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Original article

Synthesis, characterization and pharmacological screening of some novel 5-imidazopyrazole incorporated polyhydroquinoline derivatives

Piyush N. Kalaria, Shailesh P. Satasia, Dipak K. Raval*

Department of Chemistry, Sardar Patel University, Vallabh Vidyanagar 388 120, Gujarat, India

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ABSTRACT

A new category of polyhydroquinoline derivatives **8a**–**t** were synthesized in moderate to good yield (64–85%) by one-pot three-component cyclocondensation reaction of 5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde **3** with various enaminones **6a**–**h** and different active methylene compounds (malononitrile **7a**, ethylcaynoacetate **7b** and caynoacetamide **7c**) in absolute ethanol. The newly synthesized compounds were evaluated for their *in vitro* antimalarial activity against *Plasmodium falciparum*, *in vitro* antibacterial activity against a panel of pathogenic strains of bacteria and fungi and also for their antitubercular activity against *Mycobacterium tuberculosis* H37Rv strain. Two of them (**8n**, **8t**) exhibited excellent antimalarial activity. Some of them exhibited excellent antibacterial activity and moderate antituberculosis activity compared with the first line drugs.

1. Introduction

Malaria is presently prevalent in a broad band around the equator, in areas of the Americas, many parts of Asia, and much of the Africa; however, it is in sub-Saharan Africa where 85–90% of malaria fatalities occur [1]. The World Health Organization has estimated that in 2010, 216 million documented cases of malaria were reported. Around 655,000 people died from the disease, most of them were children in Africa http://en.wikipedia.org/wiki/Malaria [2]. The emergence and spread of drug-resistant malarial parasites further intensify this serious situation and has compounded the need for the development of new, cost effective antimalarial. Similarly tuberculosis is the second most common reason of death from infectious disease after those due to HIV/AIDS http://en.wikipedia.org/wiki/Tuberculosis [3]. One third of the world's population is suspected to have been infected with *Mycobacterium tuberculosis*, with new infections occurring at a rate of about one per second http://en.wikipedia.org/wiki/Tuberculosis [4]. In 2010, there were an estimated 8.8 million new cases and 1.5 million associated deaths mostly found in developing countries http://en.wikipedia.org/wiki/Tuberculosis [5]. In this context, it was thought worth to synthesize various novel compounds which may have combined applications as antimalarial, antituberculosis and antimicrobial agents.

Among the heterocyclic motifs, azoles constitute enormously important members due to their presence in a multitude of bioactive natural products as privileged pharmacophores. Imidazole and its derivatives are of great significance due to their important roles in biological systems such as antimicrobial [6], anti-inflammatory [7], antitubercular [8], analgesic, anticonvulsant [9], anticancer and anti-parkinson [10] activities. Pyrazole containing compounds have received significant interest owing to their various chemotherapeutic potentials including versatile antineoplastic activities. Many pyrazole derivatives are reported to have the broad spectrum of biological activities, such as antimicrobial [11], anti-inflammatory [12–14], antifungal [15], antitumour, cytotoxic, molecular modelling [16–18] and antiviral [19,20] activities.

Much interest has been dedicated to the synthesis of polyhydroquinoline compounds due to their diverse therapeutic and pharmacological properties, such as antitumour, antiatherosclerotic, vasodilator, geroprotective, bronchodilator and hepatoprotective activity [21]. The polyhydroquinoline ring is one of the important heterocyclic core constitutes and prominent structural motif found in numerous pharmaceutically active compounds. Specifically 4-substituteted 1, 4-dihydropyridines (1, 4-DHPs) are well known as Ca^{2+} channel blockers and have emerged as one of the most important class of drugs for the treatment of

* Corresponding author.
E-mail addresses: dipanalka@yahoo.com, dk_raval@spuvvn.edu (D.K. Raval).

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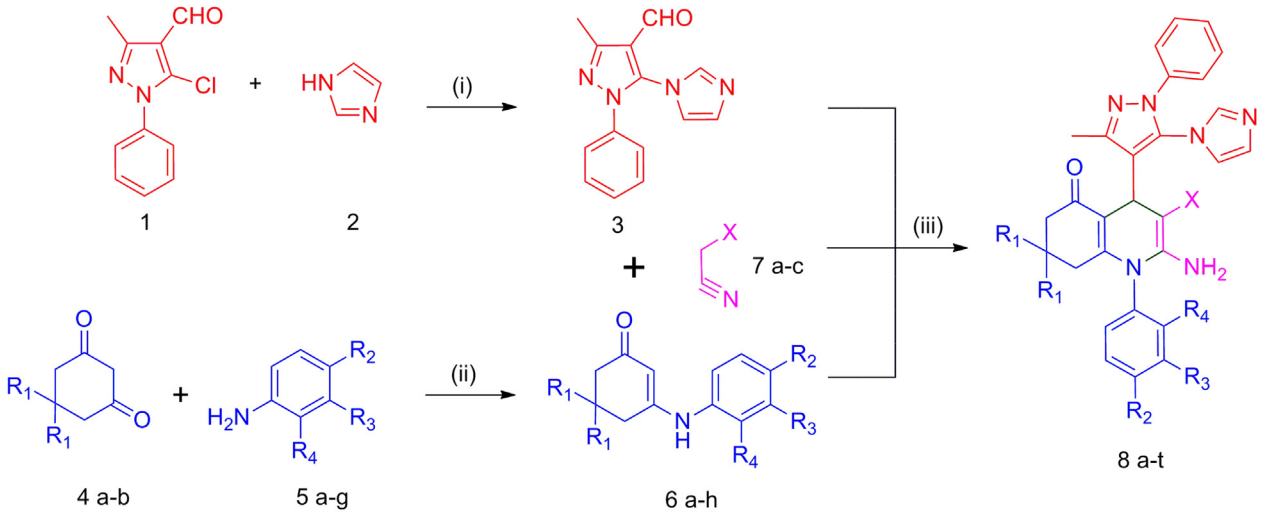
cardiovascular diseases [22]. In addition to the diverse biological activities of polyhydroquinoline, other heterocycles in association with polyhydroquinoline play an essential role in numerous biological processes and possess significant chemical and pharmacological importance. In view of the above biological importance, our aim was to develop new approaches for a variety of heterocycles incorporating polyhydroquinoline scaffold. We attempted to involve pyrazole and imidazole in one molecule which may play a vital role as an important creation part in title compounds.

