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(71) Applicant: **COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH** [IN/IN]; Anusandhan Bhawan, Rafi Marg, New Delhi 110001 (IN).

(72) Inventors: **GOYAL, Neena**; Central Drug Research Institute, Lucknow (IN). **GANGWAR, Sonali**; Central Drug Research Institute, Lucknow (IN). **KALA SADAN, Anil Kumar**; Central Drug Research Institute, Lucknow (IN). **BISWAS, Subhasish**; Central Drug Research Institute, Lucknow (IN). **DWIVEDI, Anil Kumar**; Central Drug Research Institute, Lucknow (IN). **AHMAD, Hafsa**; Central Drug Research Institute, Lucknow (IN). **GUPTA, Kailash Chand**; Indian Institute of Toxicology Research, Lucknow (IN). **KUMAR, Pradeep**; Institute of Genomics and Integrative Biology, Delhi (IN). **BHATNAGAR, Priyanka**; Institute of Genomics and Integrative Biology, Delhi (IN). **BATRA, Sanjay**; Central Drug Research Institute, Lucknow (IN).

(74) Agents: **PHILLIPS, Prashant** et al.; LAKSHMIKUMARAN & SRIDHARAN, B6/10, Safdarjung Enclave, New Delhi 110029 (IN).

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(54) Title: A NOVEL ANTILEISHMANIAL FORMULATION

(57) Abstract: A novel antileishmanial formulation containing compounds belonging to the series of (2Z,2'Z)-3,3'-(alkane-1,2-diyl-bis(azanediyl))bis(6-hydroxy-1-aryl/fluorenyl hex-2-en-1-ones) or their salts thereof showing antileishmanial activity under in vivo conditions against *L. donovani* in hamster model. These compounds or their formulation elicit antileishmanial activity both via oral and ip routes.



WO 2016/113763 A1

A NOVEL ANTILEISHMANIAL FORMULATION

FIELD OF THE INVENTION

[001] The present invention relates to a novel antileishmanial formulation. Particularly, the present invention relates to the development of a novel formulation containing compounds belonging to the series of (2Z,2'Z)-3,3'-(alkane-1,2-diylbis(azanediyl))bis(6-hydroxy-1-arylhex-2-en-1-ones) as bioactive agents against Leishmania parasites in visceral organs.

BACKGROUND OF THE INVENTION

[002] Leishmaniasis are vector born parasitic diseases caused by protozoa of the genus Leishmania. Twenty one Leishmania spp. are known to be pathogenic to human and may cause broad spectrum of clinical syndromes ranging from benign self healing cutaneous leishmaniasis (oriental sore) to fulminant mucocutaneousleishmaniasis (espundia) and fatal visceral leishmaniasis (Kala-azar). About 350 million people are at risk, as Leishmaniasis is considered to be endemic in 88 tropical and subtropical countries including southern Europe (Alvar J., Yactayo S., Bern C., 2006, Leishmaniasis and poverty, Trends in Parasitol; 22:552–557). Therefore studies on control and management of the disease have received priority at national and international levels.

[003] Vaccines against leishmaniasis are still under development (Guerin P. J., Olliaro P., Sundar S., Boelaert M., Croft S. L., Desjeux P., Wasunna M. K., Bryceson A. D., 2002, Visceral Leishmaniasis: current status of control, iagnosis, and treatment, and a proposed research and development agenda. Lancet Infect Dis; 2: 494–501.), and therefore control of leishmaniasis relies mainly on chemotherapy. The drugs recommended for the treatment of leishmaniasis are far from ideal because of high costs, high toxicity, and long-term treatment requirements (Barrett, M. P., and Croft, S. L., 2012, Management of trypanosomiasis and leishmaniasis. British Medical Bulletin; 104: 175–196). Increasing incidences of therapeutic failures and emergence of drug resistant parasites (Croft, S. L., Sundar, S. and Fairlamb, A. H., 2006, Drug Resistance in Leishmaniasis. Clin. Microbiol. Rev.; 19:111.), have made imperative the need for the development of new effective, safe and nontoxic antileishmanial drug.

[004] Further, it has been observed that the ability of the parasite to survive and replicate inside host phagocytes poses a major problem in the development of effective anti-leishmania

therapeutic agents. The intracellular localization of the parasite is able to protect it from exposure to drugs that do not readily diffuse into cells. Therefore, use of drug carriers capable of delivering anti-leishmania compounds to infected cells should improve the therapeutic efficiency of those drugs and at the same time reduce their toxicity by modifying the pharmacokinetic and bio-distribution profiles of the drug (Couvreur, P., Vauthier, C., 2006, Nanotechnology: intelligent design to treat complex disease. *Pharm Res*; 23: 1417–50). In fact, polymeric nanoparticles have already been tested as nanocarriers for the delivery of standard toxic antileishmanial drugs (Nahar M. and Jain, N. K., 2009, Preparation, Characterization and Evaluation of Targeting Potential of Amphotericin B-Loaded Engineered PLGA Nanoparticles. *Pharm. Research*; 26: 2588-2598). Sometimes, these systems have led to an increase in drug efficacy and a decrease in toxicity, resulting in an improved therapeutic ratio (Lima, S. C., Rodriguesa, V., Garridob, J., Borgesb, F., Lind, P.K. T., da Silva, A.C., 2012, *In vitro* evaluation of bisnaphthalimidopropyl derivatives loaded into pegylated nanoparticles against *Leishmania infantum* protozoa *Int. J. Antimicrob. Agents*; 39: 424–430). The delivery of a drug to a patient with controlled-release of the active ingredient has been an active area of research for decades and has been fuelled by the many recent developments in polymer science. Biodegradable particles have been developed as sustained release vehicles used in the administration of small molecule drugs, proteins and peptide drugs, and nucleic acids.

[005] Rational approach to develop new chemo-therapeutic agent is based on knowledge of molecular mechanisms, employed by the parasite for its survival at various levels of life cycle.

[006] Further, molecular structure and chemical interactions define how cells or organisms live and die. Exploiting this approach towards the development of better therapeutics against visceral Leishmaniasis (VL), we focused on *Leishmania donovani* dipeptidylcarboxypeptidase (LdDCP), an angiotensin converting enzyme (ACE)-related metallopeptidase (Goyal N., Duncan R., Selvapandian A., Debrabant A., Baig M. S., Nakhasi H.L. 2006, Cloning and characterization of angiotensin converting enzyme related dipeptidylcarboxypeptidase from *Leishmaniadonovani*. *Mol. Biochem.Parasitol.*,145:147–157), a novel drug target. The LdDCP belongs to M3 family of mono-zinc peptidases which cleaves N-benzoyl-L-glycyl-L-histidyl-L-leucine (Hip-His-Leu, HHL), a substrate for ACE to release hippuric acid. Further, captopril, a known mammalian ACE inhibitor, was able to inhibit both LdDCP enzyme activity and *in vitro* parasite replication. These observations underscored the therapeutic

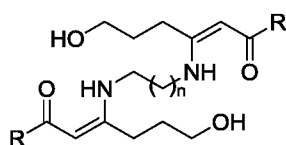
potential of parasite enzyme inhibition. Recently, three-dimensional model of LdDCP was generated based on crystal structure of *E. coli* DCP (EcDCP) by means of comparative modelling (Baig M.S., Kumar A., Siddiqi M.I., Goyal N., 2010, Characterization of dipeptidylcarboxypeptidase of *Leishmaniadonovani*: a molecular model for structure based design of antileishmanials. *J Comput Aided Mol Des*; 24:77–87) and a virtual screening approach was applied to identify potential inhibitors for LdDCP. Out of 15452 compounds, four belonging to two chemical classes were identified as potential DCP inhibitors that also exhibited *in vitro* antileishmanial activity against promastigotes (Gangwar, S., Baig, M. S., Shah, P., Biswas, S., Batra, S., Siddiqi, M. I. and Goyal, N., 2012, Identification of novel inhibitors of dipeptidylcarboxypeptidase of *Leishmaniadonovani* via ligand-based virtual screening and biological evaluation. *Chem. Biol. Drug Des.*; 79: 149–156). These compounds belong to (2Z,2'Z)-3,3'-(ethane-1,2-ylbis(azanediyl))bis(1-(4-halophenyl)-6-hydroxyhex-2-en-1-one) and 3,5-disubstituted isoxazoles. It may be worthwhile to mention that previously compounds of these chemical classes were reported to display antioxidant, hypolipidemic and antithrombotic activities (Batra, S.; Bhaduri, A. P.; Joshi, B. S.; Roy, R.; Khanna, A. K.; Chander, R. 2001, Syntheses and biological evaluation of alkanediamines as antioxidant and hypolipidemic agents *Bioorg. Med. Chem.*; 9: 3093-3099). The results of these initial experiments, therefore inferred that these chemically diverse compounds are the potent inhibitor of parasite enzyme, LdDCP with almost no effect on mammalian ACE. These compounds may offer chemical starting points to develop novel drug candidates and their formulations to treat *Leishmania* infections.

[007] Further, these compounds are not aqueous soluble rather have solubility in not pharmaceutically preferred solvents like DMSO, DMF and chloroform. In this endeavour, we also prepared the nanoparticles of the LdDCP inhibitors (Batra, S., Bhaduri, A. P., Joshi, B. S., Roy, R., Khanna, A. K., Chander, R., 2001, Syntheses and biological evaluation of alkanediamines as antioxidant and hypo-lipidemic agents. *Bioorg. Med. Chem.*; 9: 3093-3099) and their bioefficacy was evaluated for both parasite and mammalian enzyme inhibition as well as for their therapeutic potential under both *in vitro* and *in-vivo* condition against *L. donovani*, the causative agent of Indian kala-azar.

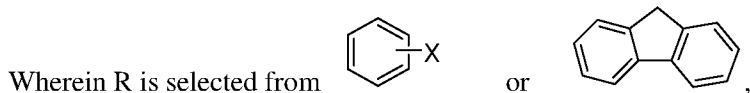
[008] These compounds have limited solubility and are soluble only in DMSO DMF and chloroform. This property seriously limits their use as pharmaceutical agent hence their development as antileishmanial therapeutic agents.

OBJECTIVES OF THE INVENTION

[009] The main objective of the present invention is to develop a novel nanoparticle formulation containing compounds belonging to the series of (2Z,2'Z)-3,3'-(alkane-1,2-diylbis(azanediyl))bis(6-hydroxy-1-aryl/fluorenyl-hex-2-en-1-ones) of general formula **1** having high order of *in vivo* antileishmanial activity.



Compound **1**



wherein X is selected from halogens (Cl, Br and F) and 'n' is the number of methylene groups (n = 1, 2 and 3).

[010] Still another objective of the present invention is to achieve enhanced antileishmanial activity of the analogues of this series of compound.

SUMMARY OF THE INVENTION

[011] The present invention provides a novel antileishmanial formulation to treat visceral leishmaniasis, commonly known as Kala –azar. A novel nanoparticle formulation containing compounds belonging to the series of (2Z,2'Z)-3,3'-(alkane-1,2-diylbis(azanediyl))bis(6-hydroxy-1-aryl/fluorenyl-hex-2-en-1-ones) of general formula **1** having high order of *in vivo* antileishmanial activity. Bio-efficacy of nanoformulation of compound of general formula **1** coated with Edragit is increased by two fold under *in vivo* condition via Oral route of administration as compared to bulk compound. Eudragit polymer is the anionic acrylic polymer that provided stability to the orally delivered biomolecules towards acidic pH, thus enhanced two fold bioefficacy of compound by oral administration as compared to bulk compound. The compounds of general formula **1** display potential antileishmanial activity via oral and intraperitoneal administration. The compounds of general formula **1** are specific inhibitor of leishmanial target enzyme dipeptidylcarboxypeptidase (IdDCP) and have no effect

on mammalian counterpart angiotensin converting enzyme (ACE) at nM concentration.

