Journal Pre-proof

Exploring the ability of dihydropyrimidine-5-carboxamide and 5-benzyl-2,4-diaminopyrimidine-based analogues for the selective inhibition of *L. major* Dihydrofolate reductase

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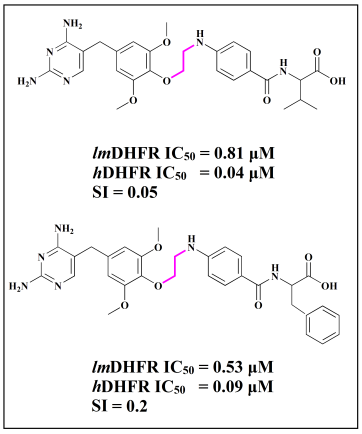

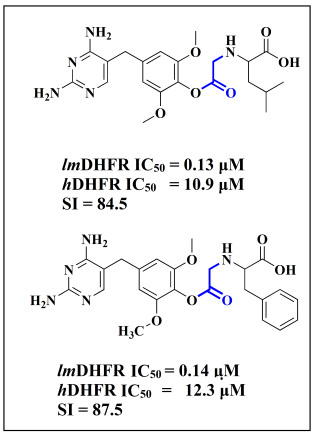

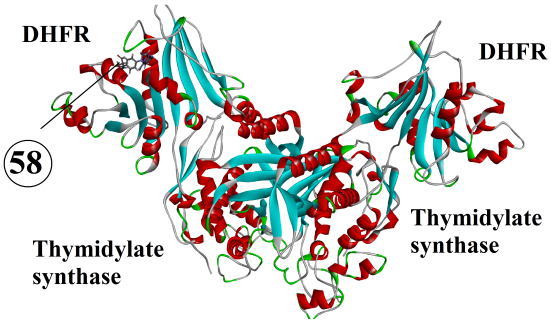

Homology modelled *L. major* DHFR-
Thymidylate synthase

Exploring the ability of dihydropyrimidine-5-carboxamide and 5-benzyl-2,4-diaminopyrimidine-based analogues for the selective inhibition of *L. major* Dihydrofolate reductase

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Abstract

To tackle leishmaniasis, search for efficient therapeutic drug targets should be pursued. Dihydrofolate reductase (DHFR) is considered as a key target for the treatment of leishmaniasis. In current study, we are interested in the design and synthesis of selective antifolates targeting DHFR from *L. major*. We focused on the development of new antifolates based on 3,4-dihydropyrimidine-2-one and 5-(3,5-dimethoxybenzyl)pyrimidine-2,4-diamine motif. Structure activity relationship (SAR) studies were performed on 4-phenyl ring of dihydropyrimidine (**26-30**) template. While for 5-(3,5-dimethoxybenzyl)pyrimidine-2,4-diamine, the impact of different amino acids (valine, tryptophan, phenylalanine, and glutamic acid) and two carbon linkers were explored (**52-59**). The synthesized compounds were assayed against *Lm*DHFR. Compound **59** with the IC_{50} value of 0.10 μM appeared as potent inhibitors of *L. major*. Selectivity for parasite DHFR over human DHFR was also determined. Derivatives **55-59** demonstrated excellent selectivity for *Lm*DHFR. Highest selectivity for *Lm*DHFR was shown by compounds **56** (SI = 84.5) and **58** (SI = 87.5). Compounds Antileishmanial activity against *L. major* and *L. donovani* promastigotes was also performed. To explore the interaction pattern of the synthesized compounds with biological macromolecules, the docking studies were carried out against homology modelled *Lm*DHFR and *h*DHFR targets.

Key words: Leishmania major DHFR, Human dihydrofolate reductase, Methotrexate mimics; antileishmanials; dihydropyrimidine-5-carboxamides; benzyl-2,4-diaminopyrimidines

1. Introduction

The diseases caused by protozoan parasites are considered neglected tropical diseases (NTDs). These are the major health issues and causes of mortality and economic hardships predominantly in developing countries. Among these NTDs, leishmaniasis is the second-highest incidence of parasitic disease in the world, second only to malaria, with high mortality and morbidity. The NTDs primarily affect the people living in poverty with inadequate sanitary conditions and close contact with the infectious vectors. There are 20 types of the parasite of Leishmania which cause Leishmaniasis belongs to genus Leishmania and family trypanosomatidae. Among these species, *L. donovani, L. tropica, L. Mexicana* and *L. braziliensis* are major causes of Leishmaniasis [1-7].

The available drugs to treat leishmaniasis have poor clinical efficacy, toxicity, and resistance problems. Therefore, there is a dire need to find new drugs to treat leishmaniasis. To tackle leishmaniasis, a search for efficient therapeutic drug targets should be pursued. Dihydrofolate reductase (DHFR) is a ubiquitous enzyme and is involved in the folate reduction pathway. Since it is involved in generating raw material for the replication of DNA, it is a key therapeutic target for the treatment of many human diseases such as bacterial, mycobacterial, fungal, protozoal infections, rheumatoid arthritis, and cancer [8-9]. Dihydrofolate reductase (DHFR) is considered as a key target for the treatment of leishmaniasis. In human beings, the activities of DHFR and thymidylate synthase (TS) are well separated. However, in contrast to human cell, leishmania have a bifunctional dihydrofolate reductase-thymidylate (DHFR-TS) [10-12]. Among diverse bioactive moieties, methotrexate (MTX, **1**) and trimethoprim (TMP, **2**) are clinically used DHFR inhibitors. However, these two inhibitors have shown poor selectivity for leishmania DHFR [13-17].

Several studies have been reported on parasite DHFR with special emphasis on the selectivity comparison with human enzyme. Cavazzuti et al., identified the inhibitors of folate-dependent parasitic enzymes (PTR1 and DHFR-TS) *via* screening of folate-based library. Quinoxaline and 2,4-diaminopteridine derivatives showed selectivity and specificity against studied parasitic enzymes compared with human enzymes. 2,4-diaminopteridine derivative (**3**, Figure 1) with *Ki* = 4 μM against *Lm*DHFR showed excellent selectivity (*Ki* for *h*DHFR = 10 μM). Identified quinoxaline derivative (**4)** and amino quinoxaline (**5**) also showed selectivity towards *Lm*DHFR. However, derivative **6** showed selectivity toward human enzyme [18]. Hardy et al., tested a library of 74 compounds against parasitic as well as *h*DHFR. Quinazoline derivative **7** showed high activity and selectivity towards *Lm*DHFR (IC_{50} for *Lm*DHFR = 0.025 μM, for *h*DHFR = 0.040 μM). While pyrimidine derivative **8** showed selectivity towards *h*DHFR [19]. Chowdhury et al., identified compound **9** as inhibitor of *h*DHFR [20].

Our research group have already reported C-5 and C-6 dihydropyrimidine (DHPM) derivatives as potent antileishmanial agents [26]. Pyrazole derivative emerged as most potent antileishmanial agent with IC_{50} value of 0.47 μg/mL (0.95 μM). The objective of the current research is to design, synthesis, and evaluation of antifolates targeting Leishmania major dihydrofolate reductase (*Lm*DHFR). In current study, we synthesized 2,4-diaminopyrimidine and dihydropyrimidine (DHPM) based methotrexate mimics and evaluate them for their potential against *L. major* DHFR. Antileishmanial activity against promastigotes of *L. major* and *L. donovani* was also performed. Interaction of the newly synthesized compounds with biological targets was also explored.

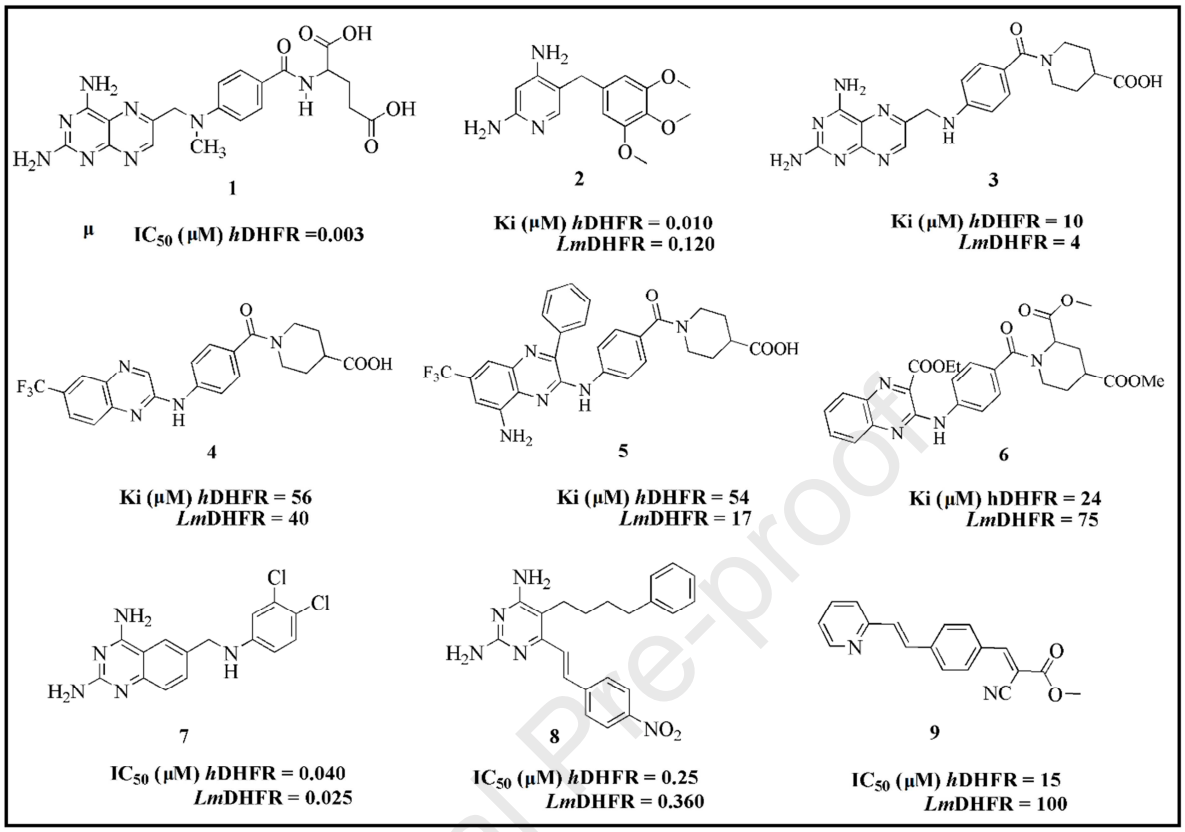


Figure 1. Potent anti-leishmanial agents reported recently

Results and discussion

2.1. Design strategy

Our design strategy of the current research is based on structural information of methotrexate. The methotrexate structure consists of pteridine ring, *p*-aminobenzoic acid (PABA) and glutamic acid (**Figure 2b**). A detailed SAR on 5-benzyl-2,4-diaminopyrimidines has already undertaken by Chowdhury et al., and Gilbert et al., [11, 20]. We selected pyrimidine-2,4-diamine from trimethoprim (5-benzyl-2,4-diaminopyrimidine derivative) and 3,4-dihydropyrimidine-2-one scaffold for C-5 modifications. Apart from glutamate tail, 1-benzoylpiperidine-4-carboxylic acid, and dimethyl 1-benzoylpiperidine-2,4-dicarboxylate mimics were also employed to explore their

effect on the selectivity between human and parasitic DHFR. In our current study, we designed two strategies to explore the structure activity relationship (SAR) to synthesize more potent antileishmanial drugs. For current study, we planned to synthesize variety of compounds containing varying linker length. In strategy 1, SAR was carried out on 4-phenyl ring of the DHPM-2-one core and glutamate core remains same. While in strategy 2, we planned SAR on 5-(3,5-dimethoxybenzyl)pyrimidine-2,4-diamine derivatives at amino acid point by using valine, tryptophan, phenylalanine, and glutamic acid. A two-carbon linker was also attached to explore the inhibition potential of these derivatives (**Figure 3c**).