In continuation to our research work [23], we report herein on the utility of 5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde as the building block for the synthesis of functionalized polyhydroquinoline scaffold. Study on their pharmacological broadcast in order to get some innovative compounds that could be optimized for potent antimicrobial, antimalarial and antituberculosis agents is also included.

2. Chemistry

The synthesis of targeted 5-imidazopyrazole incorporated polyhydroquinoline derivatives are summarized in Scheme 1. The starting material 5-chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde **1** was prepared according to Vilsmeier–Haack reaction of 3-methyl-1-phenyl-1*H*-pyrazol-5(4*H*)-one [24]. The final aldehyde 5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde **3** was prepared by refluxing compound **1** and imidazole in presence of anhydrous K_2CO_3 as basic catalyst in DMF as solvent. The required enaminones **6a**–**h** were prepared by the reaction of β-diketones **4a**–**b** with various amines under aqueous condition. The final molecules substituted 4-(5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-2-amino-1-phenyl-4,6,7,8-tetrahydroquinolin-5(1*H*)-one were synthesized by refluxing the mixture of 5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde **3**, various enaminones **6a**–**h** and different active methylene compounds (malononitrile **7a**, ethylcayno acetate **7b** and caynoacetamide **7c**) in absolute ethanol using piperidine as the basic catalyst (Scheme 1). The formation of quinoline derivatives **8a**–**t** was attempted through an *in situ* initial formation of heterylidenenitrile, containing the electron-poor C=C double bond, from the Knoevenagel condensation between aldehyde **3** and various active methylene compounds **7a**–**c** by loss of water molecules. Finally, Michael addition of enaminones **6a**–**h** to the heterylidene



Where,

Entry	4a-b R_1	5a-g R_2	5a-g R_3	5a-g R_4	7a-c X
8a	CH_3	CH_3	H	H	CN
8b	CH_3	CH_3	H	H	COOEt
8c	CH_3	CH_3	H	H	$CONH_2$
8d	CH_3	Cl	H	H	CN
8e	CH_3	Cl	H	H	COOEt
8f	CH_3	Cl	H	H	$CONH_2$
8g	CH_3	OH	H	H	CN
8h	CH_3	OH	H	H	COOEt
8i	CH_3	OH	H	H	$CONH_2$
8j	CH_3	H	H	H	CN

Entry	4a-b R_1	5a-g R_2	5a-g R_3	5a-g R_4	7a-c X
8k	CH_3	H	H	H	COOEt
8l	CH_3	H	H	H	$CONH_2$
8m	CH_3	H	H	OH	CN
8n	CH_3	H	OH	H	CN
8o	CH_3	H	OH	H	COOEt
8p	CH_3	H	OH	H	$CONH_2$
8q	CH_3	OCH_3	H	H	CN
8r	CH_3	OCH_3	H	H	COOEt
8s	H	OH	H	H	COOEt
8t	H	OH	H	H	$CONH_2$

Scheme 1. Synthesis of the substituted 4-(5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-2-amino-1-phenyl-4,6,7,8-tetrahydroquinolin-5(1*H*)-one **8a**–**t**. (i) DMF, K_2CO_3, Reflux 2 h (ii) PEG 600: deionized water (1:1), Reflux 0.5–1 h (iii) Ethanol, Piperidine, Reflux 1–3 h.

olefins gave acyclic intermediate which underwent cyclization by nucleophilic attack of the NH group on the cyano carbon, followed by tautomerization to afford cyclized polyhydroquinoline derivatives **8a**–**t**.

3. Pharmacology

3.1. In vitro antimicrobial activity

The minimal inhibitory concentration (MIC) of all synthesized compounds **8a**–**t** was determined by broth microdilution method according to National Committee for Clinical Laboratory Standards (NCCLS) [25]. Antibacterial activity was screened against three Gram positive (*Streptococcus pneumoniae* MTCC 1936, *Bacillus subtilis* MTCC 441 and *Clostridium tetani* MTCC 449) and three Gram negative (*Escherichia coli* MTCC 443, *Salmonella typhi* MTCC 98, *Vibrio cholerae* MTCC 3906) bacteria by using ampicillin, ciprofloxacin, norfloxacin and chloramphenicol as the standard antibacterial drugs. Antifungal activity was screened against two fungal species (*Candida albicans* MTCC 227 and *Aspergillus fumigats* MTCC 3008) where nystatin and griseofulvin were used as the standard antifungal agents. The strains employed for the activity were procured from the Institute of Microbial Technology, Chandigarh (MTCC-Micro Type Culture Collection). Mueller Hinton broth was used as nutrient medium to grow and dilute the drug suspension for the test. 2% aqueous DMSO was used as the diluent to get the desired concentration of compound to test upon the standard bacterial strains. The results of antimicrobial screening data are shown in Table 1.

Table 1
In vitro antimicrobial activity (MIC, μg/mL) of compounds **8a**–**t**.

