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Synthesis, characterization and docking studies of amide ligands as anti-leishmanial agents

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Abstract: Aim of this study was to synthesize new inhibitors on the basis of active site of aspartic protease enzyme and to evaluate their intended biological activity. A3D model of an enzyme was generated via homology modeling and series of novel amide ligands were synthesized by using a short high yield process, subsequently, analyzed *in-silico* and *in-vitro* anti-leishmanial activities. Characterization and identification was accomplished via NMR (H^1& C^{13}), infrared and mass spectroscopic techniques. Among all compound (4) was found to show significant activity (IC_{50} 58±0.01) against *Leishmania major* (*L. major*) species. Furthermore, docking studies confirmed the inhibition of a targeted enzyme that supported the interaction of potent compound (4) with key residues (aspartic protease) via hydrogen bonds. Present study conferred about novel compound (4) as a promising compound to antagonize *L. major* activities in future.

Keywords: Amide ligands, aspartic protease, molecular docking, anti-leishmanial activity.

INRODUCTION

Leishmania among the neglected parasitic diseases is caused by the leishmania parasite. Mainly three forms of leishmania are visceral, cutaneous and mucocutaneous, that mainly effect skin, mucous membranes, and may results in development cause of anemia, fever, inflammation of spleen and liver (Desjeux, 2004). The main species of leishmania parasite are: *L. aethopia, L. viannia, L. infantum, L. donovoni, L. major, L. tropica, L. mexicana,* (Bañuls *et al*., 2007). Phlebotomine female sand fly is main cause for disease transmission (Kamhawi, 2006), although other potential vectors have recently been implicated (Dougall *et al*., 2011).

Leishmaniasis, affects millions of people worldwide with higher prevalence in developing countries. Furthermore, approximately 350 million human beings are at risk and every year 2 million new clinical cases are reported (Alvar *et al*., 2006 a,b). Association of leishmania HIV aids as a co-infection is also emerging (Desjeux and Alvar, 2003; Curz *et al*., 2006). Highest mortality rate is associated with visceral leishmaniasis (almost 60,000 yearly), which accounts for highest second rate among all neglected parasitic diseases (Alvar *et al*., 2006, Antinori *et al*., 2012). Despite presence of therapeutic treatment options like first line pentavalent antimony compounds (Aït-Oudhia *et al*., 2011), and second line drugs including anti-fungal, allopurinol, pentamidine, miltefosine, paramomicine, amphotericin B and sitamaquine (Almeida & Santos, 2011) in the market, severe side effects, difficulties in administration, high cost and drug resistance are key areas that limit clinical success (Schubach *et al*., 1998). Thus there exists a need to develop novel drugs that offer better safety profile. Plant or marine-derived natural compounds, are important sources of anti-leishmanial agents (Saleem *et al*., 2011).

Proteases breakdown peptide bonds in polypeptides or proteins molecules. Among them proteases, cysteine, serine and aspartic proteases are of particular interest due to their role in parasitic lifecycle. They are imperative virulence factors and have been concerned a variety of adaptation mechanisms for in-host parasite survival, including host immune modulation system, destruction of host connective tissues destruction and invasion, enabling

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parasites to move to the target sites to proliferate infection (Gómez and Olivier, 2010; Swenerton *et al*., 2011). Aspartic proteases are proteolytic enzymes with a pair of aspartic acids at the site of catalysis, crucial to activity of aspartic protease (Dunn, 2002). Aspartic protease enzymes in particular have been studies widely as drug targets in helminths (Brindley *et al*., 2001) and mites (Mahmood *et al*., 2013) due to their ability to digest skin and plasma proteins. Immunocyto-chemical techniques have identified the protease enzyme as good targets in leishmanial parasite, providing useful knowledge about the crucial one the main role of aspartic protease in life cycle of parasite and in the development of leishmanial (Kaye and Scott, 2011). Based on importance of protease enzyme in leishmania protozoan, various investigators have focused on leishmanial aspartic protease to combat leishmania (Valdivieso *et al*., 2007; Valdivieso *et al*., 2010; Perteguer *et al*., 2013).

