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ORIGINAL PAPER

Synthesis, characterization, in vitro biological and molecular docking evaluation of *N,N'*-(ethane-1,2-diyl)bis(benzamides)

Hamid Aziz[1] · Aamer Saeed[1] · Farukh Jabeen[2,3] · Nazif Ullah[4] · Ashfaq Ur Rehman[5,6]

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Abstract

The present research describes the synthesis, characterization, in vitro biological and docking evaluation of *N,N'*-(ethane-1,2-diyl)bis(benzamides) **(3a-3j)**. Consequently, in in vitro *h*RBCs hemolysis assay, only the *bis*-amide **(3d)** induced 52.4% hemolysis at higher concentration (1000 µg/mL) that decreased drastically with concentration (250 µg/mL) to 27.9% (CC_{50} = 400.41). Similarly, the tested *bis*-amide **(3j)** was found to be the least toxic with 7.8% hemolysis at higher concentration (1000 µg/mL) that gradually decreases to 6.1% (CC_{50} = 19,347.83) at lower concentration (250 µg/mL). Accordingly, the tested *bis*-amides were found to be highly biocompatible against *h*RBCs at higher concentrations with much higher CC_{50} values (> 1000 µg/mL). The biocompatible *bis*-amides **(3a-3j)** were subjected to in vitro DNA ladder assay to analyze their apoptotic potential. The results obtained suggest the tested *bis*-amides **(3a-3j)** are highly degradative toward DNA causing the appearance of more than one bands or complete degradation of DNA except **(3a), (3c), (3i)** and **(3 g)**. Moreover, the synthesized *bis*-amides **(3a-3j)** were tested in in vitro *antileishmanial* assay to unveil their *leishmaniacidal* potential. The results obtained clearly indicated that some of the tested *bis*-amides displayed good dose dependent response. The tested *bis*-amides were highly active at higher concentration (1000 µg/mL) against the *leishmanial* promastigotes and their % inhibitory potential decreased drastically with concentration (250 µg/mL). Consequently, at higher concentration (1000 µg/mL), the *bis*-amide **(3f)** caused 85% inhibition and was ranked as the most effective *leishmaniacidal bis*-amides followed by the *bis*-amide **(3 g)** with 73.54% inhibition of *leishmanial* promastigotes. However, in terms of their IC_{50} values, the best *leishmaniacidal* potential was displayed by the *bis*-amide **(3f)** followed by **(3b), (3j)** and **(3 g)** with IC_{50} values increasing in the order of 633.16, 680.22, 680.22 and 712.93 µg/mL, respectively. Molecular docking studies revealed that *bis*-amides having electron-donating groups showed good binding potential against *antileishmanial* target.

✉ Hamid Aziz
hamidazizwazir@yahoo.com

[1] Department of Chemistry, Quaid-I-Azam University, Islamabad 45320, Pakistan

[2] Department of Biology, Laurentian University, 935 Ramsey Lake Road, Sudbury, ON P3E 2C6, Canada

[3] Computation, Science, Research and Development Organization, 1401, 2485 Hurontraio street, Mississauga, ON L5A2G6, Canada

[4] Department of Biotechnology, Abdul Wali Khan University, Mardan, Khyber Pukhtoonkhwa, Pakistan

[5] Department of Bioinformatics and Biostatistics, Shanghai Jiao Tong University, 800 Dongchuan Road,, Minhang District, Shanghai 200240, China

[6] Department of Biochemistry, Abdul Wali Khan University, Shankar Campus, Mardan, Khyber Pukhtoonkhwa, Pakistan

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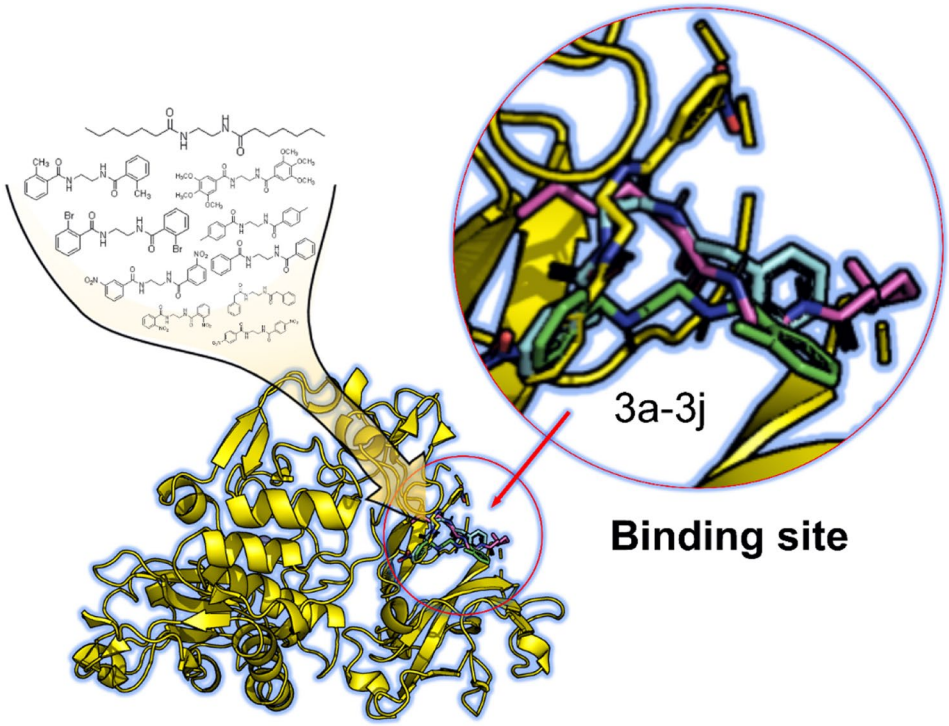
Graphic abstract



Keywords Agarose gel electrophoresis · Apoptosis · Biocompatibility · *Bis*-amides · DNA · *Leishmania tropica* · Promastigotes · Human red blood cells

Introduction

Leishmaniasis is a neglected vector-borne skin disease being caused by the bite of obligate intracellular protozoan of the genus *Leishmania. Leishmanial* parasite possesses diverse survival pathways and performs various physiological functions. Species of the genus *leishmania* transmit easily to its mammalian host and infects external as well as internal parts of the human body [1]. Primary therapies of *Leishmaniases* include Glucantime and Pentostam. Similarly, Pentamidine and Amphotericin B are used as second line treatment. Moreover, the phenomenon of emerging resistance mechanism in the established key targets affects the use of drugs negatively. This makes the drugs inefficient, troublesome, and therefore, the development of potent *antileishmanial* drugs with alternative and efficient mechanism of action is required to eradicate this disease [2, 3]. In this context, literature survey suggests nitrogen-based heterocycles as biologically relevant scaffolds having possible *leishmaniacidal* as well as biosafe nature [4, 5].

