 synthesized nanoparticles (Supplementary Figure S1). Further, TEM analysis confirms the uniform
 373 and spherical size of NPs with a diameter of $18.5\pm 2.4\text{ nm}$ (Figure 2A and 2B). The difference in
 374 diameter of the DHF-GNP from DLS and TEM analysis was ascribed to differences in DLS and TEM
 375 measurement techniques. Powdered X-Ray Diffraction (pXRD) measurement revealed Bragg's
 376 diffraction at 38.2° , 47.7° , 65.3° , and 77.1° which were attributed to (111), (200), (220), and (311)
 377 sets of lattice planes reflecting the face-centered cubic structure of metallic gold. The pXRD pattern
 378 thus confirms the crystalline nature of GNP in DHF-GNP (Figure 2C).
 379 In order to compare the biological efficacy and cytotoxicity, we prepared citrated –reduced GNP (c-
 380 GNP) to study in parallel with DHF-GNP [3]. DHF showed IC_{50} of $\sim 140\text{ }\mu\text{M}$ ($\sim 36\text{ }\mu\text{g/ml}$) against LD
 381 promastigotes (Figure 3A). This value is higher than what is reported earlier for *in vitro* studies
 382 against axenic amastigotes of LD ($\sim 1.7\text{ }\mu\text{g/ml}$, [27]). For DHF-GNP and c-GNP the IC_{50} is $\sim 40\text{ }\mu\text{M}$

383 and $\sim 10 \mu\text{M}$ respectively. Therefore, DHF-GNP is less effective than c-GNP against LD. However,
384 DHF-GNP is more efficient than DHF alone, considering $\sim 1.33 \mu\text{M}$ DHF is associated with DHF-
385 GNP ($P < 0.001$, Figure 3A). Efficacy of DHF-GNP was increased in *ex vivo* amastigote model where
386 IC_{50} is reduced to $\sim 22 \mu\text{M}$ compared to $\sim 40 \mu\text{M}$ in promastigotes model ($P < 0.001$, Figure 3B). For
387 DHF we, also see a decrease in IC_{50} against amastigotes than promastigotes ($\sim 40 \mu\text{M}$ vs. $\sim 140 \mu\text{M}$).
388 A dose-dependent steep decrease in parasite survival was observed for DHF-GNP than in DHF-alone
389 (Figure 3B). The 72 h data on dose response curve shows that IC_{50} of DHF and DHF-GNP is $\sim 60 \mu\text{M}$
390 and $\sim 18 \mu\text{M}$, respectively against promastigotes (Supplementary Figure S2A). Against amastigotes,
391 after 72 h the IC_{50} of DHF and DHF-GNP is $\sim 25 \mu\text{M}$ and $\sim 13 \mu\text{M}$ (Supplementary Figure S2B). This
392 data correlates well with early published reports on fast macrophage uptake of flavonoid-
393 functionalized GNPs in VL model [4]. It suggests that there is a possible advantage of GNP based
394 drug delivery in VL since the parasite primarily infects the macrophages of the liver and spleen.
395 Again, c-GNP was very effective against amastigotes with an IC_{50} of $\sim 10 \mu\text{M}$, after 48 h (Figure 3B)
396 as we observed in our previous studies [3].

397 Flavonoid and its derivatives are well established for their efficacy against drug-resistant LD due to
398 inhibition of multidrug transporter 1 (MDR1), thereby accumulating high intracellular drug
399 concentration [48,51]. A similar trend was observed for DHF and DHF-GNP against AmB- resistant
400 and SAG-resistant LD as compared to sensitive strains. The IC_{50} for DHF and DHF-GNP is $\sim 150 \mu\text{M}$
401 and $\sim 40 \mu\text{M}$, respectively, against the AmB-resistant parasite (Figure 3C). Against SAG-resistant
402 parasite, the IC_{50} for DHF and DHF-GNP is $\sim 160 \mu\text{M}$ and $\sim 35 \mu\text{M}$ respectively (Figure 3D) and
403 almost similar to sensitive strains. IC_{50} for AmB against AmB-resistant strain was increased ~ 5.8 fold,
404 whereas, for SAG, the IC_{50} was increased ~ 4.2 fold, which is consistent with the previously reported
405 data confirming the drug-resistance associated with these clinically isolated strains [33,52].