Entry	Gram positive bacteria			Gram negative bacteria			Fungi	
	S.P.	B.S.	C.T.	E.C.	S.T.	V.C.	C.A.	A.F.
	MTCC	MTCC	MTCC	MTCC	MTCC	MTCC	MTCC	MTCC
	1936	441	449	443	98	3906	227	3008
8a	125	**100**	**100**	200	**62.5**	250	1000	1000
8b	**100**	**250**	**250**	125	**100**	**100**	1000	>1000
8c	250	**250**	**200**	200	200	250	>1000	250
8d	125	**200**	**125**	500	500	250	>1000	500
8e	125	**100**	**125**	**100**	**100**	200	>1000	500
8f	200	**200**	**200**	250	250	500	**250**	1000
8g	200	**250**	500	200	250	250	**500**	1000
8h	250	**62.5**	**200**	200	200	200	**500**	>1000
8i	250	**200**	**250**	250	125	250	**500**	1000
8j	500	**250**	500	**100**	125	**100**	1000	500
8k	500	**250**	**200**	250	200	125	**250**	500
8l	200	**125**	**125**	200	250	250	**250**	1000
8m	125	**100**	**200**	250	250	125	1000	500
8n	200	500	**200**	200	250	**62.5**	1000	500
8o	200	500	**250**	200	200	125	**250**	1000
8p	125	**200**	**200**	125	200	200	**500**	500
8q	**100**	**125**	**100**	200	**100**	250	1000	500
8r	**100**	**125**	**250**	**100**	200	250	1000	1000
8s	250	**200**	**200**	**62.5**	200	200	**500**	500
8t	250	**200**	**200**	200	**100**	500	1000	1000
A	100	250	250	100	100	100	n.t.[a]	n.t.
B	25	50	100	25	25	25	n.t.	n.t.
C	10	100	50	10	10	10	n.t.	n.t.
D	50	50	50	50	50	50	n.t.	n.t.
E	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	100	100
F	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	500	100

S.P.: *Streptococcus pneumoniae*, B.S.: *Bacillus subtilis*, C.T.: *Clostridium tetani*, E.C.: *Escherichia coli*, S.T.: *Salmonella typhi*, V.C.: *Vibrio cholerae*, C.A.: *Candida albicans*, A.F.: *Aspergillus fumigatus*, MTCC: Microbial Type Culture Collection. A: Ampicillin, B: Ciprofloxacin, C: Norfloxacin, D: Chloramphenicol, E: Nystatin, F: Griseofulvin.
The bold characters indicate the higher or equal activity compared to standard drugs.
[a] n.t.: not tested.

3.2. In vitro antituberculosis activity

The cheering results from the antibacterial activity impelled us to opt for preliminary screening of the title compounds for their *in vitro* antituberculosis activity. Primary screening of all the newly synthesized compounds **8a**–**t** was conducted at 250 μg/mL and 100 μg/mL against *M. tuberculosis* H37Rv strain by using Lowensteine–Jensen medium (conventional method) as described by Rattan [26]. The obtained results are presented in Table 2 in the form of % inhibition. Rifampicin and Isoniazid were used as the standard drugs.

3.3. In vitro antimalarial activity

All the synthesized title compounds **8a**–**t** were evaluated for their *in vitro* antimalarial activity against *Plasmodium falciparum* strain using chloroquine and quinine as the reference compounds. The consequences of the antimalarial screening are expressed as the drug concentration resulting in 50% inhibition (IC_{50}) of parasite growth and are listed in Table 3.

4. Results and discussion

4.1. Analytical results

The structures of all the synthesized compounds were confirmed by ^1H NMR, FT-IR, ^{13}C NMR, mass and elemental analysis. The ^1H NMR spectra of compound **8a**–**t** exhibited the presence of the –CH (C4 of polyhydroquinoline ring) proton as a sharp singlet around δ 4.32–4.88 ppm and one more singlet arising at δ 7.77–7.95 ppm due to imidazole proton (N–CH–N). The aromatic protons resonate as multiplets at around δ 6.93–7.70 ppm. The IR spectrum of compounds **8a**–**t** exhibited characteristic absorption band around 1663–1646 cm^{-1} which was attributed to the presence carbonyl group (C5 of polyhydroquinoline ring). The strong absorption band was observed in the range of 1369–1376 cm^{-1} due to $-CH_3$ stretching. The characteristic absorption band in the range 3459–3333 cm^{-1} may be attributed to asymmetric and symmetric stretching of $-NH_2$. In ^{13}C NMR spectra, the carbonyl carbon (C5 of polyhydroquinoline ring) was displayed at δ 195.4–196.9 ppm. The signals at around δ 57.9–78.5 ppm, 78.3–78.6 ppm and 76.3–76.8 ppm were assigned to carbon attached to carbonitrile, ester and amide group respectively. The mass spectrum of all the compounds showed molecular ion peak at M^+ corresponding to

Table 2
In vitro antituberculosis activity (% inhibition) of compounds **8a**–**t** against *M. tuberculosis* H37Rv (at concentration 250 μg/mL and 100 μg/mL).

Entry	% Inhibition		Entry	% Inhibition	
	250 μg/mL	100 μg/mL		250 μg/mL	100 μg/mL
8a	**90**	**80**	8l	7	4
8b	12	6	8m	45	38
8c	35	30	8n	38	34
8d	**84**	76	8o	12	9
8e	63	58	**8p**	**84**	75
8f	75	66	**8q**	**91**	**88**
8g	**94**	**94**	8r	25	20
8h	20	12	8s	41	37
8i	70	61	8t	67	60
8j	33	28	Rifampicin	98	98
8k	45	37	Isoniazid	99	99

The bold characters indicate the higher or equal activity compared to standard drugs.

Table 3
In vitro antimalarial activity of compounds **8a**–**t**.

Entry	IC_{50} (μg/mL)	Entry	IC_{50} (μg/mL)
8a	1.073	8l	0.62
8b	1.79	8m	0.71
8c	1.025	**8n**	**0.034**
8d	**0.073**	**8o**	**0.072**
8e	**0.057**	8p	0.57
8f	**0.059**	**8q**	**0.086**
8g	1.23	8r	1.49
8h	**0.096**	**8s**	**0.067**
8i	**0.057**	**8t**	**0.033**
8j	1.082	Chloroquine	0.020
8k	1.47	Quinine	0.268

The bold characters indicate the higher or equal activity compared to standard drugs.

their molecular weights, which confirmed the respective chemical structures.