[012] BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Synthesis of N,N'- [bis (1-aryl-6-hydroxy-hex-2-ene-1-one-3-yl) 1,n-alkanediamines

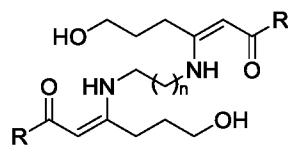
Figure 2: In-vitro release kinetics of nanoparticle preparation of compound 1c in PBS at 37°C

ABBREVIATIONS

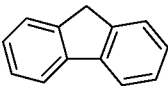
AIDS: Acquired Immune Deficiency Syndrome; **WHO:** World Health Organization; **VL:** visceral Leishmaniasis; **LdDCP:** Leishmaniadonovanidipeptidylcarboxypeptidase ; **ACE:** angiotensin converting enzyme; **DMSO:** Dimethylsulfoxide; **DCM:** Dichloromethane; **SSG:** sodium stibogluconate; **CDRI:** Central Drug Research Institute; **THF:** Tetrahydrofuran; **EE:** encapsulation efficiency ; **PBS:** phosphate buffer ; **UV/VIS:** Ultraviolet / Visible; **KBr:** Potassium Bromide; **NMR:** Nuclear Magnetic Resonance; **CDCl₃:** Deuteriated chloroform; **J:** Coupling constant; **s:** singlet; **d:** doublet; **t:** triplet; **ESI MS:** Electron Spray Ionization Mass spectroscopy; **HR MS:** High Resolution Mass spectroscopy; **IR:** Infrared; **ppm:** parts per million; **DMF:** Dimethylformamide; **THF:** Tetrahydrofuran; **PVA:** poly vinyl alcohol; **BSA:** Bovine serum Albumin; **PEG:** Polyethylene glycol.

DETAILED DESCRIPTION OF THE INVENTION

[013] Accordingly the present invention relates to a novel controlled release antileishmanial formulation comprising a compound of formula 1,



Compound 1

wherein R is selected from  or  wherein X is selected from a group consisting of Cl, Br and F; n is the number of methylene groups (n = 1, 2 and 3); encapsulated in alkyl dextran.

In an embodiment of the present invention, wherein the formulation comprising a compound selected from a group consisting of :

- i. (2Z,2Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-fluorophenyl)-6-hydroxyhex-2-en-1-one) (**1a**)

- ii. (2Z,2Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-chlorophenyl)-6-hydroxyhex-2-en-1-one) (**1b**)
- iii. (2Z,2Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1c**)
- iv. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1d**)
- v. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1e**)
- vi. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1f**)
- vii. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1g**).
- viii. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1h**).
- ix. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1i**).
- x. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1j**).
- xi. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1k**).
- xii. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1l**).

[014] In another embodiment of the present invention, wherein the alkyl dextran may be selected from a group consisting of O-butyl dextran, O-hexyl dextran, O-octyl dextran, O-dodecyl dextran, O-hexadecyl dextran.

[015] In yet another embodiment of the present invention, wherein the O-alkyl-dextran is optionally coated with Eudragit, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate trimellitate, sodium alginate.

[016] In still another embodiment of the present invention, wherein about 80% drug from the formulation is released in about 48 hours.

[017] In another embodiment of the present invention a process for preparation of formulation, wherein the process comprising the steps:

- a. preparing a suspension of the compound of general formula 1 with O-alkyldextran in a solvent selected from a group consisting of DMSO, and DMF with vigorously stirring followed by dropwise addition of water and stirring for a period ranging between 30min to 40min at a temperature ranging between 30⁰C to 35⁰C,
- b. optionally adding a solution of polymer and co-polymer to the suspension obtained in step (a) and further stirring for a period of 3hr to 5hr at a temperature ranging between 30⁰C to 35⁰C ,
- c. dialyzing the suspension obtained in step (a) or (b) for a period of 2 to 4 days, followed by the freeze drying to obtain the solid dried nanoparticle.

In another embodiment of the present invention, wherein the polymer is selected from the group consisting of PVA, PEG, and BSA.

[018] In yet another embodiment of the present invention, wherein the co-polymer is selected from the group consisting of Eudragit, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate trimellitate, and sodium alginate

[019] In yet another embodiment of the present invention, wherein the size of nanoparticle is found in the range of 190-210 nm.

[020] In another embodiment of the present invention, wherein the O-alkyldextran is prepared by the process comprising steps:

- i. treating the dextran with % solution of sodium hydroxide ranging between 10-40% in the presence of catalyst selected from the group consisting of aliquat, tetrabutylammonium bromide, tetrabutylammonium hydrogen sulfate, and tetramethylammonium chloride followed by heating in the range of 50-80⁰C to obtain a solution,
- ii. adding n-bromoalkane in THF in the step (I) solution dropwise with stirring at the temperature ranging between 50-80 °C for the time in the range of 3-6 hr, followed by concentrating the reaction mixture on rotavapor to obtain a residual mass,
- iii. dissolving the residual mass of step (ii) in diethylether followed by the filtration and washing with diethyl ether to obtain a solid,

iv. dissolving the solid of step (iii) in deionized water followed by dialysis and lyophilization for the time in the range of 12-24hr at the temperature ranging between 30-35°C to obtain solid dry product.

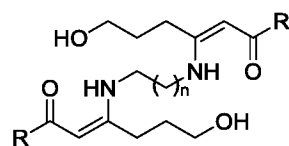
In still another embodiment of the present invention wherein *in vivo* antileishmanial activity via intraperitoneal route at dose 2.5-50mg/Kg in hamster model and has five times enhanced bioefficacy as compared to bulk drug.

[021] In yet another embodiment of the present invention a formulation as claimed in Claim 1 has *in vivo* antileishmanial activity via oral route at dose 25-100mg/Kg in hamster model and has two times enhanced bioefficacy as compared to bulk drug.

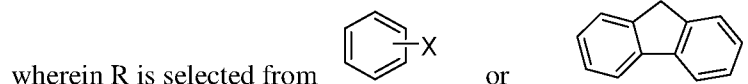
[022] In another embodiment of the present invention, wherein the n-bromoalkane is selected from the group consisting of 1-bromobutane, 1-bromohexane, 1-bromooctane, 1-bromododecane, and 1-bromohexadecane.

[023] In another embodiment of the present invention, wherein the solvent is selected from the group consisting of THF, dioxane, and n-butanol.

In another embodiment of the present invention a compound of formula 1,



Compound 1



wherein X is selected preferably from halogens (Cl, Br and F) and 'n' is the number of methylene groups (n = 1, 2 and 3),

wherein the novel compound is:

- i. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1d**)
- ii. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1e**)
- iii. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1f**)

- iv. (2Z,2Z)-3,3'-(ethane-1, 2-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1g**).
- v. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1h**).
- vi. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1i**).
- vii. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1j**).
- viii. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1k**).
- ix. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1l**).

[024] In another embodiment of the present invention, wherein Compounds of formula 1 are the specific inhibitor of parasite target enzyme dipeptidylcarboxy peptidase with no effect on mammalian counterpart angiotensin converting enzyme at nanomolar concentration.

[025] In yet another embodiment of the present invention, the compound of general formula 1 is useful as antileishmanial agent.

[026] In still another embodiment of the present invention, a pharmaceutical composition comprising therapeutically effective amount of compound of general formula 1 thereof optionally along with pharmaceutically acceptable diluents, excipients, or carriers.

[027] In yet another embodiment of the present invention, (2Z,2'Z)-3,3'-(alkane-1,2-diylbis(azanediyl))bis(6-hydroxy-1-aryl/flourene-hex-2-en-1-ones) of general formula 1 display antileishmanial activity under *in vivo* conditions in hamster model both by ip and oral route

[028] In another embodiment of the present invention the aryl group may be chosen from halo-substituted phenyl group.

[029] In yet another embodiment of the present invention the halogen group may be fluoro, chloro or bromo.

[030] In another embodiment of the present invention the alkane implies a 2, 3 or 4 carbon chain.

[031] In yet another embodiment of the present invention the compounds were prepared in dimethylsulfoxide (DMSO) for *in vitro* evaluation, and test concentrations were diluted from stocks in appropriate medium immediately before the assays.

[032] In still another embodiment of the present invention, the aqueous solution of the test compounds were prepared by suspending the accurately weighed sample in a standard suspension vehicle of 10 % Tween-80/Ethanol (70:30) in deionized water for *in vivo* part of the study. The final volume contains 10% of the vehicle for inoculation by the intraperitoneal route.

[033] In yet another embodiment of the present invention, the enhancement of the antileishmanial activity under *in vivo* conditions both by ip and oral route could be achieved via a nanoparticle-based preparation of compounds belonging to general formula 1.

[034] In another embodiment of present invention, the novel formulation containing compounds belonging to the series of (2Z,2'Z)-3,3'-(alkane-1,2-diylbis(azanediyl))bis(6-hydroxy-1-aryl/flourene-hex-2-en-1-ones) of general formula 1 is entrapped in functionalized dextran nanoparticles.

[035] In another embodiment of present invention, the novel formulation containing compounds belonging to the series of (2Z,2'Z)-3,3'-(alkane-1,2-diylbis(azanediyl))bis(6-hydroxy-1-aryl/flourene-hex-2-en-1-ones) of general formula 1 is encapsulated to O- (alkyl)_n dextran.

[036] In another embodiment of present invention, molecular weight of dextran varies from 25 to 45KDa with the number of alkyl group varies from 4-16 in O- (alkyl)_n dextran.

[037] In another embodiment of present invention, the nanoparticle preparation containing compound (2Z,2'Z)-3,3'-(alkane-1,2-diylbis(azanediyl))bis(6-hydroxy-1-aryl/flourene-hex-2-en-1-ones) of general formula 1, is coated with polymer to provide protection against acidic pH, to be used for oral administration may selected from polymer belonging to the class Eugrate, cellulose derivatives,.

[038] The present invention provides a novel antileishmanial formulation to treat visceral leishmaniasis, commonly known as Kala –azar. The present invention comprises:

Synthesis of compounds of general formula 1 reported in Batra, S., Bhaduri, A. P., Joshi, B. S., Roy, R., Khanna, A. K., Chander, R., 2001, Syntheses and biological evaluation of alkanediamines as antioxidant and hypolipidemic agents. Bioorg. Med. Chem., 9:3093-3099)

[039] The three known compounds were ;Compound **1a**:chemical name (2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(4-fluorophenyl)-6-hydroxyhex-2-en-1-one); Compound **1b**: Chemical name (2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(4-chlorophenyl)-6-hydroxyhex-2-en-1-one).; Compound**1c**: chemical name (2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(4-bromophenyl)-6-hydroxyhex-2-en-1-one).New compounds were also synthesized.

