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

Pragya Prasanna[1], Prakash Kumar[1,2], Saptarshi Mandal[3], Tanvi Payal[1,4], Saurabh Kumar[1], Prolay Das[3], V. Ravichandiran[1,5], Debabrata Mandal[1]*

[1]Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER) – Hajipur 844102, India
[2] Present addresses: CSIR-Institute of Microbial Technology, Chandigarh 160036, India
[3]Department of Chemistry, Indian Institute of Technology Patna 801106, India
[4]Present address: Cognizant Technology Solution, Hyderabad 800051, India
[5]National Institute of Pharmaceutical Education and Research (NIPER) – Kolkata 700054, India

*Corresponding Author
Dr. Debabrata Mandal
Associate Professor
Department of Biotechnology, NIPER-Hajipur
Export Promotions Industrial Park (EPIP)-Hajipur
District: Vaishali, Pin- 844102, BIHAR, INDIA.
Email: debabrataman@gmail.com
Ph # 91-8335996348
Fax: 06224 – 277225

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 IC_{50} for DHF and DHF-GNP is ~140 µM and ~40 µM respectively. In *ex vivo* amastigote model, IC_{50} 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 bioreductants 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

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

Leishmaniasis is the second-largest protozoal disease after malaria and one of the 'most neglected' diseases. It is still a major health concern in developing countries like India. An asymptomatic condition like post kala-azar dermal leishmaniasis (PKDL) poses a threat of recurrent disease outbreak. Among all types, the visceral leishmaniasis (VL) caused by *Leishmania donovani* (LD) is fatal and most challenging one to control. Although, there are significant developments of NPs as antileishmanial agents, but its application is severely restricted due to toxicity issues. In recent years, several natural and synthetic flavonoids are considered as lead compounds for developing antiprotozoal agents. Compounds like quercetin, 7, 8- dihydroxyflavone, Luteolin, Apigenin, Fisetin, and Kaempferol had been shown to inhibit LD at very low concentration *in vitro* [25,26]. Quercetin and some of its glycoside derivatives have shown to be active after oral administration on the *L. amazonensis* in vivo mice model [27,28]. 7, 8-dihydroxyflavone (DHF) is a naturally occurring flavonoid obtained from plants. It was discovered while searching for molecules that imitate the function of brain-derived neurotrophic factors [29]. DHF rescues cells from damage and death caused 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 $HAuCl_4$ (0.5 mM) and stirred for 20 min at room temperature followed by heating at 80-90^{o}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 (1×10^{6}/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.

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

Ex vivo assay against amastigotes

RAW 264.1 cells were maintained in RPMI-1640 medium and infected with promastigotes as described [3]. Parasite-infected macrophages, grown for another 24 h, were treated with varying concentrations of DHF (5-160 μM), DHF-GNP (5-160 μM) and c-GNP (5-80 μM) and chamber slides were washed and supplemented with fresh medium and kept in a CO_2 incubator for another 12 h. Untreated parasite-infected macrophages were used as control. Amastigotes from 100 macrophage nuclei per well were counted, at least, under the oil immersion objective of a light microscope (Nikon Eclipse TS100, Japan) after methanol fixation and Giemsa staining of the slides. IC_{50} for the drug was determined by standard protocol after 48 hours.

Cytotoxicity assays

Cytotoxicity was measured by MTT assay, against human THP-1 cell lines. Briefly, THP-1 cells were cultured with RPMI-1640 medium in 6 well plates (2×10^6/well) and treated with c-GNP, DHF-GNP and DHF at 5-1000 μM concentration for 48 h and cell viability was measured as described [3].

Determination of ROS in promastigotes

The generation of reactive oxygen species upon drug treatment was measured by H_2DCFDA mediated fluorimetric assay [32]. The Promastigotes (1×10^6 cells in each assay) were treated with 5 μM c-GNP, 40-280 μM DHF and 40-280 μM of DHF-GNP for 12 h and accumulated ROS was measured by H_2DCFDA with respect to untreated cells [33].