4.2. Biological section

4.2.1. Antibacterial activity

The analysis of antibacterial screening data (Table 1) revealed that all the compounds **8a**–**t** showed moderate to very good inhibitory activity. The compound **8h** (R_2 = OH, X = COOEt) showed maximum activity i.e. 62.5 μg/mL against *B. subtilis*. Majority of the compounds showed excellent activity against gram positive bacteria *B. subtilis* and *C. tetani* as compared to ampicillin (MIC = 250 μg/mL), while compounds **8b** (R_2 = CH_3, X = COOEt), **8c** (R_2 = CH_3, X = $CONH_2$), **8g** (R_2 = OH, X = CN), **8j** (R_2 = H, X = CN), **8k** (R_2 = H, X = COOEt) and **8b** (R_2 = CH_3, X = COOEt), **8i** (R_2 = OH, X = $CONH_2$), **8q** (R_2 = OCH_3, X = CN), **8r** (R_2 = OCH_3, X = COOEt) showed similar activity as that of the standard drugs against *B. subtilis* and *C. tetani*, at 250 μg/mL concentration. The compounds **8b** (R_2 = CH_3, X = COOEt), **8q** (R_2 = OCH_3, X = CN), and **8r** (R_2 = OCH_3, X = COOEt) displayed comparatively good activities i.e. 100 μg/mL against *S. pneumonia*. The compounds **8a** (R_2 = CH_3, X = CN), **8n** (R_3 = OH, X = CN) and **8s** (R_1 = H, R_2 = OH, X = COOEt) illustrated highest activity in inhibiting gram negative bacteria i.e. 62.5 μg/mL against *S. typhi*, *V. cholera* and *E.* coli. Compounds **8b** (R_2 = CH_3, X = COOEt), **8e** (R_2 = Cl, X = COOEt) and **8j** (R_2 = H, X = CN) also showed same potency against all gram negative bacteria. While compounds **8q** (R_2 = OCH_3, X = CN), **8t** (R_1 = H, R_2 = OH, X = $CONH_2$) and **8r** (R_2 = OCH_3, X = COOEt) showed same potency as that of standard drugs against *S. typhi* and *E. coli*. Remaining other compounds are moderate or less active against all gram positive and gram negative bacteria.

4.2.2. Antifungal activity

The result of antifungal study (Table 1) of the synthesized polyhydroquinoline derivatives revealed that all the compounds have poor activity against *A. fumigates*. Where as in comparison with standard fungicidal griseofulvin (MIC = 500 μg/mL), compounds **8f** (R_2 = Cl, X = $CONH_2$), **8k** (R_2 = H, X = COOEt), **8l** (R_2 = Cl, X = $CONH_2$) and **8o** (R_3 = OH, X = COOEt) contributed excellent antifungal activity i.e. 250 μg/mL against *C. albicans*. While compounds **8g** (R_2 = OH, X = CN), **8h** (R_2 = OH, X = COOEt), **8i** (R_2 = OH, X = $CONH_2$) and **8s** (R_1 = H, R_2 = OH, X = COOEt) showed same potency i.e. 500 μg/mL against *C. albicans*. All other compounds showed weak antifungal potency than nystatin and griseofulvin.

4.2.3. Antituberculosis activity

Antituberculosis screening of all the newly synthesized compounds **8a**–**t** was conducted at two concentrations i.e. 250 μg/mL and 100 μg/mL against tuberculosis H37Rv strain. The bioassay results obtained for the efficacy of all the synthesized analogues against *Mycobacterium tuberculosis* H37Rv is summarized in Table 2. The outcome of the result revealed that, compounds **8a** (R_2 = CH_3, X = CN), **8g** (R_2 = OH, X = CN) and **8q** (R_2 = OCH_3, X = CN) were found to possess excellent activity (i.e. 90%, 94% and 91% at 250 μg/mL) against *M. tuberculosis* H37Rv. Among the above three compounds, the compound **8g** (R_2 = OH, X = CN) showed brilliant activity at both the concentration i.e. 94% at 250 μg/mL and 100 μg/mL. While compounds **8d** (R_2 = Cl, X = CN) and **8p** (R_3 = OH, X = $CONH_2$) are moderately active against *M. tuberculosis* H37Rv. All other compounds showed poor inhibition of *M. tuberculosis* growth. From the above results, it can be concluded that, compound **8q** (R_2 = OCH_3, X = CN) **8g** (R_2 = OH, X = CN) may become new class of antitubercular agents in future.