Table 1. Reaction conditions for synthesis of compounds belonging to General formula 1

Starting material		Initial temp, Temp during reaction	Time	Product	Yield (%)
<i>Diol</i>	<i>diamine</i>				
2a	ethylenediamine	0°C, 30-35°C	10 h	1a	77
2b	ethylenediamine	0°C, 30-35°C	8 h	1b	58
2c	1,4-diaminobutane	0°C, 30-35°C	10 h	1c	62
2d	ethylenediamine	0°C, 30-35°C	8h	1d	65
2d	1,3-diaminopropane	0°C, 30-35°C	10 h	1e	72
2d	1,4-diaminobutane	0°C, 30-35°C	10 h	1f	56
2e	ethylenediamine	0°C, 30-35°C	10 h	1g	79
2e	1,3-diaminopropane	0°C, 30-35°C	10 h	1h	61
2e	1,4-	0°C, 30-35°C	10 h	1i	66

	diaminobutane				
2f	ethylenediamine	0°C, 30-35°C	10 h	1j	16
2f	1,3-diaminopropane	0°C, 30-35°C	10h	1k	11
2f	1,4-diaminobutane	0°C, 30-35°C	10h	1l	8

[040] In vitro evaluation of antileishmanial activity of novel compounds:

b.1 Antipromastigote assay: The *Leishmania donovani* promastigotes (MHOM/IN/Dd8/80), originally obtained from Imperial college, London, transfected with firefly luciferase gene as described earlier (Ashutosh, Gupta, S., Ramesh, Sundar, S. and Goyal, N. Use of *Leishmania donovani* field isolates expressing the luciferase reporter gene in *in vitro* drug screening Antimicrob. Agents Chemother. 2005, 49, 3776–3783) were maintained in medium 199 (Sigma) supplemented with 10-15% foetal calf serum (Gibco) and gentamycin (40µg/mL) solution (Sigma). Exponentially growing transgenic promastigotes (0.5-2 X 10⁶promastigotes/100µl/well) were seeded in 96-well flat bottom tissue culture plates (Cellstar) and allowed to grow for 72 h at 24 °C, in presence of 1-100µg/mL of test compounds (stock prepared in 100% DMSO, initial concentration, followed by serial dilution in media) in duplicate for each concentration . After 72h of incubation, 50 µl of the parasite suspension was transferred in black 96 well plate (Nunc) followed by addition of 50 µl Steady Glo reagent (Promega), incubated for 1 min with mild shaking, and read on luminometer (Berthold). The inhibition of parasite growth was determined by comparison of the luciferase activity of drug-treated parasites with that of untreated control parasites by the following formula-

$$\text{Percentage Inhibition (PI)} = \frac{N-n}{N} \times 100$$

Where, N is average relative luminescence unit (RLU) of control wells and n is average RLU of treated wells. The experiment was repeated two times and test compounds which showed PI

> 80 - 90% at <10-30µg/ml concentration was selected for further evaluation against the amastigote stage of parasite.

b.2.Antiamastigote assay: Macrophage cells (J744) were harvested from exponentially growing culture. The cells were diluted to $1-5 \times 10^6$ /mL in RPMI medium plus 10-15% FCS (Sigma, USA) and layered in 16 well chamber slides (Nunc) under a final volume of 100 µl/well and allowed to adhere for 24-30 hrs at 37°C in a 5% CO₂-95% air mixture. Adherent macrophages were infected with stationary phase promastigotes (WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) at ratio 1:5 to 1:10 for 4 -24 hours at 37°C in a 5% CO₂-95% air mixture. After incubation, non-phagocytosed parasites were removed by washing and infected cultures were incubated further at 37°C in a 5% CO₂-95% air mixture in presence of antileishmanial compound(s) for three days. The test compounds, prepared in DMSO and then diluted in complete RPMI medium, were added at two fold dilutions up to 7 points in complete medium starting from 100ug/ml concentrations after replacing the previous medium. Finally slides were fixed with 100% methanol and stained with 20% Giemsa stain for 45 minutes on day 6 (Seifert K. and Croft, S.L. 2006, *In vitro* and *in vivo* interactions between Sodium stibogluconate, miltefosine and other antileishmanial drug. Antimicrobial Agents Chemotherapy; 50: 73-79). The number of amastigotes per 500 cell nuclei was counted in each well and the parasitic burden was expressed in terms of the number of amastigote per 100 cell nuclei. Drug activity (percent inhibition) was determined by comparing amastigote count of treated and untreated wells by the general formula:

$$\text{Percent Inhibition} = \frac{N-n}{N} \times 100$$

Where, N is average number of amastigotes per 100 cell nuclei of untreated well and n is average number of amastigotes per 100 cell nuclei of treated well.

The compounds exhibited antileishmanial activity >80-100 activity in amastigote- macrophage assay at 10-30 ug/ml concentration were selected for *in vivo* bio-efficacy evaluation

b.3. Cytotoxicity assay: The macrophage cell viability was determined using the MTT assay (Mosmann, T. J., 1983, J.Immunol. Methods;65: 55-63). Exponentially growing cells (J774) ($1-2 \times 10^5$ cells/100µl/well) were incubated with test compounds. The test compounds are added at three fold dilutions up to 7 points in complete medium starting from 200 µg/ml concentrations, and were incubated at 37°C in a humidified mixture of CO₂ and 95 % air in an

incubator. Control wells containing dimethyl sulfoxide (DMSO) without compounds were also included in the experiment. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. After incubation, 25 μ l of MTT reagent (5mg/mL) in PBS medium, followed by syringe filtration was added to each well and incubated at 37°C for 2 hours. At the end of the incubation period, the supernatant were removed by inverting the plate completely without disturbing cell layer and 150 μ l of pure DMSO was added to each well. After 15 min. of shaking the readings were recorded as absorbance at 544 nm on a micro plate reader. The cytotoxic effect were expressed as 50% lethal dose (i.e., as the concentration of a compound which provoked a 50% reduction in cell viability compared to cell in culture medium alone). CC₅₀ values were estimated as described by Huner et al. (Huber, W., Koella, J.C. 1993, Acta Trop.; 55: 257-261). The selectivity index (SI) for each compound was calculated as the ratio between, cytotoxicity (CC₅₀) and activity (IC₅₀) against *Leishmania* amastigotes. Compounds with SI index > 4, comparable or less than Miltefosine were considered as safe in this cytotoxic assay.

c. *In vivo* Bioefficacy evaluation: All compounds that exhibited significant inhibition of parasite enzyme, dipeidylcarboxypeptidase (LdDCP) and antiparasitic activity under *in vitro* condition, were selected for determination of their *in vivo* therapeutic potential against *L. donovani* in golden hamster model using method of Bhatnagar et al., 1989. (Bhatnagar, S., Guru, P. Y.; Katiyar, J. C. Srivastava, R., Mukherjee, A., Akhtar, M. S., 1989, Indian J. Med. Res. 89: 439-443). Golden hamsters (Inbred strain) of either sex weighing 40-45g were infected intra-cardially with $1-3 \times 10^7$ amastigotes per animal. The infection is well adapted to the hamster model and establishes itself in 15-20 days. Pre-treatment spleen biopsy in all the animals was carried out to assess the degree of infection. The animals with +1 infection (5-15 amastigotes / 100 spleen cell nuclei) were included in the chemotherapeutic trials. The infected animals were randomized into several groups on the basis of their parasitic burdens. Five to seven animals were used for each test compounds. Aqueous solution of the test compounds were prepared by suspending the accurately weighed sample in a standard suspension vehicle of 10 % Tween-80/Ethanol (70:30) in deionized water for *in vivo* part of the study. The final volume contains 10-12% of the vehicle for inoculation by the intraperitoneal route. Drug treatment by intraperitoneal (i.p.) route was initiated after 2 days of biopsy and continued for 5 consecutive days. Post-treatment biopsies were done on day 7th and 28th day of the last drug

administration and amastigote counts are assessed by Giemsa staining. Intensity of infection in both, treated and untreated animals, as also the initial count in treated animals was compared and the efficacy was expressed in terms of percentage inhibition (PI). All three known compounds exhibited therapeutic potential and inhibited parasite multiplication ranging from 55-92% by intraperitoneal route. and hence promising as compared to standard drug, sodium stibogluconate (SSG). Thus this chemical series provides a new lead that could be exploited as new antileishmanial agents. Compound **1c** exhibited best bio-efficacy among the series therefore selected for development of nanoformulation.

c). Process of preparation of nanoparticles of compound **1c** entrapped to O-(alkyl)_n dextran for efficient drug delivery and consisting of the steps of

1. Synthesizing the O-(alkyl)_n-dextran by attaching butyl-hexadecyl chain to the dextran.
2. Entrapping model drug compound **1c** in synthesized O-butyl-dextran formulating nanoparticles.
3. Characterizing the formulated nanoparticles for particle size and zeta potential.
4. Estimating the entrapment efficiency and in-vitro release of Compound **1c** in nanoparticles.
5. Coating of 1c-nanoparticles with polymers example; Eudragit, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate,

c.1. O-alkyldextrans were prepared using a novel method. The synthesis of O-alkyldextrans was achieved in high yields using phase transfer catalysis. Briefly, to synthesize O-butyl-dextran to -hexadecyl dextran 5g- dextran was taken up in a 10-40% solution of sodium hydroxide (20ml) containing a catalytic amount of Aliquat 336 (0.5g). The reaction mixture was heated up to 60-65°C and n-bromoalkane (bromobutane, 1-bromohexane, 1-bromooctane, 1-bromododecane, or 1-bromohexadecane) (60nmol) in tetrahydrofuran (5ml) was added dropwise. The reaction mixture was then allowed to stir 3h at 60-65°C and then concentrated on a rotavapor. The residual mass was taken up in diethylether and filtered the solid on a sintered funnel. The solid was washed with diethylether (2X50ml) and then it was dissolved in deionized water (20ml). It was poured in a dialysis bag (MWCO 12kD) and subjected to dialysis for 24-36hr followed by lyophilization to obtain solid dry product in 65-82% yield.

To fabricate Compound loaded functionalized dextran nanoparticles, 50mg of Compound **1c** and 150mg of O-butyl/hexadecyl-dextran were taken up in 4ml DMSO and stirred the

suspension for 30 min. To the vigorously stirred solution, water (10ml) was added drop wise. The suspension was allowed to stir for 12h at room temperature ranging from 30-35 °C and then subjected to dialysis for 4-6h to remove un-entrapped Compound 1c. It was then freeze dried to obtain solid dry nanoparticles.

The mean particle size and zeta potential of the nanoparticles were analyzed by laser light diffraction using a Zetasizer Nano-ZS (Malvern Instruments, U.K). A known amount of nanoparticles were suspended in water (1mg/ml) and the mean particles size and charge on the nanoparticles were measured employing the following settings on the instrument: a nominal 5mW He-Ne laser operating at 633nm wavelength; 14 measurements per sample; refractive index of water, 1.33; viscosity for water, 0.89 cP. All measurements were carried out at 25°C. Zeta potential measurements were carried out on the same instrument in triplicates in automatic mode and the values are presented as the average value of 30 runs. The Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility (Swami, A., Aggarwal, A., Pathak, A., Patnaik, S., Kumar, P., Singh, Y., et al., 2007, Imidazolyl-PEI modified nanoparticles for enhanced gene delivery. *Int J Pharm*; 335:180–192).

The encapsulation efficiency of the model drug was determined by spectrophotometric estimation using Lambda Bio 20 UV/VIS Spectrophotometer (Perkin Elmer, USA). Briefly, accurately weighed drug loaded nanoparticles (~10-15 mg) were suspended in water (1mL) and after 10-20min the suspension was centrifuged at 13000rpm for 15min. The supernatant was removed and the pellet was dried and dissolved in chloroform (1mL) and measured the absorbance of the solution at 360 nm. The amount of drug (mg) was calculated from the standard curve drawn between the varied amounts of drug (mg) and absorbance (O.D.). From the following equation, the encapsulation efficiency (EE) was determined. All the measurements were conducted in triplicate.

$$\text{EE \%} = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of drug used for nanoparticles preparation}} \times 100$$

Compound 1c loaded O-butyl-dextran nanoparticles were evaluated for their *in vitro* release kinetics by dialysis method, as reported previously (Asadishad, B., Vossoughi, M., Alamzadeh, I., 2010, *In vitro* drug release behaviour and cytotoxicity of doxorubicin-loaded

gold nanoparticles in cancerous cells. *Biotechnology Letters*; 32:649-654). Briefly, 10mg NPs were suspended in 2ml phosphate buffer (PBS), transferred the solution to a dialysis bag (12kDa) and allowed to dialyze against 20ml of same buffer at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ with stirring at 50rpm in an incubator shaker (Heidolph Unimax 1010, Germany). At predetermined time-intervals, an aliquot (1ml) of the sample was withdrawn and its absorbance was measured at 360nm using Lambda Bio 20 UV/VIS Spectrophotometer (Perkin Elmer, USA) and added the same amount of fresh medium to the dialysis container. The amount of the released drug was then calculated using a previously prepared standard curve of the pure drug. The drug was slowly and consistently released (Figure 2.). More than 80% drug was released in 48 hours.