Nitrogen-based heterocycles have immense importance because of their potential applications as propellants, pyrotechnics, pharmaco and chemotherapeutics [6, 7]. Amides are nitrogen-containing heterocycles formed by coupling carboxylic acids and amines in the presence of a coupling reagent or by prior conversion of the carboxylic acid into reactive acid chlorides. Amide bond is considered as one of the most stable functionalities in organic chemistry and is widely distributed in living organisms. Amide bond constitutes key functional group in peptides, natural products and pharmaceuticals. Amide bonds because of their higher polarity, stability and conformational diversity constitute most abundant motif in synthetic organic chemistry [4, 8, 9]. Amide-based heterocycles serve as efficient precursors and intermediates in organic synthesis to synthesize various agrochemicals, lubricants and drugs. The potent antibiotic and anti-inflammatory drugs valdecoxib **(1)**, celecoxib **(2)**, and penicillin G **(3)** contain amide linkages in their structures [10–12]. Natural and synthetic benzamides are reported as potent enzyme inhibitors [13, 14], antimicrobials [15], cytotoxic [16] and anti-inflammatory agents [17]. The development of potent and selective COX-2 inhibitors having lower side effects and improved gastric safety profile is a challenging task for medicinal chemists to deal with. In this regard, benzamide derivatives are reported as in vitro COX inhibitors and in vivo anti-inflammatory agents [18]. More

specifically, peptide derivatives possessing SO_2Me and N_3 pharmacophores are rationalized as selective COX-2 inhibitors with selectivity index above 500 [19]. *N*-phenylacetamide-based functionalized carbazoles **(4)** are reported as novel leading template in the chemotherapeutic treatments of various bacterial ailments [20]. Amide-based heterocycles offer opportunity for the development of novel drugs against tuberculosis. Alanis et al. revealed the synthesis and potent antimycobacterial activity of α,β-unsaturated amides [21]. Benzothiazole-based amides are highly biocompatible and display in vitro antiproliferative potential against cancer cells [22].

To unveil the *leishmaniacidal* potential of amide containing heterocycles, Piplartine **(5)** has been reported to possess in vitro *leishmaniacidal* potential against promastigotes of *Leishmania donovani*. The tested compound showed $89.1 \pm 2.9\%$ inhibition at 100 µM concentration with IC_{50} value of 7.5 µM [23]. Similarly, amide derivatives of isoxazole **(6)** display promising antileishmanial potential against *Leishmania donovani* and could be a possible hit against *leishmaniases* [24]. 3-Nitro-1*H*-1,2,4-triazole as well as 2-nitro-1*H*-imidazole based amides **(7)** and sulfonamides are tested against intracellular amastigotes of *Trypanosoma cruzi*. Consequently, most of the tested compounds were found to be even more potent (up to 58-fold) than the reference drug Benznidazole [25]. Amides derivatives **(8)** extracted from *Piper amalago* and some of the synthetic analogues are found to be active against the promastigote and intracellular amastigote forms with IC_{50} values in nanomolar range [26]. Chiral amides **(9–10)** with two asymmetric centers and C_2 axis of symmetry were tested against *Leishmania tropica* KWH23 Promastigotes. The tested compounds particularly their deprotected analogues showed good to moderate in vitro *leishmaniacidal* potential [4]. Recently, in vitro biocompatible and *leishmaniacidal* potential of *N,N'*-(ethane-1,2-diyl)bis(3-methylbenzamide) against the promastigotes of *Leishmania tropica* has been revealed. The tested *bis*-amide displayed *leishmaniacidal* potential higher than the reference standard Glucantime. Molecular docking analysis revealed that the bisamide strongly bind to the active site of Trypanothione reductase, an enzyme involved in the redox metabolism of the *Leishmanial* parasite [27]. Figure 1 shows some potent heterocyclic analogues containing amide functionality.

Thus, considering the phenomenon of *leishmaniasis*, the lack of effective biocompatible drugs and the *leishmaniacidal* potential of heterocyclic amide analogues, there is a need for the synthesis of biocompatible *antileishmanial* amides. In this regard, the present research paper reports the facile synthesis, spectroscopic characterizations, in vitro biological and docking evaluation of *N,N'*-(ethane-1,2-diyl) bis(benzamides). The synthetic work completes in a simple and facile way with excellent yields using substituted organic acids in dry distilled THF at room temperature.

Materials and methods

All the reagents and solvents used were purchased from Sigma-Aldrich and were used without any further purifications. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance AVIII spectrometer operating at 400 and 100 MHz for ^1H and ^{13}C NMR, respectively. Deuterated methanol (CD_3OD-d_4) was used as a solvent and TMS as an internal reference. Chemical shifts values are reported in ppm (δ), relative integral, multiplicity (*s*, singlet,

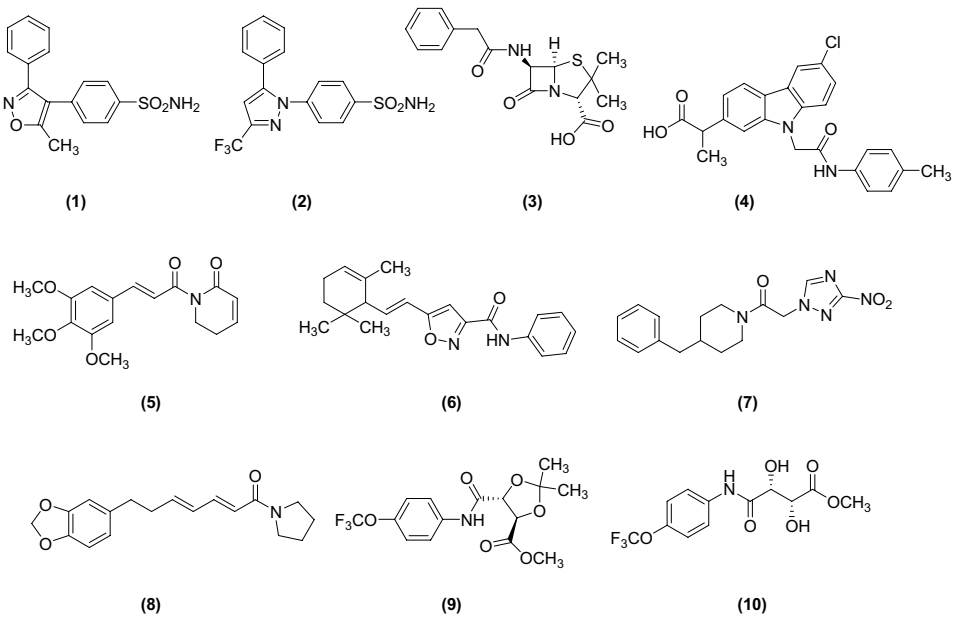


Fig. 1 Some literature reported biologically relevant and potent amide containing heterocycles

d, doublet, dd, doublet of doublets, *t*, triplet, *m*, multiplet; *b*, broad peak), coupling constant (*J*, *Hz*) and the atom assignment. ^{13}C NMR data are reported as the chemical shift value (δ) and assignment of the atom. FT-IR spectra were recorded on the Vertex 70 Bruker apparatus. % Elemental analysis (CHNS) was performed to find out the percentage of each element present in the synthesized compounds. The progress of the reaction was checked by thin layer chromatography (TLC) on 2.0 cm × 5.0 cm aluminum sheets precoated with silica gel 60F254 with a layer thickness of 0.25 mm (Merck).