4.2.4. Antimalarial activity

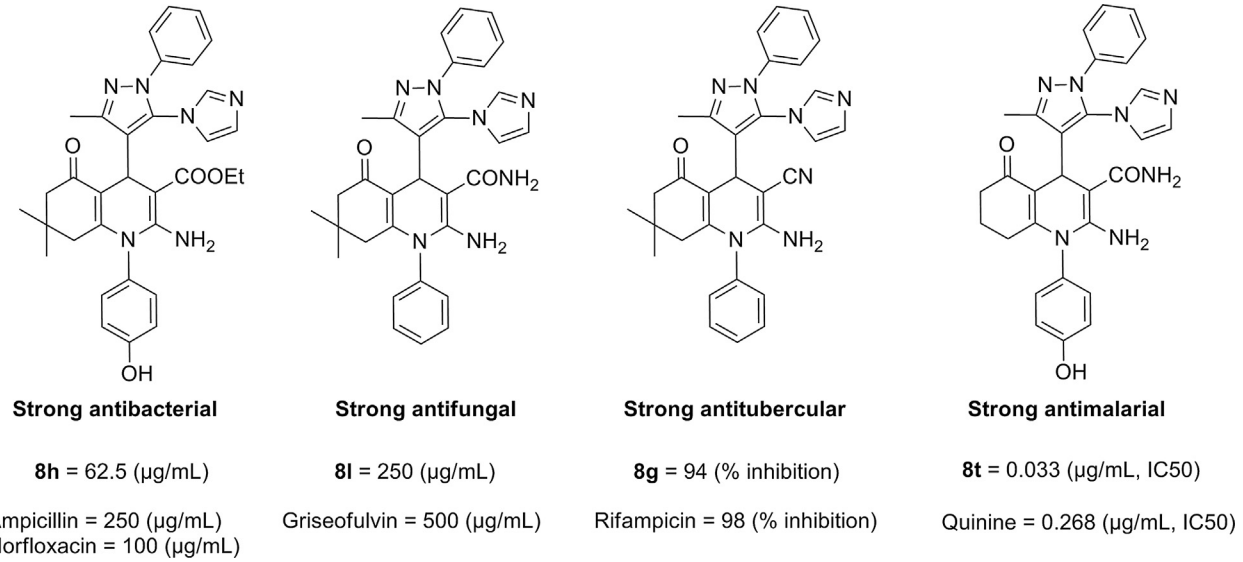
All the synthesized compounds **8a**–**t** were evaluated for their antimalarial activity against chloroquine and quinine sensitive strain of *Plasmodium falciparum*. All experiments were performed in duplicate and a mean value of IC_{50} is mentioned in Table 3. As shown in Table 3, the compounds **8d** (R_2 = Cl, X = CN), **8e** (R_2 = Cl, X = COOEt), **8f** (R_2 = Cl, X = $CONH_2$), **8h** (R_2 = OH, X = COOEt), **8i** (R_2 = OH, X = $CONH_2$), **8n** (R_3 = OH, X = CN), **8o** (R_3 = OH, X = COOEt), **8q** (R_2 = OCH_3, X = CN), **8s** (R_2 = OH, X = COOEt) and **8t** (R_1 = H, R_2 = OH, X = $CONH_2$) were found to have IC_{50} in the range of 0.033–0.096 against *P. falciparum* strain. These compounds displayed marvellous activity against *P. falciparum* strain as compared to quinine IC_{50} 0.268. Moreover compounds **8n** (R_3 = OH, X = CN) and **8t** (R_1 = H, R_2 = OH, X = $CONH_2$) were found to possess moderate activity i.e. IC_{50} 0.033 and 0.034 aligned with chloroquine. Remaining all other compounds were found to be less active against *P. falciparum* strain against chloroquine and quinine as the standard drugs.

4.2.5. Structure–activity relationship (SAR)

The results of the biological evaluation revealed that the activity was considerably affected by various substituents on the aromatic ring present at first position in polyhydroquinoline derivatives. The hydroxyl group present at various positions in phenyl ring played an important role for the divergence in activity [27]. We observed that the presence of hydroxyl group at *ortho* position showed significant potency against *Bacillus subtilis* and *Clostridium tetani*; while at *meta* position, it showed maximum activity against *V. cholerae* and *C. tetani* as well as increased activity against *Plasmodium falciparum*. The hydroxyl group present at *para* position offered higher antifungal activity against *C. albicans*. Chloro substitution at *para* position on phenyl ring showed excellent antimalarial activity against *P. falciparum*. Whereas methoxy group present at *para* position on phenyl ring showed very good antituberculosis activity against *Mycobacterium tuberculosis* H37Rv as well as showed best antibacterial activity against all 3 g positive bacteria. Carbonitrile, ester and amide groups present at third position in polyhydroquinoline ring were also found responsible for variation in activity. Particularly the carbonitrile group was found accountable for increasing antituberculosis activity against *M. tuberculosis* H37Rv whereas ester and amide groups were responsible for increased antifungal activity against *C. albicans*. The strongly active compounds of the series with reference to the standard drugs are shown in Scheme 2.

5. Conclusion

The objective of the present study was to synthesize and examine the antimicrobial, antimalarial and antituberculosis



Scheme 2. Strong antibacterial, antifungal, antitubercular and antimalarial compounds from the series.

activities of some newly functionalized pyrazole incorporated polyhydroquinoline compounds in search of new structural leads looking promising as potent antimicrobial, antimalarial and antituberculosis agents. This synthetic approach allows the incorporation of three potent bioactive nuclei in a single scaffold through an easy way. Except only two compounds, all the compounds were found to be active against *B. subtilis* and *C. tetani* and nearly about half of the compounds showed excellent antifungal activity against *Candida albicans*. The compounds **8a**, **8d**, **8g**, **8p** and **8q** exhibited good antituberculosis activities. Majority of the compounds showed excellent activity against strains of *P. falciparum* as compared to quinine.

6. Experimental section

6.1. Chemistry

All reactions were performed with commercially available reagents. They were used without further purification. The solvents used were of analytical grade. All reactions were monitored by thin-layer chromatography (TLC) on aluminum plates coated with silica gel 60 F_{254}, 0.25 mm thickness (Merck). Detection of the components was made by exposure to iodine vapours or UV light. Melting points were taken in melting point apparatus μThermo-Cal_{10} (Analab Scientific Pvt. Ltd, India) and are uncorrected. The IR spectra were recorded in KBr on a Perkin–Elmer Spectrum GX FT-IR Spectrophotometer (Perkin–Elmer, USA) and only the characteristic peaks are reported in cm^{-1}. Mass spectra were recorded on Shimadzu LCMS 2010 spectrometer (Shimadzu, Tokyo, Japan) purchased under PURSE program of DST at Sardar Patel University, Vallabh Vidyanagar. ^1H and ^{13}C Nuclear Magnetic Resonance spectra were recorded in DMSO-d_6 on a Bruker Avance 400F (MHz) spectrometer (Bruker Scientific Corporation Ltd., Switzerland) using residual solvent signal as an internal standard at 400 MHz and 100 MHz respectively. Chemical shifts are reported in parts per million (ppm). Splitting patterns were designated as follows: s, singlet; d, doublet; dd, doublet of doublet and m, multiplet. The elemental analysis was carried out by using Perkin–Elmer 2400 series-II elemental analyzer (Perkin–Elmer, USA) and all compounds are within ±0.4% of the theoretical compositions. Yields are not optimized. Ampicillin, griseofulvin, isoniazid and nystatin were purchased from local market.