In recent past research to identify anti-leishmanial compounds with better activity and safety profiles have been conducted. Among the compound identified, diterpene amides have proven as compounds with promising anti protozoal activity (Pirttimaa *et al*., 2016). We therefore decided to investigate the chemical synthesis and biological evaluation of amide ligand toward leishmania both *in-silico* and *in- vitro*. We synthesized and tested a series of amides nature compounds on culture of Leishmania among these"(9H-fluoren-9-yl) methyl (1-amino-1-oxo-3-(3-trityl-2, 3-di-hydro-1H-imidazole-4-yl) propan-2-yl) carbamate" shows tremendous as anti-leishmanial agents, which is leading to open new channel for novel drug development for leishmaniasis.

MATERIALS AND METHODS

Chemicals/instruments

Chemicals and solvents required for experiments were purchased from Sigma-Aldrich (UK). Glassware required for performing chemical reaction was further dried at 80 °C in an oven, for 4 h prior to use. Proton and carbon (NMR) spectra were taken by using $CDCl_3$ & MeoD as a solvent on Bruker spectrometer (300MHz) by using main reference as tetramethylsilane. Furthermore chemical shifts are in δ scale (ppm). FT-IR data was taken on Perkin Elmer apparatus. On other hand Mass spectra of series of compounds were recorded on microTOF-Q mass spectrophotometer. To find out the percentage of each element present in the synthesized compound, elemental analysis was performed by using elemental analyzer (GmbH -vario EL III). Compounds melting points were calculated by Gallenkemp apparatus.

General method for synthesis

A five compounds series (1-5) were synthesized with modifications to previously reported (Lei *et al*., 2008) (Scheme 1). Briefly, to a 250 mL round-bottomed flask, activeingredient (1.0 g, 2.5 mM), pyridine (125 µL), t-butoxycarbonyl anhydride (0.75 g, 3.25 mM), ammonium bicarbonate (0.25 g, 3.15 mM) and 12 mL dioxane were poured. For 24 h, the mixture of reaction was stir at 25 ^{0}C and sufficient quantity of water (25 mL) was added. After filtration, resultant products was filtered, and washed successively by distill water (3x 25 mL) and further the compounds were dried under vacuum in order to get solid compounds (1-5) table 1.

Homology, modeling & in-silico, anti-leishmanial activity

By using homology modeling procedures, 3D model of *Leishmania major* aspartic protease was generated, having template PDB ID: as 3S8I. Target protein & template has 52.80% amino acids sequence identity. Swiss model sever was used to build model (Peitsch 1996; Guex *et al*., 1999; Schwede *et al*., 2003). Different analysis programs was used to check model quality (QMEAN4; Benkert *et al*., 2011). By using GROMOS model energy was minimized (Guex and Peitsch, 1997).

Auto Dock Vina was used to perform molecular docking (Trott and Olson, 2010). Marvin Sketch 16.5.2 was used to draw ligands structures (Stowe *et al*., 2016). Optimization of ligands were done in Auto Dock 4.2 by using "prepare ligands". All the B-chain, water molecules and co-factor were removed for enzyme, however, hydrogen atoms were added which is a crucial step for the partial atomic charges calculations. The file was saved in PDB format. This refined PDB file of ligand & enzyme was opened, in AutoDock Tools (ADT) and were converted in to PDBQT file using Auto Dock, grid box of 50 Å (x, y, z) having spacing of 1.0 Å was designed using Auto Dock Toll and was specially centered in the active site region containing catalytic residues Asp-Thr-Gly having size (X=14; Y=14; Z=18 & center X=5.555; Y=20.163; Z=-20.455). Docking studies were conducted to find binding interactions with lowest energy by usingAutoDock and (LGA) (Morris *et al*., 1998).Discovery studio visualizer, Pymol and Auto Dock were used for visualizing the model and creating the illustrations (Systemes *et al*., 2015, Pettersen *et al*., 2004).