Synthesis of *N,N′*-(Ethane-1,2-diyl)bis(benzamides) (3a-3j)

The synthesis of *bis*-amides **(3a-3j)** was performed at room temperature in dry distilled THF as reported elsewhere [27]. Briefly, substituted organic acids (5 mmol) were treated with thionyl chloride (5.5 mmol) in dry distilled THF (10 mL) at room temperature for half an hour (hr). A solution of ethylenediamine (2.50 mmol) dissolved in THF (5 mL) was added dropwise to the reaction mixture, and the mixture was stirred for next half an hr. During the course of the reaction, TLC in *n*-hexane/ethyl acetate (1:1) solvent system was constantly used to monitor the progress of the reaction. Once the reaction was completed, THF was rotary evaporated to obtain a solid product. The solid product obtained was purified by washing it with sodium carbonate solution (2%), distilled water and was later on recrystallized from methanol at room temperature.

N,N′-(Ethane-1,2-diyl)bis(4-nirobenzamide) (3a)

Yield (90%): m.p. 250 ℃; R_f; 0.80 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 358.31; IR (ATR) ν: 3299.63 (NH), 3077.92 (C=C–H), 1636.55 (C=O, amide), 1616.67, 1551.52, 1522.59, 1453.83 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.08 (b, s, 2H, NH), 8.40 (d, 4H, *J* = *8.10 Hz,* Ar–H), 8.23 (d, 4H, *J* = *8.10 Hz,* Ar–H), 3.53 (s, 4H, C-H); ^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 168.70 (C=O), 151.93 (Ar), 140.33 (Ar), 128.43 (Ar), 121.23 (Ar), 39.73 (C-N); Anal. Calcd. For $C_{16}H_{14}N_4O_6$: C, 53.63%; H, 3.94%; N, 15.64%; Found: C, 53.60%; H, 3.96%; N, 15.65%.

N,N′-(Ethane-1,2-diyl)bis(phenylacetamide) (3b)

Yield (80%): m.p. 163 ℃; R_f; 0.60 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 296.36; IR (ATR) ν: 3307.27 (NH), 3027.10 (C=C–H), 2928.33 (–C-–H), 1660.51 (C=O, amide), 1625.13, 1494.37, 1454.38, 1452.37 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.0 (b, s, 2H, NH), 7.97 (d, 4H, *J* = *8.20 Hz,* Ar–H), 7.52 (t, 2H, *J* = *8.20 Hz,* Ar–H), 7.46 (t, 4H, *J* = *8.20 Hz,* Ar–H), 3.62 (s, 4H, C-H), 3.56 (s, 4H, C-H); ^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 170.76 (C=O), 135.62 (Ar), 132.69 (Ar), 129.56 (Ar), 128.30 (Ar), 40.89 (C–C), 39.23 (C-N); Anal. Calcd. For $C_{18}H_{20}N_2O_2$: C, 72.95%; H, 6.80%; N, 9.45%; Found: C, 72.86%; H, 6.85%; N, 9.49%.

N,N′-(Ethane-1,2-diyl)bis(3-nirobenzamide) (3c)

Yield (85%): m.p. 225 ℃; R_f; 0.69 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 358.31; IR (ATR) ν: 3234.45 (NH), 3075.74, 2974.83 (C = C-H), 2865.48 (-C-H), 1637.83 (C = O, amide), 1623.64, 1559.84, 1523.67, 1450.85 (Ar–C = C stretch), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.09 (b, s, 2H, NH), 8.91 (s, 2H, Ar–H), 8.47 (d, 2H, *J* = *8.60 Hz,* Ar–H), 8.37 (d, 2H, *J* = *8.60 Hz,* Ar–H), 7.73 (t, 2H, *J* = *7.80 Hz,* Ar–H), 3.52 (s, 4H, C-H); ^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 167.63 (C = O), 148.53 (Ar), 135.31 (Ar), 133.63 (Ar), 129.83 (Ar), 124.53 (Ar), 122.43 (Ar), 39.80 (C-N); Anal. Calcd. For $C_{16}H_{14}N_4O_6$: C, 53.63%; H, 3.94%; N, 15.64%; Found: C, 53.58%; H, 3.99%; N, 15.84%.

N,N′-(Ethane-1,2-diyl)bis(2-nirobenzamide) (3d)

Yield (82%): m.p. 256 ℃; R_f; 0.80 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 358.31; IR (ATR) ν: 3314.39 (NH), 3080.99, 2946.59 (C=C–H), 2867.60 (–C–H), 1636.53 (C=O amide), 1615.54, 1553.74, 1521.69, 1450.92 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.09 (b, s, 2H, NH), 8.40 (d, 2H, *J* = *8.29 Hz,* Ar–H), 8.24 (d, 2H, *J* = *8.29 Hz,* Ar–H), 7.86 (t, 2H, *J* = *8.20 Hz,* Ar–H), 7.80 (t, 2H, *J* = *8.20 Hz,* Ar–H), 3.63 (s, 4H, C-H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 170.83 (C=O), 147.23 (Ar), 135.13 (Ar), 133.13 (Ar), 128.43 (Ar), 127.95 (Ar), 121.23 (Ar), 39.73 (C-N); Anal. Calcd. For $C_{16}H_{14}N_4O_6$: C, 53.63%; H, 3.94%; N, 15.64%; Found: C, 53.62%; H, 3.95%; N, 15.64%.

N,N′-(Ethane-1,2-diyl)bis(4-methylbenzamide) (3e)

Yield (80%): m.p. 237 ℃; R_f; 0.82 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 296.36; IR (ATR) ν: 3305.57 (NH), 3043.82, 2939.27 (C=C–H), 2815.19, 2853.97 (–C–H), 1628.60 (NCO), 1613.21, 1573.59, 1536.47, 1440.16 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.0 (b, s, 2H, NH), 7.74 (d, 4H, *J* = *8.10 Hz,* Ar–H), 7.29 (d, 4H, *J* = *8.10 Hz,* Ar–H), 3.62 (s, 4H, C-H), 2.41 (s, 6H, C-H); ^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 170.77 (C=O), 143.42 (Ar), 132.75 (Ar), 130.17 (Ar), 128.36 (Ar), 40.92 (C–N), 21.42 (C-H); Anal. Calcd. For $C_{18}H_{20}N_2O_2$: C, 72.95%; H, 6.80%; N, 9.45%; Found: C, 72.88%; H, 6.85%; N, 9.57%.