6.1.1. General procedure for the synthesis of 5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde (**3**)

5-Chloro-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde **1** (1.1 g, 5 mmol), appropriate imidazole **2** (0.51 g, 7.5 mmol) and anhydrous potassium carbonate (0.6 g, 10 mmol) in dimethylformamide (5 mL) were charged in a 50 mL round bottom flask with mechanical stirrer and condenser. The reaction mixture was refluxed for 2 h and the progress of the reaction was monitored by TLC. After the completion of reaction (as evidenced by TLC), the reaction mixture was cooled to room temperature and then poured into ice cold water (50 mL) with continuous stirring followed by neutralization with 1 N HCl until pH 7. The separated precipitates of 5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1*H*-pyrazole-4 carbaldehyde **3** were filtered, thoroughly washed with water, dried, and recrystallized from ethanol.

Yield 79%; m.p. 204 °C; ^1H NMR (400 MHz, DMSO-d_6): δ 2.59 (s, 3H, CH_3), 7.07–7.46 (m, 7H, Ar–H), 7.94 (s, 1H, imidazole), 9.74 (s, 1H, CHO); ESI-MS (*m*/*z*): Calcd. 252.2, found 253.0 (M^+)

6.1.2. Synthesis of the substituted 3-(phenylamino)cyclohex-2-enone (substituted enaminones) (**6a–h**)

Cyclohexane − 1,3 − dione or dimedone (10 mmol), substituted amines (10 mmol) and PEG (polyethylene glycol):deionised water (5 mL:5 mL) were charged in a 50 mL round bottom flask with mechanical stirrer and condenser. The reaction mixture was refluxed for 0.5–1 h. After the completion of reaction (checked by TLC), the substituted enaminones (**6 a-h**) were filtered and washed with deionised water. The further purification was carried out by leaching in equal volume ratio of water and methanol (10:10 mL) to obtain the pure solid sample.

6.1.3. Synthesis of the substituted 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-phenyl-4,6,7,8-tetrahydroquinolin-5(1H)-one (**8a–t**)

A 50 mL round bottom flask, fitted with a reflux condenser, was charged with a mixture of 5-(1*H*-imidazol-1-yl)-3-methyl-1-phenyl-1*H*-pyrazole-4-carbaldehyde **3** (1 mmol), malononitrile or caynoacetamide or ethylcayno acetate (1 mmol) **7a–c**, substituted enaminones **6a–h** (1 mmol), and catalytic amount of piperidine (2–3 drops) in ethanol (10 mL). The mixture was heated under reflux for 1–3 h and the progress of the reaction was monitored by TLC. After the completion of reaction, the reaction mixture was cooled to room temperature and stirred magnetically for further 10 min. The solid

mass separated was collected by filtration, washed well with ethanol (10 mL) and crystallized from hot chloroform (10 mL). The physicochemical and spectroscopic characterization data of the synthesized compounds **8a**–**t** are given below.

*6.1.3.1. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-7,7-dimethyl-5-oxo-1-p-tolyl-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (**8a**).* Yield 80%; m.p. 278 °C; IR (KBr, ν_{max}, cm^{-1}): 3459 & 3333 (asym. & sym. stretching of – NH_2), 2950 (Ar C–H), 2921 (C–H asym. stretching of CH_2 group), 2177 (C≡N stretching), 1641(C=O stretching), 1371 (–CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.2 (CH_3), 22.2 (CH_3), 27.3, 28.0 (2C, CH_3), 28.5 (C_4), 32.4 ($C(CH_3)_2$), 41.5, 49.9 ($2(CH_2)_2$), 58.3 (C–CN), 108.1, 121.0, 121.9, 122.5, 122.7, 127.8, 129.67, 129.8, 130.7, 132.4, 134.9, 134.9, 138.1, 147.5, 150.8, 151.3 (16C, Ar–C), 195.7 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.93 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 1.84 (dd, J = 26.8, 17.2 Hz, 2H, CH_2), 2.15 (dd, J = 20.4, 16.4 Hz, 2H, CH_2), 2.48 (s, 3H, CH_3), 2.50 (s, 3H, CH_3), 4.03 (s, 2H, NH_2), 4.62 (s, 1H, CH), 7.10–7.40 (m, 11H, Ar–H), 7.84 (s, 1H, imidazole); ESI-MS (*m*/*z*): Calcd. 529.26, found 530.4 (M^+); Anal. Calcd. (%) for $C_{32}H_{31}N_7O$:72.57; H, 5.90; N, 18.51. Found: C, 72.42; H, 5.79; N, 18.69.

*6.1.3.2. Ethyl 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-7,7-dimethyl-5-oxo-1-p-tolyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**8b**).* Yield 79%; m.p. 247 °C; IR (KBr, ν_{max}, cm^{-1}): 3418 & 3362 (asym. & sym. stretching of –NH_2), 2961 (Ar C–H), 2930 (C–H asym. stretching of CH_2 group), 1645 (C=O stretching), 1372 (–CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.5 (CH_3), 15.1 (CH_3), 21.3 (CH_3), 25.3, 27.8 (2C, CH_3), 28.8 (C_4), 32.2 ($C(CH_3)_2$), 41.7, 50.1 ($2(CH_2)_2$), 58.6 (OCH_2), 76.4 (C–COOEt), 110.8, 122.4, 122.5, 122.6, 127.4, 129.3, 129.5, 131.2, 132.4, 133.7, 138.4, 139.8, 148.4, 150.7, 152.8, (15C, Ar–C), 169.3 (COOEt), 196.0 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.81 (s, 3H, CH_3), δ 0.86 (s, 3H, CH_3), δ 0.95 (t, J = 7.2 Hz, 3H, CH_3), 1.70 (d, J = 17.6 Hz, 1H, CH_2), 1.94 (d, J = 17.2 Hz, 1H, CH_2), 2.14 (s, 2H, CH_2), 2.41 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 3.44 (m, 2H, CH_2), 4.84 (s, 1H, CH), 6.70 (s, 2H, NH_2), 6.96–7.40 (m, 11H, Ar–H), 7.86 (s, 1H, imidazole); ESI-MS (*m*/*z*): Cacld. 576.28, found 577.2 (M^+); Anal. Calcd. (%) for $C_{34}H_{36}N_6O_3$: C, 70.81; H, 6.29; N, 14.57. Found: C, 70.70; H, 6.17; N, 14.72.