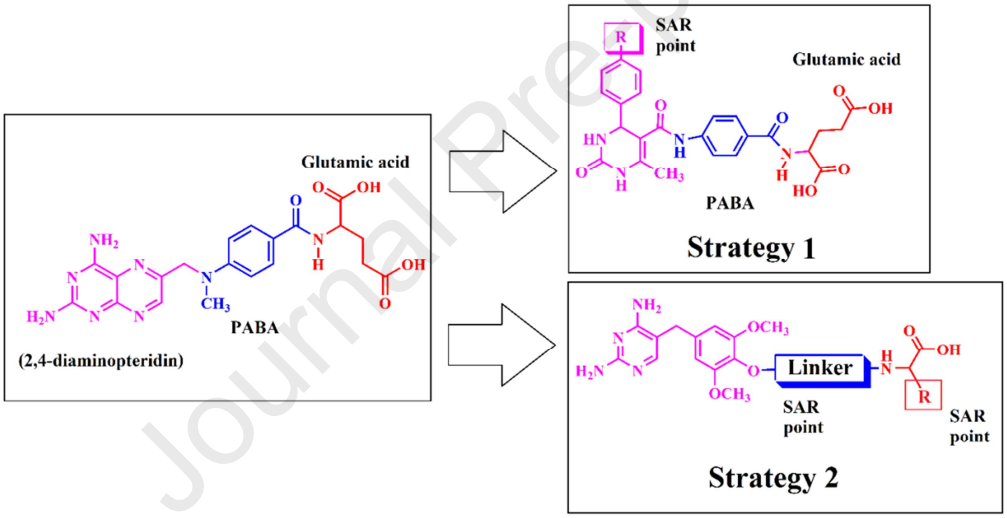
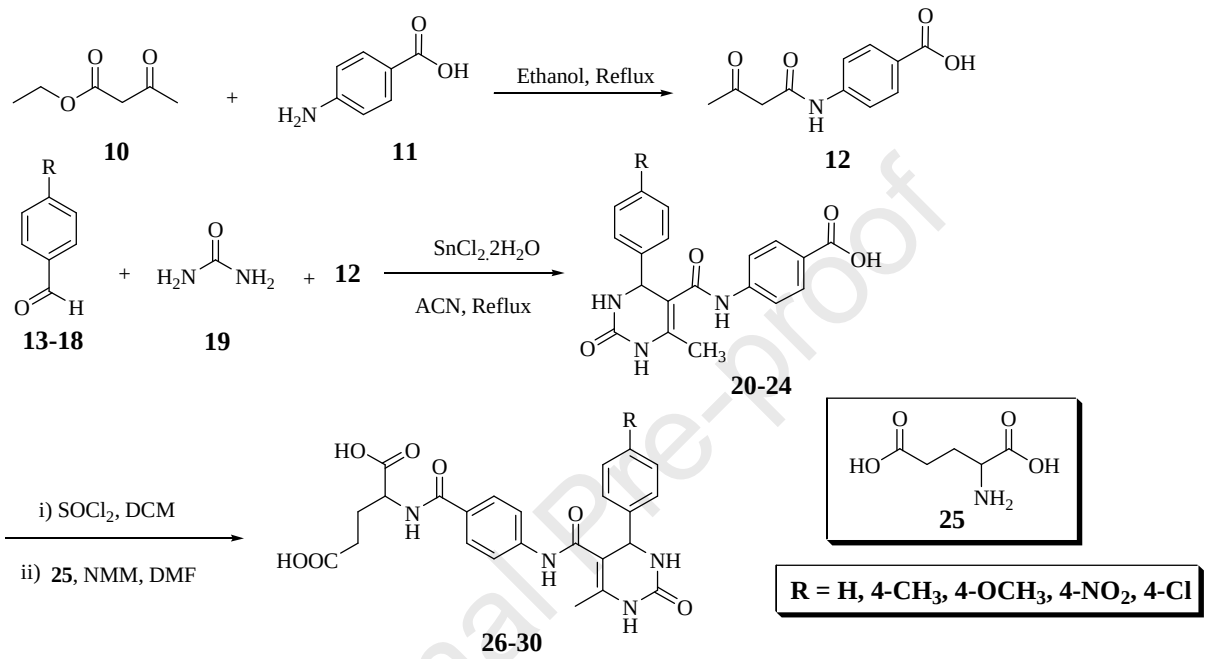


Figure 2. Design strategy for current research

2.2. Chemistry

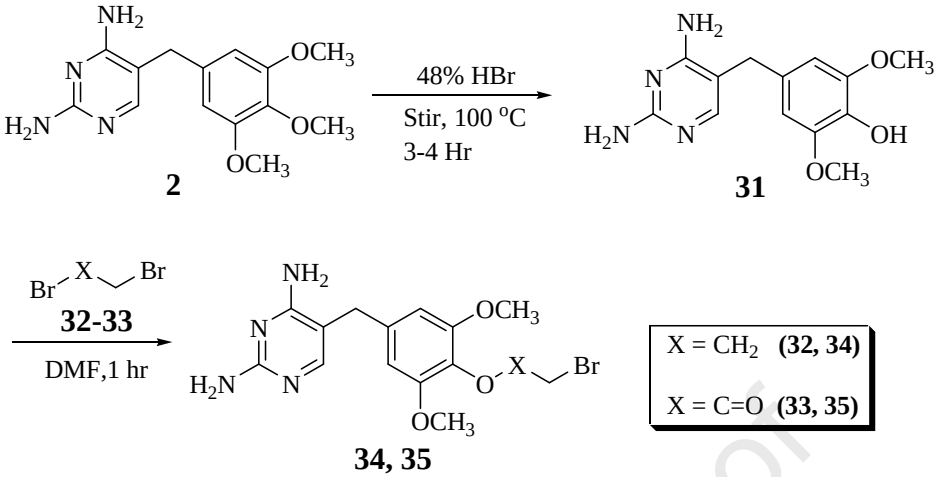
The general synthetic rout for the synthesis of compounds **26-30** is outlined in **Scheme 1**. Compound **12** was synthesized in 51.3 % yield by refluxing a mixture of 4-aminobenzoic acid (PABA, **11**) and ethyl acetoacetate (**10**) in absolute ethanol. 2-oxo-3,4-dihydropyrimidine-5-carboxylates (**20-24**) were synthesized by using our previously reported Biginelli protocol [21].

Acyl chloride derivatives of **20-24** were synthesized by the reaction with thionyl chloride in dichloromethane (DCM). These acyl chloride derivatives were then reacted with glutamic acid (**25**) to obtain target compounds (**26-30**).

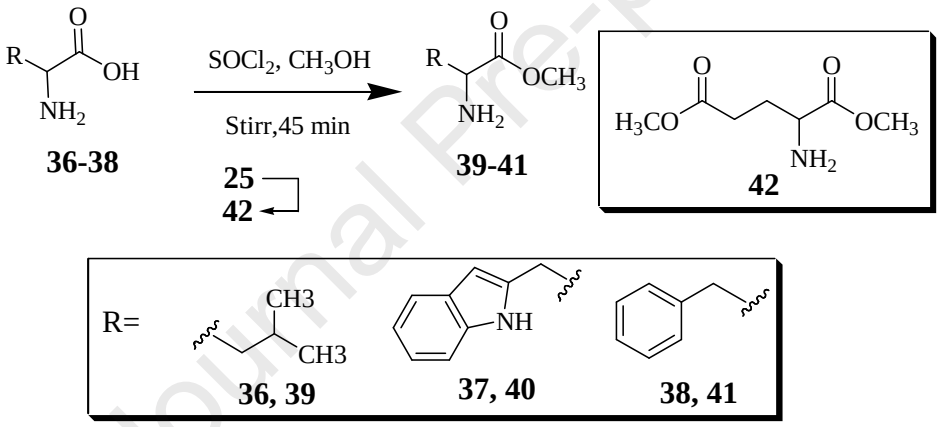


Scheme 1: Synthesis of dihydropyrimidine based methotrexate mimics

A multistep protocol for the synthesis of trimethoprim derivatives **56-63** is outlined in **Schemes 2-4**. Hydroxy trimethoprim derivative (**31**) was obtained by using our previously reported method by using 48% HBr and trimethoprim (**2**) [22-24]. Compound **40** was then reacted with 1,2-dibromoethane (**32**) and bromoacetyl bromide (**33**) in DMF to afford **34-35 (Scheme 2)**. Methyl esters (**39-42**) of amino acids (**25**, **36-38)** were synthesized by reaction of amino acids by drop-wise addition of thionyl chloride in methanol at 0 ^{o}C **(Scheme 3)**.

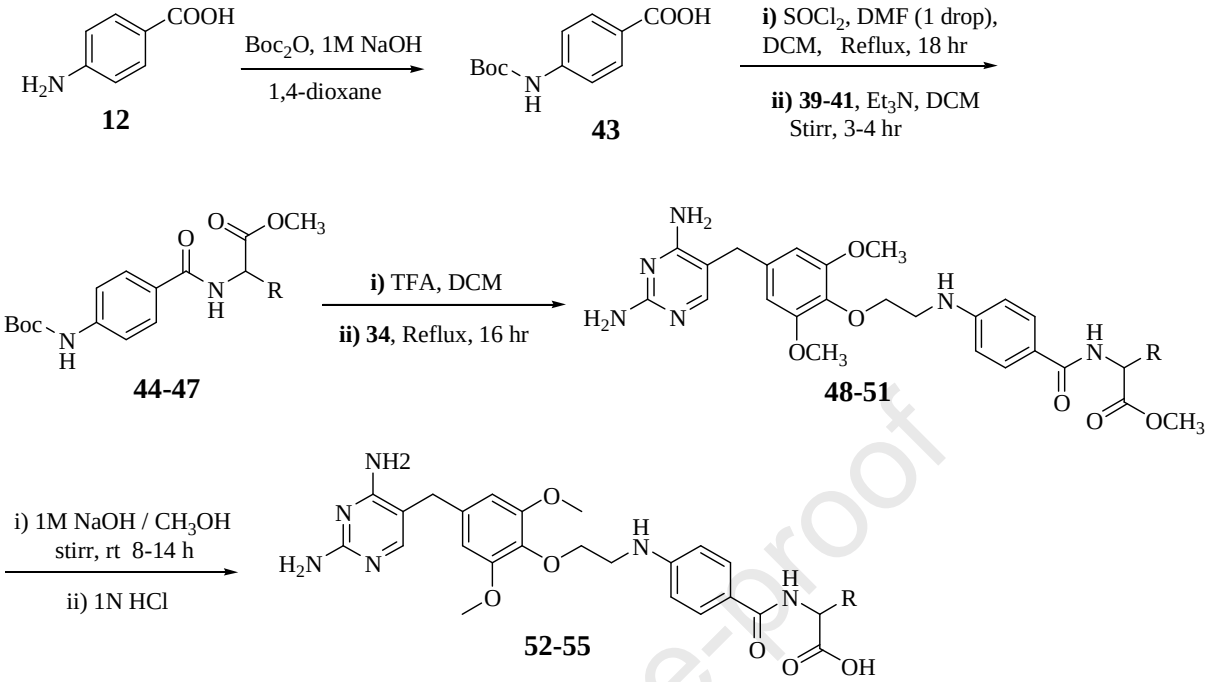


Scheme 2: Synthesis of trimethoprim derivatives 34-35



Scheme 3: Esterification of amino acids

Boc-protection of 4-aminobenzoic acid (**11**) formed **43**. Coupling of amino acid methyl esters (**39-42**) yielded **44-47**. Deprotection of Boc-amine using trifluoroacetic acid (TFA) and subsequent reaction with **34-35** in DMF afforded **48-55**. Free acids (**56-63**) were obtained by alkaline hydrolysis of **48-55 (Scheme 2)** [25-26].