Determination of RNS in amastigotes

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

DPPH assay

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

Arginase assay

To evaluate the effect of DHF and NPs on LD arginase, arginase assay was performed as described previously [35]. Promastigotes were treated with IC_{50} doses of c-GNP, DHF and DHF-GNP and cell lysates were prepared by resuspending harvested cells in lysis buffer with 0.1% of Triton X-100 [36]. 25 µl of cell lysates were solubilized with 25 µl of lysis buffer containing: 0.1% Triton x-100, 10 mM $MnCl_2$ and 50 mM Tris-HCl (pH 7.5). Arginase was activated by heating for 7 min at 56^{o}C. L-arginine hydrolysis was done by incubating the activated lysates with 50 µl of L-arginine (pH 9.7) at 37^{o}C for 60 min. The reaction was stopped by the addition of 400 µl acid solution (H_2SO_4 (96%)/ H_3PO_4 (85%)/H_2O (1:3:7, v/v/v). Then 20 µl of 9% ISPF (α-isonitrosopropiophenone) (dissolved in 100% ethanol) was added, heated at 100^{o}C for 45 min and spectrophotometric reading was taken at 540 nm [37]. Assay without L-arginine was used as a negative control.

Supplementation assay

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

Superoxide dismutase (SOD) assay

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

Ascorbate peroxidase (APx) assay

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

Isolation of total RNA

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

287 treated with 10μM c-GNP, 140 μM DHF, and 40 μM DHF-GNP for 6 hours. Treated cells were
288 centrifuged at 4200g for 10 minutes, washed thrice with PBS, and then total RNA was isolated by the
289 addition of TRIZOL solution. Pellet of RNA was air-dried, re-suspended in 100 μL of RNase-free
290 water, and treated with DNase I (1U/μl) at 37°C for 30 minutes. Digested RNA was loaded on
291 RNeasy Mini Kit columns, and RNA was eluted in 30 μl of RNase-free water. RNA quality was
292 checked by gel electrophoresis and quantified by Nanodrop spectrophotometer (Thermo scientific,
293 Nanodrop 2000, USA). For isolation of RNA from macrophage-infected parasites, RAW 264.1 cells
294 (1×10^6/well) were grown in a 6 well plate and infected with 1×10^7 parasites for 12 hours. Non-
295 phagocytic cells were washed out; fresh medium was added and then incubated further for 12 hours.
296 Parasite-infected macrophages were treated with c-GNP (20 μM), DHF (40 μM), and DHF-GNP (22
297 μM) for 6 hours, and then RNA was isolated from the cell pellet as described above. The isolated
298 RNA was converted into cDNA with the help of BioRad cDNA synthesis kit following the
299 manufacturer's protocol.

300 **Semi-quantitative PCR for gene expression studies**

301 Reverse transcription was performed using 1 μg of total RNA by cDNA synthesis kit (Roche, USA)
302 according to the manufacturer's instruction. The synthesized cDNAs (from RNA of promastigotes)
303 were amplified by PCR (primer detail is mentioned in Supplementary Table 1) for specific genes viz.
304 arginase, ornithine decarboxylase (ODC), trypanothione reductase (TryR), spermidine synthase
305 (SPD), S-adenosylmethionine decarboxylase (AdoMetDC), trypanothione synthase (TryS), multi drug
306 transporter 1 (MDR1), amino acid permease 3 (AAP3), glyceraldehyde 3 phosphate dehydrogenase
307 (GAPDH) and α-tubulin. The synthesized cDNAs (from RNA of amastigote infected macrophages)
308 were amplified by PCR for genes viz. IFN-γ, inducible nitric oxide synthase (iNOS), IL-10.GAPDH
309 was used as a loading control for both promastigotes and macrophage models. For promastigote
310 model α-tubulin is also used as additional loading control. The PCR mixture (25 μl) contains 0.6-0.8
311 μM of forward and reverse primer, 0.5 mM of each dNTP, 2 mM $MgCl_2$, 0.5 μg 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.