406 Cytotoxicity studies on THP-1 cells show that CC_{50} (cytotoxic concentration for 50% viability of
407 THP-1) values of c-GNP are $\sim 25 \mu\text{M}$. However, with DHF-GNP even with $1000 \mu\text{M}$, the cell

408 viability is ~70% (Figure 4C). Also, at 160 μ M concentrations, the survival of c-GNP and DHF-GNP
 409 treated THP-1 cells was ~24% and ~80%, respectively ($P < 0.001$, Figure 4C). Therefore, synthesis of
 410 GNP with DHF, instead of, sodium citrate increases the biocompatibility of GNP significantly without
 411 compromising the biological efficacy.

412 Studies with DHF and other flavonoids of the similar structure have shown that arginase of
 413 *Leishmania amazonensis* species is inhibited by these flavonoids, whereas the mammalian counterpart
 414 is un-inhibited [53]. Arginase, the first enzyme in the polyamine biosynthesis pathway, converts L-
 415 arginine to ornithine. Conversion of ornithine to putrescine by ornithine decarboxylase (ODC) is the
 416 rate-limiting step in polyamine biosynthesis [38]. DHF and DHF-GNP treated parasites show ~2.1
 417 and ~4.4 fold decrease in arginase expression, respectively, as measured by semi-quantitative PCR
 418 (Figure 4A). Parasites treated under the same conditions do not show any significant change in the
 419 gene expression of ODC, SpdS and AdoMetDC, which are downstream enzymes in the polyamine
 420 biosynthesis pathway. Consequently, arginase assay with DHF and DHF-GNP-treated cell lysates
 421 show a gradual decrease in arginase activity in a time-course study. There were ~20%, ~45%, and
 422 65% inhibition of arginase activity after 24, 48, and 72 h of DHF treatment (Figure 5A). For DHF-
 423 GNP the inhibition was ~31%, ~63%, and ~83% ($P < 0.001$, Figure 5A) under similar conditions.
 424 Therefore, DHF-GNP is a more potent inhibitor of arginase than DHF alone. In short, gene expression
 425 and enzyme studies prove that inhibition of arginase is the possible cause of parasite death *in vitro*.
 426 This observation was further confirmed by supplementation with ornithine, the product of arginase
 427 enzyme, in DHF, and DHF-GNP treated parasites. Preincubation of DHF and NP-treated cells with 1
 428 mM ornithine increases cell survival from ~49% to ~82% for DHF and ~45% to ~92% for DHF-GNP
 429 after 48 h ($P < 0.001$, Figure 5B). Following a similar trend, 1 mM ornithine supplementation increases
 430 cell survival from ~31% to ~72% for DHF and ~23% to ~85% for DHF-GNP after 72 h ($P < 0.001$,
 431 Figure 5C). Therefore, parasite survival can be simply increased by supplementation of ornithine, the
 432 product of arginase enzyme, in the growth medium. This indicates inhibition of arginase as the

primary reason for DHF and DHF-GNP induced cell death *in vitro*. This observation was further supported by an increase in cell survival with increasing doses of ornithine. Preincubation of DHF-treated cells with 0.05, 0.25, and 1 mM of ornithine increases parasite survival from ~31% to ~47%, ~63%, and ~64% respectively after 72 h (Figure 5D). For DHF-GNP treated cells under similar conditions, the parasite survival increases from ~24% to ~55%, ~82%, and ~86% ($P < 0.001$, Figure 5D). These data indicate the importance of ornithine, the product of arginase, and consequently, the role of arginase inhibition in DHF and DHF-GNP induced cell death. Also, these results show DHF-GNP is more potent inhibitor of arginase than DHF alone.