Sample preparation (Anti-leishmanial activity)

1mg of each compound as well as Amphotericin B standard drug was dissolved and diluted in 1 mL of DMSO solvent, further at 3000 rpm Log phase parasite were centrifuged for a period of 3 min. In a fresh medium culture, parasites were diluted to final density 2×10^6 cells/mL. To various wells of 96 well plates, 180 ìL of medium was transferred. After that compounds were transferred in medium, diluted serially by adding 20 ìL. In all parasite culture wells 100 ìL were added. For negative and positive control, three rows were kept. Serial dilution of DMSO was made in medium for negative control, and

for control positive different dilution of standard drug was added. For 72 h plates were incubated at 24 ^{0}C. Neubaur counting chamber was used to examine the culture microscopically, and compounds IC_{50} values having anti-leishmanial activity was checked. Experiments done in triplicate. IC_{50} of the compounds were calculated by using prism software (zahi *et al.,* 1999; Al-kahraman *et al*., 2010).

STATISTICAL ANALYSIS

All numerical values were given as Mean ± SEM. Conventional statistical methods were used to compare the results and level of significance was set at $p < 0.05$.

RESULTS

Spectroscopic detail

Five novel compounds (1-5) were synthesized whose spectroscopic detail is given below (table 1):

(9H–fluoren-9-yl) methyl (1-amino-1-oxo-3phenyl propan-2-yl) carbamate

Yield 82%, white colour solid,[1] HNMR(300MHz, $CDCl_3$) δ7.89(s,NH,1H), 7.72(d,*J*=6Hz,2Ar-H), 7.49(d,*J*=6Hz, 2Ar-H), 7.42(d,*J*=6Hz,2Ar-H,), 7.36(t,*J*=6Hz,2ArH), 7.29-7.18(m,ArH,NH_2,7H), 4.85(s,CH), 4.64(t,*J*=6Hz, CH_2,2H), 4.34(s,CH), 3.68(m, CH_2).

R= (1) Phenylalanine, R= (2) (Lysine)NHBOC , R= (3) (Tyrosine)Otbu ,R= (4) (Histidine)NTrityl , R= (5) Glutamine

Scheme 1: Scheme for synthesis for the newly synthesized compounds (1-5).

^{13}CNMR δ170.2(C=O), 156.0(CONH), 143.6(2Ar-C), 141.2(2Ar-C), 136.9(Ar-C), 128.3(2Ar-CH), 127.5(2Ar-CH,), 126.9(2Ar-CH), 126.7(2Ar-CH), 125.5(3Ar-CH), 119.7(2Ar-CH), 77.3(CH_2), 66.9(CH), 48.7(CH), 47.0(CH_2). (ῡ,cm^{-1})FTIR;3316 (NH_2), 3204 (HN), 1681(CO), 2972 (CH,arom), 1531(C=C, arom), 1420(CH).HREI MS: m/z 387.1[M+1] % Composition Calculated; $C_{24}H_{22}N_2O_3$: C 74.57; H 5.72; N 7.17; O 12.42.

(9H–fluoren-9-yl)methyltert-butyl(6-amino-6-oxo-hexane-1,5 diyl) di carbamate

Yield 93%, off white color solid, ^{1}H NMR (300 MHz, $CDCl_3$) δ7.81(d, *J*=9Hz,NH,2H), 7.75(d, *J*=6Hz,NH_2,2H), 7.58(d, *J*=6Hz,2Ar-H),7.40(t, *J*=9Hz,2Ar-H), 7.33(d, *J*=6Hz,2ArH), 7.31(d, *J*=6Hz,2Ar-H), 7.28(t, *J*=9Hz, NH_2,2H), 5.63(d, *J*=6Hz,CH_2,2H), 4.43(s, CH), 4.20(s, CH), 3.11(d, *J*=6Hz,CH_2,2H), 1.43(m,CH_3,9H), 1.27(m,CH_2,4H).

