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Original article

Synthesis and Antileishmanial activity of Piperoyl-Amino Acid Conjugates

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ABSTRACT

Based on reported antileishmanial activity of naturally occurring alkaloid piperine and amino acid esters, their conjugates were synthesized by the hydrolysis of piperine to piperic acid followed by reaction with amino acid methyl esters. These conjugates were further converted to compounds with free carboxyl group and those with reduced double bonds. The synthesized compounds were evaluated for activity against promastigote and amastigote forms of *L. donovani in vitro*. All the compounds showed better activity than either piperine or the amino acid methyl esters. Piperoyl-valine methyl ester was the most active compound showing an IC_{50} of 0.075 mM against the amastigotes. Two active compounds were evaluated for *in vivo* activity in golden hamster model of leishmaniasis.

1. Introduction

Leishmaniasis is a neglected protozoal disease and is a major cause of concern in developing countries. The disease is caused by different species of protozoa *Leishmania* and transmitted by bite of *Phlebotomine* sand flies. It is mainly manifested in three forms - visceral leishmaniasis (VL), also known as *kala-azar* caused by *L. donovani*; cutaneous leishmaniasis (CL) caused by *L. major*, *L. donovani*, *L. tropica* and *L. aethiopica*; muco-cutaneous leishmaniasis (MCL) caused by *L. braziliensis*. VL is the most severe form of leishmaniasis and is usually fatal in the absence of treatment. Most of the first line drugs available for the treatment of leishmaniasis such as sodium stibogluconate, miltefosine, pentamidine *etc* cause serious side effects and toxicity [1–3]. Moreover, resistance developing to first line agents is now increasing even up to an extent of >60% of the patients in some endemic areas [1]. Thus, there is an urgent need for safe, inexpensive and orally available drugs. Various classes of natural products such as alkaloids, flavonoids, lignans, terpenes *etc* have been shown to possess antileishmanial activity [4].

Piperine **1**, the most common and abundant alkaloid of the genus *Piper*, has shown inhibitory activity against promastigote form of *L. donovani in vitro* [5]. Reports have suggested that this activity is enhanced if piperine is delivered in oil-in-water emulsion form or as mannose-coated liposomes [6,7]. More recently, piperine and its derivatives have been evaluated for inhibitory effects against epimastigote and amastigote forms of the protozoan parasite *Trypanosoma cruzi* [8,9].

Amino acid esters and amides have been found to possess inhibitory activity against intracellular as well as isolated amastigotes of *L. amazonensis* and *L. mexicana*. Amastigotes of leishmania protozoa replicate within the macrophage phagolysosome. Amino acid esters have been found to accumulate in these phagolysosomes. Once inside, the acidic pH of these organelles causes accumulation of charged amino acids, which leads to osmotic disruption of these phagolysosomes resulting in killing of the infected cells. Inside the amastigotes, amino acid esters are hydrolyzed by parasitic enzymes resulting in free amino acids accumulation which kills the parasite [10]. However there are no reports of antileishmanial activity of amino acid esters against *L. donovani*.

In our continuous search for new antileishmanial compounds, we aimed to make use of abundantly available natural product piperine for generation of new antileishmanial compounds. We report in this paper, synthesis and evaluation of antileishmanial

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activity of i) piperoyl-amino acid methyl ester conjugates **3**-**7** ii) piperoyl-amino acid conjugates **8**-**12** and iii) tetrahydropiperoyl-amino acid methyl ester conjugates **13**-**17**. Lee *et al* reported central nervous system depressant action of conjugates of piperic acid with alanine and phenyl alanine together with some non-natural amino acids [11].

2. Results and discussion

2.1. Chemistry

Reported activities of piperine and amino acid esters suggested that their conjugates may show enhanced antileishmanial activity. The amide bond of piperine can be hydrolyzed to obtain piperic acid (**2**) and the resulting carboxylic acid group can be used as a site for linking it covalently with amino acid esters via amide bond formation.

Piperine (**1**) was isolated from chloroform extract of *Piper nigrum* by column chromatography using *n*-hexane-ethyl acetate gradient elution (0-30%). Piperic acid (**2**) was prepared by alkaline hydrolysis of piperine by reported procedures [12]. An alternative method for preparation of piperic acid involved direct saponification of the chloroform extract of *P. nigrum*. The piperic acid liberated was washed with water and recrystallized from methanol. The yield of piperic acid obtained by two methods was similar, no chromatographic purification was required in the alternative method.

Piperoyl-amino acid conjugates with carboxyl protected amino acids (amino acid methyl esters) were prepared via piperic acid mesylate obtained by reaction of piperic acid with methanesulfonyl chloride (CH_3SO_2Cl) in dry CH_2Cl_2 at 0 °C. This mesylate was reacted with amino acid methyl esters to yield the desired piperoyl-amino acid methyl ester conjugates **3**-**7** in yields ranging from 40-75% [12] (scheme1).