Further, these compounds are not aqueous soluble rather have solubility is not pharmaceutically preferred solvents like DMSO, DMF and chloroform. In this endeavour, we also prepared the nanoparticles of the LdDCP inhibitors (Batra, S., Bhaduri, A. P., Joshi, B. S., Roy, R., Khanna, A. K., Chander, R., 2001, Syntheses and biological evaluation of alkanediamines as antioxidant and hypo-lipidemic agents. *Bioorg. Med. Chem.*; 9: 3093-3099) and their bioefficacy was evaluated for both parasite and mammalian enzyme inhibition as well as for their therapeutic potential under both *in vitro* and *in-vivo* condition against *L. donovani*, the causative agent of Indian kala-azar.

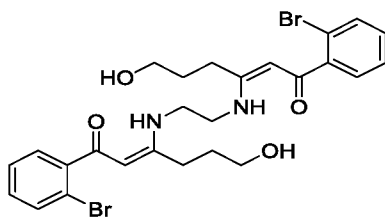
Coating: 0.50 g Compound 1c was dissolved in 15ml DMSO. To this, 1.5 g of O-alkyl-Dextran dissolved in 40ml water was added. The reaction was stirred for 2-3hr. Subsequently, the solution was added to 50ml of 1% PVA, BSA, PEG solution having 0.04% Eudragit dissolved in it. The reaction mixture was allowed to stir for 12-14hr at room temperature ranging from 30-35 oC, followed by dialysis for 4-7hr. It was then freeze dried to obtain solid dry nanoparticles.

The following examples are given by way of illustration of the present invention and should not be construed to limit the scope of the present invention;

[041] Example 1:

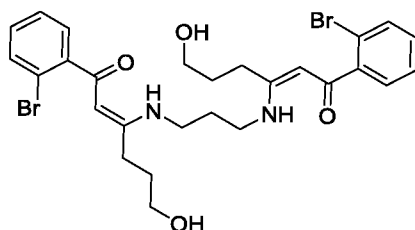
Preparation of general formula 1 is reported in Batra, S., Bhaduri, A. P., Joshi, B. S., Roy, R., Khanna, A. K., Chander, R., 2001, , Syntheses and biological evaluation of alkanediamines as antioxidant and hypolipidemic agents. *Bioorg. Med. Chem.*; 9:3093-3099. The spectral data of the new compounds (**1d-1l**) are provided.

Example 1.1.



(2Z,2'Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1d**). 65% as an off white solid; R_f = 0.52 (MeOH/ CHCl_3 , 10:90, v/v); mp 139-140 °C; IR(KBr) ν_{max} = 3451, 1590 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ = 1.77-1.84 (m, 4H, 2 x CH_2), 2.49 (t, 4H, J =8.0 Hz, 2 x CH_2), 3.05 (brs, 2H, 2 x OH), 3.63-3.67 (m, 4H, 2 x CH_2), 3.72-3.73 (m, 4H, 2 x CH_2), 5.32 (s, 2H, 2 x CH), 7.19 (dt, 2H, J_1 = 1.8 Hz, J_2 = 7.6 Hz, ArH), 7.30 (dt, 2H, J_1 = 1.0 Hz, J_2 = 7.6 Hz, ArH), 7.38 (dd, 2H, J_1 = 1.7 Hz, J_2 = 7.5 Hz, ArH), 7.56 (d, 2H, J = 7.9 Hz, ArH), 11.38 (brs, 2H, 2 x NH); ^{13}C NMR (CDCl_3 , 100 MHz) δ = 27.6, 28.7, 31.3, 42.7, 61.5, 95.7, 119.6, 127.4, 129.2, 130.2, 133.4, 143.4, 170.0, 190.2; ESI-MS m/z = 593.2 $[\text{M}+\text{H}]^+$; ESI-HR-MS m/z = 593.0656, calcd. for $\text{C}_{26}\text{H}_{30}\text{Br}_2\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 593.0651.

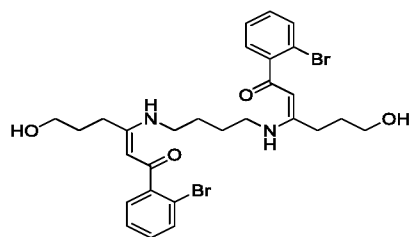
Example 1.2.



(2Z,2'Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one)(**1e**).

72% as an off white solid; R_f = 0.48 (MeOH/ CHCl_3 , 10:90, v/v); mp 61-62 °C; IR(KBr) ν_{max} = 3461, 1594 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ = 1.85 (brs, 4H, 2 x CH_2), 2.07-2.11 (m, 2H, CH_2), 2.48-2.53 (m, 4H, 2 x CH_2), 2.92 (brs, 2H, 2 x OH), 3.63-3.67 (m, 8H, 4 x CH_2), 5.34 (s, 2H, 2 x CH), 7.17-7.22 (m, 2H, ArH), 7.32 (d, 2H, J = 7.2 Hz, ArH), 7.39 (d, 2H, J = 6.9 Hz, ArH), 7.57 (d, 2H, J = 7.6 Hz, ArH), 11.37 (brs, 2H, 2 x NH); ^{13}C NMR (CDCl_3 , 100 MHz) δ = 28.8, 29.7, 31.2, 39.8, 61.4, 95.7, 119.5, 127.4, 129.1, 130.3, 133.4, 143.4, 170.0, 190.2; ESI-MS m/z = 507.3 $[\text{M}+\text{H}]^+$; ESI-HR-MS m/z = 607.0809, calcd. for $\text{C}_{27}\text{H}_{32}\text{Br}_2\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 607.0807.

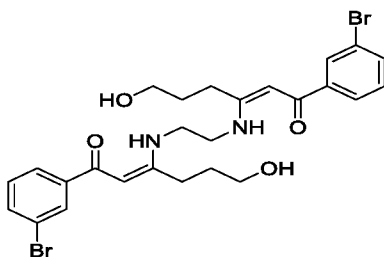
Example 1.3.



(2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (1f).

56% as an off white solid; R_f = 0.49 (MeOH/ CHCl_3 , 10:90, v/v); mp 95-96 °C; IR(KBr) ν_{max} = 3452, 1588 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ = 1.85 (brs, 8H, 4 x CH_2), 2.43-2.49 (m, 4H, 2 x CH_2), 2.58 (brs, 2H, 2 x OH), 3.47 (d, 4H, J = 3.9 Hz, 2 x CH_2), 3.66-3.69 (m, 4H, 2 x CH_2), 5.29 (s, 2H, 2 x CH), 7.16-7.21 (m, 2H, ArH), 7.31 (d, 2H, J = 7.0 Hz, ArH), 7.39 (d, 2H, J = 6.2 Hz, ArH), 7.56 (d, 2H, J = 7.9 Hz, ArH), 11.38 (brs, 2H, 2 x NH); ^{13}C NMR (CDCl_3 , 100 MHz) δ = 27.4, 28.4, 30.8, 42.5, 61.0, 94.9, 119.3, 127.1, 128.9, 129.8, 133.1, 143.4, 169.4, 189.4; ESI-MS m/z = 621.1 $[\text{M}+\text{H}]^+$; ESI-HR-MS m/z = 621.0968, calcd. for $\text{C}_{28}\text{H}_{34}\text{Br}_2\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+$ 621.0964.

Example 1.4.

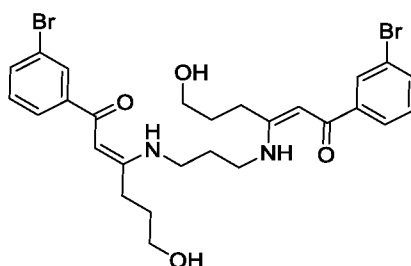


(2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (1g).

(2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (1g). 69% as an off white solid; R_f = 0.50 (MeOH/ CHCl_3 , 10:90, v/v); mp 121-122 °C; IR(KBr) ν_{max} = 3458, 1589 cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) δ = 1.78-1.83 (m, 4H, 2 x CH_2), 2.5 (t, 4H, J = 8.0 Hz, 2 x CH_2), 3.11 (brs, 2H, 2 x OH), 3.62-3.66 (m, 4H, 2 x CH_2), 3.72-3.74 (m, 4H, 2 x CH_2), 5.66 (s, 2H, 2 x CH), 7.23-7.29 (m, 2H, ArH), 7.54 (d, 2H, J = 7.4 Hz, ArH), 7.73 (d, 2H, J = 7.7 Hz, ArH), 7.93 (s, 2H, ArH), 11.66 (brs, 2H, 2 x NH); ^{13}C NMR (CDCl_3 , 100 MHz) δ = 27.6, 28.7, 31.3, 42.7, 61.5, 91.3, 122.7, 125.5, 130.0, 130.1, 133.5, 142.6, 170.0,

186.0; ESI-MS $m/z = 593.3$ $[M+H]^+$; ESI-HR-MS $m/z = 593.0658$, calcd. for $C_{26}H_{30}Br_2N_2O_4$ $[M+H]^+$ 593.0651.

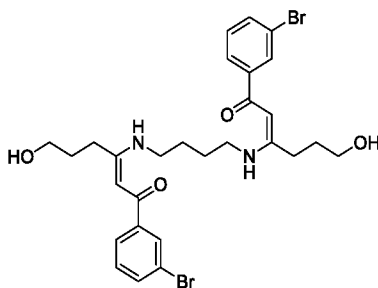
Example 1.5.



(2Z,2'Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (1h).

61% as an off white solid; $R_f = 0.42$ (MeOH/ $CHCl_3$, 10:90, v/v); mp 63-64 °C; IR(KBr) $\nu_{max} = 3467, 1584$ cm^{-1} ; ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.84$ (brs, 6H, 3 x CH_2), 2.10 (brs, 2H, 2 x OH), 2.52-2.57 (m, 4H, 2 x CH_2), 3.65 (s, 4H, 2 x CH_2), 3.67 (s, 4H, 2 x CH_2), 5.70 (s, 2H, 2 x CH), 7.31 (d, 2H, $J = 7.9$ Hz, ArH), 7.57 (d, 2H, $J = 7.4$ Hz, ArH), 7.77 (d, 2H, $J = 7.5$ Hz, ArH), 7.96 (s, 2H, ArH), 11.62 (brs, 2H, 2 x NH); ^{13}C NMR ($CDCl_3$, 100 MHz) $\delta = 29.1, 29.5, 31.6, 39.7, 61.4, 91.8, 122.7, 125.6, 130.1, 133.7, 142.3, 170.5, 186.4$; ESI-MS $m/z = 607.3$ $[M+H]^+$; ESI-HR-MS $m/z = 607.0802$, calcd. for $C_{27}H_{32}Br_2N_2O_4$ $[M+H]^+$ 607.0807.

Example 1.6.

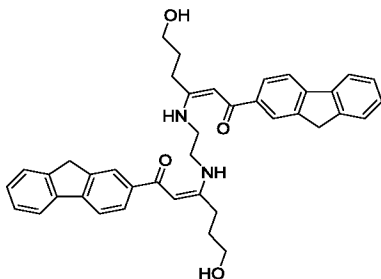


(2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (1i).