^{13}C NMR, δ 174.1(C=O),156.2(2C=O-O),143.6(2Ar-C), 141.3(2Ar-C,), 127.7(2Ar-CH), 127.1(2Ar-CH), 125.0 (2Ar-CH), 119.9(2Ar-CH), 79.3(tc,1C), 67.0(CH_2), 66.8 (CH), 47.0(CH), 31.7(CH_2), 29.6(CH_2,2C), 28.4(CH_3,3C), 22.3(CH_2). (ῡ,cm^{-1}) FTIR; 3320 (NH_2), 3207(HN), 1680(CO), 2966(CH, arom), 1525(C=C, arom), 1418(C-H). HR.EI MS: m/z 468.2[M+1]. % Composition Calculated; $C_{26}H_{33}N_3O_5$: C, 66.71; H, 7.04; N, 8.96; O, 17.12.

(9H–fluoren-9-yl) methyl (1-amino-3-(4-tert-butoxy) phenyl) 1-oxopropan-2-yl) carbamate

Yield 90%, white colour solid, ^{1}H NMR (300 MHz, $CDCl_3$), δ 7.91(s,NH,1H), 7.75(d, *J*=9Hz,2Ar-H), 7.55(d, *J*=9Hz,2Ar-H), 7.40(t, *J*=6Hz,2Ar-H),7.31(dd, *J*=9Hz, 2Ar-H),7.11(d, *J*=6Hz,2Ar-H), 7.09(d, *J*=6Hz NH_2), 6.90(d, *J*=9Hz,CH_2), 5.43(s,CH), 4.46(s, CH), 4.19(t, *J*=6Hz,CH_2), 3.12(d, *J*=6Hz,CH_2), 1.32(m,CH_3,9H), ^{13}C NMR, δ 173.0(C=O), 154.4(CONH), 154.0(Ar-C), 143.6(2Ar-CH), 141.3(2Ar-C), 129.7(2Ar-CH), 127.7(Ar-C), 127.1(2Ar-CH), 125.2(2Ar-CH),124.9(2Ar-CH), 124.3(2Ar-CH), 120.0(2Ar-CH), 78.4(1C), 66.9 (CH_2), 47.1(CH), 37.7(CH_2), 28.8(CH_3,3C). (ῡ,cm^{-1})FTIR; 3318(NH_2), 3204(NH), 1683(CO), 2976(CH, arom), 1528(C=C,arom), 1412(CH).HR-EI MS: m/z 459.2 [M+1]. % Composition Calculated; $C_{28}H_{30}N_2O_4$: C, 73.32; H, 6.58; N, 6.10; O, 13.94

(9H–fluoren-9-yl) methyl (1-amino-1-oxo-3-(3-trityl-2,3 di-hydro-1H-imidazole-4-yl)propan-2-yl) carbamate

Yield 48%, pale yellow colour solid, ^{1}H NMR (300 MHz,$CDCl_3$), δ 7.74(d, *J*=9Hz,2Ar-H), 7.59(t,*J*=6Hz,2Ar-H), 7.41-7.28(m,NH,Ar-H,14H), 7.10(m,NH_2,Ar-H,5H), 6.70(s,CH), 4.46(m,CH), 4.36(d,*J*=9Hz,CH_2), 4.20 (m,CH), 3.71(s,CH_2,8H).^{13}C NMR,δ 173.7(CO), 155.2 (CONH), 143.8(2Ar-C), 142.1(2Ar-C), 141.4(3Ar-C), 129.6 (6Ar-CH), 128.4(Ar-C), 127.5(6Ar-CH), 126.9 (7Ar-CH), 125.2(2Ar-CH), 119.9(2Ar-CH), 77.9(C), 67.0(CH_2), 56.2(CH.CH_2), 47.1(CH), 30.7(CH_2). (ῡ,cm^{-1}); FTIR3310(NH_2), 3200(HN), 1685(CO), 2975 (CH,arom), 1523(C=C,arom) ,1420(CH). HR-EI MS m/z 620.2[M+1].