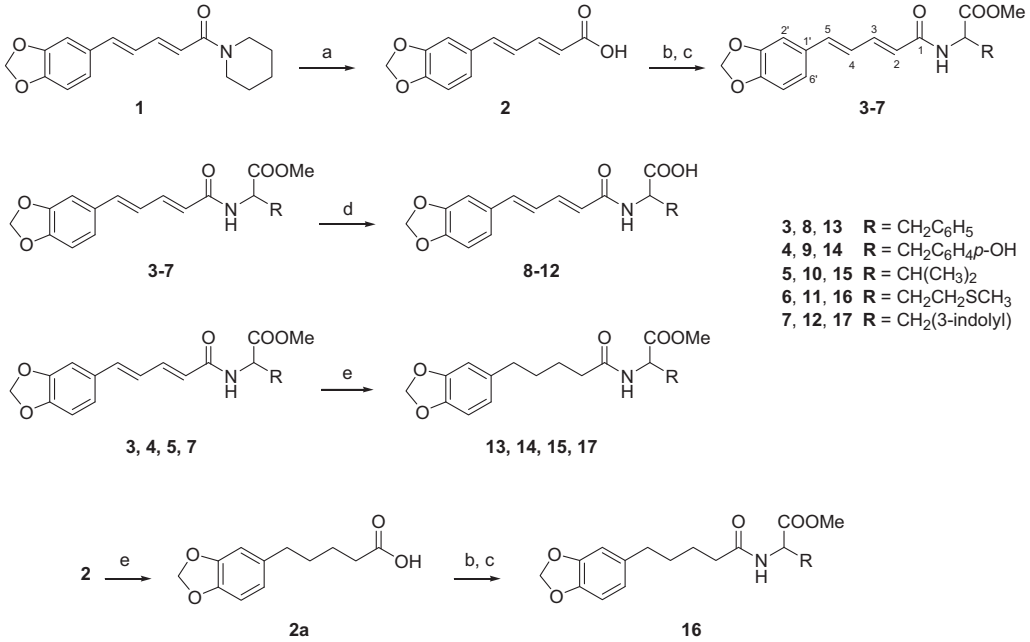
Compounds **8**-**12** were prepared by deprotection of carboxyl group of compounds **3**-**7** by microwave assisted solid phase reaction with Al_2O_3 (40% KF) in 70-80% yields [13,14]. Disappearance of methoxy signal in ^1H NMR of compounds **3**-**7** confirmed the conversion of ester group into free carboxyl group in compounds **8**-**12**.

The third series comprised of saturated derivatives of compounds **3**-**7** *i.e*. tetrahydropiperoyl-amino acid methyl ester conjugates **13**-**17**. Two alternate strategies were tried for preparation of these compounds (i) hydrogenation of compounds **3**-**7** of series 1, (ii) hydrogenation of piperic acid to tetrahydropiperic acid followed by reaction with amino acid methyl esters. Compounds **13**-**15** and **17** were synthesized by catalytic hydrogenation of compounds **3**-**5** and **7**. Efforts to prepare **16** by catalytic hydrogenation of **6** were not successful; this could be ascribed to catalytic poisoning by sulfur. Therefore, for synthesizing **16**, tetrahydropiperic acid (**2a**) was prepared by catalytic hydrogenation of piperic acid (**2**), it was then coupled to methionine methyl ester by the same method as used for preparation of compounds of series 1 [9]. As a result of reduction of conjugated double bonds, proton signals in the olefinic region of compounds **3**-**7** disappeared while additional signals in the aliphatic region for eight protons were observed confirming hydrogenation of the double bond.

2.2. Antileishmanial activity

Parasites of the *Leishmania* genus are known to exist as flagellated extracellular promastigotes and intracellular amastigotes. All the synthesized compounds were evaluated for antileishmanial activity against both these forms *in vitro*. The results are summarized in Table 1.

All the compounds showed higher activity against the amastigote form compared to promastigote form (Fig. 1); IC_{50} values were approximately three times lower against amastigotes. Piperine **1** showed an IC_{50} value of 0.752 and 2.558 mM against promastigotes and amastigotes, respectively. Piperoyl-amino acid ester conjugates (**3**-**7**) showed substantial increase in activity as compared to either piperine or amino acid esters. All the compounds were found to be active in IC_{50} range of 0.075-0.310 mM against amastigotes while IC_{50} was considerably higher *i.e*. 0.473-1.312 mM against promastigotes. This indicates that amastigotes are more sensitive to these compounds than promastigotes. Compounds **3**-**7** were found to be



Scheme 1. Reagents and conditions: (a) 20% KOH, CH_3OH, 80 °C, 3 days, 88% (b) CH_3SO_2Cl, Et_3N, DCM, 0 °C, 30 min, 85% (c) Amino acid methyl ester, Et_3N, DCM, RT, 40-75% (d) KF-Al_2O_3 (40%), Microwave, 1000 W, 3-4 min, 70-80% (e) 5% Pd/C, H_2 (40 Psi), MeOH, 30 min, 75-80%

Table 1
Antileishmanial activity and cytotoxicity of piperoyl-amino acid conjugates.

Compound	IC_{50} (mM)		Cytotoxicity (%)	
	Amastigotes	Promastigotes	At IC_{50}	At 2 × IC_{50}
1	0.752	2.558	7	10
2	0.395	1.756	4.5	8
3	0.197	0.834	4	7
4	0.210	0.791	3.5	6
5	0.075	0.875	3	3.8
6	0.168	0.823	4	7.5
7	0.119	0.855	3	5.2
8	0.256	0.927	1.2	8.5
9	0.249	0.761	4	6
10	0.310	1.030	5.5	9
11	0.237	1.312	1.8	7.7
12	0.236	1.025	3.6	10
13	0.212	0.669	5.5	7.2
14	0.202	0.536	5	7.6
15	0.240	0.722	4.5	7.7
16	0.221	0.607	3	5
17	0.176	0.473	2.5	5
L-Phenylalanine methyl ester (**18**)	1.537	2.161	5.7	8.2
L-Tyrosine methyl ester (**19**)	1.396	2.091	0.3	5
L-Valine methyl ester (**20**)	1.760	2.348	4	6
L-Methionine methyl ester (**21**)	1.898	1.931	1.5	7.3
L-Tryptophan methyl ester (**22**)	1.421	1.366	1.8	5.3
Miltefosine	0.018	0.033	29	35