N,N′-(Ethane-1,2-diyl)bis(2-methylbenzamide) (3f)

Yield (80%): m.p. 265 ℃; R_f; 0.70 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 296.36; IR (ATR) ν: 3315.47 (NH), 3056.56, 2952.65 (C=C–H), 2822.15, 2858.86 (–C–H), 1658.56 (C=O, amide), 1618.27, 1577.58, 1565.43, 1442.65 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.0 (b, s, 2H, NH), 7.86 (d, 2H, *J* = *8.29 Hz,* Ar–H), 7.28 (t, 2H, *J* = *8.20 Hz,* Ar–H), 7.42 (t, 2H, *J* = *8.20 Hz,* Ar–H), 7.27 (d, 2H, *J* = *8.29 Hz,* Ar–H); 3.50 (s, 4H, C-H), 2.27 (s, 6H, C-H); ^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 170.10 (C=O), 137.25 (Ar), 135.43 (Ar), 132.41 (Ar), 129.45 (Ar), 127.67 (Ar), 125.93 (Ar), 39.25 (C–N), 17.23 (C–H); Anal. Calcd. For $C_{18}H_{20}N_2O_2$: C, 72.95%; H, 6.80%; N, 9.45%; Found: C, 72.90%; H, 6.74%; N, 9.56%.

N,N′-(Ethane-1,2-diyl)bis(benzamide) (3 g)

Yield (80%): m.p. 175 ℃; R_f; 0.52 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 268.31; IR (ATR) ν: 3294.04 (NH), 3063.22 (C=C–H), 2945.41 (–C–H), 1631.86 (C=O, amide), 1603.64, 1579.15, 1551.75 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.0 (b, s, 2H, NH), 7.83–7.80 (m, 4H, Ar–H), 7.54–7.50 (m, 2H, Ar–H), 7.47–7.43 (m, 4H, Ar–H), 3.62 (s, 4H, C–H); ^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 170.76 (C=O), 135.62 (Ar), 132.69 (Ar), 129.56 (Ar), 128.30 (Ar), 40.89 (C–N); Anal. Calcd. For $C_{16}H_{16}N_2O_2$: C, 71.62%; H, 6.01%; N, 10.44%; Found: C, 71.55%; H, 6.15%; N, 10.43%.

N,N′-(Ethane-1,2-diyl)bis(2-bromobenzamide) (3 h)

Yield (90%): m.p. 251.60 ℃; R_f; 0.60 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 426.1; IR (ATR) ν: 3273.53 (NH), 3069.35 (C=C–H), 2943.68 (–C–H), 1639.99 (C=O, amide), 1589.32, 1538.64, 1464.81, 1451.21 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.10 (b, s, 2H, NH), 7.86 (d, 2H, *J* = *8.29 Hz,* Ar–H), 7.42 (t, 2H, *J* = *8.20 Hz,* Ar–H), 7.28 (t, 2H, *J* = *8.20 Hz,* Ar–H), 7.27 (d, 2H, *J* = *8.29 Hz,* Ar–H), 3.50 (s, 4H, C-H);^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 169.63 (C=O), 136.83 (Ar), 134.43 (Ar), 131.83 (Ar), 129.73 (Ar), 127.93 (Ar), 120.72 (Ar), 39.71 (C–N); Anal. Calcd. For $C_{16}H_{14}Br_2N_2O_2$: C, 45.10%; H, 3.31%; N, 6.57%; Found: C, 45.0%; H, 3.36%; N, 6.62%.

N,N′-(Ethane-1,2-diyl)bis(3,4,5-trimethoxybenzamide) (3i)

Yield (75%): m.p. 117 ℃; R_f; 0.70 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 448.71; IR (ATR) ν: FT-IR (ATR, cm^{-1}): 3353.68 (NH), 3069.35, 2980.43 (C=C–H), 2943.68, 2895.89 (-C-H), 1644.76 (C=O, amide), 1583.33, 1546.84, 1490.81, 1460.25 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.10 (b, s, 2H, NH), 6.93 (s, 4H, Ar–H), 3.78 (s, 18H, C-H), 3.53 (s, 4H, C–H);^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 169.63 (C=O), 150.93 (Ar), 142.63 (Ar), 128.53 (Ar), 105.13 (Ar), 56.56 (O–C), 39.73 (C–N); Anal. Calcd. For $C_{22}H_{28}N_2O_8$: C, 58.92%; H, 6.29%; N, 6.25%; Found: C, 58.82%; H, 6.32%; N, 6.32%.

N,N′-(Ethane-1,2-diyl)bis(heptylamide) (3j)

Yield (90%): m.p. 254.6 ℃; R_f; 0.50 (*n*-hexane/ethyl acetate 1:1); color: white powder; Mol. wt: 284.44; IR (ATR) ν: 3293.90 (NH), 3087.02, 2953.54 (C=C–H), 2819.90, 2856.31 (–C–H), 1635.13 (C=O, amide), 1553.79, 1469.78, 1448.72 (Ar–C=C), cm^{-1}; ^{1}H NMR (CD_3OD-d_4, 400 MHz) δ: 11.10 (b, s, 2H, NH), 3.50 (s, 4H, C-H), 2.21 (t, 4H, *J* = *7.30 Hz*, C–H), 1.60 (q, 4H, *J* = *7.20 Hz*, C-H), 1.36 (q, 4H, *J* = *7.20 Hz*, C-H), 1.26–1.30 (m, 8H, C-H), 0.90 (t, 6H, *J* = *7.50 Hz*, C-H); ^{13}C NMR (CD_3OD-d_4, 100 MHz) δ: 172.73 (C=O), 39.23 (C-N), 36.53 (C-H), 31.73 (C-H), 27.45 (C-H), 24.34 (C-H), 22.83 (C-H), 14.13 (C-H); Anal. Calcd. For $C_{16}H_{32}N_2O_2$: C, 67.56%; H, 11.34%; N, 9.85%; Found: C, 67.49%; H, 11.40%; N, 9.83%.

In vitro biological assays

% *h*RBCs Hemolysis assay

Cytotoxic testing of the synthesized *bis*-amides **(3a-3j)** was performed to confer their biosafe nature using fresh human red blood cells (*h*RBCs) hemolysis assay [27]. Briefly, 5 mL of the *h*RBCs was diluted with phosphate buffer saline (PBS) and were centrifuged at 1500 rpm for 10 min (mins) to collect the erythrocytes. The blood suspensions were incubated for 3 h at 37 ℃ using 250 ppm, 500 ppm and 1000 ppm solutions of the *bis*-amides **(3a-3j)**. The incubated mixtures were again centrifuged at 1500 rpm for 10 min, and the hemolysis was observed through naked eye. Hundred microliters of the supernatant from each concentration was poured into 96-well plate to assess the amount of hemoglobin released in the supernatants measured at 576 nm using UV–visible spectrophotometry. Triton X-100 (0.5%) was used as positive control, while methanol (5%) was taken as negative control. % Hemolysis was calculated from Eq. (1) given below.

$$\%\ \text{Hemolysis} = \left[\frac{\text{OD at576nm in bisamide solution} - \text{ODat576nminPBS}}{\text{ODat576nmin0.5\%Triton}X - 100 - \text{ODat576nminPBS}}\right] \times 100 \quad ((1))$$

whereas OD = optical density, PBS = phosphate buffer saline.