Supplementation with putrescine, the substrate for ODC, provides partial recovery in cell survival for DHF after 48 h (~49% to ~59%) and 72 h (~31% to ~50%). For DHF-GNP treated cells, the recovery with putrescine was ~45% to ~60% after 48 h and ~22% to ~52% after 72 h (Figure 5A and 5B). This indicates a possible secondary response involving inhibition of ODC in parasite death since arginase and ODC are the first two enzymes in the polyamine biosynthesis pathway. Supplementation with spermidine, the product of ODC, does not provide any significant change in parasite survival under similar conditions. Since the growth medium is already enriched with L-arginine (70 g/L), supplementation of arginine does not cause any significant change in survival after DHF and DHF-GNP treatment (Figure 5B and 5C) as expected.

The enzymes TryS and reductase TryR are involved in the biosynthesis of trypanathione (major thiol of *Leishmania* along with glutathione) and maintain the reduced status of LD under oxidative stress along with other two enzymes Superoxide dismutase (SOD) and Ascorbate peroxidase (APx). Gene expression study showed very little change in the mRNA level of SOD, APx, and TryR after DHF and DHF-GNP treatment compared to untreated cells (Figure 4A). In parallel, SOD and APx assay with parasite cell lysate do not show any significant change in activity either (Supplementary Figure S5A and 5B, respectively). However, substantial inhibition for SOD with AmB [3] and for APx with miltefosine [39] was observed under similar conditions. Flavonoids contain both antioxidant and

458 prooxidant properties, and the switch from one form to another depends on the positions of hydroxyl
459 groups and redox status of chelated metal [54]. The antioxidant property, as measured by DPPH
460 scavenging assay, was abolished entirely in DHF-GNP, whereas DHF showed a dose-dependent
461 increase (Supplementary Figure 4A). Conjugation of phenolic –OH with GNP may be the reason for
462 the loss of antioxidant activity. In parallel, we found slightly increased prooxidant activity of DHF-
463 GNP compared to DHF as measured by ROS (Supplementary Figure S4B). Overall, gene expression
464 and enzyme-based studies prove that oxidative stress is not involved in DHF and DHF-GNP mediated
465 parasite death *in vitro*. Decreased expression of MDR1 (~2.1 and ~3.4 fold reduction in DHF and
466 DHF-GNP treated cells compared to untreated cells, Fig 4A) further confirms the role of these
467 transporters, which is inhibited by many flavonoids [51]. We have not found any change in gene
468 expression of amino acid permease 3 (AAP3), known for L-arginine transport in LD [55], in DHF and
469 DHF-GNP treated parasites (Fig 4A).

470 Oxidation of L-arginine by host inducible nitric oxide synthase (iNOS) produces nitric oxide (NO),
471 which contributes to parasite killing. In contrast, hydrolysis of L-arginine by host arginase blocks NO
472 generation and provides polyamines supporting parasite proliferation [56,57]. NO is a potent
473 cytotoxin involved in clearance or inhibition of intracellular pathogens like *Leishmania*. We found a
474 dose-dependent increase of reactive nitrogen species (RNS) after treatment with DHF and DHF-GNP
475 on macrophages infected with LD amastigotes. There was ~2.7 and ~3.2 fold increase in NO
476 production after DHF and DHF-GNP treatment (Supplementary Figure S3). Pre-treatment of cells
477 with 1 mM DPI, an inhibitor of iNOS, abolishes NO production indicating the role of increased iNOS
478 activity for death of amastigotes. This is further confirmed by an increased mRNA level of iNOS for
479 DHF and DHF-GNP treated cells (~2.1 and ~3.5 fold, respectively, Figure 4B) compared to the
480 untreated control. *Leishmania*-infected macrophages generally activate T_h1 cytokines and down-
481 regulate T_h2 cytokine to produce NO via iNOS, thereby facilitating parasite killing [58]. We found
482 ~2.8 and ~3.7 fold increase in mRNA level of IFN- γ , a T_h1 cytokine, for DHF and DHF-GNP treated