% Composition Calculated: $C_{40}H_{36}N_4O_3$: C, 77.39;H,5.75; N,9.08; O,7.72

(9H-fluoren-9-yl) methyl (1,5-diamino-1,5-dioxopentan-2-yl) carbamate

Yield 85%, White colour powder, ^{1}H NMR (300 MHz,$CDCl_3$), δ 7.80(s,NH), 7.71(d *J*=6Hz, 2Ar-H), 7.55(d, *J*=9Hz,2Ar-H), 7.39(m,2Ar-H), 7.33(m,2Ar-H), 7.26(d, *J*=6Hz, NH_2,4H), 4.46(d, *J*=6Hz,CH_2), 4.29(s,CH), 4.16(d, *J*=6Hz ,CH), 2.07(m, CH_2,4H).

Table 1: Physical properties of the novel synthesized compounds (1-5).

Sr. No	Structure	Molecular weight	Formula	Melting point	Solubility
1		386.44	$C_{24}H_{22}N_2O_3$	206°C	DMSO
2		467.56	$C_{26}H_{33}N_3O_5$	76°C	DMSO
3		458.55	$C_{28}H_{30}N_2O_4$	125°C	DMSO
4		620.74	$C_{40}H_{36}N_4O_3$	90°C	DMSO
5		367.40	$C_{20}H_{21}N_3O_4$	170°C	DMSO

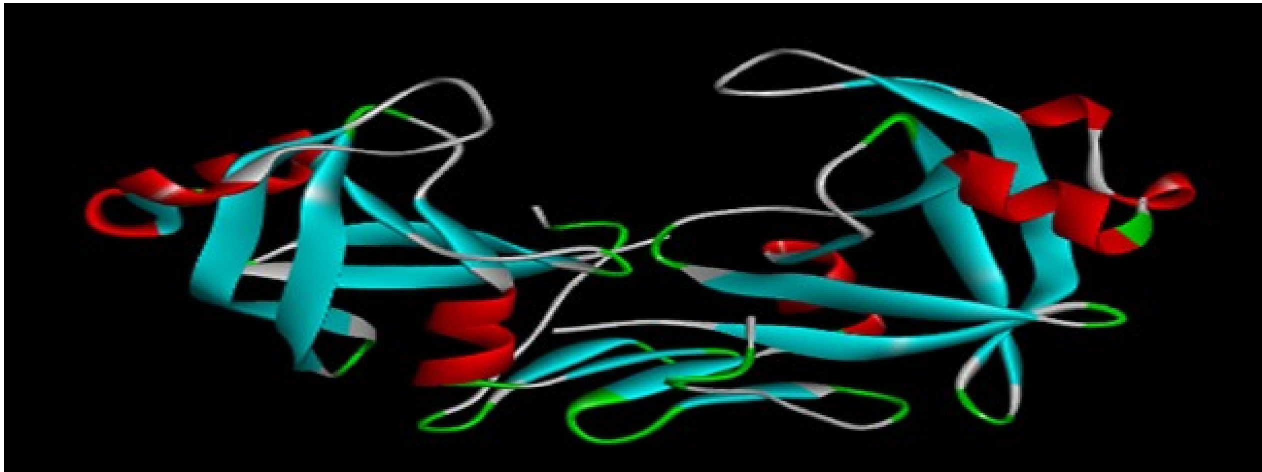

Fig. 1: A 3D generated homology model of *Leishmania major* aspartic protease

^{13}C NMR, δ 173.5(CO), 172.9(CO), 156.2(CONH), 143.5(2Ar-C), 141.3(2Ar-C), 127.7(2Ar-CH), 127.0(2Ar-CH), 125.3(2Ar-CH), 119.9(2Ar-CH), 67.0(CH_2), 62.2(CH), 47.1(CH), 31.7(CH_2), 28.0(CH_2). FTIR ($\bar{\upsilon}$, cm^{-1}); 3317(NH_2), 3203(NH), 1679(CO), 2965(CH, arom), 1520(C=C, arom), 1422(C-H). HR-EIMS m/z 367.1 [M+1]. % Composition Calculated: $C_{20}H_{21}N_3O_4$: C, 64.36; H ,5.76; N, 11.42; O, 17.42

Homology model of leishmania major aspartic protease

A 3D model *Leishmania major* aspartic protease was generated by using homology modeling procedures (fig. 1).