*6.1.3.3. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-7,7-dimethyl-5-oxo-1-p-tolyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**8c**).* Yield 71%; m.p. 230 °C; IR (KBr, ν_{max}, cm^{-1}): 3409 & 3371 (asym. & sym. stretching of – NH_2), 2955 (Ar C–H), 2925 (C–H asym. stretching of CH_2 group), 1641 (C=O stretching), 1375 (–CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.5 (CH_3), 21.0 (CH_3), 26.6, 27.6 (2C, CH_3), 28.9 (C_4), 32.1 ($C(CH_3)_2$), 41.8, 50.1 ($2(CH_2)_2$), 78.3 (C–$CONH_2$), 109.6, 117.9, 119.7, 122.4, 128.5, 129.3, 129.9, 130.0, 132.3, 133.4, 136.5, 138.5, 147.9, 149.8, 151.1 (15C, Ar–C), 171.1 ($CONH_2$), 195.7 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.84 (s, 3H, CH_3), 0.88 (s, 3H, CH_3), 1.73 (d, J = 17.2 Hz, 1H, CH_2), 1.96 (d, J = 17.2 Hz, 1H, CH_2), 2.16 (s, 2H, CH_2), 2.44 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 4.69 (s, 1H, CH), 5.74 (s, 2H, NH_2), 6.73 (s, 2H, NH_2), 7.01–7.64 (m, 11H, Ar–H), 7.91 (s, 1H, imidazole); ESI-MS (*m*/*z*): Calcd. 547.27, found 547.9 (M^+); Anal. Calcd. (%) for $C_{32}H_{33}N_7O_2$:C, 70.18; H, 6.07; N, 17.90. Found: C, 70.24; H, 5.95; N, 17.79.

*6.1.3.4. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-chlorophenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (**8d**).* Yield 80%; m.p. 285 °C; IR (KBr, ν_{max}, cm^{-1}): 3432 & 3356 (asym. & sym. stretching of – NH_2), 2953 (Ar C–H), 2929 (C–H asym. stretching of CH_2 group), 2187 (C≡N stretching), 1644(C=O stretching), 1376 (–CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.1 (CH_3), 27.3, 27.9 (2C, CH_3), 28.5 (C_4), 32.3 ($C(CH_3)_2$), 41.5, 49.8 ($2(CH_2)_2$), 58.1 (C–CN), 108.5, 121.0, 121.9, 122.5, 122.7, 127.8, 129.7, 129.8, 130.7, 132.4, 134.9, 135.1, 138.1, 147.5, 150.8, 151.2 (16C, Ar–C), 195.7 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 1.74 (d, J = 17.2 Hz, 1H, CH_2), 1.93 (d, J = 17.2 Hz, 1H, CH_2), 2.11 (s, 2H, CH_2), 2.38 (s, 3H, CH_3), 4.33 (s, 1H, CH), 5.48 (s, 2H, NH_2), 7.08–7.65 (m, 11H, Ar–H), 7.82 (s, 1H, imidazole); ESI-MS (*m*/*z*): Cacld. 549.2, found 550.1 (M^+), 551.2 (M+2); Anal. Calcd. (%) for $C_{34}H_{36}N_6O_3$:C, 67.69; H, 5.13; N, 17.82. Found: C, 67.72; H, 5.01; N, 17.67.

*6.1.3.5. Ethyl 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-chlorophenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**8e**).* Yield 78%; m.p. 234 °C; IR (KBr, ν_{max}, cm^{-1}): 3412 & 3369 (asym. & sym. stretching of –NH_2), 2955 (Ar C–H), 2925 (C–H asym. stretching of CH_2 group), 1639 (C=O stretching), 1371 (–CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.6 (CH_3), 14.9 (CH_3), 25.0, 27.5 (2C, CH_3), 28.8 (C_4), 32.1 ($C(CH_3)_2$), 41.6, 49.7 ($2(CH_2)_2$), 58.5 (OCH_2), 76.5 (C–COOEt), 110.3, 121.9, 122.4, 122.5, 127.5, 128.9, 128.9, 129.1, 130.3, 138.1, 139.5, 139.9, 148.1, 150.1, 153.1, (15C, Ar–C), 169.1 (COOEt), 196.1 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83 (s, 3H, CH_3), δ 0.88 (s, 3H, CH_3), δ 0.95 (t, J = 7.2 Hz, 3H, CH_3), 1.72 (d, J = 17.6 Hz, 1H, CH_2), 1.97 (d, J = 17.2 Hz, 1H, CH_2), 2.17 (s, 2H, CH_2), 2.40 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 3.42 (m, 2H, CH_2), 4.88 (s, 1H, CH), 6.76 (s, 2H, NH_2), 6.93–7.43 (m, 11H, Ar–H), 7.89 (s, 1H, imidazole); ESI-MS (*m*/*z*): Calcd. 596.2, found 597.0 (M^+), 598.2 (M+2); Anal. Calcd. (%) for $C_{33}H_{33}ClN_6O_3$: C, 66.38; H, 5.57; N, 14.07. Found: C, 66.30; H, 5.44; N, 14.15.