DNA ladder assay

DNA extraction DNA was extracted from collected lesion sample using standard procedure of organic phenol chloroform [28]. Accordingly, 750 µL of the blood sample was mixed with an equal volume of lysis buffer (0.1 mM EDTA, 12 mM $NaHCO_3$ and 155 mM NH_4Cl). Proteinase K (Biomatik Corporation, Canada) was added to a 0.5 mg/mL of final concentration, and sample was incubated at 56 ºC for 24 h. DNA was extracted successively with one volume of phenol at pH 8.0, one volume of phenol/chloroform and one volume of chloroform. The DNA was precipitated from the aqueous phase with ethanol and the pellet washed with 70% ethanol. The sample was air-dried and finally resuspended in 40 µL of the buffer.

DNA treatment The extracted human DNA was treated with the synthesized *bis*-amides **(3a-3j)**. Ten microliters of normal DNA was treated with 1 µL (1000 µg/mL) of the drugs and was incubated for 1 h at 37 ºC. H_2O_2 was used as positive control, and untreated DNA was used as negative control.

Gel electrophoresis The treated DNA was then visualized on 1.6% Agarose gel electrophoresis containing 5 µL of ethidium bromide by keeping the voltage at 72 V for 2 h. The gel was visualized under Gel documentations system Bio-Rad.

Antileishmanial assay

Antileishmanial assay of the synthesized *bis*-amides **(3a-3j)** was performed using a literature reported methodology [27]. For this purpose, *Leishmania tropica* promastigotes were cultured in RPMI medium (supplemented with 10% FBS and 1% antibiotic Streptomycin/Penicillin) unless the culture count using Neubauer chamber reached 4–5 million/mL. The synthesized bisamides **(3a-3j)** were added to 96-well plate in three different concentrations (1000 ppm, 500 ppm, and 250 ppm) screened in triplicates. Amphotericin B was used as a reference drug, while methanol (5%) being non-toxic to *leishmanial parasites* was used as a negative control. A 100 µL of the complete culture (10^6 cells/mL) was added to the 96-well plate containing bisamides **(3a-3j)** at three different concentrations and incubated at 25 ºC for 72 h. The viability of the promastigote was assessed by tetrazolium-dye (MTT) colorimetric method. A 100 µL of the MTT dye was added to each well, and the plates were incubated at 37 ºC for 3 h. Finally, 40 µL of the DMSO was added as stop solution and the readings were taken using an ELISA plate reader at 570 nm.

Molecular docking analysis

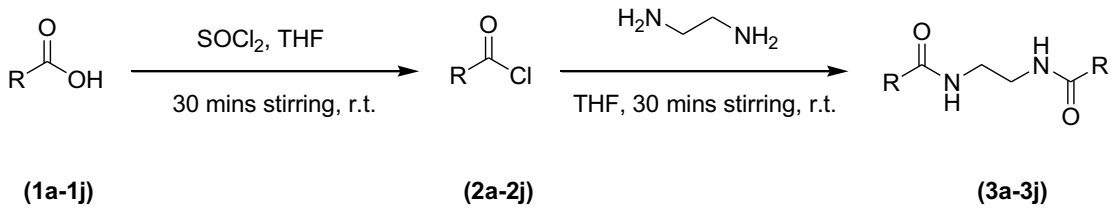
Molecular docking analysis has been shown to play an essential role in predicting the binding modes of ligands to proteins. MOE-dock module implemented in the Molecular Operating Environment (MOE) software package [29] was used to dock all the synthesized *bis*-amides **(3a-3j)** against *antileishmanial* target protein to further evaluate their *leishmaniacidal* potential. The structural coordinates of *antileishmanial* target protein (PDB code 1LML) [30] were retrieved from Protein Data Bank (www.rcsb.org). All the solvent molecules were removed and were subjected to 3D protonation and energy minimization up to 0.05 Gradient using **MMFF94s** forcefield implemented in MOE. Since the 1LML is devoid of a co-crystalized ligand so, the active site was indicated using the site-finder wizard of MOE by the creation of an alpha center followed by dummies. The alpha spheres were created inside the cleft in the form of a compact cluster; similarly, dummy atoms were created at the site of alpha sphere to carry out docking on the dummy atoms as the receptor was devoid of its co-crystallized ligand.

The 3D structural coordinates for all the *bis*-amides **(3a-3j)** were built using Molecular Builder Module in MOE. Finally, refined structures were used for docking study using the default parameters of MOE; placement: Triangle Matcher, rescoring 1: London dG, refinement: Forcefield, rescoring 2: GBVI/WSA. Before running the docking protocol, all the *bis*-amides **(3a-3j)** were then docked into predicted active site of protein and 10 conformations for each *bis*-amides were allowed to be generated. The ligands are flexible during docking so that to obtain the minimal energy conformation. The ligands were ranked based on docking score; lower scores indicate more favorable poses. Finally, the predicted protein–ligand interaction (PLI) was analyzed for molecular interactions using **PyMol** v 1.7.

Results and discussion

Synthesis and chemistry

The synthetic route employed for the synthesis of *bis*-amides **(3a-3j)** is provided below in Scheme 1. Briefly, various organic acid chlorides **(2a-2j)** were synthesized from organic acids **(1a-1j)** at room temperature using dry THF and thionyl chloride by constantly stirring them for half an hr. To the freshly synthesized acid chlorides **(2a-2j)**, a solution of ethylenediamine in dry THF was slowly added in situ and the reaction was allowed to stir at room temperature for another half an hour to synthesize the corresponding *bis*-amides **(3a-3j)**. Upon completion, THF was removed under reduced pressure to get solid *bis*-amides **(3a-3j)** in excellent yields (75–90%) as given in Table 1. The obtained solid



Scheme 1 Synthetic route employed for the synthesis of the target *bis*-amides **(3a-3j)**

Table 1 Structures of the synthesized variously substituted *bis*-amides **(3a-3j)**

S. No	Compound	R	S. No	Compound	R
1	**3a**	4-NO_2-Ar	6	**3f**	2-CH_3-Ar
2	**3b**	Ar-CH_2	7	**3 g**	Ar
3	**3c**	3-NO_2-Ar	8	**3 h**	2-Br-Ar
4	**3d**	2-NO_2-Ar	9	**3i**	3,4,5-triOCH_3-Ar
5	**3e**	4-CH_3-Ar	10	**3j**	C_6H_{11}

bis-amides **(3a-3j)** were purified by washing with 2% solution of sodium carbonate, distilled water and finally recrystallized from methanol at room temperature.