Analysis of 3D generated model quality

Quality of 3D generated homology model of *Leishmania major* aspartic protease was checked by swiss model server.QMEAN4 and QMEAN6 values for model was also calculated (fig. 2).

Docking of Compound (4) on L. major aspartic protease active site

Different docking software like Auto Dock Tool, Discovery studio visualize and Pymol were used for visualization of enzyme active site and docking of compound on enzyme active site (fig. 3).

Table 2: *In vitro* studies about anti-leishmanial activity of newly synthesized compounds

Compounds	Anti-leishmanial activity of (1-5) on *L. major* IC_{50} µg /m L± S.D
1	0.72 ± 0.05
2	0.68 ± 0.02
3	0.65 ± 0.08
4	0.58 ± 0.01
5	0.66 ± 0.07
DMSO (-ve Control)	0.98 ± 0.01
Amphotericin B (Standard drug)	0.56 ± 0.03

Note: % Inhibition activity: 0.99±0.00 = non-significant, 0.95-0.80 = low, 0.79-0.70 = moderate, 0.69-0.60 = good, <0.59-0.56 =significant.

Compound (4) binding interactions with enzyme active site residues

L. major aspartic protease contains three amino acids (Aspartic acid, Threonine and Glycine) in its active site cavity. Compound (4) shows strong interactions via hydrogen bonding with the aspartic acid, one of the key amino acid necessary for catalysis by the enzyme (fig. 4).

Biological activity anti-leishmanial

In vitro anti-leishmanial activity of newly synthesized compounds (1-5) was summarized in table 2 (Zahi *et al.,* 1999; Al-kahraman *et al*., 2010).

DISCUSSION

A series of five amide ligands (1-5) (table 1) were synthesized starting from the mixing of active ingredient, pyridine, t-butoxycarbonyl anhydride, ammonium bicarbonate and dioxane for 24 h in round bottom flask. Finally products filtered, successively washed with distilled water, dried to furnish amide ligands (1-5) respectively. The compounds (1-5) were subjected to FTIR, which showed strong absorption peaks in reign between 3200 and 3400 cm^{-1} which were indicate appearance of N-H stretching vibrations. The carbonyl absorption band appeared at about 1700 cm^{-1} and bands in the region peaks 1600-1500 cm^{-1}indicated the presence of a benzene ring. The spectra of ^{1}H NMR of ligands (1-5) revealed that signals due to the aromatic protons appeared around δ 7-8 ppm, and aliphatic protons resonated at δ~2-4 ppm. In the ^{13}C NMR spectra of ligands, (1-5) the carbonyl carbon signal was located at δC170-175 ppm. Aromatic carbon signals were observed in the region δC140-120 ppm. Sp3-Carbons resonated around δC30-15 ppm. Mass spectra peaks of ligands confirmed the molecular masses of compounds.

Inhibition of aspartic protease enzyme in leishmania will disrupt the cell division of the parasite leading to parasitic death (Valdivieso *et al*., 2007).Thus organic molecules may be designed & synthesized, which can bind to active site of leishmanial aspartic protease leading to its inhibition. *In silico* techniques like (QSAR), homology modeling, molecular docking are in use for the drug designing and discovery process of therapeutic agents (Jabeen *et al*., 2014). Synthesized series of compounds were computationally docked into the main cavity of active site of leishmanial aspartic protease enzyme, essential for the proliferation of parasite. By using procedures of homology modelling, *L. major* aspartic protease 3D model was produced by using amino acids similarity between this protein and its close crystallized