more active against amastigotes while compounds **13**-**17** were more active against promastigotes. Maximum effectiveness against amastigotes was found with compound **5**, conjugate of piperic acid and valine methyl ester, which showed an IC_{50} of 0.075 mM against amastigotes. Other than valine, conjugates of piperic acid with methionine methyl ester (**6**) and tryptophan methyl ester (**7**) also showed promising activity against amastigotes with IC_{50} values of 0.168 and 0.119 mM, respectively. Modification by deprotection of carboxyl group of amino acid functionality in compounds **8**-**12** resulted in decrease in antileishmanial activity against amastigotes (IC_{50} range 0.236-0.310 mM). Though much more potent than piperine and amino acid methyl esters, compounds of series 2 (**8**-**12**) were less active than compounds of series 1. Compounds of series 3 with reduced double bonds were more active than their unsaturated analogues **3**-**7** against promastigotes, tetrahydropiperoyl tryptophan methyl ester **17** being most active with an IC_{50} value of 0.473 mM. These observations indicate that reduction of conjugated double bonds in piperine subunit decreases the antileishmanial activity of the conjugates against amastigotes where as activity against promastigotes is enhanced. Hydrolysis of ester functionality in amino acid subunit decreases the

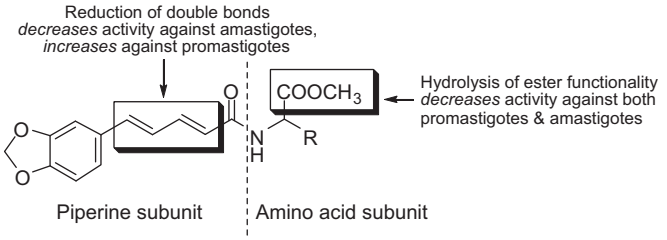


Fig. 2. Piperoyl-amino acid methyl ester conjugates

antileishmanial activity of the conjugates against both the forms of parasite (Fig. 2).

Cytotoxicity of all the synthesized compounds was determined against PBMC (peripheral blood mononuclear cells) at two concentrations (IC_{50} and twice that of IC_{50}). None of the compounds showed more than 10% killing at a concentration twice that of IC_{50} (Table 1).

Compounds **3** and **5**, which showed higher activity against the amastigote form, were evaluated *in vivo* and the results are shown in Table 2. A dose of 250 mg/kg was chosen based on pilot studies. Since the oral absorption of these compounds is not known, therefore these were administered intraperitoneally (i.p.).

Compound **3** showed significant reduction in spleen parasitic burden (35%) and spleen weight (30%) in *in vivo* assay. Though **5** showed reduction in parasitic burden (24%) but reduction in spleen weight was not observed. Standard antileishmanial, miltefosine showed significant reduction in spleen parasitic burden (87%) and spleen weight (43%) in *in vivo* study.

Table 2
Effect of compounds on spleen weight and parasitic burden in hamster model of visceral leishmaniasis.

Groups	Spleen weight in mg (% reduction)[a]	Spleen parasitic burden × 10^9 (% reduction)[a]
Vehicle	503 ± 34	0.54 ± 0.04
3 (250 mg/kg, i.p.)	353 ± 42[b] (30%)	0.35 ± 0.06[b] (35%)
5 (250 mg/kg, i.p.)	516 ± 20	0.41 ± 0.02 (24%)
Miltefosine (12.5 mg/kg, p.o.)	287 ± 23[b] (43%)	0.07 ± 0.00[b] (87%)

[a] Results are expressed as mean ± SEM (n = 5)
[b] $p<0.05$ vs vehicle treated group

2.3. Molecular docking against putative targets

Compound **5** which showed good antileishmanial activity was docked against *L. donovani* targets to generate the appropriate binding orientation and conformation to the active site of these targets. The docking study of compound **5** was performed against the target adenine phosphoribosyltransferase (APRT), PDB ID 1QB8 [15] using AutoDock 4.2 [16] program. The enzyme 1QB8 has role in

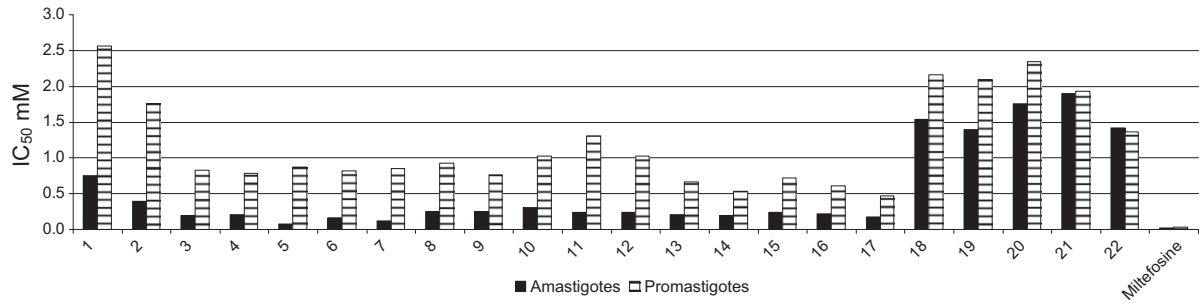


Fig. 1. IC_{50} values of piperoyl-amino acid conjugates and standard

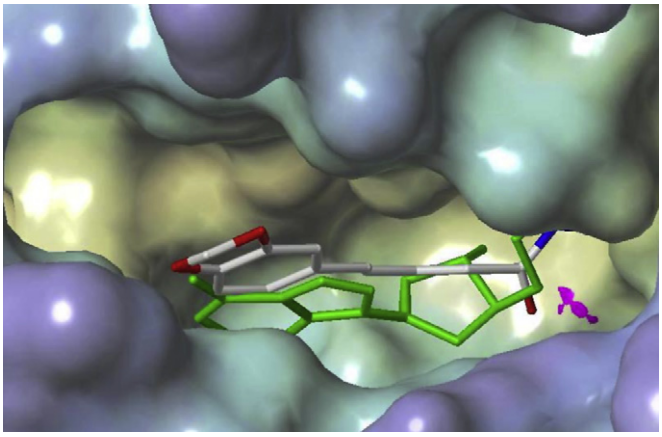


Fig. 3. Docked pose of Compound **5** into active site of 1QB8 protein. Green color sticks represent co-crystallized AMP structure and white color sticks represent AutoDock 4.2 conformation. Magenta color area represents Mg^{2+} ion (generated by MOLCAD program)