66% as an off white solid; $R_f = 0.41$ (MeOH/ $CHCl_3$, 10:90, v/v); mp 69-70 °C; IR(KBr) $\nu_{max} = 3471, 1580$ cm^{-1} ; ^1H NMR ($CDCl_3$, 300 MHz) $\delta = 1.82-1.88$ (m, 8H, 4 x CH_2), 2.46-2.51 (m, 4H, 2 x CH_2), 2.63 (brs, 2H, 2 x OH), 3.46 (d, 4H, $J = 4.1$ Hz, 2 x CH_2), 3.68-3.72 (m, 4H, 2 x CH_2), 5.62 (s, 2H, 2 x CH), 7.24-7.29 (m, 2H, ArH), 7.53 (d, 2H, $J = 7.8$ Hz, ArH), 7.74 (d, 2H,

$J = 7.7$ Hz, ArH), 7.94 (s, 2H, ArH). 11.64 (brs, 2H, 2 x NH); ^{13}C NMR (CDCl_3 , 100 MHz) $\delta = 27.6, 28.7, 31.3, 42.7, 61.5, 91.3, 122.7, 125.5, 130.0, 130.1, 133.5, 142.5, 186.0$; ESI-MS m/z 621.3 $[\text{M}+\text{H}]^+$; ESI-HR-MS $m/z = 621.0968$, calcd. for $\text{C}_{28}\text{H}_{34}\text{Br}_2\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+ 624.0964$.

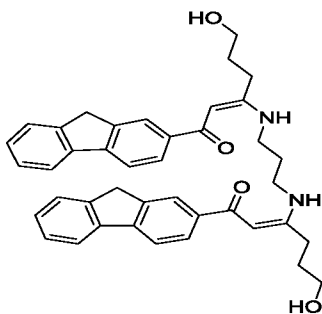
Example 1.7.



(2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (1j).

(2Z,2'Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1j**). 16% as an off white solid; $R_f = 0.51$ (MeOH/ CHCl_3 , 10:90, v/v); mp 82-84 °C; IR (KBr) $\nu_{\text{max}} = 3452, 1586$ cm^{-1} ; ^1H NMR (CDCl_3 , 300 MHz) $\delta = 1.94$ (brs, 2H, CH_2), 2.50-2.55 (m, 4H, 2 x CH_2), 2.97-3.01 (m, 4H, 2 x CH_2), 3.46-3.52 (m, 4H, 2 x CH_2), 3.71-3.75 (m, 4H, CH_2 and 2 x OH), 3.93 (s, 4H, 2 x CH_2), 5.81 (s, 2H, 2 x CH), 7.30-7.41 (m, 4H, ArH), 7.56 (d, $J = 7.3$ Hz, 2H, ArH), 7.77-7.82 (m, 4H, ArH), 7.91 (d, $J = 7.8$ Hz, 2H, ArH), 8.06 (s, 2H, ArH), 11.70 (brs, 2H, 2 x NH); ^{13}C NMR (CDCl_3 , 100 MHz) $\delta = 28.4, 31.3, 36.6, 43.0, 60.2, 91.0, 119.8, 120.7, 123.6, 125.4, 126.0, 127.1, 127.5, 128.9, 140.6, 143.2, 143.5, 144.1, 169.1, 186.4$; ESI-MS $m/z = 613.2$ $[\text{M}+\text{H}]^+$; ESI-HR-MS $m/z = 613.3059$, calcd. for $\text{C}_{40}\text{H}_{40}\text{N}_2\text{O}_4$ $[\text{M}+\text{H}]^+ 613.3066$.

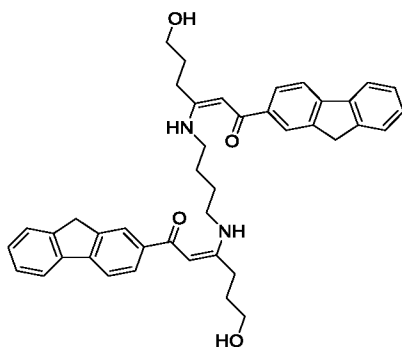
Example 1.8.



(2Z,2'Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (1k).

11% as an off white solid; $R_f = 0.50$ (MeOH/CHCl₃, 10:90, v/v); mp 76-78°C; IR(KBr) $\nu_{\max} = 3411, 1592 \text{ cm}^{-1}$; ¹H NMR (CDCl₃, 400 MHz) $\delta = 1.80$ (brs, 4H, 2 x CH₂), 2.45 (m, 2H, CH₂), 3.40-3.44 (m, 4H, 2 x CH₂), 3.63-3.66 (m, 4H, 2 x CH₂), 3.85-3.92 (m, 4H, 2 x CH₂), 3.89 (s, 4H, 2 x CH₂), 5.75 (s, 2H, 2 x CH), 7.24-7.35 (m, 4H, ArH), 7.48-7.50 (d, $J = 7.3 \text{ Hz}$, 2H, ArH), 7.70-7.76 (m, 4H, ArH), 7.78-7.82 (d, $J = 7.8 \text{ Hz}$, 2H, ArH), 8.04 (s, 2H, ArH), 11.61 (brs, 2H, 2 x NH); ¹³C NMR (CDCl₃, 100 MHz) $\delta = 27.8, 31.4, 37.0, 42.6, 61.6, 91.4, 95.4, 119.5, 120.4, 123.6, 125.1, 126.0, 126.9, 127.3, 137.4, 138.4, 139.0, 139.5, 141.1, 143.1, 144.1, 168.9, 188.0$; ESI-MS m/z 627.0 [M+H]⁺; ESI-HR-MS m/z 627.3219, calcd. for C₄₁H₄₂N₂O₄ [M+H]⁺ 627.3223.

Example 1.9.



(2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (11).

8% as an off white solid; $R_f = 0.49$ (MeOH/CHCl₃, 10:90, v/v); mp 74-76°C; IR(KBr) $\nu_{\max} = 3426, 1522 \text{ cm}^{-1}$; ¹H NMR (CDCl₃, 400 MHz) $\delta = 1.80$ (brs, 4H, 2 x CH₂), 2.10 (m, 4H, 2 x CH₂), 2.47-2.51 (m, 4H, 2 x CH₂), 3.56-3.68 (m, 8H, 4 x CH₂), 3.87 (s, 4H, 2 x CH₂), 5.76 (s, 2H, 2 x CH), 7.26-7.32 (m, 4H, ArH), 7.48-7.50 (d, $J = 7.3 \text{ Hz}$, 2H, ArH), 7.72-7.75 (m, 4H, ArH), 7.82 (d, $J = 7.8 \text{ Hz}$, 2H, ArH), 7.97 (s, 2H, ArH), 11.53 (brs, 2H, 2 x NH); ¹³C NMR (CDCl₃, 100 MHz) $\delta = 29.1, 31.7, 37.0, 61.4, 91.9, 119.6, 120.5, 123.6, 125.2, 126.0, 126.9, 127.4, 138.6, 141.0, 143.2, 144.2, 144.3, 169.7, 188.3$; ESI-MS m/z 641.0 [M+H]⁺; ESI-HR-MS m/z 641.3385, calcd. for C₄₂H₄₄N₂O₄ [M+H]⁺ 641.3379.

Example 2:

Process of preparation of nanoparticles of compound **1c** entrapped to O-(alkyl)_n dextran for efficient drug delivery and consisting of the steps of

1. Synthesizing the O-(alkyl)_n-dextran by attaching butyl chain to the dextran.
2. Entrapping our model drug compound **1c** in synthesized O-butyl-dextran formulating nanoparticles.
3. Characterizing the formulated nanoparticles for particle size and zeta potential.
4. Estimating the entrapment efficiency and in-vitro release of Compound **1c** from nanoparticles.
5. Coating of 1c-nanoparticles with polymer, Eucrate.

[042] Example 2.1. Chemical synthesis of O-alkyldextran herein prepared herein O-butyl-dextran

O-alkyldextrans were prepared using a novel method. The synthesis of O-alkyldextrans was achieved in high yields using phase transfer catalysis. Briefly, to synthesize O-butyl-dextran, 5g dextran was taken up in a 20% solution of sodium hydroxide (20mL) containing a catalytic amount of aliquat 336 (0.5g). The reaction mixture was heated upto 65⁰C and n-bromobutane (60nmol) in THF(5mL) was added dropwise. The reaction mixture was then allowed to stir 3h at 60⁰C and then concentrated on a rotavapor. The residual mass was taken up in diethylether and filtered the solid on a sintered funnel. The solid was washed with diethylether (2X50mL) and then it was dissolved in deionized water(20mL). It was poured in a dialysis bag (MWCO 12kD) and subjected to dialysis for 24hr followed by lyophilization to obtain solid dry product in 65-82% yield.

[043] Example 2.2. Encapsulation of Compound 1c to O-butyl dextran

Method for encapsulation of Compound 1c is developed for the first time. To fabricate Compound **1c** loaded functionalized dextran nanoparticles, 50mg Compound **1c** and 150mg of O-butyl -dextran were taken up in 4mL DMSO and stirred the suspension for 30 min. at the temperature 35⁰C. To the vigorously stirred solution, water (10mL) was added drop wise. The suspension was allowed to stir for 12h at 35⁰C and then subjected to dialysis for 4h at temperature 35⁰C to remove un-entrapped Compound **1c**. It was then freeze dried to obtain solid dry compound 1c loaded nanoparticles.

Results: Compound **1c**-DextranC4 NPs prepared had an electric charge of -4.5 ± 1.32 mV and a size of 200 ± 10.84 nm. The entrapment efficiency was 40% and was observed to be the most stable formulation.

[044] Example 2.3.Characterization of Nanoparticles

Size and zeta potential measurements

The mean particle size and zeta potential of the nanoparticles were analyzed by laser light diffraction using a ZetasizerNano-ZS (Malvern Instruments, U.K). A known amount of nanoparticles were suspended in water (1mg/mL) and the mean particles size and charge on the nanoparticles were measured employing the following settings on the instrument: a nominal 5mW He-Ne laser operating at 633nm wavelength; 14 measurements per sample; refractive index of water, 1.33; viscosity for water, 0.89 cP. All measurements were carried out at 25°C. Zeta potential measurements were carried out on the same instrument in triplicates in automatic mode and the values are presented as the average value of 30 runs. The Smoluchowski approximation was used to calculate zeta potential from the electrophoretic mobility (Swami, A., Aggarwal, A., Pathak, A., Patnaik, S., Kumar, P., Singh, Y., et al., 2007, Imidazolyl-PEI modified nanoparticles for enhanced gene delivery. Int. J. Pharm.; 335:180–192).

Results: Compound **1c**-DextranC4 NPs prepared had an electric charge of -4.5 ± 1.32 mV.

[045] Example 2.4.Encapsulation Efficiency

The encapsulation efficiency of the model drug was determined by spectrophotometric estimation using Lambda Bio 20 UV/VIS Spectrophotometer (Perkin Elmer, USA). Briefly, accurately weighed drug loaded nanoparticles (10 mg) were suspended in water (1mL) and after 10min, the suspension was centrifuged at 13000rpm for 15min. The supernatant was removed, pellet was dried and dissolved in chloroform (1mL) and measured the absorbance of the solution at 360 nm. The amount of drug (mg) was calculated from the standard curve drawn between the varied amounts of drug (mg) and absorbance (O.D.). From the following equation, the encapsulation efficiency (EE) was determined. All the measurements were conducted in triplicate.

$$\text{EE \%} = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of drug used for nanoparticles preparation}} \times 100$$

Results: The entrapment efficiency of prepared Compound 1c-DextranC4 NPs was 40%

Table1: Characteristics of Compound 1c-DextranC4 Nanoparticles

Sample	Size(nm) PDI	Zeta Potential(mV)	Entrapment Efficiency
Compound 1c-DextranC4 NPs	200 ± 10.84 0.12	-4.5 ± 1.32	40%

[046] Example 2.5: *In vitro* drug release study

Compound 1c loaded O-butyl-dextran nanoparticles were evaluated for their *in vitro* release kinetics by dialysis method, as reported previously (Asadishad, B., Vossoughi, M., Alamzadeh, I., 2010, In vitro drug release behaviour and cytotoxicity of doxorubicin-loaded gold nanoparticles in cancerous cells. *Biotechnology Letters*; 32:649-654). Briefly, 10mg NPs were suspended in 2mL phosphate buffer (PBS), transferred the solution to a dialysis bag (12kDa) and allowed to dialyze against 20mL of same buffer at 37°C±0.5°C with stirring at 50rpm in an incubator shaker (HeidolphUnimax 1010, Germany). At predetermined time-intervals, an aliquot (1mL) of the sample was withdrawn and its absorbance was measured at 360nm using Lambda Bio 20 UV/VIS Spectrophotometer (Perkin Elmer, USA) and added the same amount of fresh medium to the dialysis container. The amount of the released drug was then calculated using a previously prepared standard curve of the pure drug. The drug was slowly and consistently released (Figure 2.). More than 80% drug was released in 48 hours.