Scheme 4: Synthesis of trimethoprim-based methotrexate mimics with ethylene linker (**52-55**).

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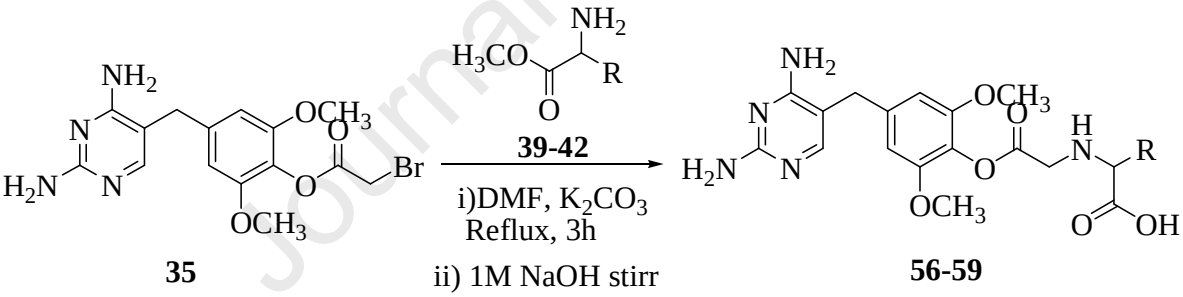


Scheme 5: Synthesis of trimethoprim-based methotrexate mimics with oxoethyl linker (**56-59**).

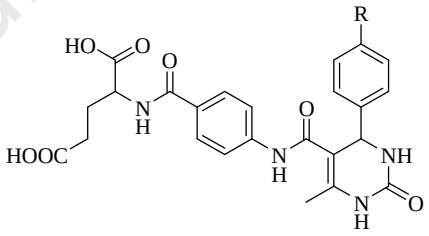
2.3. *In vitro L. major* dihydrofolate reductase (*Lm*DHFR) inhibition

The synthesized compounds were evaluated for enzyme inhibition assays against leishmania major DHFR isolated at promastigote stage. The inhibitory potential of both series given as IC_{50} value is presented in Tables **1**. From dihydropyrimidine series (**26-30**), the tested compounds **26, 28** and **29** showed excellent inhibition in submicromolar range with IC_{50} value of 0.38, 0.25 and

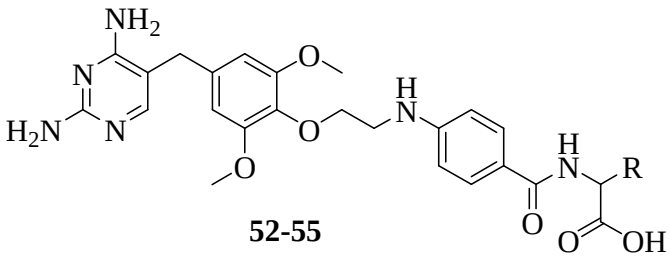
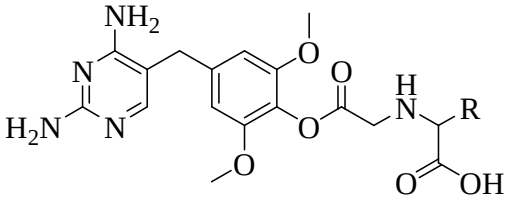
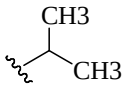
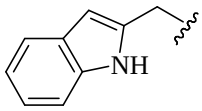
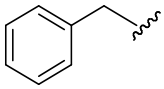
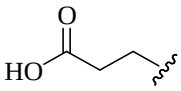
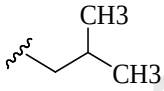
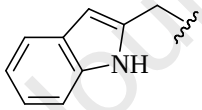
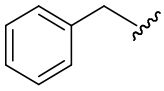
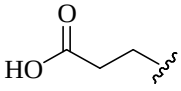
148 0.19 μM, respectively. While the compounds **52-55** showed good *Lm*DHFR inhibition with IC_{50}
149 value in low micromolar range (0.21-0.81 μM). Tested oxoethyl derivatives **56-59** showed
150 excellent potency for *Lm*DHFR. These determined IC_{50} values of these derivatives are from 0.10
151 μM to 0.18 μM.

152 Subsequently, to demonstrate the selectivity for parasite enzyme over human enzyme, we
153 performed *in-vitro* inhibition studies on recombinant human DHFR (*rh*DHFR).
154 Dihydropyrimidine derivatives **26-30** showed good selectivity with the selectivity index in the
155 range of 2.2-3.2. PABA containing trimethoprim derivatives **52-55** showed poor selectivity for
156 *Lm*DHFR. However, acetyl spacer containing trimethoprim derivatives **56-59** displayed
157 comparatively low affinity with *rh*DHFR and high selectivity for *Lm*DHFR. Highest selectivity
158 was shown by compound **56** (SI = 84.5) and **58** (SI = 87.5). The selectivity index of compounds
159 **57** and **59** is 10.2 and 16.3 respectively.

160 **Table 1:** Enzyme inhibition activities of the synthesized compounds



No.	R	*Lm*DHFR IC_{50} (μM)	*h*DHFR IC_{50} (μM)	*SI*
26	H	**0.38 ± 0.03**	0.84 ± 0.55	2.2
27	4-CH_3	1.91 ± 0.07	ND	-
28	4-OCH_3	1.63 ± 0.11	ND	-
29	4-NO_2	**0.25 ± 0.01**	0.81 ± 0.03	3.2
30	4-Cl	**0.19 ± 0.01**	0.53 ± 0.01	2.8

 **52-55**		 **56-59**		
52		0.81 ± 0.10	0.04 ± 0.00	0.05
53		ND	0.03 ± 0.00	-
54		0.53 ± 0.13	0.09 ± 0.01	0.2
55		0.21 ± 0.10	**0.01** ± 0.00	0.05
56		0.13 ± 0.04	10.98 ± 0.05	**84.5**
57		0.18 ± 0.03	1.83 ± 0.03	**10.2**
58		0.14 ± 0.01	12.26 ± 0.13	**87.5**
59		0.10 ± 0.01	1.63 ± 0.11	**16.3**
Methotrexate		-	0.005	
TMP		20.5	1.03	

Selectivity index = IC_{50} ratio (*h*DHFR/*lm*DHFR)

2.4. *In vitro* antileishmanial assay against promastigotes of *L. major* and *L. donovani*

In-vitro antileishmanial activity was carried out against promastigotes of *L. major* and *L. donovani*. The IC_{50} values of the antileishmanial activity of series **26-30** and **52-59** are enlisted in **Tables 2**. The synthesized compounds (**26-30**) showed good to excellent antiparasitic activities. The compounds were efficient against the two species of the parasite *L. major* and *L. donovani* and except compound **28**, all other showed inhibition in low micro-molar concertation. The compound **27** showed IC_{50} values against *L. major* in 2.21$\pm$ 0.01 µM. This compound showed IC_{50} values of 2.56 $\pm$ 0.01 µM against *L. donovani*. Nitrophenyl derivative **29** showed excellent antileishmanial activities with low IC_{50} values of 1.23 $\pm$ 0.66 µM against *L. major* and 0.94 $\pm$ 0.11 µM against *L. donovani*. Compound **30** also showed good IC_{50} values of 2.68 $\pm$ 0.15 µM against L. major and 3.01 $\pm$ 0.09 µM against *L. donovani*.

The compounds having diaminopyrimidine (**52-59)** showed excellent activities against both the tested parasitic species *L. major* and *L. donovani*. The compound **52** showed with low IC_{50} values of 0.67 $\pm$ 0.01 µM against *L. major* and IC_{50} values of 0.73 $\pm$ 0.01 µM against *L. donovani*. The compound **53** showed with low IC_{50} values of 0.83 $\pm$ 0.01 µM against *L. major* and IC_{50} values of 1.36 $\pm$ 0.06 µM against *L. donovani*. The compound **54** showed with low IC_{50} values of 0.98 $\pm$ 0.02 µM against *L. major* and IC_{50} values of 0.95 $\pm$ 0.04 µM against *L. donovani*. The compound **55** showed with low IC_{50} values of 0.19+0.01 µM against *L. major* and IC_{50} values of 0.11 $\pm$ 0.01 µM against *L. donovani*.

However, compounds without PABA linker (**56-59)** were not able to show high potency compared with $X=CH_2$. Compound **59** from third series showed the activities against with IC_{50} values of 12.34 $\pm$ 1.01 µM against the *L. major* and 8.94 $\pm$ 0.83 µM against the *L. donovani*.

185 Other compounds of the scheme showed larger inhibitory concentrations as compared to
186 compound **59**.

187 **Table 2:** Results (μM) in of *in-vitro* anti-leishmanial of compounds **26-29**, **52-59.**

No.	Antileishmanial activity (Promastigotes) IC_{50} (μM)	
	L. major	***L. donovani***
26	5.48 ± 0.23·	5.82 ± 0.19
27	2.21 ± 0.12	2.56 ± 0.01
28	15. 1 ± 1.11	17.4 ± 0.79
29	1.23 ± 0.06	0.94 ± 0.11
30	2.68 ± 0.15	3.01 ± 0.09
52	0.67 ± 0.01	0.73 ± 0.01
53	0.83 ± 0.01	1.36 ± 0.06
54	0.98 ± 0.02	0.95 ± 0.04
55	0.19 ± 0.01	0.11 ± 0.01
56	24.49 ± 1.08	28.51 ± 1.23
57	26.67 ± 0.97	39.10 ± 1.41
58	22.29 ± 0.85	42.88 ± 1.71
59	12.34 ± 1.01	8.94 ± 0.83
Amphotericin B	0.60 ± 0.05	-
Sodium stibogluconate	-	3.27 ± 0.23

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189

2.5. Docking study

Docking studies of the synthesized compounds were performed using Molecular Operating Environment (MOE 2016.0802). We docked the synthesized compounds against leishmania major (*Lm*DHFR). As, the crystal structure of *Lm*DHFR is not available, a three-dimensional structure of bifunctional dihydrofolate reductase-thymidylate synthase (*Lm*DHFR-TS) was constructed by using Swiss-Model webserver (https://swissmodel.expasy.org/). Best model was selected from five models and refinement of the constructed model (94.6 % amino acids were positioned in favored regions, 4.1 % in allowed regions and 1.4 % in outlier region) was validated by Ramachandran plot (**Figure S-1**). Moreover, we docked methotrexate into the DHFR region. The amino acid residues present in the modelled *Lm*DHFR active site were compared with the literature [10-11, 27]. The ribbon diagram of homology modelled *Lm*DHFR with MTX is shown in **Figure 3**.