Results and Discussion

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

nm in UV-Visible spectroscopy, along with a change from colourless to a red wine colour confirms the reduction and synthesis of GNP. Further, a separate peak at 350 nm, which comes from DHF, confirms the association of DHF in DHF-GNP (Figure 1A). Measurement of concentration in UV-visible spectroscopy for unbound and washed DHF showed conjugation efficiency of ~8-10% DHF in DHF-GNP. Therefore, 40 µM gold is associated with 1.33 µM DHF in DHF-GNP. From here on, the given concentration of DHF-GNP in each assay will indicate the concentration of GNP in it. The synthesized GNP is stable at room temperature for 6 months with no change in biological activity. In FT-IR analysis, a broadened O-H stretch at ~3000 cm^{-1} was observed due to H-bonding, which is reduced in DHF-GNP and shifted towards ~3400 cm^{-1}. This is, possibly, due to the association of -OH group of catechol ring of DHF in DHF-GNP (Figure 1B). Further, numerous bending vibrations of DHF, which were seen in the fingerprint region of 600-1400 cm^{-1}, are reduced in DHF-GNP due to non-covalent attachment in GNP (Figure 1B). DLS studies revealed the diameter of DHF-GNP as 35±7.4 nm with PDI 0.19±0.01, which indicates well-nigh uniformity in the particle size. The notable high value of the zeta potential of DHF-GNP (-34.1 mV) indicates the excellent stability of our synthesized nanoparticles (Supplementary Figure S1). Further, TEM analysis confirms the uniform and spherical size of NPs with a diameter of 18.5±2.4 nm (Figure 2A and 2B). The difference in diameter of the DHF-GNP from DLS and TEM analysis was ascribed to differences in DLS and TEM measurement techniques. Powdered X-Ray Diffraction (pXRD) measurement revealed Bragg's diffraction at 38.2°, 47.7°, 65.3°, and 77.1° which were attributed to (111), (200), (220), and (311) sets of lattice planes reflecting the face-centered cubic structure of metallic gold. The pXRD pattern thus confirms the crystalline nature of GNP in DHF-GNP (Figure 2C).

In order to compare the biological efficacy and cytotoxicity, we prepared citrated –reduced GNP (c-GNP) to study in parallel with DHF-GNP [3]. DHF showed IC_{50} of ~140 µM (~36 µg/ml) against LD promastigotes (Figure 3A). This value is higher than what is reported earlier for *in vitro* studies against axenic amastigotes of LD (~1.7 µg/ml, [27]. For DHF-GNP and c-GNP the IC_{50} is ~40 µM

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

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

Cytotoxicity studies on THP-1 cells show that CC_{50} (cytotoxic concentration for 50% viability of THP-1) values of c-GNP are ~25 µM. However, with DHF-GNP even with 1000 µM, the cell

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

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

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

Oxidation of L-arginine by host inducible nitric oxide synthase (iNOS) produces nitric oxide (NO), which contributes to parasite killing. In contrast, hydrolysis of L-arginine by host arginase blocks NO generation and provides polyamines supporting parasite proliferation [56,57]. NO is a potent cytotoxin involved in clearance or inhibition of intracellular pathogens like *Leishmania.* We found a dose-dependent increase of reactive nitrogen species (RNS) after treatment with DHF and DHF-GNP on macrophages infected with LD amastigotes. There was ~2.7 and ~3.2 fold increase in NO production after DHF and DHF-GNP treatment (Supplementary Figure S3). Pre-treatment of cells with 1 mM DPI, an inhibitor of iNOS, abolishes NO production indicating the role of increased iNOS activity for death of amastigotes. This is further confirmed by an increased mRNA level of iNOS for DHF and DHF-GNP treated cells (~2.1 and ~3.5 fold, respectively, Figure 4B) compared to the untreated control. Leishmania-infected macrophages generally activate T_h1 cytokines and down-regulate T_h2 cytokine to produce NO via iNOS, thereby facilitating parasite killing [58]. We found ~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 oa 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, Nω-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 μM), DHF-GNPs (5-80 μM) measured by percentage inhibition of DPPH scavenging activity. (B) Determination of reactive oxygen species in LD promastigotes treated with DHF (40-280 μM), DHF-GNP (40-280 μM) and c-GNP (5 μM) for 12 hours.

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