cells compared to untreated control (Figure 4B). In contrast, there was ~1.2 and ~2.3 fold decrease in mRNA level of IL-10, a T_h2 cytokine, under similar conditions. Therefore, gene expression data correlates well with an increased T_h1 vs. decreased T_h2 response leading to increased host iNOS activity and consequent parasite killing. In short, DHF-GNP kills promastigotes by inhibiting arginase and MDR1 whereas it activates host iNOS to kill the amastigotes (Figure 6).

Conclusion

The low bioavailability of flavonoids has restricted its use in animal studies and future drug development. Flavonoids were chemically modified [59] or formulated with NP-based delivery methods to improve the bioavailability [60,61]. Moreover, GNP-based delivery was used for flavonoids with improved bioavailability [15,62]. These studies encouraged us to develop a GNP-based delivery system for a flavonoid, DHF, which is very commonly used for neurological diseases but rarely been tested for antimicrobial studies. The functionalization of DHF with GNP increases its water solubility and stability. Only ~8-10% of DHF was found to be associated with GNP. However, the antileishmanial efficacy of DHF-GNP is much higher than the DHF. This is possible because GNP, as such, like c-GNP, is very effective against promastigotes and amastigotes forms of LD. The biggest advantage of synthesized DHF-GNP is its high biocompatibility, which is almost comparable with free DHF. Drug-resistance against standard chemotherapeutic agents is very common in leishmaniasis. Therefore, the use of DHF-GNP, which shows equal efficiency against both sensitive and drug-resistant parasites with high biocompatibility, may provide a new opportunity in the treatment of VL and CL where NP-based delivery has shown promises [63,64]. In this study, we found inhibition of arginase as the major cause of parasite death *in vitro* after DHF and DHF-GNP treatment. This was confirmed by gene expression and enzyme-based assays for arginase followed by supplementation assay in the presence of ornithine, where an increase in cell survival was observed in a dose-dependent manner. There are reports of natural and synthetic molecules with antileishmanial effect targeting the parasite arginase. This is possible since the structure of host and parasite arginase

differs considerably [65]. To date, based on crystal structure-based studies, two arginine analogs with the highest inhibition for parasite arginase was reported [66]. A synthetic analogue of arginine, N ω -hydroxy-L-arginine (NOHA), showed a 4-fold reduction in lesion size and as much as 4 million fold reduction in parasite burden in mice model [67]. This shows future of arginase inhibitors as potential drug targets against leishmaniasis. Our result on DHF-GNP provides enough evidence to encourage its future investigation as a potential antileishmanial agent targeting the parasite arginase.

Author's contributions

PP, PK, SM, TP and DM designed research; PP, PK, SM, TP, SK performed research; PP, PD, VR and DM analyzed data; PP, PD, DM wrote the paper. All authors read and approved the final manuscript.

Consent for publication

All authors agree to be published.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

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Ethics approval and consent to participate

Not applicable

Competing Interests

The authors report no conflicts of interest in this work.

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Figure Legends for Main manuscript and supplementary Figures

Figure 1. (A) The UV-Visible absorbance spectra of DHF, DHF-GNP, and c-GNP. (B) Fourier-transform infrared spectra of DHF-GNPs compared with pure DHF.

Figure 2. Transmission electron microscopy (TEM) micrographs of DHF-GNP at (A) 100nm and (B) 20nm scale. (C) X-ray diffraction pattern of DHF, DHF-GNP and c- GNPs.

Figure 3. Dose-response curve of DHF, DHF-GNP, and c-GNP after 48 h treatment against (A) promastigotes *in vitro* and (B) intracellular amastigotes *ex vivo*. Dose-response curve against (C) amphotericin B-resistant promastigotes and (D) SAG-resistant promastigotes.