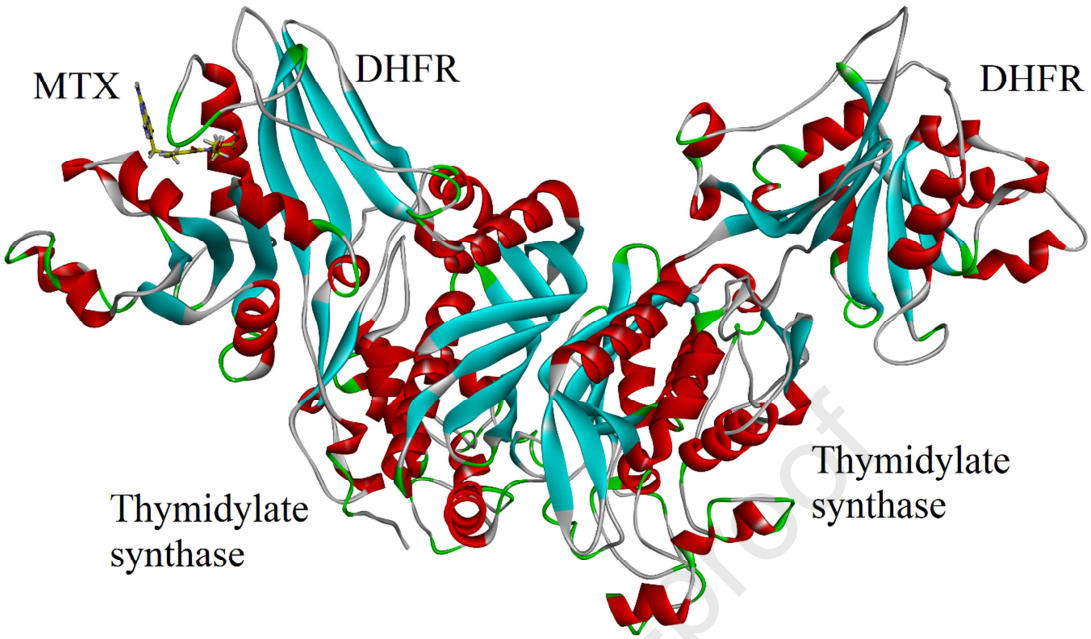

Figure 3: MTX bound to homology modelled *L. major* DHFR.

After validation of docking accuracy (details in **Supplementary material**), we docked the tested compounds into the active site of modeled *Lm*DHFR. The 3-D interaction plot of compound **55**, **59** and **29** are shown in **Figure 5a-c**. Compound **55** forms hydrogen bond interactions with Ala32 and Thr83. Phe91 forms π-π stacking interactions with phenyl ring of trimethoprim. Ile45 also forms π-π T-shaped interactions. While Met53 involved in π-sulfur type of interactions to stabilize the ligand-enzyme complex (**Figure 5a**). Ala32, Gly42 and Ser86 forms hydrogen bond interactions with compound **59**. Phe91 forms π-π stacking interaction with pyrimidine ring. Met53 forms π-sulfur type of interactions. Val87 also forms π-σ type of interactions (**Figure 5b**). Dihydropyrimidine analogue 29 forms hydrogen bond interactions with Val49, Thr83, Gln160 and Arg191. Ile45 forms π-alkyl interactions (**Figure 5c**).

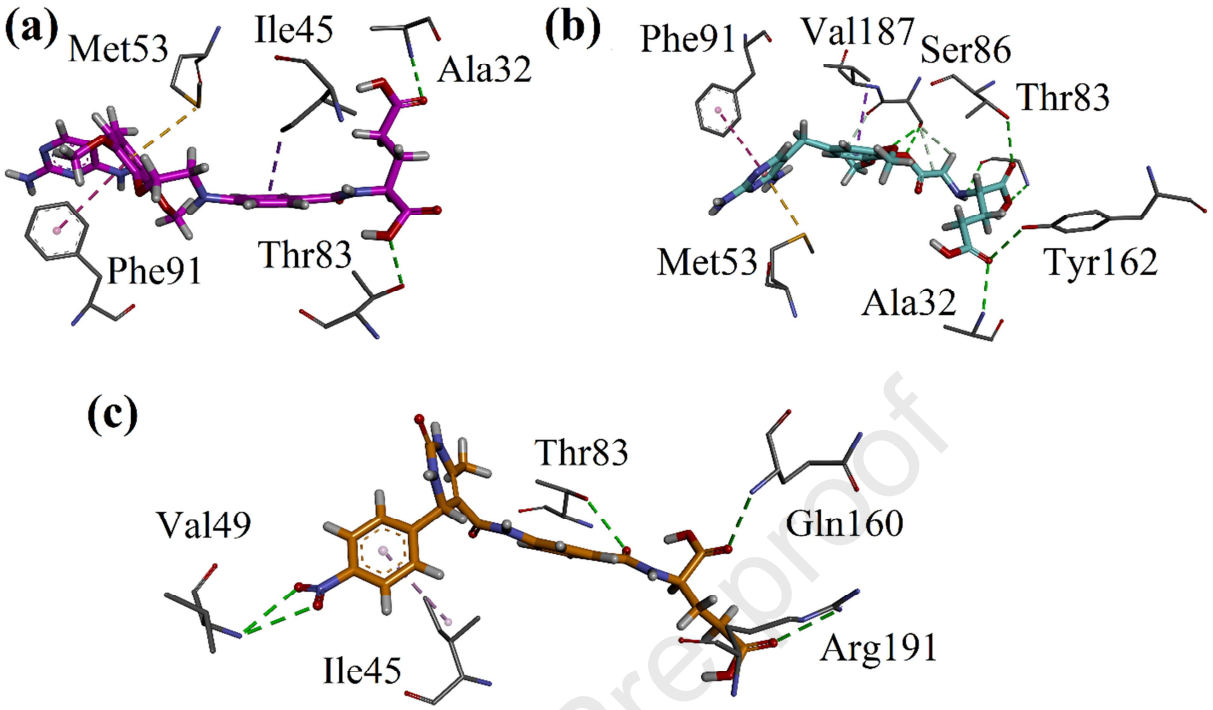

Figure 5. The 3-D interaction plot of compound (**a**) **55**; (**b**) **59** and (**c**) **29** into the homology modelled *Lm*DHFR

3. Conclusions

A series of new 13 compounds were designed and synthesized having dihydropyrimidine and 5-(3,5-dimethoxybenzyl)pyrimidine-2,4-diamine core. Our current research describes the synthesis and biological evaluation of some structural mimics of methotrexate. The focus of the current research was on the structural replacement of pteridine core of methotrexate with 5-benzyl-2,4-diaminopyrimidine core (from trimethoprim) and dihydropyrimidine core and 5-carboxamido)benzamido)pentanedioic acid glutamate core remains same.. For dihydropyrimidine derivatives (**26-30**), the structure activity relationship was performed on 4-phenyl ring of the DHPM-2-one core. These derivatives (**26-30)** showed good selectivity (SI = 2.2-3.2) for parasite enzyme (*Lm*DHFR) over human enzyme. While, for 5-benzylpyrimidine-

2,4-diamine derivatives, the impact of different amino acids (valine, tryptophan, phenylalanine, and glutamic acid) and two carbon linkers between 4-aminobenzoic acid and 5-(3,5-dimethoxybenzyl)pyrimidine-2,4-diamine core were explored. Compounds **52-55** showed poor selectivity for *Lm*DHFR While compounds **56-59** have shown excellent inhibition potential and selectivity for *Lm*DHFR. The interaction pattern of the synthesized compounds homology modelled *Lm*DHFR and *h*DHFR was explored by using docking simulations. the interaction pattern of the synthesized compounds against targets. In conclusion, dihydropyrimidine5-carboxamides and 5-benzylpyrimidine-2,4-diamine are important scaffolds parasitic dihydrofolate reductase inhibition and their further structural modification may lead to the synthesis of more potent antileishmanials.

4. Material and methods

All the reagents and solvents were purchased from standard commercial vendors and were used without any further purification. All the substituted aromatic aldehydes, ethyl acetoacetate, urea, *L*-glutamic acid, PABA, 1,2-dibromoethane and bromoacetyl bromide were purchased from Sigma Aldrich. Amino acids were purchased from Scharlau Labs. ^{1}H and ^{13}C-NMR spectra were recorded in deuterated solvents on a Bruker spectrometer at 300 and 75 MHz respectively using tetramethyl silane (TMS) as internal reference. Chemical shifts are given in δ scale (ppm). The progress of all the reactions was monitored by TLC on 2.0 x 5.0 cm aluminum sheets pre-coated with silica gel $60F_{254}$ with a layer thickness of 0.25 mm (Merck). LC-MS spectra were obtained

using Agilent technologies 1200 series high performance liquid chromatography comprising of G1315 DAD (diode array detector) and ion trap LCMS G2445D SL. Elemental analyses were conducted using Elemental Vario EI III CHN analyzer. Final products were checked for their purity on Schimadzu HPLC system using C_{18} RP-column and isocratic solvent system (mentioned in experimental) part at room temperature.

4.1. General method for the synthesis of 4-(3-oxobutanamido)benzoic acid (12)

Compound **12** was synthesized by refluxing a mixture of 4-aminobenzoic acid (20 mmol) and ethyl acetoacetate (20 mmol) in absolute ethanol. After completion of reaction (TLC), the reaction mixture was cooled and solid formed was filtered. The crude product was purified by silica gel column chromatography using *n*-hexane : EtOAc (7:1). Yield = 51 %; ^{1}H NMR = (300 MHz, DMSO - d_6) δ_H (ppm): 12.33 (s, 1H, OH), 9.08 (s, 1H. NH), 8.11 (d, 2H, *J* = 8.1 Hz, ArH), 7.80 (d, 2H, *J* = 8.1 Hz, ArH), 3.32 (s, 2H, CH_2), 2.21 (s, 3H, CH_3).

4.2. General method for the synthesis of compounds (20-24)

A mixture of aromatic aldehydes **(13-18,** 10 mmol**)** and 4-(3-oxobutanamido)benzoic acid (**12,** 10 mmol) and urea (**19**, 10.2 mmol) stirred under reflux in acetonitrile using tin(II) chloride dihydrate ($SnCl_2.2H_2O$). After the completion of the reaction (TLC), the mixture was poured into ice. The resulting solid was washed with water and recrystallize with appropriate solvent to furnish **20-24**.

4.2.1. 4-(6-Methyl-2-oxo-4-phenyl-1,2,3,4-tetrahydropyrimidine-5-carboxamido)benzoic acid (20)

White solid; R_f = 0.41 (CH_2Cl_2 /CH_3OH 5:1); Yield = 53 %; m.p. 212-214 ºC; ^{1}H NMR = (300 MHz, DMSO - *d6*) *δH* (ppm): 12.35 (s, 1H, OH), 9.16 (s, 1H. NH), 8.40 (s, 1H, NH), 8.11 (d, 2H, *J* = 7.8 Hz, Ar-H), 7.86 (d, 2H, *J* = 7.8 Hz, Ar-H), 7.69 (s, 1H, NH), 7.18-7.11 (m, 5H, ArH), 5.07 (d, 1H, *J* = 3.3, CH), 2.24 (s, 3H, CH_3); LC-MS: m/z = 351.2 [M+H]+; Analysis calculated for $C_{19}H_{17}N_3O_4$: C, 64.95; H, 4.88; N, 11.96; Observed;; C, 64.99; H, 4.86; N, 11.94.