Efficiency of the current synthetic approach was compared with some relevant reported methodologies to argue optimum conditions employed for the synthesis of *bis*-amides (Table 2). Hence, the ideal conditions developed included adding ethylenediamine in situ to the synthesized acid chlorides in THF at room temperature. The reaction mixture was stirred for 30 min at room temperature (Entry 1) to obtain the target *bis*-amides in excellent yields (75–90%). Table 2 contains comparative efficiency of the current work with some literature available methodologies employed for the synthesis of amides. Consequently, direct amidation has been successfully achieved using carbonyldiimidazole (Entry 2). The optimum conditions include stirring equimolar 2-naphthoic acid with carbonyldiimidazole dissolved in dry THF (30 mL) at room temperature. The reaction mixture was stirred for 15 min, and then, aniline dissolved in dry THF (30 mL) was added slowly [31]. The resulting mixture was refluxed for 3 h to get the crude products in higher yields (76–91%). Moreover, amides are directly synthesized from a range of carboxylic acids and amines using $B(OCH_2CF_3)_3$. The products obtained can be purified easily using commercial resins. Briefly, equimolar solution of the respective acid and amine in acetonitrile (2 mL, 0.5 M) was stirred at 80 °C for 5–24 h in the presence of $B(OCH_2CF_3)_3$ to get the products in up to 99% yields (Entry 3) [32]. Similarly, direct amidation of carboxylic acids and amines using $TiCl_4$ in pyridine is reported. The reaction works with a range of substrates and provides the respective amides in moderate to excellent yields (56–98%). The yield decreases by using sterically hindered carboxylic acid and amines. The method involves adding $TiCl_4$ (3 mmol) and amine (1 mmol) to a solution of the corresponding acid (1 mmol) in pyridine (10 mL). The reaction mixture was then heated at 85 ℃ for 120 min to afford the corresponding amides (Entry 4) [33]. The highly efficient green synthesis of *bis*-amides using polyphosphoric acid supported on silica-coated $NiFe_2O_4$ nanoparticle ($NiFe_2O_4$@SiO_2-PPA) in methanol has been described (Entry 5). The optimum conditions include refluxing aldehydes (1 mmol), amides (2 mmol), the catalyst $NiFe_2O_4$@SiO_2-PPA (0.1 gm) and methanol (1 mL). The synthesized pure symmetrical *bis*-amide are obtained in good to excellent yields (93–52%). In this synthetic methodology, aldehydes with electron withdrawing

Table 2 Comparison of the efficiency of the current methodology adopted for the synthesis of *bis*-amides **(3a-3j)** with reported methodology

S. No	Solvent	Catalyst	Temperature (℃)	Time (hrs)	Yield (%)	Reference
1	THF	…	25	0.5	75–90	[27]
2	THF	Carbonyldiimidazole	66	3	76–91	[31]
3	CH_3CN	$B(OCH_2CF_3)_3$	80	5–24	Up to 99	[32]
4	Pyridine	$TiCl_4$	85	2	56–98	[33]
5	CH_3OH	$NiFe_2O_4$@SiO_2-PPA	64	0.40–2.1	5–932	[34]
6	…	…	185	3.5–5	20–74	[35]
7	Toluene	Nanoparticles	110	3–8	77–97	[36]
8	CH_3OH	…	25	24	45–82	[37]

groups reacted faster than the aldehydes with electron donating groups [34]. Similarly, the synthesis of symmetrical *bis*-amides of malonic acid has been performed. Briefly, dimethyl/ethyl malonate (5.6 mmol) and the corresponding amine (11.0 mmol) are stirred at 185 °C for 3.5–5 h to obtain solid products in 20–74% yields (Entry 6). At the optimum conditions, the yield was found to be highest for unsubstituted aniline and lowest for anilines bearing electron donating groups [35]. Moreover, silica-bonded S-sulfonic acid nanoparticles are reported as efficient catalysts for the synthesis of symmetrical *bis*-amides (Entry 7). Reaction of aldehydes (1 mmol), amide (2 mmol) and nanoparticles (0.08 gm) in toluene (7 mL) heated under reflux affords the symmetrical *bis*-amides in good to excellent yields (77–98%). Under optimum conditions, benzaldehydes substituted with either electron-donating or electron withdrawing groups underwent the reaction in good to excellent yields. The reaction was found to be compatible with aliphatic aldehydes [36]. An efficient four-component Ugi synthesis of ferrocenyl *bis*-amides in a mild and neutral conditions has been successfully described. The optimum conditions of this synthetic strategy include stirring equimolar mixture of ferrocene carboxaldehydes, acids and amines in methanol (3 mL) for 24 h at room temperature (Entry 8). The ferrocenyl *bis*-amides are obtained in good yields (70–45%) upon completion of the reaction. However, the yield of the synthesized ferrocenyl *bis*-amides is improved greatly (68–82%) by using 2-aminopyridine and 2-aminopyrimidine as heterocyclic amines as well as indole-3-carboxylic acid as heterocyclic acids [37].

Subsequently, to compare the efficiency and generalize the synthetic methodology employed for the synthesis of *bis*-amides, the reaction was performed at optimum conditions (Entry 1, Table 2). Accordingly, *bis*-amides were synthesized in excellent yields (75–90%) by first stirring organic acids with excess of thionyl chloride at room temperature for 30 min. Upon completion of the reaction, a solution of ethylene diamine in dry THF was added slowly in situ to the synthesized acid chlorides and the reaction mixture was stirred for next 30 min. After stirring the reaction mixture for the required time, THF was removed under reduced pressure to obtain solid precipitates of *bis*-amides upon necessary purification.

Characterizations

Structural confirmation and assignments of the synthesized *bis*-amides **(3a-3j)** are based on their % elemental analysis (CHNS) data and various spectroscopic technique (FT-IR, ^{1}H NMR,^{13}C NMR). All of the synthesized *bis*-amides **(3a-3j)** gave satisfactory % analysis data for the elements C, H and N which was highly aligned with their proposed structural formulae. The NH protons appears as broader and intense band at 3305.57 cm^{-1} in the FT-IR spectrum of the *bis*-amide **(3e)**. Similarly, FT-IR spectrum of the *bis*-amide **(3e)** displays sharp band at 1628.60 cm^{-1} for the amide bond. This amide bond is clearly visible at 170.77 ppm in the ^{13}C NMR spectrum. Similarly, the *p*-substituted phenyl ring resulted in two distinct doublets at 7.29 and 7.74 ppm in the ^{1}H NMR spectrum of *bis*-amide **(3e)**. The aliphatic chain of the bisamide *bis*-amides appears as intense peaks in the ^{1}H and ^{13}C NMR spectra at 3.62 and 40.92 ppm, respectively.