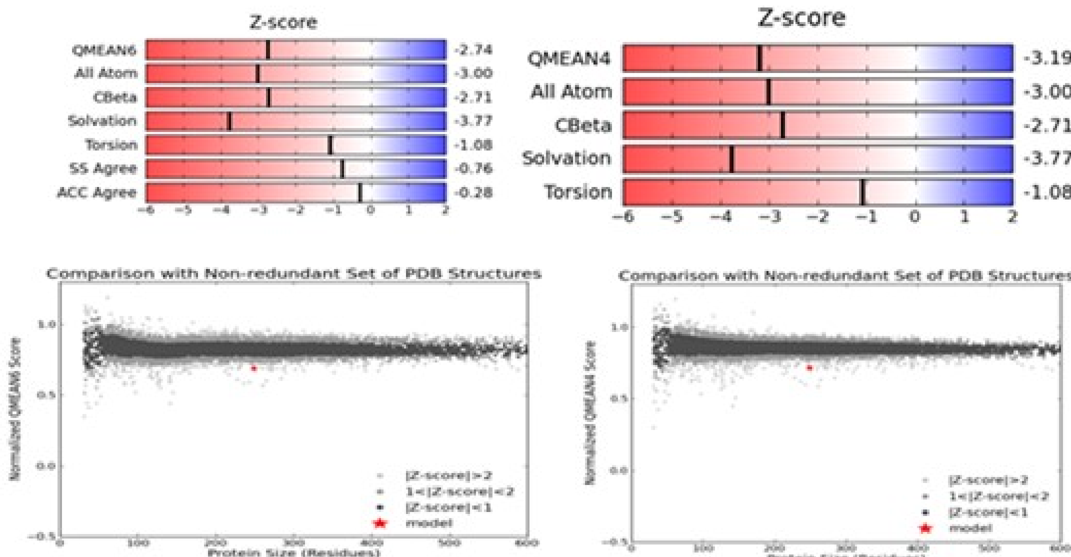

Fig. 2: Model structure quality of 3D generated homology model of *Leishmania major* aspartic protease.

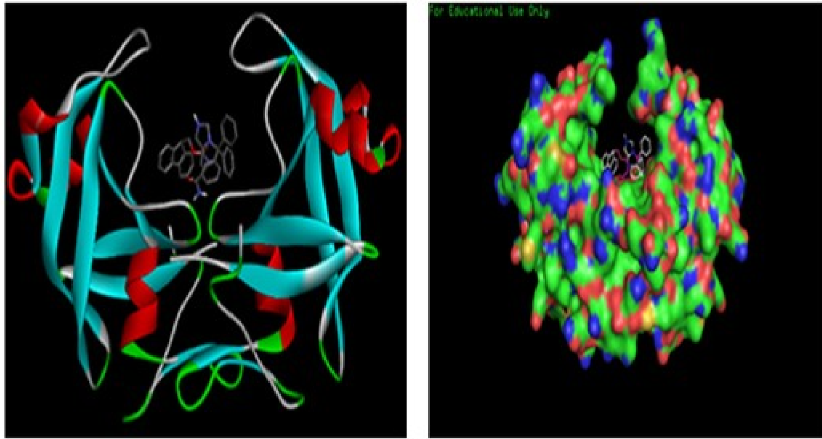

Fig. 3: Computer generated molecular model of synthesized compound **(4)** on the main active site of leishmanial aspartic protease