4.2.2. 4-(6-methyl-2-oxo-4-p-tolyl-1,2,3,4-tetrahydropyrimidine-5-carboxamido)benzoic acid (21)

White solid; R_f = 0.50 (CH_2Cl_2/ CH_3OH 5:1); Yield = 55 %; m.p. 219-221ºC; ^{1}H NMR= (300 MHz, DMSO- *d6*) *δH* (ppm): 12.37 (s, 1H, OH), 9.13 (s, 1H. NH), 8.42 (s, 1H, NH), 8.13 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.88 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.65 (s, 1H, NH), 7.06. (d, 2H, *J* = 7.5 Hz, Ar-H), 6.84 (d, 2H, *J* = 7.5 Hz, Ar-H), 5.05 (d, 1H, *J* = 3.3 Hz, CH), 2.43 (s, 3H, CH_3), 2.24 (s, 3H, CH_3); LC-MS: m/z = 365.2 $[M+H]^+$; Analysis calculated for $C_{20}H_{19}N_3O_4$: C, 65.74; H, 5.24; N, 11.50; Observed;; C, 64.71; H, 5.26; N, 11.51.

4.2.3. 4-(4-(4-Methoxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamido) benzoic acid (22)

Light yellow solid; R_f = 0.51 (CH_2Cl_2/CH_3OH 5:1); Yield = 61 %; m.p. 231-233 ºC; ^{1}H NMR= (300 MHz, DMSO- *d6*) *δH* (ppm): 12.39 (s, 1H, OH), 9.14 (s, 1H. NH), 8.44 (s, 1H, NH), 8.13 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.87 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.65 (s, 1H, NH), 7.14. (d, 2H, *J* = 7.8 Hz, Ar-H), 6.80 (d, 2H, *J* = 7.8 Hz, Ar-H), 5.03 (d, 1H, *J* = 3.0 Hz, CH), 3.83 (s, 3H, OCH_3), 2.25 (s, 3H, CH_3); LC-MS: m/z = 381.13 [M+H]+; Analysis calculated for $C_{20}H_{19}N_3O_5$: C, 62.99; H, 5.02; N, 11.02; Observed;; C, 62.95; H, 5.04; N, 11.05.

4.2.4. 4-(6-Methyl-4-(4-nitrophenyl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamido) benzoic acid (23)

Light yellow solid; R_f = 0.54 (CH_2Cl_2/CH_3OH 5:1); Yield = 48 %; m.p. 222-224 ºC; ^{1}H NMR= (300 MHz, DMSO- d_6) δ_H (ppm): 12.38 (s, 1H, OH), 9.14 (s, 1H. NH), 8.41 (s, 1H, NH), 8.21 (d, 2H, *J* = 8.7 Hz, Ar-H), 8.12 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.86 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.64 (s, 1H, NH), 7.53 (d, 2H, *J* = 8.7 Hz, Ar-H), 5.07 (d, 1H, *J* = 3.0 Hz, CH), 2.25 (s, 3H, CH_3); LC-MS: m/z = 397.11 [M+H]+; Analysis calculated for $C_{19}H_{16}N_4O_6$: C, 57.58; H, 4.07; N, 14.14; Observed;; C, 57.52; H, 4.09; N, 14.16.

4.2.5. 4-(4-(4-Chlorophenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxamido) benzoic acid (24)

Lemon colored solid; R_f = 0.56 (CH_2Cl_2/CH_3OH 5:1); Yield = 60 %; m.p. 206-208 ºC ; ^{1}H NMR= (300 MHz, DMSO-d_6) δ_H (ppm): 12.35 (s, 1H, OH), 9.15 (s, 1H. NH), 8.41 (s, 1H, NH), 8.12 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.94 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.66 (s, 1H, NH), 7.16 (d, 2H, *J* = 8.4 Hz, Ar-H), 6.91 (d, 2H, *J* = 8.4 Hz, Ar-H), 5.06 (d, 1H, *J* = 2.7 Hz, CH), 2.23 (s, 3H, CH_3); LC-MS: m/z = 385.2 $[M+H]^+$; Analysis calculated for $C_{19}H_{16}ClN_3O_4$: C, 59.15; H, 4.18; N, 10.89; Observed; : C, 59.19; H, 4.15; N, 10.87;

4.3. General method for the synthesis of target compounds 26-30

To a solution of compounds **20-24** (5 mmol) in DCM, 1 drop of DMF was added followed by freshly distilled thionyl chloride (5 mmol) was added dropwise. The solution was heated to reflux. After the completion of reaction, solvent and excess thionyl chloride were removed by distillation. The crude solid residues were used for further reactions.

The obtained acid chloride solid residues and *L*-glutamic acid (**25**) were dissolved in DMF in the presence of *N*-methylmorpholine (NMM). The resulting solutions were heating to reflux. After completion of reaction (consumption of starting material), the mixture was poured into ice. The resulting solid was washed with water.

4.3.1. (4-(4-Methyl-2-oxo-6-phenylhexahydropyrimidine-5-carboxamido) benzoyl) glutamic acid (26)

Yellow solid; R_f = 0.41 (CH_2Cl_2 /CH_3OH/GAA; 7 : 3 : 0.2); Yield = 47 %; m.p. 151-153 ºC; HPLC purity = 98.8 % (C_{18} RP, 1% TFA in MeOH / H_2O-92:8), T_R = 8.2 min. ^{1}H NMR = (300 MHz, DMSO - d_6) δ_H (ppm): 12.27 (s, 2H, 2 × COOH), 9.16 (s, 1H. NH), 8.43-8.37 (m, 2H, CONH and CONHCH), 8.12 (d, 2H, *J* = 8.1 Hz), 7.74 (d, 2H, *J* = 8.1 Hz), 7.54 (s, 1H, NH), 7.13-7.01 (m, 5H, ArH), 5.11 (s, 1H, *J* = 3.0 Hz, CH), 4.53-4.46 (m, 1H, CH), 2.40 (t, 2H, *J* = 6.9 Hz, CH_2), 2.26 (s, 3H, CH_3), 2.16-1.99 (m, 2H, CH_2); ^{13}C-NMR, (75 MHz, DMSO-d_6): δC (ppm); 17.4, 26.36 (2C), 30.44, 47.2, 54.2, 56.5, 57.1, 117.5 (2C), 121.6, 125.7, 128.3 (2C), 128.2, 129.9, 137.2, 141.4, 150.8 (2C), 167.1, 177.3 (2C), 178.3; LC-MS: m/z = 480.2 [M+H]+; Analysis calculated for $C_{24}H_{24}N_4O_7$: C, 60.00; H, 5.03; N, 11.66; Observed; C, 59.77; H, 5.41; N, 11.58

4.3.2. (4-(4-Methyl-2-oxo-6-(p-tolyl) hexahydropyrimidine-5-carboxamido) benzoyl) glutamic acid (27)

Off-white solid; R_f = 0.48 (CH_2Cl_2 /CH_3OH/GAA; 7 : 3 : 0.2); Yield = 42 %; m.p. 163-165 ºC; HPLC purity = 97.7 % (C_{18} RP, 1% TFA in MeOH / H_2O-92:8),. T_R = 10.6 min. ^{1}H NMR=(300 MHz, DMSO- d_6) δ_H (ppm): 12.29 (s, 2H, 2 × COOH), 9.15 (s, 1H. NH), 8.42 (br s, 2H, CONH and CONHCH), 8.10 (d, 2H, *J* = 8.1 Hz), 7.73 (d, 2H, *J* = 8.1 Hz), 7.56 (s, 1H, NH), 7.10 (d, 2H, *J* = 7.5 Hz), 6.80 (d, 2H, *J* = 7.5 Hz), 5.13 (s, 1H, *J* = 3.3 Hz, CH), 4.53-4.46 (m, 1H, CH), 2.39 (t, 2H, *J* = 6.9 Hz, CH_2), 2.45 (s, 3H, CH_3), 2.25 (s, 3H, CH_3), 2.16-1.99 (m, 2H, CH_2);

339 ^{13}CNMR, (75 MHz, DMSO-*d6*): *δC* (ppm); ^{13}C-NMR, (75 MHz, DMSO-*d6*): *δC* (ppm); 17.6,
340 27.35 (3C), 31.41, 47.8, 54.6, 56.8, 58.1, 117.9 (2C), 122.4, 125.5, 124.5 (2C), 128.7, 129.8,
341 137.7, 142.3, 151.7 (2C), 167.9, 177.6 (2C), 179.6. LC-MS analysis: m/z = 494.180$[M+H]^+$;
342 Analysis calculated for $C_{25}H_{26}N_4O_7$: C, 60.72; H, 5.30; N, 11.33 Observed; C, 60.52; H, 5.66; N,
343 11.32

344 **4.3.3. (4-(4-(4-Methoxyphenyl)-6-methyl-2-oxohexahydropyrimidine-5-carboxamido)**
345 **benzoyl) glutamic acid (28)**

346 Light yellow solid; R_f = 0.43 (CH_2Cl_2 /CH_3OH/GAA; 7 : 3 : 0.2); Yield = 47 %; m.p. 184-186
347 ºC; HPLC purity = 98.0 % (C_{18} RP, 1% TFA in MeOH / H_2O-92:8). T_R = 9.5 min. ^{1}H NMR=
348 (300 MHz, DMSO- *d6) δH* (ppm): 12.28 (s, 2H, 2 × COOH), 9.15 (s, 1H. NH), 8.40 (br s, 2H,
349 CONH and CONHCH), 8.11 (d, 2H, *J* = 8.1 Hz), 7.74 (d, 2H, *J* = 8.1 Hz), 7.55 (s, 1H, NH), 7.16
350 (d, 2H, *J* = 7.8 Hz), 6.78 (d, 2H, *J* = 7.5 Hz), 5.11 (s, 1H, *J* = 3.0 Hz, CH), 4.52-4.45 (m, 1H,
351 CH), 2.40 (t, 2H, *J* = 6.6 Hz, CH_2), 2.84 (s, 3H, OCH_3), 2.24 (s, 3H, CH_3), 2.15-1.99 (m, 2H,
352 CH_2); ^{13}C-NMR, (75 MHz, DMSO-*d6*): *δC* (ppm): 18.2, 28.3 (3C), 3.4, 48.3, 54.9, 56.9, 58.7,
353 117.9 (2C), 123.3, 125.3, 124.6(2C), 128.9, 129.6, 137.3, 142.3, 151.9 (2C), 168.1, 178.3 (2C),
354 179.9; LC-MS: m/z = 510.175 [M+H]+; Analysis calculated for $C_{25}H_{26}N_4O_8$;C, 58.82: H, 5.13;
355 N, 10.98; Observed; C, 58.63: H, 5.53; N, 10.96

356 **4.3.4. (4-(4-Methyl-6-(4-nitrophenyl)-2-oxo-hexahydropyrimidine-5-carboxamido) benzoyl)**
357 **glutamic acid (29)**

358 Light brown solid; R_f = 0.40 (CH_2Cl_2 /CH_3OH/GAA; 7 : 3 : 0.2); Yield = 41 %; m.p. 144-146 ºC;
359 HPLC purity = 97.3% (C_{18} RP, 1% TFA in MeOH / H_2O-92:8), T_R = 8.2 min. ^{1}H NMR= (300
360 MHz, DMSO- *d6) δH* (ppm): 12.30 (s, 2H, 2 × COOH), 9.14 (s, 1H. NH), 8.49 (d, 1H, *J* = 7.5 Hz.