salvage pathway by converting adenine into adenosine-5-monophosphate (AMP). APRT is not essential for purine salvage in *L. donovani* promastigote form (found in sandfly vector), but may play an essential role in the infective amastigote form of the *L. donovani* that lives in the host macrophage cell. So, for this parasite APRT is a target for antileishmanial chemotherapy [17,18]. The compound **5** was built using SYBYL7.1 molecular modeling package installed on a Silicon Graphics Fuel Work station running IRIX 6.5. Tripos force field, Gasteiger Huckel, partial atomic charges [19] and Powell's conjugate gradient method were used for minimization of all molecules with 0.05 kcal/mol energy gradient convergence criterion [20]. The magnesium (Mg^{2+}) is required for activity of this enzyme. The compound **5** was showing H-bonding interaction to Ala150, Thr151, Gly152, Thr154 and Ala155 residues. It was found that Mg^{2+} ions coordinated with electron-rich carbonyl oxygen and -NH group (distance from Mg^{2+} 2.04 Å and 3.09 Å, respectively) of compound **5**. These interactions have good agreement to experimental data [15]. The Compound **5** docked well in terms of location (near to AMP and Mg^{2+}) and H-bond interactions (shown in Figs. 3 and 4) that implies these interactions are important for binding of this compound to the active site of *L. donovani* protein 1QB8.

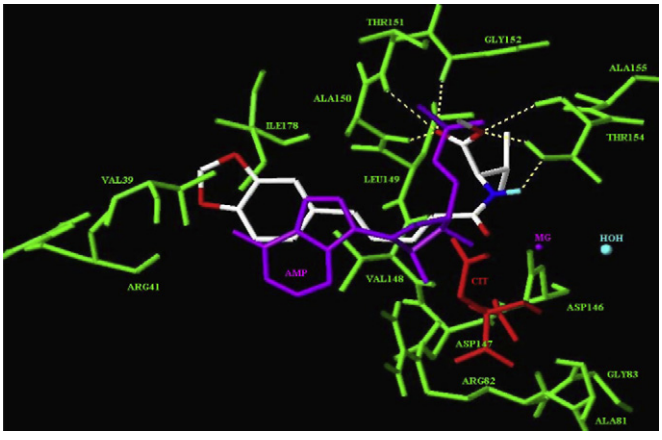


Fig. 4. Docking interactions of Compound **5** into active site of 1QB8 protein. White color-sticks represent AutoDock 4.2 conformation and purple color sticks represents co-crystallized AMP structure. The dotted lines show H-bonding with active site residues (Green color). Magenta, Red and Cyan colors represent Mg^{2+}, Citrate and water molecule respectively (generated by Sybyl7.1 program).

3. Conclusions

In the present study, several piperoyl-amino acid ester conjugates were synthesized and evaluated for antileishmanial activity *in vitro* and *in vivo*. Piperoyl-valine methyl ester showed an IC_{50} of 0.075 mM against the amastigotes and was further evaluated *in vivo* in the golden hamsters. Molecular docking studies indicated that **5** binds to the active site of *L. donovani* protein 1QB8.

4. Experimental

4.1. Chemicals and instruments

All solvents used were of analytical grade (CDH Laboratory Reagents, India). TLC plates of silica gel GF_{254} (Merck, Germany) were used for analysis. Silica gel 60-120 mesh (CDH Laboratory Reagents, India) was used for column chromatography. Melting points were recorded on capillary melting point apparatus and are uncorrected. $^1H/^{13}C$ NMR spectra are recorded on 300/75 MHz Bruker FT-NMR (Avance DPX300) spectrometer using tetramethylsilane as internal standard and the chemical shifts are reported in δ units. Mass spectra were recorded on auto sampler/direct injection LCMS (APCI/ESI).

4.2. Chemistry

4.2.1. Procedure for preparation of piperic acid (**2**) from piperine (**1**)

Piperine **1** (11.8 g) was isolated from 20 g chloroform extract of *P. nigrum*. Piperine **1** (11.4 g, 0.04 mol) was refluxed with methanolic KOH (20%, 500 mL) for 3 days. After completion of hydrolysis, methanol was distilled under reduced pressure. The resulting reaction mixture was suspended in hot water and acidified with HCl to pH<1. Yellow precipitate obtained was collected by filtration, washed with cold water and recrystallized from methanol to yield crystals of piperic acid **2** (7.9 g, yield 88%); R_f 0.30 (CH_2Cl_2:MeOH = 9.5:0.5); m.p. 212-215 °C (Lit. 215-216 °C). The yield of piperic acid from crude extract was 39.5%. This procedure was then scaled up to 50 g of piperine to obtain 36 g of piperic acid which was used for further reactions.

4.2.2. Alternative method for preparation of piperic acid (**2**) from chloroform extract of P. nigrum

Chloroform extract of *P. nigrum* (20 g) was dissolved in chloroform (150 mL), washed with cold aqueous NaOH (2 N, 150 mL) three times. Remaining organic layer was evaporated to dryness. Dried extract was refluxed with methanolic KOH (40%, 200 mL) for 2 days and the reaction mixture was washed with ether and acidified with conc. HCl to pH<1. Precipitates of piperic acid obtained were filtered and washed with water. Piperic acid was recrystallized from methanol (8 g, yield 40%) and confirmed by co-TLC with standard.