[047] Example 2.6: Preparation of Eudragit coated O-butyl-Dextran-Compound 1c nanoparticles

0.5 g Compound 1c was dissolved in 15mL DMSO. To this, 1.5 g of O-butyl-dextran dissolved in 40mL water was added. The reaction was stirred for 2-3hr. Subsequently, the solution was added to 50mL of 1% PVA solution having 0.04% Eudragit dissolved in it. Eudragit polymer is the anionic acrylic polymer with methacrylic acid and ethyl acrylic acid as the functional groups, and is resistant towards acidic pH, thus providing stability to the orally delivered biomolecules. Therefore, it was selected for providing the stable enteric coating to the NPs. The reaction mixture was allowed to stir for 12 hr at room temperature ranging from 35°C, followed by dialysis for 4hr. It was then freeze dried to obtain solid dry compound 1c loaded nanoparticles with coating of eudragit.

[048] Example 3: *In vitro* antileishmanial activity assay**[049] Example 3.1: Antipromastigote assay:**

Parasite: The *Leishmania donovani* promastigotes (MHOM/IN/Dd8/80), originally obtained from Imperial college, London, transfected with fire fly luciferase gene as described earlier (Ashutosh, Gupta, S., Ramesh, Sundar, S. and Goyal, N. Use of *Leishmania donovani* field isolates expressing the luciferase reporter gene in *in vitro* drug screening. Antimicrob. Agents Chemother. 2005, 49, 3776–3783) are being maintained in medium 199 (Sigma) supplemented with 10% foetal calf serum (Gibco) and gentamycin (40 µg/mL) solution (Sigma). Exponentially growing transgenic promastigotes (1×10^6 promastigotes/100 µl/well) were seeded in 96-well flat bottom tissue culture plates (Cellstar) and allowed to grow for 72 h at 24 °C, in presence of 1-100 µg/mL of test compounds (stock prepared in 100% DMSO, initial concentration, 1 mg/ml followed by dilution in media) in duplicate for each concentration. After 72h of incubation, 50 µl of the parasite suspension was transferred in black 96 well plate (Nunc) followed by addition of 50 µl Steady Glo reagent (Promega), incubated for 1 min with mild shaking, and read on luminometer (Berthold). The inhibition of parasite growth was determined by comparison of the luciferase activity of drug-treated parasites with that of untreated control parasites by the following formula-

$$\text{Percentage Inhibition (PI)} = \frac{N-n}{N} \times 100$$

Where, N is average relative luminescence unit (RLU) of control wells and n is average RLU of treated wells. The experiment was repeated two times and test compounds which showed PI > 80 - 90% at 25 µg/mL concentration were selected for further evaluation against the amastigote stage of parasite.

Results: Compounds belonging to General formula 1 showed *in vitro* antileishmanial activity with IC₅₀ against promastigotes in the range of 1-27 µg/mL. Compound 1h, 1i and 1j exhibited minimum IC₅₀ against promastigotes (1-2 µg/mL) while compound 1c and 1e exhibited maximum IC₅₀ against promastigotes in range of 20-27 µg/mL. Compounds 1k and 1l did not show any anti leishmanial activity (Table 2).

[050] Example 3.2: Anti-amastigote assay:

Parasite:

The WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) was obtained from Imperial College, London (UK), and being maintained in this laboratory as promastigotes *in vitro* and as amastigotes in golden hamsters. Promastigotes of *L. donovani* were cultivated in medium 199 (Sigma-Aldrich) supplemented with 0.1% gentamycin (Biovaccines Private Ltd., Chevella, Andhra Pradesh, India) and 10% Fetal Calf Serum

[051] Procedure:

Macrophage cells (J744) were harvested from exponentially growing culture. The cells were diluted to 1×10^6 /mL in RPMI medium plus 10% FCS (Sigma, USA) and layered in 16 well chamber slides (Nunc) under a final volume of 100 μ l/ well and allowed to adhere for 24hrs at 37°C in a 5% CO₂-95% air mixture. Adherent macrophages were infected with stationary phase promastigotes at ratio 1:10 for 24 hours at 37°C in a 5% CO₂-95% air mixture. After incubation, non-phagocytosed parasites were removed by washing and infected cultures were incubated further at 37°C in a 5% CO₂-95% air mixture in presence of antileishmanial compound(s) for three days. The test compounds, prepared in DMSO and then diluted in complete RPMI medium, were added at two fold dilutions up to 7 points in complete medium starting from 100 μ g/mL concentrations after replacing the previous medium. Finally slides were fixed with 100% methanol and stained with 20% Giemsa stain for 45 minutes on day 6 (Seifert K. and Croft, S.L. 2006, *In vitro* and *in vivo* interactions between Sodium stibogluconate, miltefosine and other antileishmanial drug. Antimicrobial Agents Chemotherapy; 50: 73-79). The number of amastigotes per 500 cell nuclei was counted in each well and the parasitic burden was expressed in terms of the number of amastigote per 100 cell nuclei. Drug activity (percent inhibition) was determined by comparing amastigotes count of treated and untreated wells by the general formula:

$$\text{Percent Inhibition} = \frac{N-n}{N} \times 100$$

Where, N is average number of amastigotes per 100 cell nuclei of untreated well and n is average number of amastigotes per 100 cell nuclei of treated well.

Results: All compounds exhibited antileishmanial activity in amastigote- macrophage assay. Compound 1e, 1g, 1i and 1j showed minimum IC₅₀ value ((3-7 μ g/ml) while 1d showed maximum IC₅₀ value against amastigotes.

[052] Example 3.3: Cytotoxicity assay:

The macrophage cell viability was determined using the MTT assay (Mosmann, T. J., 1983, Immunol. Methods; 65: 55-63). Exponentially growing cells (J774) (1×10^5 cells/100 μ l/well) were incubated with test compounds for 72 hours. The test compounds are added at three fold dilutions up to 7 points in complete medium starting from 200 μ g/mL concentrations, and were incubated at 37°C in a humidified mixture of CO₂ and 95 % air in an incubator. Podophyllotoxin was used as a reference drug, and control wells containing dimethyl sulfoxide (DMSO) without compounds were also included in the experiment. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. After incubation, 25 μ l of MTT reagent (5mg/mL) in PBS medium, followed by syringe filtration was added to each well and incubated at 37°C for 2 hours. At the end of the incubation period, the supernatant were removed by inverting the plate completely without disturbing cell layer and 150 μ l of pure DMSO are added to each well. After 15 min. of shaking the readings were recorded as absorbance at 544 nm on a micro plate reader. The cytotoxic effect were expressed as 50% lethal dose (i.e., as the concentration of a compound which provoked a 50% reduction in cell viability compared to cell in culture medium alone). CC₅₀ values were estimated as described by Huber, W., Koella, J.C. 1993, Acta Trop.; 55: 257-261). The selectivity index (SI) for each compound was calculated as the ratio between, cytotoxicity (CC₅₀) and activity (IC₅₀) against *Leishmania amastigotes*.

Results: Compounds 1b, 1e, 1h-j have shown SI index >6. Compounds 1a, 1f, and 1g exhibited SI index less than 3.5- 4.5, while compound 1c showed SI index, 2.7.

[053] Example 4: *In Vivo* Antileishmanial activity in Hamster model:

The modified method of Bhatnagar et al., 1989 (Bhatnagar, S., Guru, P. Y.; Katiyar, J. C. Srivastava, R., Mukherjee, A., Akhtar, M. S., 1989, Indian J. Med. Res. 89: 439-443) was used for in-vivo screening. Golden hamsters (Inbred strain) of either sex weighing 40-45g were infected intra-cardially with 1×10^7 amastigotes per animal. Pre-treatment spleen biopsy in all the animals was carried out to assess the degree of infection on day 20 of infection. The animals with +1 infection (5-15 amastigotes / 100 spleen cell nuclei) were included in the chemotherapeutic trials. The infected animals were randomized into several groups on the basis of their parasitic burdens. six animals were used for each test sample. Drug treatment by intraperitoneal (i.p.) route and oral route was initiated after 2 days of biopsy and continued for 5 consecutive days. Post-treatment biopsies were done on day 7th and 28th day of the last drug

administration and amastigote counts are assessed by Giemsa staining. Intensity of infection in both, treated and untreated animals, as also the initial count in treated animals was compared and the efficacy was expressed in terms of percentage inhibition (PI) using the following formula:-

$$PI = 100 - [ANAT \times 100 / (INAT \times TIUC)]$$

Where PI is Percent Inhibition of amastigotes multiplication, ANAT is Actual Number of Amastigotes in treated animals, INAT is Initial Number of Amastigotes in Treated animals and TIUC is Times Increase of parasites in Untreated Control animals.

For the *in vivo* evaluation, aqueous solution of the test compounds was prepared by suspending the accurately weighed sample in a standard suspension vehicle of 10 % Tween-80/Ethanol (70:30) in ddH₂O. The final volume contains 10% of the vehicle for inoculation by the intraperitoneal route.

Results: By ip route, out of 12 compounds, 1c, 1i and 1j exhibited significant parasite inhibition in the range of 80-90% on day 7th of treatment. Interestingly this antileishmanial activity was maintained on day 28th also. The activity was comparable to standard reference drug (Sodium Stibogluconate (SSG)). Rest compounds exhibited moderate activity in the range of 55-65% inhibition (Table 2).

All three compounds 1c, 1i and 1j were evaluated for their bio-efficacy by oral route. All compounds exhibited significant *in vivo* antileishmanial activity by oral route also. Compound 1c showed 87% parasite inhibition on day 7th which was maintained up to day 28th in range of more than 75% (Table 3). Other two compounds exhibited very significant antileishmanial activity by oral route on day 7th (Table 4) in the range of 90% but the activity was reduced to less than 70% on day 28th. Therefore compound 1c was selected for development of formulation.

The two nanoformulations of compound 1c were developed. Formulation1, 1c-dextranC4 nanoparticles exhibited significantly enhanced (five folds) therapeutic efficiency as compared to bulk drug by ip route. This formulation exhibited more than 84% parasite inhibition at one fifth ip dose i.e. 10mg/kg on both day 7th and day 28th of treatment.. The dose, is half of standard drug the dose, sodium stibogluconate (SSG), still showing same range of parasite inhibition (Table3). Thus, the compound 1c- dextranC4 nanoparticles has better therapeutic potential as compared standard drug SSG.

Formulation2 1c-dextranC4 eutragit coated nanoparticles also exhibited dose benefit of two fold hence enhanced therapeutic efficiency as compared to bulk drug. At 50mg/kg dose, the formulation exhibited 80% parasite inhibition, comparable to therapeutic efficacy of 100mg/kg dose of bulk drug. The activity was maintained up to 28th day of treatment. The activity was also comparable to mefloquine, the only oral antileishmanial drug available.