CONH), 8.36 (br s, 1H, CONHCH), 8.23 (d, 2H, *J* = 8.7 Hz), 8.15 (d, 2H, *J* = 8.1 Hz), 7.76 (d, 2H, *J* = 8.1 Hz), 7.59 (s, 1H, NH), 7.48 (d, 2H, *J* = 8.7 Hz), 5.15 (s, 1H, *J* = 3.3 Hz, CH), 4.52-4.45 (m, 1H, CH), 2.40 (t, 2H, *J* = 6.6 Hz, CH_2), 2.25 (s, 3H, CH_3), 2.15-1.99 (m, 2H, CH_2); ^{13}C-NMR, (75MHz, DMSO-*d6*): *δC* (ppm): 18.5, 28.5 (3C), 30.5, 48.5, 54.6, 57.3, 59.5, 118.5 (2C), 123.5, 125.6, 124.7(2C), 127.9, 130.6, 137.5, 142.5, 152.5 (2C), 168.5, 178.7 (2C), 180.9; LC-MS: m/z = 525.150 $[M+H]^+$; Analysis calculated for $C_{24}H_{23}N_5O_9$;C, 54.86: H, 4.41; N, 13.33; Observed; C, 58.64: H, 5.53; N, 10.96.

4.3.5. (4-(4-(4-Chlorophenyl)-6-methyl-2-oxohexahydropyrimidine-5-carboxamido) benzoyl) glutamic acid (30)

Lemon colored solid; R_f = 0.48 (CH_2Cl_2 /CH_3OH/GAA; 7 : 3 : 0.2); Yield = 50 %; m.p. 156-158 ºC ; HPLC purity = 100.0 %. (C_{18} RP, 1% TFA in MeOH / H_2O-92:8), T_R = 11.9 min. ^{1}H NMR= (300 MHz, DMSO-*d6*) *δH* (ppm): 12.27 (s, 2H, 2 × COOH), 9.16 (s, 1H. NH), 8.43 (br s, 2H, CONH and CONHCH), 8.12 (d, 2H, *J* = 8.4 Hz), 7.78 (d, 2H, *J* = 8.4 Hz), 7.54 (s, 1H, NH), 7.20 (d, 2H, *J* = 8.1 Hz), 6.99 (d, 2H, *J* = 8.1 Hz), 5.10 (s, 1H, *J* = 3.3 Hz, CH), 4.51-4.46 (m, 1H, CH), 2.41 (t, 2H, *J* = 7.2 Hz, CH_2), 2.25 (s, 3H, CH_3), 2.17-1.99 (m, 2H, CH_2); ^{13}C-NMR, (75 MHz, DMSO-*d6*): *δC* (ppm): 18.7, 28.5 (3C), 30.7, 48.8, 54.9, 57.8, 59.7, 118.9 (2C), 123.9, 125.8, 124.3(2C), 128.2, 130.8, 137.7, 142., 152.9 (2C), 169.2, 178.9 (2C), 180.1 LC-MS analysis: m/z= 515.14 $[M+H]^+$; Theoretical perusal Calculated for : $C_{24}H_{23}ClN_4O_7$; C, 55.98 ; H, 4.50 ; N, 10.88 ; Observed; ; C, 55.72 ; H, 4.89 ; N, 10.90

4.4. General method for the synthesis of hydroxy-trimethoprim (31)

Hydroxy trimethoprim derivative **31** was obtained by using our previously reported procedure. Yield = 52 %; m.p. 282-285ºC ; ^{1}H NMR= (300 MHz, DMSO-d_6): δ (ppm): 8.53 (s, 1H), 7.87 (s, 2H), 7.66 (s, 1H), 7.38 (s, 2H), 6.69 (s, 2H), 3.73 (s, 6H), 3.53 (s, 2H).

4.5. General method for the synthesis of trimethoprim derivatives 34-35

To a stirred solution of hydroxy-TMP (10 mmol) and potassium carbonate in DMF, 1,2-dibromoethane (**32**) / bromoacetyl bromide (**33**) were added dropwise at room temperature. The solution was stirred at 70-80 ^{o}C. After completion of the reaction, the solvent was evaporated under vacuum and the residue was dissolved in ethyl acetate. The organic layer was washed with sodium bicarbonate and brine. The organic layer was dried over anhydrous magnesium hydroxide. Finally, the solvent was evaporated under vacuum. The crude solid was purified using silica gel column chromatography using *n*-hexane / EtOAc (7:1).

4.5.1. 5-(4-(2-Bromoethoxy)-3,5-dimethoxybenzyl)pyrimidine-2,4-diamine (34)

Yield = 56 %; ^{1}H NMR= (300 MHz, DMSO- d6) δH (ppm): 7.79 (s, 1H), 7.67 (s, 2H, NH_2), 7.35 (s, 2H, NH_2), 6.70 (s, 2H), 4.82 (t, 2H, J = 6.9 Hz), 3.92 (t, 2H, J = 6.9 Hz), 3.73 (s, 6H, OCH_3), 3.53 (s, 2H). LC-MS: m/z = 383.1 $[M+H]^+$; Analysis calculated for $C_{15}H_{19}BrN_4O_3$: C, 47.01; H, 5.00; N, 14.62; Observed; C, 47.05; H, 4.09; N, 14.61.

4.5.2. 4-((2,4-Diaminopyrimidin-5-yl)methyl)-2,6-dimethoxyphenyl 2-bromoacetate (35)

Yield = 62 %; ^{1}H NMR= (300 MHz, DMSO-d6) δH (ppm): 7.78 (s, 1H), 7.66 (s, 2H, NH_2), 7.34 (s, 2H, NH_2), 6.72 (s, 2H), 4.55 (s, 2H), 3.76 (s, 6H, OCH_3), 3.54 (s, 2H). LC-MS: m/z =

396.043 $[M+H]^+$; Analysis calculated for $C_{15}H_{17}BrN_4O_4$: C, 45.36; H, 4.31; N, 14.10; Observed; C, 45.39; H, 4.29; N, 14.08.

4.6. General method for the synthesis of amino acid esters (39-42)

The amino acids (**25, 36-38**) (20.0 mmol) were taken in a round bottom flask in methanol under N_2, thionyl chloride (22 mmol), 44 mmol in the case of glutamic acid **(25)**, was added dropwise to the mixture. The mixture was stirred for 2 hours. The reaction mixture was concentrated under vacuum. To the concentrated mixture concentrated $NaHCO_3$ solution was added until pH is 8-9. The organic layer was extracted, and amino esters **(39-42)** were obtained**.**

4.7. General method for the synthesis of NH-Boc protected compounds 44-47

In a round bottom flask 4-amino benzoic acid (**12**, 20 mmol), was taken in 1, 4-dioxane (25 mL) and 1M NaOH (1 mL) was added. The mixture was stirred at 0 ^oC. To the solution, Boc_2O (1.2 eq, 24 mmol) was added and mixture was stirred at low temperature than at room temperature for 4 hours. The precipitates of compound **43** was obtained.

In a next step, thionyl chloride (2.5 eq.) was added dropwise to the solution of compound **43** (10 mmol) in DCM (25 mL) and 1 drop of DMF and refluxed for 18 hours under nitrogen. After completion of reaction, the solvent was evaporated under vacuum and acyl chloride derivative was obtained. The crude product was used without further purification in the next reactions.

The synthesized acyl chloride derivative and amino acid esters **39-42** (5.0 mmol) were added in DCM (10 mL) as solvent. To the mixture triethylamine base (3 eq) was added. The mixture was stirred for 3-4 hours. After the completion of the reaction the mixture diluted with DCM and added 1M HCl. The mixture was extracted. The organic layer was proceeded under vacuum evaporation. The crystals of compounds (**44-47**) were obtained.

Methyl 2-(4-(tert-butoxycarbonyl) benzamido)-3-methylbutanoate (44)

^{1}H NMR= (300 MHz, DMSO- d_6) δ_H (ppm): 8.30 (s, 1H, CONH), 8.14 (s, 1H, NH-Boc), 8.01 (d, 2H, J = 8.4 Hz), 7.76 (d, 2H, J = 8.4 Hz), 4.88 (d, 1H, J = 7.2 Hz), 3.65 (s, 3H, OCH_3), 2.31 (m, 1H), 1.42 (s, 9H), 0.89 (s, 6H, $2CH_3$). LC-MS: m/z = 351.2 $[M+H]^+$; Analysis calculated for $C_{18}H_{26}N_2O_5$: C, 61.70; H, 7.48; N, 7.91; Observed; C, 47.05; H, 4.09; N, 14.61.

Methyl 2-(4-(tert-butoxycarbonyl)benzamido)-3-(3a,7a-dihydro-1H-indol-3-yl)propanoate (45)

^{1}H NMR= (300 MHz, DMSO- d_6) δ_H (ppm): 10.56 (s, 1H, NH-indole), 8.43 (d, 1H, J = 7.2 Hz, CONHCH), 8.16 (s, 1H, NH-Boc), 8.03 (d, 2H, J = 8.4 Hz), 7.75 (d, 2H, J = 8.4 Hz), 7.41 (m, 2H, Ar-H), 7.26 (s, 1H, Ar-H), 7.14 (m, 2H, Ar-H), 5.01 (dd, 1H, J = 10.2, 9.9 Hz, CH), 3.67 (s, 3H, OCH_3), 3.41 (dd, 1H, J = 16.5, 10.5 Hz, CHH), 3.08 (dd, 1H, J = 16.8, 9.6 Hz, CHH), 1.44 (s, 9H). LC-MS: m/z = 439.21 $[M+H]^+$; Analysis calculated for $C_{24}H_{29}N_3O_5$: C, 65.54; H, 6.65; N, 9.56; Observed; : C, 6.52; H, 6.67; N, 9.52;

Methyl 2-(4-(tert-butoxycarbonyl)benzamido)-3-phenylpropanoate (46)

^{1}H NMR= (300 MHz, DMSO- d_6) δ_H (ppm): 8.33 (br s, 1H, CONHCH), 8.14 (s, 1H, NH-Boc), 8.01 (d, 2H, J = 8.4 Hz), 7.78 (d, 2H, J = 8.4 Hz), 7.11 (m, 5H, Ar-H), 4.99 (dd, 1H, J = 12.3, 7.8 Hz, CH), 3.62 (s, 3H, OCH_3), 4.77 (dd, 1H, J = 17.1, 12.3 Hz, CHH), 2.75 (dd, 1H, J = 17.1, 7.5 Hz, CHH), 1.42 (s, 9H, 3 × CH_3). LC-MS: m/z = 398.4 $[M+H]^+$; Analysis calculated for $C_{22}H_{26}N_2O_5$: C, 66.32; H, 6.58; N, 7.03; Observed: C, 66.36; H, 6.56; N, 7.01.

Dimethyl 2-(4-(tert-butoxycarbonyl)benzamido)pentanedioate (47)

^{1}H NMR= (300 MHz, DMSO- d_6) δ_H (ppm): 8.32 (d, 1H, J = 7.5 Hz CONHCH), 8.12 (s, 1H, NH-Boc), 7.97 (d, 2H, J = 8.1 Hz), 7.79 (d, 2H, J = 8.1 Hz), 4.52-4.42 (m, 1H, CH), 3.63 (s, 3H, OCH_3), 3.53 (s, 3H, OCH_3), 2.41 (t, 2H, J = 6.6 Hz, CH_2), 2.16-1.99 (m, 2H, CH), 1.45 (s, 9H, 3 × CH_3). LC-MS: m/z = 394.17 $[M+H]^+$; Analysis calculated for $C_{19}H_{26}N_2O_7$: C, 57.86; H, 6.64; N, 7.10; Observed; C, 57.69; H, 4.07; N, 14.61;

4.8. General method for the synthesis of target compounds 52-55

For deprotection of amino group, compounds **44-47** were taken in a round bottom flask and 25 mL of DCM solvent (7 ml) was added. Then added trifluoroacetic acid (6 eq; 20.0 mmol) and water in 1:1 ratio at 0 ^oC. The reaction was stirred for 2 hours and after completion of the reaction deprotected amino acid product was obtained under vacuum evaporation.