Figure 4. Semi-quantitative PCR for genes of polyamine biosynthesis pathway of promastigotes (A) and for genes of IFN- γ , iNOS, IL-10 of macrophages (B). (C) Cytotoxicity assay on THP-1 cells after 48 h treatment with different concentrations of DHF, DHF-GNP, and c-GNP.

Figure 5. (A) Time course of arginase assay with DHF and DHF-GNP treated promastigote cell lysates. Supplementation assay with pre-incubation of 1 mM arginine (Arg), ornithine (Orn), putrescine (Put), spermidine (Spd) for DHF, and DHF-GNP and c-GNP treated cells (B) after 48 h and (C) 72 h. (D) Supplementation assay with different doses of ornithine (0.05, 0.25, and 1 mM) for DHF and DHF-GNP and c-GNP treated cells after 48 h.

Figure 6. Graphical abstract showing the role DHF and DHF-GNP against promastigotes (targeting arginase and MDR1) and against amastigotes with elevated T_H1 cytokine mediated iNOS activity followed by RNS-mediated killing.

Supplementary Figure 1. Characterization of DHF-GNPs by Zeta potential studies (A) and by DLS studies (B).

Supplementary Figure 2. Dose-response curve of DHF, DHF-GNP, and c-GNP after 72 h treatment against (A) promastigotes *in vitro* and (B) intracellular amastigotes *ex vivo*

Supplementary Figure 3. Estimation of reactive nitrogen species in macrophages by Griess reagent after DHF, DHF-GNP, and c-GNP treatment.

Supplementary Figure 4. (A) Antioxidant activities of DHF (5-80 μ M), DHF-GNPs (5-80 μ M) measured by percentage inhibition of DPPH scavenging activity. (B) Determination of reactive oxygen species in LD promastigotes treated with DHF (40-280 μ M), DHF-GNP (40-280 μ M) and c-GNP (5 μ M) for 12 hours.

Supplementary Figure 5. Enzyme assay with LD promastigotes lysates after treatment with DHF (120-240 μ M), DHF-GNP (40-80 μ M) and c-GNP (20 μ M). (A) Superoxide dismutase assay and (B) Ascorbate peroxidase assay.

Figures

Figure 1

(A) The UV-Visible absorbance spectra of DHF, DHF-GNP, and c-GNP. (B) Fourier-transform infrared spectra of DHF-GNPs compared with pure DHF.

Figure 2

Transmission electron microscopy (TEM) micrographs of DHF-GNP at (A) 100nm and (B) 20nm scale. (C) X-ray diffraction pattern of DHF, DHF-GNP and c- GNPs.

Figure 3

Dose-response curve of DHF, DHF-GNP, and c-GNP after 48 h treatment against (A) promastigotes in vitro and (B) intracellular amastigotes ex vivo. Dose-response curve against (C) amphotericin B-resistant promastigotes and (D) SAG-resistant promastigotes.

Figure 4

Semi-quantitative PCR for genes of polyamine biosynthesis pathway of promastigotes (A) and for genes of IFN- γ , iNOS, IL-10 of macrophages (B). (C) Cytotoxicity assay on THP-1 cells after 48 h treatment with different concentrations of DHF, DHF-GNP, and c-GNP.

Fig.5

Figure 5

(A) Time course of arginase assay with DHF and DHF-GNP treated promastigote cell lysates. Supplementation assay with pre-incubation of 1 mM arginine (Arg), ornithine (Orn), putrescine (Put), spermidine (Spd) for DHF, and DHF-GNP and c-GNP treated cells (B) after 48 h and (C) 72 h. (D) Supplementation assay with different doses of ornithine (0.05, 0.25, and 1 mM) for DHF and DHF-GNP and c-GNP treated cells after 48 h.

Fig.6

Figure 6

Graphical abstract showing the role DHF and DHF-GNP against promastigotes (targeting arginase and MDR1) and against amastigotes with elevated Th1 cytokine mediated iNOS activity followed by RNS-mediated killing.

Supplementary Files

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