Table 2: Screening results of antileishmanial compounds, 1(a-l)

No	Compound code	IC ₅₀ (µg/mL)		SI index	<i>In vivo</i> evaluation at 50mg/kgX5, ip dose	
		Promastigote	Amastigote		% inhibition	
					7 th day	28 th day
1	1a	16.02	16.29	4.0	68.66±11.54	61.5±6.16
2	1b	12.44	8.41	6.2	66.5±4.94	39±8.48
3	1c	21.77	14.29	2.7	80±6.0	83.4±6.42
4	1d	17.61	24.59	1.38	55±3.61	ND
5	1e	27.18	6.69	6.0	ND	ND
6	1f	13.84	11.86	3.9	ND	ND
7	1g*	6.8	3.43	4.37	58.5±8.66	69 ±10.39
8	1h	1.57	8.84	7.26	73.0±8.87	58.0±4.35
9	1i	1.43	5.16	12.10	87.53±3.79	80.6±4.03
10	1j	1.03	6.36	12.12	89.6±3.86	84.3±3.67
11	1k	>100	ND	ND	ND	ND
12	1l	>100	ND	ND	ND	ND
13	SSG*	4.06			81.37±5.49	85.37±9.37

*- compounds were tested at 25mg/Kg ip X 5 days; SSG = Sodium stibogluconate – (20mg/kg x 5days, i.p.)

Table3. *In vivo* efficacy of Compound 1c and its nanoparticle formulations both by IP and Oral route.

Compound	Route	Dose mg/kg	In vivo efficacy	
			7 th day	28 th day
1c	IP	50	80.72±8.59	83.4±6.54
		25	78.57±6.65	73.0±5.81
		10	72.23±6.59	70.16±13.64
Ic	Oral	100	87.33±5.80	75.55±8.38
		50	69.42±5.39	77.66±13.52
		25	42.66±2.05	28.5±8.50
Compound1c-DextranC4 Nanoparticles	1P	10	88.66±3.09	89.0±2.00
		5	80.75±2.86	86.0±2.16
		2.5	78.0±2.00	74.33±5.79
Compound1c-DextranC4 Nanoparticles	Oral	50	80.0±8.86	78.33±8.99
		25	77.66±4.1	62.87±4.50
		10	44.57±8.98	34.0±7.70
Meltifosine	Oral	25	91.57±3.20	96.5±0.55
SSG*	IP	20	81.37±5.49	85.37±9.37

*SSG: Sodium Stibogluconate

Table 4. Comparison of *In vivo* efficacy of Compounds, 1i and 1j both by IP and Oral routes.

Compound	Route	Dose mg/kg	In vivo efficacy	
			7 th day	28 th day
1j	IP	50	87.53±8.59	80.6±4.03
	Oral	100	91.0±3.87	70.00±11.60
Ij	1P	50	89.66±3.86	84.3±3.67
	Oral	100	90.20±5.07	54.50±13.40
Meltifosine	Oral	25	91.57±3.20	96.5±0.55
SSG*	IP	20	81.37±5.49	85.37±9.37

*SSG: Sodium Stibogluconate

Example 5: Effect of identified compounds on target enzymes; Leishmanial dipeptidylcarboxypeptidase (LdDCP) and mammalian angiotensin converting enzyme (ACE)

Recombinant leishmanial DCP was expressed in *E. coli* BL 21 pLysS (DE3) and purified to homogeneity as described earlier (Goyal N., Duncan R., Selvapandiyar, A., Debrabant, A., Baig, M.S., Nakhasi H.L. 2006, Cloning and characterization of angiotensin converting enzyme related dipeptidylcarboxypeptidase from *Leishmaniadonovani*. *Mol.Biochem.Parasitol.*;145:147–157). Enzyme activity was measured according to the method of Cushman and Cheung, using N-benzoyl-L-glycyl-L-histidyl-L-leucine (HHL), a routine substrate used for ACE (Cushman D.W., Cheung H.S., 1971, Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *BiochemPharmacol*; 20:1637–1648). Activity of LdDCP was carried out spectrophotometrically, which is based on the direct measurement of released hippuric acid from HHL, which absorbs at 228 nm. Inhibitory activity of all selected compounds against LdDCP activity was measured at 1 μ M concentrations. To determine K_i value of compounds, different concentrations of inhibitors were incubated with the enzymes (LdDCP and ACE) for 5 min prior to the addition of the substrate (three separate experiments). Rat serum was taken as source of mammalian ACE. Protein concentration was determined by Bradford method using bovine serum albumin as standard (Bradford M.M. 1976, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*; 72:248–254).

Results: Four compounds 1c, 1h, 1i and 1j inhibited specifically leishmanial LdDCP with K_i values in the range of 25–28 nM while same compounds inhibited mammalian ACE at thousand times higher concentration that is in μ M range (Table 5). The data clearly suggest that the identified compounds 1c, 1h, 1i, and 1j are the specific inhibitor of leishmanial DCP.

Table 5: Effect of identified compounds on Leishmanialdipeptidylcarboxypeptidase and mammalian ACE

Compound	K _i value	
	DCP (μM)	ACE (μM)
1c	0.025±5	147.6±6.2
1h	0.025±7.07	7±1.4
1i	0.0233±2.8	15.1±1.4
1j	0.02835±7	39±6.3
Captopril	0.035.8	0.0143*

*captopril is known inhibitor of ACE. The data in table is reported value for human testicular ACE (Deddish PA, Wang LX, Jackman HL, Michel B, Wang J, Skidgel, RA, Erdo's EG (1996) J PharmacolExpTher 279:1582

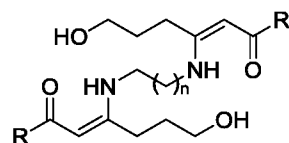
[054]ADVANTAGES OF THE PRESENT INVENTION

1. The compounds described herein are readily synthesized using cheap starting materials via only three step process.
2. The formulations described herein are readily prepared using cheap starting materials.
3. The compounds of general formula 1 display potential antileishmanial activity via oral and intraperitoneal administration.
4. The compounds of general formula 1 are specific inhibitor of leishmanial target enzyme dipeptidylcarboxypeptidase (ldDCP) and has no effect on mammalian counterpart angiotensin converting enzyme (ACE) at nM concentration.
5. The bio-efficacy of the nano formulations of compound of general formula 1 is increased by more than **5-fold** as compared to bulk compound under *in vivo* conditions via intraperitoneal route of administration.
6. Bio-efficacy of nanoformulation of compound of general formula 1 coated with Edragit is increased by two fold under *in vivo* condition via Oral route of administration as

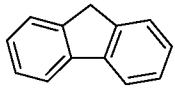
compared to bulk compound. Eudragit polymer is the anionic acrylic polymer that provided stability to the orally delivered biomolecules towards acidic pH, thus enhanced two fold bioefficacy of compound by oral administration as compared to bulk compound.

We CLAIM:

1. A novel controlled release antileishmanial formulation comprising a compound of formula 1



Compound 1

wherein R is selected from  or ,

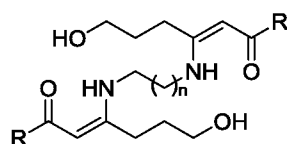
wherein X is selected from a group consisting of Cl, Br and F; n is the number of methylene groups (n = 1, 2 and 3); encapsulated in alkyl dextran.

2. A formulation as claimed in claim 1, wherein the formulation comprising a compound selected from a group consisting of :
 - i. (2Z,2Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-fluorophenyl)-6-hydroxyhex-2-en-1-one) (**1a**)
 - ii. (2Z,2Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-chlorophenyl)-6-hydroxyhex-2-en-1-one) (**1b**)
 - iii. (2Z,2Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1c**)
 - iv. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1d**)
 - v. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1e**)
 - vi. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1f**)
 - vii. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1g**).
 - viii. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1h**).

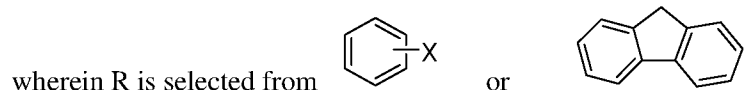
- ix. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1i**).
 - x. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1j**).
 - xi. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1k**).
 - xii. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1l**).
3. A formulation as claimed in claim 1, wherein the alkyl dextran is selected from a group consisting of O-butyl dextran, O-hexyl dextran, O-octyl dextran, O-dodecyl dextran, O-hexadecyl dextran.
 4. A formulation as claimed in claim 1, wherein the O-alkyl-dextran is optionally coated with Eudraigt, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate trimellitate, sodium alginate.
 5. A formulation as claimed in claim 1, wherein about 80% drug from the formulation is released in about 48 hours.
 6. A formulation as claimed in Claim 1 the formulation has *in vivo* antileishmanial activity via intraperitoneal route at dose 2.5-50mg/Kg in hamster model and has five times enhanced bioefficacy as compared to bulk drug.
 7. A formulation as claimed in Claim 1, wherein the formulation has *in vivo* antileishmanial activity via oral route at dose 25-100mg/Kg in hamster model and has two times enhanced bioefficacy as compared to bulk drug.
 8. A process for preparation of formulation as claimed in claim1, wherein the process comprising the steps:
 - a. preparing a suspension of the compound of general formula 1 with O-alkyl dextran in a solvent selected from a group consisting of DMSO, and DMF with vigorously stirring followed by dropwise addition of water and stirring for a period ranging between 30min to 40min at a temperature ranging between 30°C to 35°C,

- b. optionally adding a solution of polymer and co-polymer to the suspension obtained in step (a) and further stirring for a period of 3hr to 5hr at a temperature ranging between 30°C to 35°C ,
 - c. dialyzing the suspension obtained in step (a) or (b) for a period of 2 to 4 days, followed by the freeze drying to obtain solid dried nanoparticle.
9. The process as claimed in claim 8, wherein the polymer is selected from the group consisting of PVA, PEG, and BSA.
10. The process as claimed in claim 8, wherein the co-polymer is selected from the group consisting of Eudragit, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate trimellitate, and sodium alginate.
11. The process as claimed in claim 8, wherein the size of nanoparticle is found in the range of 190-210 nm.
12. The process as claimed in claim 8, wherein the O-alkyldextran is prepared by the process comprising steps:
- i. treating the dextran with % solution of sodium hydroxide ranging between 10-40% in the presence of catalyst selected from the group consisting of aliquat, tetrabutylammonium bromide, tetrabutylammonium hydrogen sulfate, and tetramethylammonium chloride followed by heating in the range of 50-80°C to obtain a solution,
 - ii. adding n-bromoalkane in THF in the step (I) solution dropwise with stirring at the temperature ranging between 50-80 °C for the time in the range of 3-6 hr, followed by concentrating the reaction mixture on rotavapor to obtain a residual mass,
 - iii. dissolving the residual mass of step (ii) in diethylether followed by the filtration and washing with diethyl ether to obtain a solid,
 - iv. dissolving the solid of step (iii) in deionized water followed by dialysis and lyophilization for the time in the range of 12-24hr at the temperature ranging between 30-35°C to obtain solid dry product.

13. The process as claimed in claim 12, wherein the n-bromoalkane is selected from the group consisting of 1-bromobutane, 1-bromohexane, 1-bromooctane, 1-bromododecane, and 1-bromohexadecane.
14. The process as claimed in claim 12, wherein the solvent is selected from the group consisting of THF, dioxane, and n-butanol,
15. A compound of formula 1,



Compound 1



wherein X is selected preferably from halogens (Cl, Br and F) and 'n' is the number of methylene groups (n = 1, 2 and 3); wherein the novel compound comprising:

- i. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1d**)
- ii. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1e**)
- iii. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1f**)
- iv. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1g**).
- v. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1h**).
- vi. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1i**).
- vii. (2Z,2Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1j**).
- viii. (2Z,2Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1k**).

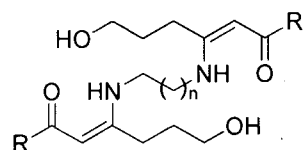
- ix. (2Z,2Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**11**).
16. A compound as claimed in claim 15, wherein Compounds of formula 1 are the specific inhibitor of parasite target enzyme dipeptidylcarboxy peptidase with no effect on mammalian counterpart angiotensin converting enzyme at nanomolar concentration.