To a solution of compounds **34** in DCM added compounds **44-47** and mixture was refluxed for 16 hours. After the completion of the reaction the mixture was washed with water and ethyl acetate. The mixture was washed and filtered, and precipitate was obtained under vacuum evaporation to crude products. The crude mixture was purified using silica gel column chromatography using *n*-hexane / EtOAc (1:1) to obtain compounds **48-51.**

To a stirred solution of esters **48-51** (5 mmol) in methanol (20 mL), 4 mL of sodium hydroxide (1M) was added and the reaction mixture was stirred over night at room temperature. Finally, the mixture was neutralized using 1 M HCl solution and concentrated in vacuum. The solution was further acidified to a pH of 2-3 with 1M HCl and resulting product was extracted with 3 x 15 mL of ethyl acetate. Combined organic layer was washed with brine washed with brine (2 x 10 mL) and dried over anhydrous $MgSO_4$. The resulting precipitated carboxylic acids were collected by filtration.

(4-((2-(4-((2,4-Diaminopyrimidine-5-yl)methyl)-2,6-dimethoxyphenoxy)ethyl)amino) benzoyl)valine (52)

White solid; R_f = 0.48 (CH_2Cl_2/CH_3OH/GAA; 8 : 1 : 0.2); Yield = 45 %; m.p. 131-133 °C; HPLC purity = 99.1 %. (C_{18} RP, 1% TFA in MeOH / H_2O-90:10), T_R = 16.5 min. ^{1}H NMR = (300 MHz, DMSO-*d6*) δ_H (ppm): 12.26 (s, 1H, OH-valine), 8.29 (s, 1H, -NH), 7.98 (s, 2H, NH_2), 7.81 (d, 2H, *J* = 7.8 Hz), 7.68 (s, 1H), 7.42 (s, 2H, NH_2), 6.87 (d, 2H, *J* = 7.8 Hz), 6.69 (s, 2H), 5.10 (d, 1H, *J* = 6.9 Hz), 4.88 (s, 1H, NH), 4.44 (t, 2H, *J* = 6.9 Hz), 3.80 (s, 6H), 3.63 (s, 2H), 3.42 (t, 2H, *J* = 6.9 Hz), 2.41-2.32 (m, 1H, CH), 0.99 (t, 6H, J = 6.9 Hz). ^{13}C-NMR, (75 MHz, DMSO-*d6*): *δC* (ppm): 18.5 (2c), 30.5, 36.6, 42.4, 56.3(2c), 62.86, 6., 101.8 (C), 105.3, 112.5 (2C), 122.6, 128.9, 130.3 (2C), 134.6, 150.9, 153.5 (2C), 161.3, 162.5, 167.7, 172.9. LC-MS analysis: m/z = 538.25 [M+H] +; Analysis calculated for $C_{27}H_{34}N_6O_6$: C, 60.21; H, 6.36; N, 15.60; Observed; C, 60.29; H, 6.35; N, 15.57.

(4-((2-(4-((2, 4-Diaminopyrimidine-5-yl)methyl)-2,6dimethoxyphenoxy)ethyl) amino) benzoyl) tryptophan (53)

Pale white solid; R_f = 0.53 (CH_2Cl_2/CH_3OH/GAA; 8 : 1 : 0.2); Yield = 41 %; m.p. 163-165 °C ; HPLC purity = 99.4 % (C_{18} RP, 1% TFA in MeOH / H_2O-90:10), T_R = 17.3 min. ^{1}H NMR = (300 MHz, DMSO-*d6*) δ_H (ppm): 12.54 (s, 1H, OH-tryp), 10.59 (s, 1H, NH-imd), 8.33 (d, 1H, *J* = 7.5, CON*H*CH), 7.96 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.89 (br s, 2H, NH_2), 7.68 (s, 1H, Ar-H), 7.41 (m, 2H, Ar-H), 7.34 (br s, 2H, NH_2), 7.26 (s, 1H, Ar-H), 7.14 (s, 2H, Ar-H), 6.93 (d, 2H, *J* = 8.4 Hz, Ar-H), 6.67 (s, 2H, Ar-H), 5.00 (dd, 1H, *J* = 10.2, 9.9 Hz, CH), 4.87 (s, 1H, NH), 4.47 (t, 2H, J = 6.9 Hz), 3.83 (s, 6H, OCH_3), 3.63 (s, 2H, CH_2), 3.51 (t, 2H, *J* = 6.9 Hz), 3.36 (dd, 1H, *J* = 16.5, 10.5 Hz, CHH), 3.08 (dd, 1H, *J* = 16.8, 9.6 Hz, CHH). ^{13}C-NMR, (75 MHz, DMSO-*d6*): *δC* (ppm): 25.8, 35.8, 42.7, 56.5 (2c), 59.3, 69.5, 98.4, 102.6 (2C), 105.4, 111., 112.69 (2C),

119.3, 120.9, 121.8, 121.5, 127.6(2C), 129.5, 130.3(2C), 133.5, 133.8, 135.7, 150., 153.13 (2C), 161.9, 163.6, 166.4, 175.6. LC-MS: m/z = 625.26 [M+H]+ ; Analysis calculated for ($C_{33}H_{35}N_7O_6$): C,63.35;H , 5.64; N, 15.67; Observed; C, 63.30; H, 5.66; N, 15.72

(4-((2-(4-((2,4-Diaminopyrimidine-5-yl)methyl)-2,6-dimethoxyphenoxy)ethyl)amino) benzoyl) phenylalanine (54)

Light yellow solid; R_f = 0.42 (CH_2Cl_2/CH_3OH/GAA; 8 : 1 : 0.2); Yield= 43 %; m. p. 137-139 ºC; HPLC purity = 100.0 %. (C_{18} RP, 1% TFA in MeOH / H_2O-90:10), T_R = 22.9 min. ^{1}H NMR (300 MHz, DMSO- *d6*) δ_H (ppm): 12.62 (s, 1H, OH), 8.33 (br s, 1H, CON*H*CH), 7.95 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.88 (br s, 2H, NH_2), 7.67 (s, 1H, Ar-H), 7.36 (br s, 2H, NH_2), 7.14 (m, 5H, Ar-H), 6.89 (d, 2H, *J* = 8.4 Hz, Ar-H), 6.68 (s, 2H, Ar-H), 4.79 (dd, 1H, *J* = 12.3, 7.8 Hz, CH), 4.85 (s, 1H, NH), 4.46 (t, 2H, J = 7.2 Hz), 3.74 (s, 6H, OCH_3), 3.57 (s, 2H, CH_2), 3.51 (t, 2H, *J* = 6.9 Hz), 3.13 (dd, 1H, *J* = 17.1, 12.3 Hz, CHH), 2.71 (dd, 1H, *J* = 17.1, 7.5 Hz, CHH). ^{13}C-NMR, (75 MHz, DMSO-*d6*): δ_C (ppm): 35.7, 42.3, 55.0 (2c), 59.2, 70.1, 103.6 (2C), 104.4, 113.4 (2C), 124.1, 125, 126, 128.1, 133.1 (2C), 133.4, 135.8, 151, 152.8 (2C), 160-161(2C), 163.5, 167.1, 179.5. LC-MS analysis: m/z = 586.25 $[M+H]^+$; Analysis calculated for ($C_{31}H_{34}N_6O_6$): C, 63.47; H,5.84; N, 14.33; Observed; C, 63.51, H; 5.86; N, 14.27.

4-((2-(4-((2,4-Diaminopyrimidine-5-yl)methyl)-2,6-dimethoxyphenoxy) ethyl)amino) benzoyl) Glutamic acid (55)

Light brown crystals; R_f = 0.39 (CH_2Cl_2/CH_3OH/GAA; 8 : 1 : 0.2); Yield = 39 %; m.p. 114-115 ºC; HPLC purity = 98.5 %. (C_{18} RP, 1% TFA in MeOH / H_2O-90:10), T_R = 11.4 min.^{1}H NMR=

(300 MHz, DMSO *d6*) *δH* (ppm): 12.35 (s, 2H, OH-tryp), 8.31 (br s, 1H, CON*H*CH), 7.88 (br s, 2H, NH_2), 7.72 (d, 2H, *J* = 8.1 Hz, Ar-H), 7.67 (s, 1H, Ar-H), 7.40 (br s, 2H, NH_2), 6.81 (d, 2H, *J* = 8.1 Hz, Ar-H), 6.65 (s, 2H, Ar-H), 4.82 (s, 1H, NH), 4.49-4.45 (m, 1H, CH), 4.06 (t, 2H, J = 6.9 Hz), 3.80 (s, 6H, OCH_3), 3.54 (s, 2H, CH_2), 3.41 (t, 2H, *J* = 6.9 Hz), 2.41 (t, 2H, *J* = 6.6 Hz, CH_2), 2.18-1.98 (m, 2H, CH_2). ^{13}C-NMR, (75 MHz, DMSO-*d6*): *δC* (ppm): 26.0, 30.2, 36.8, 43.4, 56.1(2c), 69.2, 102.5(2C), 105.1, 112.2(2C), 122.8, 129.8, 130.2(2C), 135.4, 151.1, 152.9(2C), 160-161.(2C), 162.5, 167.5, 174.8, 178.4. LC-MS analysis: m/z = 569.23 [M+H] +; Analysis calculated for ($C_{27}H_{32}N_6O_8$): C, 57.04; H, 5.67; N, 14.78 Observed; C, 57.07; H, 5.65; N, 14.72.

4.9. General method for the synthesis of target compounds 56-59

To a solution of compounds **35** in DCM added compounds **39-42** and mixture was refluxed for 16 hours. After the completion of the reaction the mixture was washed with water and ethyl acetate. The mixture was washed and filtered, and precipitate was obtained under vacuum evaporation to obtain desired esters. To a stirred solution of synthesized esters in methanol (20 mL), 4 mL of sodium hydroxide (1M) was added and the reaction mixture was stirred over night at room temperature. Finally, the mixture was neutralized using 1 M HCl solution and concentrated in vacuum. The solution was then acidified to a pH of 2 with 1M HCl and resulting product was extracted with 3 x 15 mL of ethyl acetate. Combined organic layer was washed with brine and dried over anhydrous MgSO4. The resulting products were filtered, and solvent was removed under reduced pressure.

(2-(4-((2,4-Diaminopyrimidine-5-yl)methyl)-2,6-dimethoxyphenoxy)-2-oxoethyl)leucine (56)

White solid; R_f = 0.51 (CH_2Cl_2/CH_3OH/GAA; 4 : 1 : 0.2); Yield = 59 %; m.p. 143-145 ºC; HPLC purity = 97.1%. (C18 RP, 1% TFA in MeOH / H_2O-85:15), T_R = 10.2 min. ^{1}H NMR = (300 MHz, DMSO-*d*$_6$) δ_H (ppm): 12.21 (s, 1H, OH-valine), 7.84 (s, 2H, NH_2), 7.68 (s, 1H), 7.42 (s, 2H, NH_2), 6.69 (s, 2H), 5.10 (s, 1H, NH), 4.14 (d, 1H, *J* = 7.2 Hz, CH), 3.93 (s, 2H, $COCH_2$), 3.80 (s, 6H, 3 × OCH_3), 3.52 (s, 2H, CH_2), 2.41-2.32 (m, 1H, CH), 0.99 (t, 6H, J = 6.9 Hz, CH_3). ^{13}C-NMR (75 MHz, DMSO-*d*$_6$): δ_C (ppm): 22.8, 25.6, 36.4, 41.4, 47.3, 55.1(2c), 65.2, 103.5(2C), 106.1, 113.2(2C), 125.8, 125.4, 152.1 (2C), 162.2, 169.5, 175.8. LC-MS analysis: m/z= 433.196 [M+H]+; Analysis Calculated for ($C_{20}H_{27}N_5O_6$): C, 55.42; H, 6.28; N, 16.16 Observed; C, 56. 41; H, 6.55; N, 15.58.