4.2.3. General procedure for preparation of amides (**3**-**7**) from piperic acid

Piperic acid **2** (218 mg, 1 mmol) was stirred in dry CH_2Cl_2 (10 mL) for 10 minutes at -15 °C. Triethylamine (0.26 mL, 2 mmol) was added to the reaction mixture followed by methanesulfonyl chloride (0.15 mL, 1.5 mmol) and reaction mixture was stirred for 1 h. Solution of amino acid methyl ester (1 mmol) in CH_2Cl_2 was then slowly added to the reaction mixture. It was then allowed to stir for 30 min at 0 °C followed by stirring at room temperature for 4-5 h. Reaction was monitored by TLC using mobile phase - $CHCl_3$:MeOH (9.5:0.5). Reaction mixture was extracted with CH_2Cl_2 and was washed with $NaHCO_3$ and 2 N HCl. CH_2Cl_2 layer was dried over anhydrous sodium sulfate. The crude product was purified by silica gel column chromatography using $CHCl_3$:MeOH with increasing

gradient (0-0.5%). All the conjugates were characterized by ^{1}H, ^{13}C NMR, IR and MS data.

*4.2.3.1. Piperoyl-L-phenylalanine methyl ester (**3**).* Off-white solid; yield 70%; m.p. 117-118 °C; IR (KBr) υ_{max} 3314, 1749, 1646, 1608, 1531, 1504, 1247, 1218, 997, 928 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.40 (dd, 1H, J = 10.5 Hz, 14.8 Hz, −C*H*═CH−CO), 7.31-6.69 (9H, olefinic and Ar-*H*), 6.65 (dd, 1H, J = 10.5 Hz, 15.4 Hz, ═C*H*−CH═CH−CO), 5.91 (s, 2H, OCH_2O), 5.89 (d, 1H, J = 14.8 Hz, ═CH−CO), 5.03-4.97 (m, 1H, NH−C*H*−CO), 3.73 (s, 3H, $-COOCH_3$), 3.20 (d, 2H, J = 5.5 Hz, CH_2−Ar); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.6, 166, 148.7 (2C), 142.4, 140, 136.4, 131.3, 129.8 (2C), 129.1, 127.6 (2C), 125, 123.2, 122.8, 109, 106.3, 101.8, 53.7, 52.8, 38.4; CIMS: m/z 379 [M+1]$^+$

*4.2.3.2. Piperoyl-L-tyrosine methyl ester (**4**).* Light yellow solid; yield 50%; m.p. 188-189 °C; IR (KBr) υ_{max} 3518, 1724, 1608, 1506, 1244 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.36 (dd, 1H, J = 15.2 Hz, 10.6 Hz, −C*H*═CH−CO), 7.14-6.77 (m, 8H, olefinic and Ar-*H*), 6.70 (dd, 1H, J = 10.6 Hz, 15.2 Hz, ═C*H*−CH═CH−CO), 6.10 (d, 1H, J = 15.2 Hz, ═CH−CO), 5.98 (s, 2H, OCH_2O), 5.03-4.99 (m, 1H, NH−C*H*−CO), 3.74 (s, 3H, $COOCH_3$), 3.19 (d, 2H, J = 5.0 Hz, CH_2−Ar); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 173.1, 166.2, 156.7, 148.7 (2C), 141.1, 139.3, 131.6, 130 (2C), 128.1, 125.9, 124.3, 123.6, 115.9 (2C), 109.3, 106.5, 102.1, 54.9, 52.6, 36.8; CIMS: m/z 418 [M+Na+1]$^+$

*4.2.3.3. Piperoyl-L-valine methyl ester (**5**).* Light green solid; yield 75%; m.p. 124-125 °C; IR (KBr) υ_{max} 3293, 1741, 1647, 1606, 1253, 1037 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.36 (dd, 1H, J = 10.4 Hz, 14.4 Hz, −C*H*═CH−CO), 7.08-6.63 (5H, olefinic and Ar-*H*), 6.01 (d, 1H, J = 14.7 Hz, ═CH−CO), 5.99 (s, 2H, OCH_2O), 4.71-4.67 (m, 1H, NH−C*H*−CO), 3.75 (s, 3H, $COOCH_3$), 2.23-2.15 (m, IH, C*H*$(CH_3)_2$), 0.96 (d, 3H, J = 8.1 Hz, CH_3), 0.92 (d, 3H, J = 7.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.2, 166.4, 148.7 (2C), 142.4, 139.9, 131.3, 125, 123.2, 123, 109, 106.3, 101.8, 57.6, 52.7, 32.1, 19.4, 18.4; CIMS: m/z 354 [M+Na+1]$^+$

*4.2.3.4. Piperoyl-L-methionine methyl ester (**6**).* Off-white solid; yield 68%; m.p. 121-122 °C; IR (KBr) υ_{max} 3286, 1720, 1610, 1252, 1149, 1097, 1038 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.37 (dd, 1H, J = 10.5 Hz, 14.8 Hz, −C*H*═CH−CO), 6.97 (s, 1H, Ar−H), 6.89 (d, 1H, J = 7 Hz, Ar−H), 6.97-6.63 (m, 2H, olefinic and Ar-*H*), 6.36 (d, 1H, J = 7.7 Hz, Ar−H), 6.00 (d, 1H, J = 14.8 Hz, ═CH−CO), 5.92 (s, 2H, OCH_2O), 4.87-4.80 (m, 1H, NH−C*H*−CO), 3.77 (s, 3H, $COOCH_3$), 2.60-2.47 (m, 2H, $-CH_2-CH_2S-$), 2.23-2.20 (m, 2H, $-CH_2S-$), 2.10 (s, 3H, $-SCH_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.6, 166.1, 148.7 (2C), 142.5, 140.1, 131.2, 125, 123.2, 122.7, 109, 106.3, 101.8, 53.1, 52.1, 32.4, 30.5, 16; CIMS: m/z 386.1 [M+Na+1]$^+$