In vitro biological assays

% *h*RBCs Hemolysis assay

The cytotoxicity of the tested *bis*-amides **(3a-3j)** was investigated using *h*RBCs assay as described previously for the evaluation of cytotoxic effects of the *antileishmanial* compounds [38]. The release of hemoglobin from *h*RBCs is an indication of the cytotoxicity of the bisamide measured by UV–visible spectroscopy and compared with Triton X-100 (0.5%). The % hemolysis of *h*RBCs using three different concentrations (1000, 500 and 250 µg/mL) of the tested *bis*-amides **(3a-3j)** and their corresponding CC_{50} values are given in Tables 3 and 4. Accordingly, the tested *bis*-amides **(3a-3j)** were found to be highly biocompatible with much lower % *h*RBCs hemolysis at lower concentrations (250 µg/mL). However, with the increase in the concentration of the tested *bis*-amides **(3a-3j)**, % *h*RBCs hemolysis increased gradually. In terms of % hemolysis at higher concentrations (1000 µg/mL), the *bis*-amides **(3b)** and **(3d)** induced 55.3% and 52.4% *h*RBCs hemolysis, respectively, and were ranked as highly cytotoxic followed by *bis*-amides **(3 h)** and **(3e)** as shown in Table 2. Similarly, the *bis*-amide **(3j)** induced 7.8% hemolysis at higher concentrations (1000 µg/mL) and was found to be the least toxic followed by *bis*-amides **(3a),**

Table 3 % Hemolysis and CC_{50} (µg/mL) values obtained for the slightly toxic *bis*-amides **(3b, 3d, 3e, 3 h)** in *h*RBCs hemolysis assay

S. No	Compound	% Hemolysis (1000 µg/mL)	% Hemolysis (500 µg/mL)	% Hemolysis (250 µg/mL)	CC_{50} (µg/mL)
1	**3b**	55.3	52.4	30.7	621.77
2	**3d**	52.4	43.3	27.9	400.41
3	**3e**	40.2	31.2	30.2	1735.71
4	**3 h**	41.9	38.1	5.8	1089.83

Table 4 % Hemolysis and CC_{50} (µg/mL) values obtained for the tested no-toxic *bis*-amides **(3a, 3c, 3f, 3 g, 3i, 3j)** in *h*RBCs hemolysis assay

S. No	Compound	% Hemolysis (1000 µg/mL)	% Hemolysis (500 µg/mL)	% Hemolysis (250 µg/mL)	CC_{50} (µg/mL)
1	**3a**	10.4	7.1	6.8	8794.11
2	**3c**	17	8.3	5.8	3173.20
3	**3f**	27	20	17.1	2736.84
4	**3 g**	11.5	10	8.2	1.04E + 10
5	**3i**	27.4	24.4	21	131,757.6
6	**3j**	7.8	6.6	6.1	19,347.83

(3 g), (3c), (3f) and **(3i)** as shown in Table 3. The same trends are obvious from their CC_{50} values which show that the *bis*-amides **(3d)** and **(3b)** (CC_{50} 400.41, 621.77 µg/mL) are slightly cytotoxic when compared to the remaining bisamides having much higher CC_{50} values (> 1000 µg/mL) and were ranked as non-toxic to *h*RBCs.

DNA ladder assay

DNA ladder assay was adopted to study the effect of the tested *bis*-amides **(3a-3j)** on apoptosis and determine the possible mode of cellular death (apoptosis). For this purpose, human DNA was exposed to the tested bisamides for 1 h at 37 ºC. Briefly, one of the hallmarks of the confirmation of late apoptosis is the appearance of unclear DNA fragment of nuclear DNA on agarose gel. According to the results obtained, the well no.1 of the agarose gel (Fig. 2) shows the normal and untreated human DNA, while well no. 2 shows DNA treated with H_2O_2 as positive control which induces breakage in DNA strands and result in appearance of different bands. In comparison with the positive control, *bis*-amides **(3a), (3c), (3i)** and **(3 g)** were found non-toxic to DNA, while the remaining bisamides were highly degradative toward DNA causing the appearance of more than one bands and complete degradation (appearance of a smear of DNA). The smear-like appearance of the DNA treated with *bis*-amides **(3b)** and **(3d)** can be observed in the following figure.

Antileishmanial assay

Antileishmanial potential observed for the tested *bis*-amides **(3a-3j)** is presented in Table 5 in terms of their % inhibition and IC_{50} values. In terms of their % inhibition at highest concentration (1000 µg/mL), the *bis*-amide **(3f)** was found to be the most effective one against promastigotes of *Leishmania tropica* which caused 85% growth inhibition followed by 73.54% inhibition of the *bis*-amide **(3 g)**. For the tested *bis*-amides **(3a-3j)**, the percent inhibitory response was found to be highly dependent upon the concentration of the tested *bis*-amides **(3a-3j)** and decreases drastically at lower concentrations except for *bis*-amide **(3c)** which decreased gradually with the decrease in concentration. In terms of their IC_{50} values, *bis*-amide **(3f)** was evaluated to possess good *leishmanicidal* potential (IC_{50} = 633.16 µg/mL) followed by *bis*-amides **(3b), (3j)** and **(3 g)**. Surprisingly, the IC_{50} values were found to be similar (IC_{50} = 680.22 µg/mL) for *bis*-amides **(3b)** and **(3j)** though their percent inhibition was found to be different (71.11%, 67.42%) at the tested concentrations.

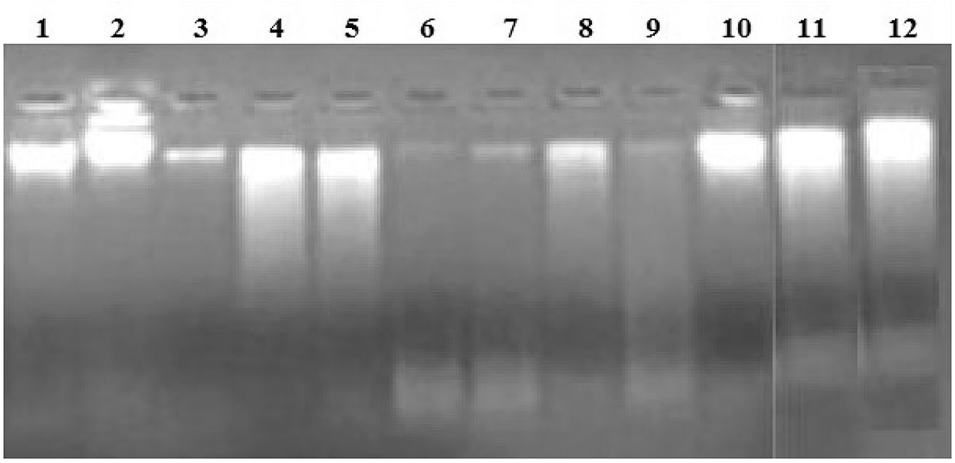


Fig. 2 DNA damage caused after treatment with the *bis*-amides 1: untreated DNA (negative control), 2: H_2O_2-treated DNA (positive control), 3: **(3a),** 4: **(3b)**, 5: **(3d)**, 6: **(3j)**, 7: **(3 h),** 8: **(3f),** 9: **(3e),** 10: **(3c),** 11: **(3i),** 12: **(3 g)**

Table 5 % Inhibition and IC_{50} (µg/mL) values obtained for the tested *bis*-amides **(3a-3j)** against *Leishmania tropica* promastigotes

S. No	Compound	% Inhibition (1000 µg/mL)	% Inhibition (500 µg/mL)	% Inhibition (250 µg/mL)	IC_{50} (µg/mL)
1	**3a**	37.26	31.56	26.94	5673.86
2	**3b**	71.11	24.56	26.45	680.22
3	**3c**	66.56	43.23	38.98	691.21
4	**3d**	42.31	25.73	21.36	1286.48
5	**3e**	45.83	33.38	25.26	1146.51
6	**3f**	85.89	26.36	24.80	633.16
7	**3 g**	73.54	25.64	22.97	712.93
8	**3 h**	13.65	10.92	0.750	43,356.53
9	**3i**	42.32	23.09	18.72	1258.13
10	**3j**	67.42	36.01	32.23	680.22