(2-(4-((2,4-Diaminopyrimidine-5-yl)methyl)-2,6-dimethoxyphenoxy)-2-oxoethyl)tryptophan (57)

Brown solid; R $_f$ = 0.52 (CH_2Cl_2/CH_3OH/GAA; 4 : 1 : 0.2); Yield = 53 %; m. p 179-181 ºC; HPLC purity = 100.0 % (C18 RP, 1% TFA in MeOH / H_2O-85:15), T_R = 9.1 min. ^{1}H NMR = (300 MHz, DMSO- *d*$_6$) δ_H (ppm); 12.51 (s, 1H, OH), 10.56 (s, 1H, NH-imd), 7.87 (br s, 2H, NH_2), 7.68 (s, 1H, Ar-H), 7.41 (m, 2H, Ar-H), 7.34 (br s, 2H, NH_2), 7.26 (s, 1H, Ar-H), 7.14 (s, 2H, Ar-H), 6.67 (s, 2H, Ar-H), 5.00 (dd, 1H, *J* = 10.2, 9.9 Hz, CH), 4.98 (s, 1H, NH), 3.97 (s, 2H, $COCH_2$), 3.81 (s, 6H, OCH_3), 3.63 (s, 2H, CH_2), 3.36 (dd, 1H, *J* = 16.5, 10.5 Hz, CHH), 3.08 (dd, 1H, *J* = 16.8, 9.6 Hz, CHH). ^{13}C-NMR, (75 MHz, DMSO-*d*$_6$): δ_C (ppm): 29.8, 36.8, 46.3, 55.8 (2C), 64.2, 99.2, 102.5(2C), 105, 111.2, 119.5, 120.7, 121.4, 127.8 (2C), 128.1 (2C) , 133.8, 136.5, 152.2, 160-161.0, 162.5, 168.5, 171.8. LC-MS analysis: m/z = 520.21 $[M+H]^+$; Analysis calculated for ($C_{26}H_{28}N_6O_6$): C, 59.99; H, 5.42; N, 16.14; Observed; C, 59.92; H, 5.45; N, 16.19

(2-(4-((2,4-Diaminopyrimidine-5-yl)methyl)-2,6-dimethoxyphenoxy)-2-oxoethyl)phenyl-alanine (58)

Yellow solid; R_f = 0.54 (CH_2Cl_2/CH_3OH/GAA; 4 : 1 : 0.2); Yield = 49 %; m.p. 153-155 ºC; HPLC purity = 99.4 % (C18 RP, 1% TFA in MeOH / H_2O-85:15), T_R = 13.9 min.^{1}H NMR= (300 MHz, DMSO- d_6) δ_H (ppm): 12.58 (s, 1H, OH), 7.87 (br s, 2H, NH_2), 7.68 (s, 1H, Ar-H), 7.38 (br s, 2H, NH_2), 7.21-7.10 (m, 5H, Ar-H), 6.69 (s, 2H, Ar-H), 5.27 (s, 1H, NH), 4.78 (dd, 1H, *J* = 12.3, 7.8 Hz, CH), 4.96 (s,), 3.99 (s, 2H, $COCH_2$), 3.73 (s, 6H, OCH_3), 3.50 (s, 2H, CH_2), 3.14 (dd, 1H, *J* = 17.1, 12.3 Hz, CHH), 2.73 (dd, 1H, *J* = 17.1, 7.5 Hz, CHH). ^{13}C-NMR, (75 MHz, DMSO-d_6): δ_C (ppm): 36.7-36.8 (2C), 46.3, 55.8 (2C), 64.2, 102.5 (2C), 105.1, 111.2, 127.8, 128.1, 152.2 (2C), 160 (2C), 161.0 (2C), 164.5, 168.5, 171.8. LC-MS: m/z= 481.2 $[M+H]^+$; Analysis calculated for ($C_{24}H_{27}N_5O_6$): C, 59.87;H, 5.65 ; N, 14.54 : Observed; ; C, 5 9.92; H, 5.63 ; N, 14.56

(2-(4-((2,4-Diaminopyrimidine-5-yl)methyl)-2,6-dimethoxyphenoxy)-2-oxoethyl)glutamic acid (59)

Light yellow solid; R_f =0.41 (CH_2Cl_2/CH_3OH/GAA; 4 : 1 : 0.2); Yield = 37 %; m.p. 129-131 ºC; HPLC purity = 100.0 % (C18 RP, 1% TFA in MeOH / H_2O-98:2), T_R = 8.3 min. ^{1}H NMR = (300 MHz,DMSO-d_6 δ_H (ppm): 12.36 (s, 2H, OH), 7.88 (br s, 2H, NH_2), 7.69 (s, 1H, Ar-H), 7.39 (br s, 2H, NH_2), 6.69 (s, 2H, Ar-H), 5.19 (s, 1H, NH), 4.52-4.39 (m, 1H, CH), 4.00 (t, 2H, J = 6.9 Hz), 3.75 (s, 6H, OCH_3), 3.53 (s, 2H, CH_2), 2.36 (t, 2H, *J* = 6.6 Hz, CH_2), 1.98- 1.62 (m, 2H, CH_2). ^{13}C-NMR, (75MHz, DMSO-d_6): δ_C (ppm): 27.7, 30.07, 36.7-36.8 (2C), 46.3, 55.8 (2C), 66.2, 102.5 (2C), 105.1, 127.8, 135.5, 152.2 (2C), 160-161.0 (2C), 162.5 (1C), 168.5 (1C), 174.8 (1C), 178.4 (1C); LC-MS analysis: m/z = 463.17 $[M+H]^+$; Analysis calculated for $C_{20}H_{25}N_5O_8$: C,51.83; H, 5.44; N, 15.11 Observed; C, 51.79; H, 5.46; N, 15.18.

4.7 *L. major* DHFR (*Lm*DHFR) enzyme inhibitory Assay

The DHFR in protozoa is bifunctional dimeric enzyme as compared to monomeric in bacteria and mammals. By using previously reported method, DHFR has been cloned and over-expressed from *Leishmania major*, it is an essential enzyme at least in the promastigotes stage [28-32]. The promastigotes (10^9/ml of buffer [10 mM sodium phosphate, pH 7.0, 1 mM dithiothreitol, 1 mM EDTA) are sonicated and centrifuged (10,000 x g for 20 min), and the supernatant of the sonic extract of these organisms was used to determine DHFR activity. Mixture of 850 µl was prepared by using; 80 µM of sodium phosphate buffer of pH 7.0, 60 µM of NADPH, 60 µM of dihydrofolic acid, 250 µM of EDTA and 250 µM of dithriothreitol. Furthermore, the concentrations 10, 0.10 and 0.010 µg/ml of 2,4-diaminoquinazolines was added and settled down the mixture for 5-10 min. Reaction started by addition of 150 µl of crude DHFR supernatant to mixture. One cuvette did not receive drug (positive control) and for negative control other cuvette did not receive the drug or enzymes. The reaction incubated for 5 days and the initial velocity DHFR activity was measured by initial rate of decrease in NDPH at A340 using spectrophotometer. The net reaction rate was recorded as

The rate in the presence of drug – the rate of negative control

Percentage was calculated as

Positive control rate – negative control rate×100

4.9. *In vitro* antileishmanial activity

Antileishmanial activity was performed in National Institute of Health (NIH) Islamabad using pre-established culture of clinical L. major and L. donovani isolates according to our previously reported method. Antileishmanial activity of the compounds was assayed using a pre-established

599 culture of clinical isolate of *L. major* and *L. donovani* obtained from National Institute of Health
600 (NIH), Islamabad, Pakistan [26]. Complete procedure is presented in Supporting Information.

601 ## 4.10. Homology-modelling of *Lm*DHFR

602
603 The amino acid sequence of dihydrofolate reductase-thymidylate synthase (accession: XP-
604 001680857.1.) of *Leishmania major* was downloaded in FASTA format for structural modeling
605 from NCBI (https://www.ncbi.nlm.nih.gov/protein/XP_001680857.1) and submitted to the
606 Swiss Model [33]. Swiss Model use more than 50 templates for modeling *Leishmania major*
607 protein. Based on maximum sequence identity and GMQE values, we selected four homologous
608 proteins belonging to *Trypanosoma cruzi* DHFR-TS. The selection was based on sequence
609 similarity based (SSB) and Ligand Similarity based (LSB). The selected templates are: 3IRM
610 (SSB), 3INV (SSB), 3CL9 (LSB) and 3HBB (LSB) with sequence identity 68.50, 68.50, 68.31
611 and 68.31, respectively. 3CL9 and 3HBB have methotrexate as native ligand.

612 The resultant structures were then viewed by using Chimera1.11 to study molecular features and
613 interactive visualization of protein structure [34]. After confirmation of its molecular feature
614 protein validation by using online server RAMPAGE, Mol probity
615 (http://molprobity.biochem.duke.edu/) an online tool for confirmation of bad angles and poor
616 rotamers that was alright We calculated RAMPAGE values for the modelled protein structures
617 using Ramachandran Plot analysis [35]. Protein structures will be considered as better structures
618 if their RAMPAGE values are greater than 80% [36].

619 ## 4.10. Docking studies

620 Molecular Operating Environment (MOE 2016.0802) was used for carrying out the Docking
621 studies [37]. Docking studies on *Lm*DHFR was carried out on constructed homology model.

While crystal structure of *h*DHFR in complex with methotrexate (MTX) and NADPH was obtained from Protein Data Bank (PDB accession code 1DLS). Docking reliability on homology modelled *Lm*DHFR and *h*DHFR was assessed by redocking as well as cross-docking protocol [38-40].

Our previously reported methods were used for preparation of ligands, enzymes downloading, energy minimization, 3D protonation and determination of binding site [41-45]. Builder option in MOE was used to draw the ligand structures. Ligand.mdb data base of compounds was built. By using MMFF94X force field the compounds were then energy minimized upto 0.001 Gradient. The enzyme structure was opened in MOE window. The water molecules (if present) were removed. At 300 K temperature with pH = 7, and salt concentration of 0.1 the 3D protonation was done for all atoms in implicit solvated environment. The complete structure was energy minimized using MMFF94X force field followed by the docking of all the compounds into the binding sites of the prepared enzymes. Default docking parameters were set and for each compound, ten different conformations were created. Lowest binding energy ligand enzyme complexes were analyzed by MOE ligand interaction module while, for 3-D interaction plot, discovery studio visualizer was used [46].

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Highlights

- Two novel series of structural mimics of methotrexate were designed and synthesized
- Selectivity for parasite enzyme over human enzyme was evaluated.
- These series were evaluated for their potential against *L. major* /*donovani* promastigotes
- Compounds showed excellent selectivity for *Lm*DHFR over *h*DHFR
- Homology model of L. major DHFR-TS was constructed
- Docking studies

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

There is no conflict of interest