*4.2.3.5. Piperoyl-L-tryptophan methyl ester (**7**).* Off-white solid; yield 61%; m.p. 129-130 °C; IR (KBr) υ_{max} 2919, 2853, 1739, 1613, 1281, 1062 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.09 (NH), 7.55-6.59 (11H, olefinic and Ar-*H*), 6.04 (d, 1H, J = 14.9 Hz, ═CHCO), 5.97 (s, 2H, OCH_2O), 5.10-5.04 (m, 1H, NH−C*H*−CO), 3.70 (s, 3H, $COOCH_3$), 3.38 (d, 2H, J = 4.9 Hz, $-CH_2$ −3-Indolyl); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.3, 166.3, 148.8, 148.7, 142.3, 139.9, 136.7, 131.6, 128.2, 125, 123.4, 123.2, 123, 122.7, 120.2, 119.2, 111.8, 110.5, 109, 106.3, 101.8, 53.7, 52.9, 28.3; CIMS: m/z 419 [M+1]$^+$

*4.2.4. General procedure for deprotection of carboxyl moiety: Synthesis of compounds (**8**-**12**)*

Neutral alumina (15 g) was mixed with KF (10 g) in 200 mL of water for the preparation of KF/Al_2O_3. Water was then removed at 50-60 °C in rotary evaporator, this reagent was further dried in vacuum oven for 12 h. Compounds **3**-**7** (1 mmol) were added to KF/Al_2O_3 (1 g, 40% KF) and this dry mixture was stirred at room temperature in a round bottom flask to ensure uniform mixing of solid support KF/Al_2O_3 with substrate. The round bottom flask was then fitted with septum, placed in microwave oven and irradiated at 100% power for 3-4 min. After cooling, 5 mL water was added to the reaction mixture, stirred for 5 min and filtered. The filtrate was neutralized by adding aqueous HCl and the precipitate thus obtained was filtered and dried to obtain compounds **8**-**12**. All the compounds were characterized by ^{1}H, ^{13}C NMR, IR and MS data.

*4.2.4.1. Piperoyl-L-phenylalanine (**8**).* Yellow solid; yield 78%; m.p. 100-101 °C; IR (KBr) υ_{max} 3466, 1717, 1264 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.62 (d, 1H, J = 7.8 Hz), 6.43-5.98 (m, 12H, OCH_2O, olefinic and Ar-*H*), 5.31 (d, 1H, J = 14.9 Hz, olefinic), 3.8-3.6 (m, 1H, CH), 2.31-2.05 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 173.5, 165.8, 148.2, 148.1, 140.5, 138.7, 137.9, 131, 129.3 (2C), 128.5 (2C), 126.8, 125.3, 124, 123, 108.8, 105.9, 101.5, 54, 37.1; CIMS: m/z 366.1 [M+1]$^+$

*4.2.4.2. Piperoyl-L-tyrosine (**9**).* Brown solid; yield 77%; m.p. 127-128 °C; IR (KBr) υ_{max} 3100, 1595, 1253, 1193, 1148, 1036 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.24 (br s), 8.38 (1H, NH), 7.16-6.12 (11H, olefinic and Ar-*H*), 6.04 (s, 2H, OCH_2O), 4.47-4.40 (m, 1H, NHC*H*CO), 3.01-2.74 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 173.6, 165.5, 156.2, 148.2, 148.1, 140.2, 138.5, 131.1, 130.3 (2C), 128, 125.5, 124.3, 123, 115.3 (2C), 108.7, 106, 101.6, 54.3, 36.4; CIMS: m/z 382.1 [M+1]$^+$

*4.2.4.3. Piperoyl-L-valine (**10**).* Light yellow solid; yield 81%; m.p. 151-152 °C; IR (KBr) υ_{max} 3200, 1705, 1608, 1255 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.36 (dd, 1H, J = 10.9 Hz, 14.8 Hz, −C*H*═CHCO), 6.94-6.51 (5H, olefinic and Ar-*H*), 6.02 (d, 1H, J = 14.9 Hz, ═CHCO), 5.95 (s, 2H, OCH_2O), 4.70-4.66 (m, 1H, NHC*H*CO), 2.31-2.24 (m, 1H), 1.01-0.96 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.5, 165.8, 148.2, 148.1, 140.2, 138.5, 131.1, 125.5, 124.4, 123, 108.7, 106, 101.6, 52.6, 30.2, 19.5, 18.4; CIMS: m/z 318.1 [M+1]$^+$

*4.2.4.4. Piperoyl-L-methionine (**11**).* White solid; yield 75%; m.p. 169-170 °C; IR (KBr) υ_{max} 3300, 3273, 1707, 1612, 1512, 1255, 1039 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 8.44 (d, 1H, J = 7.6 Hz, NH), 7.19 (dd, 1H, J = 9.4 Hz, 14.8 Hz, −C*H*═CHCO), 7.03-6.85 (5H, olefinic and Ar-*H*), 6.20 (d, 1H, J = 14.8 Hz, ═CHCO), 6.05 (s, 2H, OCH_2O), 4.44-4.41 (m, 1H, NHC*H*CO), 2.53 (m, 2H, CH_2), 2.05-1.93 (5H, SCH_2, SCH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 173.8, 165.8, 148.2, 148.1, 140.4, 138.6, 131.1, 125.4, 124.1, 123, 108.8, 106, 101.6, 51.4, 31, 30, 14.8; CIMS: m/z 349.9 [M+1]$^+$