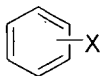
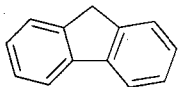
AMENDED CLAIMS

received by the International Bureau on 20 June 2016 (20.06.2016)

We CLAIM:

1. A novel controlled release antileishmanial formulation comprising a compound of formula 1

**Compound 1**

wherein R is selected from  or ,

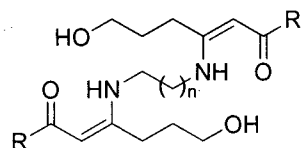
wherein X is selected from a group consisting of Cl, Br and F; n is the number of methylene groups (n = 1, 2 and 3); encapsulated in alkyl dextran.

2. A formulation as claimed in claim 1, wherein the formulation comprising a compound selected from a group consisting of :
 - i. (2Z,2'Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-fluorophenyl)-6-hydroxyhex-2-en-1-one) (**1a**)
 - ii. (2Z,2'Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-chlorophenyl)-6-hydroxyhex-2-en-1-one) (**1b**)
 - iii. (2Z,2'Z)-3,3'-(Ethane-1,2-diylbis(azanediyl))bis(1-(4-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1c**)
 - iv. (2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1d**)
 - v. (2Z,2'Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1e**)
 - vi. (2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(2-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1f**)
 - vii. (2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1g**).
 - viii. (2Z,2'Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1h**).

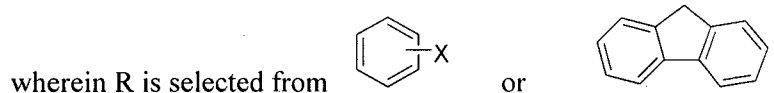
- ix. (2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1i**).
 - x. (2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1j**).
 - xi. (2Z,2'Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1k**).
 - xii. (2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1l**).
3. A formulation as claimed in claim 1, wherein the alkyl dextran is selected from a group consisting of O-butyl dextran, O-hexyl dextran, O-octyl dextran, O-dodecyl dextran, O-hexadecyl dextran.
 4. A formulation as claimed in claim 1, wherein the O-alkyl-dextran is optionally coated with Eudragit, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate trimellitate, sodium alginate.
 5. A formulation as claimed in claim 1, wherein about 80% drug from the formulation is released in about 48 hours.
 6. A formulation as claimed in Claim 1 the formulation has *in vivo* antileishmanial activity via intraperitoneal route at dose 2.5-50mg/Kg in hamster model and has five times enhanced bioefficacy as compared to bulk drug.
 7. A formulation as claimed in Claim 1, wherein the formulation has *in vivo* antileishmanial activity via oral route at dose 25-100mg/Kg in hamster model and has two times enhanced bioefficacy as compared to bulk drug.
 8. A process for preparation of formulation as claimed in claim 1, wherein the process comprising the steps:
 - a. preparing a suspension of the compound of general formula 1 with O-alkyl dextran in a solvent selected from a group consisting of DMSO, and DMF with vigorously stirring followed by dropwise addition of water and stirring for a period ranging between 30min to 40min at a temperature ranging between 30°C to 35°C,

- b. optionally adding a solution of polymer and co-polymer to the suspension obtained in step (a) and further stirring for a period of 3hr to 5hr at a temperature ranging between 30°C to 35°C ,
 - c. dialyzing the suspension obtained in step (a) or (b) for a period of 2 to 4 days, followed by the freeze drying to obtain solid dried nanoparticle.
9. The process as claimed in claim 8, wherein the polymer is selected from the group consisting of PVA, PEG, and BSA.
10. The process as claimed in claim 8, wherein the co-polymer is selected from the group consisting of Eudragit, cellulose acetate succinate, hydroxy propyl methyl cellulose phthalate, hydroxy propyl methyl cellulose acetate succinate, polyvinyl acetate phthalate, cellulose acetate trimellitate, and sodium alginate.
11. The process as claimed in claim 8, wherein the size of nanoparticle is found in the range of 190-210 nm.
12. The process as claimed in claim 8, wherein the O-alkyldextran is prepared by the process comprising steps:
- i. treating the dextran with % solution of sodium hydroxide ranging between 10-40% in the presence of catalyst selected from the group consisting of aliquat, tetrabutylammonium bromide, tetrabutylammonium hydrogen sulfate, and tetramethylammonium chloride followed by heating in the range of 50-80°C to obtain a solution,
 - ii. adding n-bromoalkane in THF in the step (i) solution dropwise with stirring at the temperature ranging between 50-80 °C for the time in the range of 3-6 hr, followed by concentrating the reaction mixture on rotavapor to obtain a residual mass,
 - iii. dissolving the residual mass of step (ii) in diethylether followed by the filtration and washing with diethyl ether to obtain a solid,
 - iv. dissolving the solid of step (iii) in deionized water followed by dialysis and lyophilization for the time in the range of 12-24hr at the temperature ranging between 30-35°C to obtain solid dry product.

13. The process as claimed in claim 12, wherein the n-bromoalkane is selected from the group consisting of 1-bromobutane, 1-bromohexane, 1-bromooctane, 1-bromododecane, and 1-bromohexadecane.
14. The process as claimed in claim 12, wherein the solvent is selected from the group consisting of THF, dioxane, and n-butanol,
15. A compound of formula 1,



Compound 1



wherein X is selected preferably from halogens (Cl, Br and F) and 'n' is the number of methylene groups (n = 1, 2 and 3); wherein the novel compound comprising:

(2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(3-bromophenyl)-6-hydroxyhex-2-en-1-one) (**1i**).

(2Z,2'Z)-3,3'-(ethane-1,2-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1j**).

(2Z,2'Z)-3,3'-(propane-1,3-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1k**).

(2Z,2'Z)-3,3'-(butane-1,4-diylbis(azanediyl))bis(1-(9H-fluoren-2-yl)-6-hydroxyhex-2-en-1-one) (**1l**).

Scheme-1

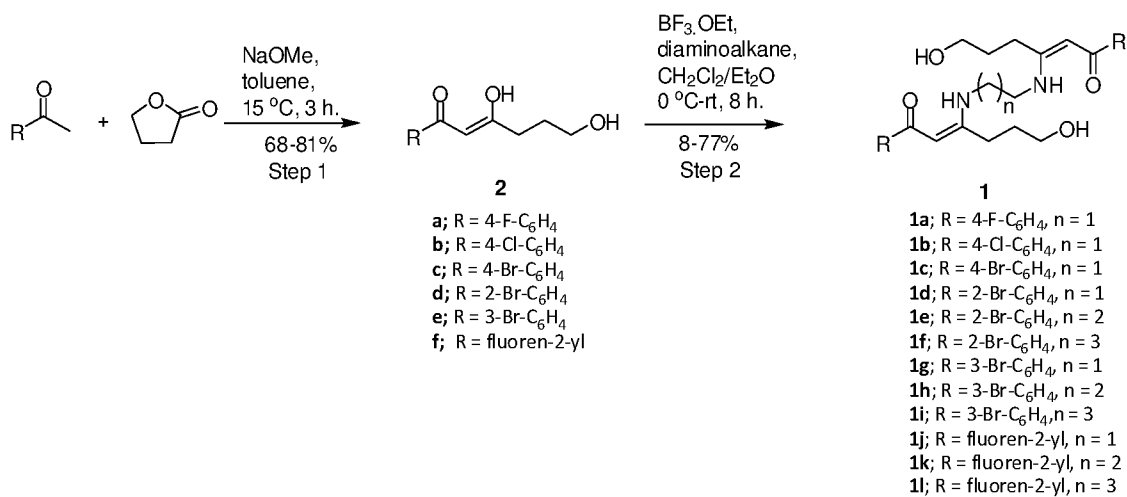


Figure-1

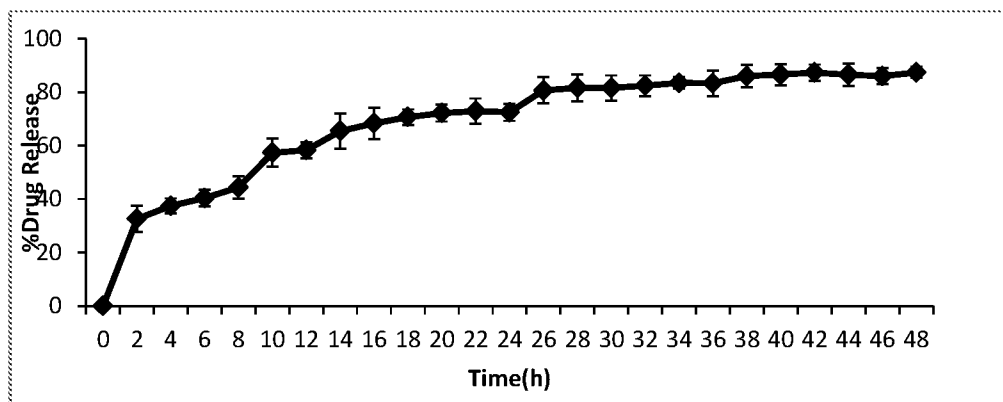


Figure-2

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2016/050019

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	A61K9/00	A61K47/36
	A61K31/135	C07C225/14
		C07C225/16
		A61P33/02
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
A61K C07C		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SONALI GANGWAR ET AL: "Identification of Novel Inhibitors of Dipeptidylcarboxypeptidase of Leishmania donovani via Ligand-Based Virtual Screening and Biological Evaluation", CHEMICAL BIOLOGY & DRUG DESIGN., vol. 79, no. 2, 28 February 2012 (2012-02-28), pages 149-156, XP055264013, GB	15,16
A	ISSN: 1747-0277, DOI: 10.1111/j.1747-0285.2011.01262.x cited in the application table 1	1-14
	----- -/--	
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
11 April 2016		18/04/2016
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Kollmannsberger, M

INTERNATIONAL SEARCH REPORT

International application No

PCT/IN2016/050019

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ANTHONY D. DUONG ET AL: "Electrospray Encapsulation of Toll-Like Receptor Agonist Resiquimod in Polymer Microparticles for the Treatment of Visceral Leishmaniasis", MOLECULAR PHARMACEUTICS, vol. 10, no. 3, 4 March 2013 (2013-03-04), pages 1045-1055, XP055181864, ISSN: 1543-8384, DOI: 10.1021/mp3005098 the whole document -----	1-14
A	SIJUMON KUNJACHAN ET AL: "Chitosan-based macrophage-mediated drug targeting for the treatment of experimental visceral leishmaniasis", JOURNAL OF MICROENCAPSULATION., vol. 28, no. 4, 17 May 2011 (2011-05-17), pages 301-310, XP055264021, GB ISSN: 0265-2048, DOI: 10.3109/02652048.2011.559281 the whole document -----	1-14
A	SOFIA COSTA LIMA ET AL: "In vitro evaluation of bisnaphthalimidopropyl derivatives loaded into pegylated nanoparticles against Leishmania infantum protozoa", INTERNATIONAL JOURNAL OF ANTIMICROBIAL AGENTS, vol. 39, no. 5, 1 May 2012 (2012-05-01), pages 424-430, XP055264011, AMSTERDAM, NL ISSN: 0924-8579, DOI: 10.1016/j.ijantimicag.2012.01.003 the whole document -----	1-14
A	ALICE MACHADO-SILVA ET AL: "New perspectives for leishmaniasis chemotherapy over current anti-leishmanial drugs: a patent landscape", EXPERT OPINION ON THERAPEUTIC PATENTS., vol. 25, no. 3, 20 December 2014 (2014-12-20), pages 247-260, XP055264040, GB ISSN: 1354-3776, DOI: 10.1517/13543776.2014.993969 the whole document -----	1-16