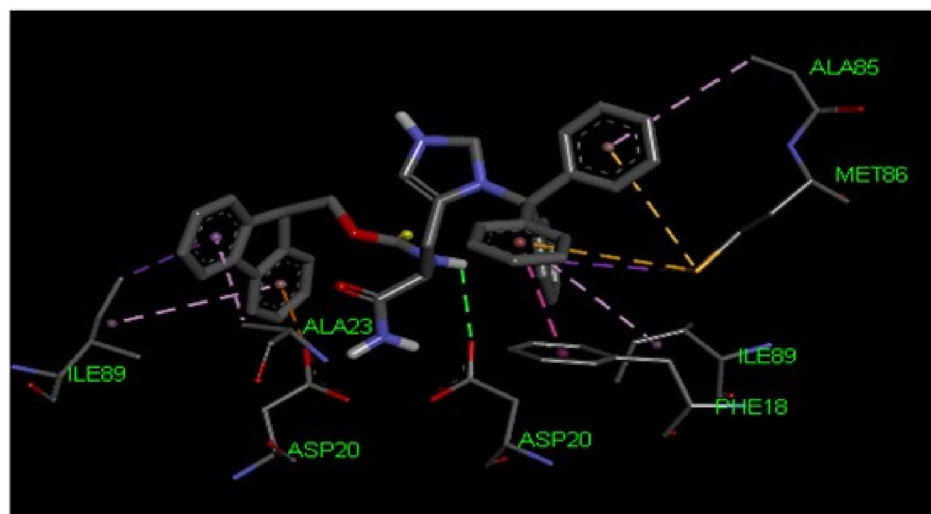

Fig. 4: Computer generated molecular model of compound (4) showing H- bonding binding with Asp20 present in the active site of leishmanial aspartic protease

template 3S8I.Global model quality estimation scores are QMEANscore4, 0.774; Zscore,-3.326, within normal range for homology based structure models are accepted (fig. 2). Modeled protein is a dimer shape (fig. 1), which is double Asp-Threonine-Gly triad amino acid sequence in the active site in almost similar manner to that exhibited by homologous protein 3S8I.This fact suggest that inhibitors are small proteinase will be normally bind the active site, thus inhibiting proteinase activity.

In current study, Auto Dock Tools (Trott and Olson, 2010) was carried out to perform molecular docking of small ligands on aspartic protease by treating the ligands as conformationally flexible. Compound (4) showed plausible docking with acceptable statistics in the active site of aspartic protease (fig. 3). The aspartic protease enzyme is a dimer consisting of two active sites having amino acid residues Aspartic acid-Threonine-Glycine triad (Asp-Thr-Gly) in active site. Residues Phe18, Asp20, Thr21, Gly22, Ala23, Ala85, Met86, Ile89 forming the active site of enzyme were seen surrounding the active compound inside the cavity of active side. In a typical aspartic protease enzyme a pair of Aspartic Acid (Asp) residues generally in Aspartic acid-Threonine-Glycine triad (Asp-Thr-Gly) in the active site play important functional roles in the catalytic function of the enzyme. These residues are conserved in leishmanial aspartic protease. Thus, interaction of the ligands with these residues in leishmanial aspartic proteases can inactivate the enzyme, leading to the inhibition of catalysis. During this investigation a strong Hydrogen bonding presented binding interactions of compound (4) with active site residue Asp20 (fig. 4), that shows possible alteration in the active site cleft, thus leading to disruption of catalytic function of leishmanial aspartic protease. Furthermore, compound (4) was found to interact

favorably with leishmanial aspartic protease with a very good docking and Glide score of -1.6 kcal/mol.

In present study anti-leishmanial activity of amide compounds (1-5) was checked according to the method of (zahi *et al.,* 1999; Al-kahraman *et al*., 2010) against *L. major* culture. As evidence from (table 2) amide compounds have shown excellent anti-leishmanial activity towards *L. major*. Compound (4) has shown significant remarks towards *L. major* having IC_{50} value 0.58 ± 0.01, in comparison to standard drug IC_{50} range (0.56 ± 0.03). Rest of the compounds have shown good and moderate activity towards *L. major*. Remarkable *in - vitro* anti-leishmanial activity, as well as docking studies of compound (4) may lead to be candidate for novel anti-leishmanial agents.

CONCLUSION

It was concluded that synthesized compound (4) presented activity towards *L. major* with significant activity, could be used as scaffold to design further compounds. This activity was supported by (*in silico* analysis) as a result of binding of ligands with aspartic protease active site residues through hydrogen bonding.

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