*4.2.4.5. Piperoyl-L-tryptophan (**12**).* Brown solid; yield 76%; m.p. 148-149 °C; IR (KBr) υ_{max} 3402, 1738, 1648, 1488, 1441, 1245, 1037 cm^{-1}; ^{1}H NMR (CD_3OD, 300 MHz) δ 7.57-6.72 (11H, olefinic and Ar-*H*), 6.07 (d, 1H, J = 15.1 Hz, ═CHCO), 5.90 (s, 2H, OCH_2O), 4.96-4.85 (m, 1H, NHC*H*CO), 3.34 (d, 2H, J = 2.3 Hz, $-CH_2$ −3-Indolyl); ^{13}C NMR (CD_3OD, 75 MHz) δ 173.1, 166.6, 147.5, 140.6, 138.3, 135.8, 130, 126.7, 123.6, 122.8, 122.6, 122.2, 121.6, 121.4, 120.2, 117.6, 117.1, 110.1, 108.8, 107.1, 100.5, 52.7, 26.4; CIMS: m/z 405.1 [M+1]$^+$

*4.2.5. Synthesis of compounds (**13**-**17**)*

Amides (**3**-**5** and **7**) were dissolved in methanol and Pd/C (5%) was added to it. The reaction mixture was subjected to catalytic hydrogenation at 40 psi for 30 min. The reaction mixture was filtered, the filtrate was collected and dried to yield compounds **13**-**15** and **17**. For synthesis of compound **16**, tetrahydropiperic acid **2a** was obtained by catalytic hydrogenation of piperic acid. Piperic acid **2** (4.36 g, 20 mmol) was treated with 5% Pd/C in methanol and subjected to catalytic hydrogenation at 40 psi for 30 min. The solution was filtered and the filtrate was concentrated to obtain tetrahydropiperic acid **2a** in 85% yield, it was coupled with methionine methyl ester using the same method as used for the preparation of compounds **3**-**7**.

*4.2.5.1. Tetrahydropiperoyl-L-phenylalanine methyl ester (**13**).* Yellow oil; yield 80%; IR (KBr) υ_{max} 3407, 1728, 1603, 1255, 1054 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 7.28-6.58 (8H, Ar-*H*), 5.90 (s, 2H, OCH_2O), 4.92-3.86 (m, 1H, NH−C*H*−CO), 3.72 (s, 3H, $COOCH_3$), 3.18-3.04 (m, 2H, CH_2−Ph), 2.54-2.49 (m, 2H, $-CH_2Ar$), 2.20-2.15 (m, 2H, $-CH_2CO$), 1.59-1.58 (m, 4H, $-CH_2-CH_2-$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.9, 172.7, 147.5 (2C), 136.5, 136.3, 129.7 (2C), 129.1 (2C), 127.6, 121.6, 109.3, 108.6, 101.2, 53.4, 52.8, 38.4, 36.8, 35.8, 31.6, 25.5; CIMS: *m/z* 384.1 $[M+1]^+$

*4.2.5.2. Tetrahydropiperoyl-L-tyrosine methyl ester (**14**).* Colorless oil; yield 75%; IR (KBr) υ_{max} 3481, 3284, 1729, 1646, 1506, 1290 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.93-6.57 (7H, Ar-*H*), 5.90 (s, 2H, OCH_2O), 4.89-4.82 (m, 1H, NH-C*H*-CO), 3.72 (s, 3H, $COOCH_3$), 3.10-2.93 (m, 2H, CH_2Ph), 2.57-2.47 (m, 2H, CH_2−Ar), 2.38-2.30 (m, 2H, CH_2CO), 1.65-1.55 (m, 4H, $-CH_2-CH_2-$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 178.9, 173, 156.1, 148, 146.1, 136.5, 136.7, 130.7, 128.4, 127.4, 121.6, 116.1, 109.3, 108.6, 101.2, 53.7, 52.9, 35.7, 32, 31.5, 31.3, 25.5; CIMS: *m/z* 400 $[M+1]^+$

*4.2.5.3. Tetrahydropiperoyl-L-valine methyl ester (**15**).* Yellow oil; yield 80%; IR (KBr) υ_{max} 3457, 1741, 1653, 1506, 1253, 1154 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.72-6.59 (3H, Ar-*H*), 5.90 (s, 2H, OCH_2O), 4.59-4.55 (m, 1H, NH−C*H*−CO), 3.73 (s, 3H, $COOCH_3$), 2.56-2.52 (m, 2H, $-CH_2Ar$), 2.27-2.08 (m, 2H, $-CH_2CO$), 2.19-2.08 (m, 1H, −C*H*$(CH_3)_2$), 1.67-1.60 (m, 4H, $-CH_2-CH_2-$), 0.93-0.88 (m, 6H, $2CH_3$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.2 (2C), 148, 146.1, 136.5, 121.6, 109.3, 108.6, 101.2, 57.3, 52.6, 37, 35.9, 31.7 (2C), 25.6, 19.4, 18.3; CIMS: *m/z* 336.1 $[M+1]^+$

*4.2.5.4. Tetrahydropiperoyl-L-methionine methyl ester (**16**).* Brown oil; yield 67%; IR (KBr) υ_{max} 3304, 1737, 1644, 1532, 1444, 1250, 1038 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.72-6.59 (m, 3H, Ar-*H*), 5.90 (s, 2H, OCH_2O), 4.75-4.68 (m, 1H, NH−C*H*−CO), 3.75 (s, 3H, $COOCH_3$), 2.57-2.47 (m, 2H, CH_2Ar), 2.26-2.00 (m, 9H, $-CH_2-CH_2-S-CH_3$, $-CH_2CO$), 1.67-1.60 (m, 4H, $-CH_2-CH_2$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173 (2C), 148 (2C), 136.5, 121.6, 109.3, 108.6, 101.2, 53, 52, 36.9, 35.8, 32.3, 31.7, 30.5, 25.5, 16; CIMS: *m/z* 368 $[M+1]^+$