Molecular docking study

The synthesized *bis*-amides **(3a-3j)** were evaluated for their *antileishmanial* potential by molecular docking approach using MOE software to explore the possible binding mode against the *antileishmanial* target. Generally, the synthesized *bis*-amides **(3a-3j)** possess differently substituted groups, e.g., electron-withdrawing groups (EWG's) and electron-donating groups (EDG's). Molecular docking results revealed good potential for *bis*-amides which possess EDG's against *antileishmanial* target. The surface representation of each targeted enzyme with zoomed the active site is depicted in Fig. 3a. The first-ranked *bis*-amide (**3f)** showed fit-well binding mode in the predicted active site of the targeted protein and was observed in adopting favorable interactions with critical residues which might have important role in enhancing the enzyme potential, i.e., electrically positively, negatively charged, and polar uncharged side chain residue R414, D491 and T417 (Fig. 3b) with an average binding affinity of **– 0.7375** kJ/mol. Second- and third-ranked *bis*-amides (**3b, 3j and 3c**) shared almost similar binding mode with R414 (Fig. 3c–e) with an average binding affinity of **– 0.45** kJ/mol, -0.475 kJ/mol and **– 0.38** kJ/mol. Besides, *bis*-amide (**3b)** showed an additional interaction with T417, while *bis*-amide **(3j)** showed two H-bond with R414. The highest potential for *bis*-amide **(3f)** might be due to the opposite side attached methyl group which is EDG's and have good potential for activating the corresponding compound and hence raising the enzyme activity, while the slight less potential for *bis*-amides **(3b)** and **(3j)** might be due to the phenyl group attached at opposite site, which might have less potential for activating the overall bisamide and hence

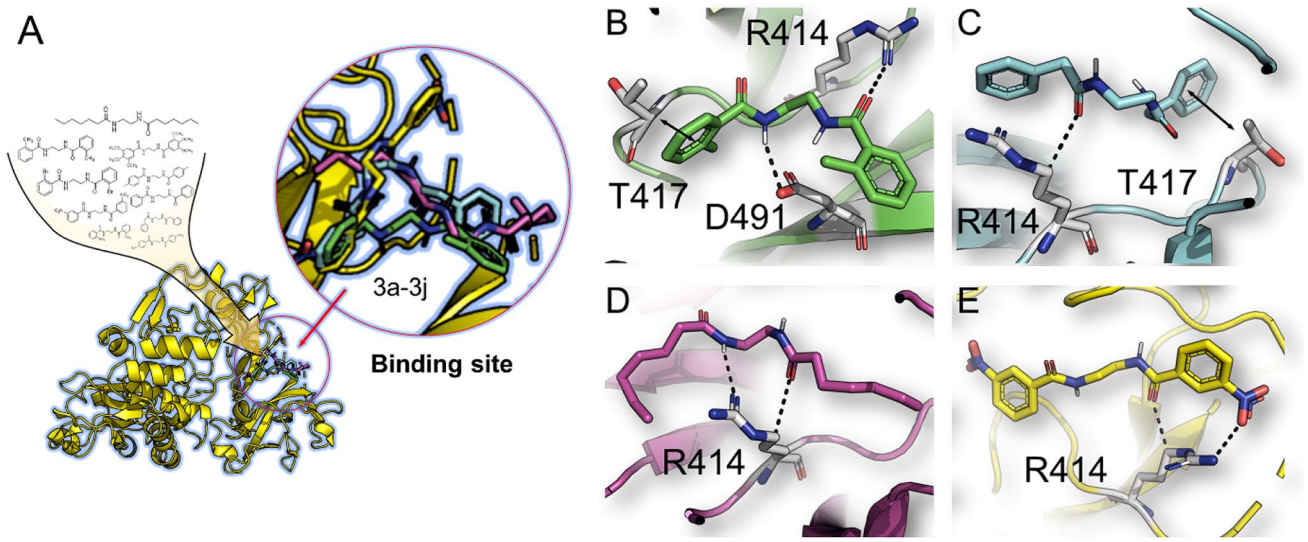


Fig. 3 Protein–ligand Interaction (PLI) profiles of the synthesized *bis*-amides **(3a-3j)** against *antileishmanial* target protein. **Figure (3a)** Indicates the surface of the *antileishmanial* enzyme. **Figure (3b-3b)** Indicates PLI profile for the tested *bis*-amides **(3f), (3b), (3j)** and **(3c)**. Double-sided arrows represent the arene–arene interaction

less activity. Mostly, the carbonyl oxygen of each bisamide was observed in adopting essential interaction with active site residues.

Conclusion

The present research article successfully describes the efficient synthesis, spectroscopic characterizations, in vitro biological and computational studies of C_2 symmetric simple *N,N'*-(ethane-1,2-diyl)bis(benzamides) **(3a-3j)**. Biocompatibility of the synthesized *bis*-amides **(3a-3j)** was assessed by analyzing their hemolytic effects on human red blood cells (*h*RBCs). The tested *bis*-amides having low % hemolysis and higher IC_{50} values were ranked as biocompatible against *h*RBCs. Consequently, all the tested *bis*-amides were found to be highly biocompatible except **(3d)** which induced 52.4% hemolysis at 1000 µg/mL (CC_{50} = 400.41 µg/mL) and hence ranked as slightly toxic. Similarly, DNA ladder assay was opted to check the apoptotic nature of the tested *bis*-amides **(3a-3j).** The results obtained revealed that most of the tested *bis*-amides are highly toxic in nature causing the complete degradation of DNA except **(3a), (3c), (3i)** and **(3 g)**. *Leishmaniacidal* potential of the tested *bis*-amides **(3a-3j)** against the promastigotes of *leishmania tropica* was assessed in terms of their % inhibitions and IC_{50} values. In terms of their % inhibitions, the *bis*-amides **(3f)** and **(3 g)** displayed excellent *leishmanicidal* potential of 85.0% and 73.54%, respectively. In terms of their IC_{50} values, **(3f)** with IC_{50} value of 633.16 µg/mL was ranked as the most potent *leishmaniacidal* bisamide. However, the *bis*-amides **(3b)** and **(3j)** received the second highest *leishmanicidal* potential with almost similar IC_{50} values of 680.22 µg/mL. The next highest *leishmanicidal* potential was displayed by the *bis*-amides **(3c)** and **(3 g)** with IC_{50} values of 691.21 µg/mL and 712.93 µg/mL, respectively. Computational molecular docking analysis revealed bearing *bis*-amides bearing electron-donating groups possess strong binding to the active site and hence display strong inhibitory potential against *antileishmanial* target. Thus, based on the results stated above, the synthesized *bis*-amides **(3a-3j)** could be concluded as a safer and better *antileishmanial* scaffolds and need to be investigated further for better results.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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