*4.2.5.5. Tetrahydropiperoyl-L-tryptophan methyl ester (**17**).* Brown oil; yield 89%; IR (KBr) υ_{max} 3402, 3306, 1738, 1657, 1652, 1501, 1245, 1038 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.51 (NH), 7.51-6.04 (8H, Ar-*H*), 5.87 (s, 2H, OCH_2O), 4.96-4.90 (m, 1H, NH−C*H*−CO), 3.66 (s, 3H, $COOCH_3$), 3.35-3.24 (m, 2H, $-CH_2$−3-Indolyl), 2.47-2.43 (m, 2H, CH_2Ar), 2.13-2.09 (m, 2H, $-CH_2CO$), 1.56-1.49 (m, 4H, $-CH_2-CH_2-$); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.2, 173.1, 148, 146, 136.7, 136.6, 128.2, 123.3, 122.7, 121.6, 120.1, 119, 111.9, 110.4, 109.4, 108.6, 101.2, 53.4, 52.9, 36.8, 35.8, 31.6, 28.1, 25.4; CIMS: *m/z* 423 $[M+1]^+$

4.3. Antileishmanial activity

4.3.1. In vitro promastigote assay

Antileishmanial activity of the compounds was tested *in vitro* using modified MTT ([3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay [21] against a culture of *L. donovani* promastigotes grown in phenol red free RPMI-1640 (Sigma, USA), supplemented with 10% FCS (Sigma, USA) at 26 °C. *L. donovani* (5×10^5 cells/mL) promastigotes from logarithmic phase culture were grown in 96 well plate for 48 h before treatment with drugs. Drug dilutions were prepared in DMSO and concentration (100-500 μg/mL) of each drug was used in triplicate. The standard miltefosine was used at reported IC_{50} value. After treatment with drugs plate was kept at 26 °C for 48 h. MTT to a final concentration of 400 μg/mL was added and incubated for 4 h at 37 °C. These cells were centrifuged at 2000 rpm for 5 min, supernatant was removed and the resultant formazan formed in live promastigotes was dissolved in DMSO (100 μL) and absorbance was read at OD_{492} nm. The mean percentage of post treatment viable cells was calculated relative to control and results were expressed as concentration inhibiting the parasitic growth.

4.3.2. In vitro amastigote assay

L. donovani amastigotes isolated from hamster spleen or axenic culture of amastigotes (transformed from promastigotes *in vitro*) was acclimatized to gradual change in temperature (from 26 °C to 37 °C) and pH (from 7.2 to 5.5) [22]. *L. donovani* amastigotes (5×10^5 cells/mL) from logarithmic phase culture were grown in 96 well plate for 48 h at 37 °C before the treatment with drugs. Drug dilutions were prepared in DMSO and appropriate concentration of each drug was used in triplicate (20-100 μg/mL). Modified MTT assay as described above was used for evaluation of drug activity against *L. donovani* amastigotes. The mean percentage of post treatment viable amastigotes was calculated relative to control and results were expressed as concentration inhibiting the parasitic growth.

4.3.3. In vitro cytotoxicity assay

In vitro cytotoxicity was determined against PBMC (peripheral blood mononuclear cells) separated from heparinized blood of a normal healthy individual by Ficoll-Hypaque (Sigma, USA) density gradient centrifugation. Modified MTT ([3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay [21] was used for *in vitro* cytotoxicity. Briefly, the assay was performed in 96-well tissue culture plates. Cells were seeded to the wells of 96-well plate (1×10^5 cells/well) and were exposed to drug compounds (IC_{50} concentration and twice the concentration of IC_{50} were used) in triplicate. After treatment with drugs, plate was kept at 37 °C for 48 h. MTT to a final concentration of 400 μg/mL was added and incubated for 4 h at 37 °C. These cells were centrifuged at 2000 rpm for 5 min, supernatant was removed and resultant formazan formed in live PBMC was dissolved in DMSO (100 μL) and absorbance was read at OD_{492} nm. The mean percentage of post treatment viable cells was calculated relative to control (unexposed to drugs).

4.3.4. In vivo testing

Male Golden Syrian Hamsters, weighing 50-60 g were obtained from Central Animal Facility, National Institute of Pharmaceutical Education and Research (NIPER). Animals were housed in a room at a temperature of 24 ± 2 °C and 12 h dark and light cycle. Standard diet and water were allowed *ad libitum*. All the procedures performed in the study were approved by Institutional Animal Ethics Committee (IAEC), NIPER.

Visceral leishmaniasis (VL) was induced by intracardiac injection of *L. donovani* amastigotes (1×10^7) in golden hamsters. After 20 days of infection, hamsters were administered compounds by intraperitoneal route at 250 mg/kg for a period of ten days. Hamsters were sacrificed next day after the last dose for spleen weight and spleen parasitic burden. Spleen parasitic burden was assessed from geimsa stained impression smears [23]. Compounds were dissolved in 50% DMSO and vehicle treated control group received DMSO. Miltefosine was used as standard antileishmanial drug.

4.3.5. Statistical analysis

Data on spleen weight and parasitic burden are expressed as mean ± SEM. Data was analyzed using one way analysis of variance followed by Dunnett's test. Differences were considered to be significant if $p<0.05$. Statistical analysis was performed using Sigma Stat 2 statistical software.

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