*6.1.3.6. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-chlorophenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**8f**).* Yield 72%; m.p. 245 °C; IR (KBr, ν_{max}, cm^{-1}): 3414 & 3381 (asym. & sym. stretching of –NH_2), 2947 (Ar C–H), 2932 (C–H asym. stretching of CH_2 group), 1645 (C=O stretching), 1371 (–CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.5 (CH_3), 26.9, 27.67 (2C, CH_3), 28.8 (C_4), 31.9 ($C(CH_3)_2$), 41.6, 49.7 ($2(CH_2)_2$),78.3 (C–$CONH_2$), 109.5, 115.5, 119.9, 122.5, 127.7, 128.3, 129.7, 129.9, 129.9, 132.6, 136.0, 138.3, 148.1, 150.3, 150.9 (15C, Ar–C), 172.2 ($CONH_2$), 195.6 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 1.75 (d, J = 17.2 Hz, 1H, CH_2), 1.93 (d, J = 17.2 Hz, 1H, CH_2), 2.13 (s, 2H, CH_2), 2.51 (s, 3H, CH_3), 4.70 (s, 1H, CH), 5.76 (s, 2H, NH_2), 6.75 (s, 2H, NH_2), 7.06–7.64 (m, 11H, Ar–H), 7.92 (s, 1H, imidazole); ESI-MS (*m*/*z*): Calcd. 567.2, found 568.0 (M^+), 569.3 (M+2); Anal. Calcd. (%) for $C_{31}H_{30}ClN_7O_2$:C, 65.54; H, 5.32; N, 17.26. Found: C, 65.41; H, 5.24; N, 17.38.

*6.1.3.7. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-hydroxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (**8g**).* Yield 84%; m.p. 283 °C; IR (KBr, ν_{max}, cm^{-1}): 3441 & 3361 asym. & sym. stretching of –NH_2), 2945 (Ar C–H), 2926 (C–H asym. stretching of CH_2 group), 2171 (C≡N stretching), 1646(C=O stretching), 1375 (–CH_3 stretching). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.5 (CH_3), 27.2, 28.3 (2C, CH_3), 28.5 (C_4), 32.4 ($C(CH_3)_2$), 41.6, 49.8 ($2(CH_2)_2$), 58.5 (C–CN), 108.1, 117.5, 118.7, 121.1, 122.6, 122.8, 127.9, 129.8, 130.0, 130.8, 135.3, 138.3, 147.4, 150.9, 151.6, 155.1 (16C, Ar–C), 195.6 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.87 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 1.77 (d, J = 17.2 Hz, 1H, CH_2), 1.94 (d, J = 17.2 Hz, 1H, CH_2), 2.15 (s, 2H, CH_2), 2.36 (s, 3H, CH_3), 4.30 (s, 1H, CH), 5.50 (s, 2H, NH_2), 7.13–7.66 (m, 11H, Ar–H), 7.77 (s, 1H, OH), 7.95 (s, 1H, imidazole); ESI-MS (*m*/*z*): Calcd. 531.2, found 532.2 (M^+); Anal. Calcd. (%) for $C_{31}H_{29}N_7O_2$:C, 70.04; H, 5.50; N, 18.44. Found: C, 70.21; H, 5.41; N, 18.49.

*6.1.3.8. Ethyl 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-hydroxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**8h**).* Yield 79%; m.p. 290 °C; IR (KBr, ν_{max}, cm^{-1}): 3415 & 3363 (asym. & sym. stretching of $-NH_2$), 2961 (Ar C–H), 2922 (C–H asym. stretching of CH_2 group), 1641 (C=O stretching), 1372 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.7 (CH_3), 15.1 (CH_3), 25.4, 28.0 (2C, CH_3), 28.9 (C_4), 32.0 ($C(CH_3)_2$), 41.9, 50.2 ($2(CH_2)_2$), 58.5 (OCH_2), 76.3 (C–COOEt), 111.1, 117.6, 118.5, 122.1, 122.6, 127.4, 129.3, 130.1, 138.3, 139.3, 139.4, 148.0, 150.1, 152.8, 156.9 (15C, Ar–C), 169.4 (COOEt), 196.2 (C=O); ^1H NMR (400 MHz, DMSO-d_6): δ 0.82 (s, 3H, CH_3), δ 0.88 (s, 3H, CH_3), δ 0.93 (t, J = 7.2 Hz, 3H, CH_3), 1.69 (d, J = 17.6 Hz, 1H, CH_2), 1.96 (d, J = 17.2 Hz, 1H, CH_2), 2.13 (s, 2H, CH_2), 2.42 (s, 3H, CH_3), 2.47 (s, 3H, CH_3), 3.44 (m, 2H, CH_2), 4.86 (s, 1H, CH), 6.73 (s, 2H, NH_2), 6.95–7.42 (m, 11H, Ar–H), 7.87 (s, 1H, imidazole), 7.93 (s, 1H, OH); MS: ESI-MS (*m/z*): Calcd. 578.3, found 578.1 (M^+); Anal. Calcd. (%) for $C_{33}H_{34}N_6O_4$:C, 68.49; H, 5.92; N, 14.52. Found: C, 68.41; H, 5.96; N, 14.52.

*6.1.3.9. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-hydroxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**8i**).* Yield 73%; m.p. 248 °C; IR (KBr, ν_{max}, cm^{-1}): 3412 & 3385 (asym. & sym. stretching of $-NH_2$), 2950 (Ar C–H), 2929 (C–H asym. stretching of CH_2 group), 1639 (C=O stretching), 1371 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.7 (CH_3), 26.8, 27.8 (2C, CH_3), 28.9 (C_4), 32.3 ($C(CH_3)_2$), 41.9, 50.2 ($2(CH_2)_2$),78.6 (C–$CONH_2$), 110.1, 112.6, 114.8, 120.0, 122.7, 130.1, 130.2, 130.6, 131.9, 136.6, 138.2, 148.3, 151.1, 151.4, 155.4 (15C, Ar–C), 172.4 ($CONH_2$), 195.6 (C=O); ^1H NMR (400 MHz, DMSO-d_6): δ 0.85 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 1.76 (d, J = 17.2 Hz, 1H, CH_2), 1.94 (d, J = 17.2 Hz, 1H, CH_2), 2.14 (s, 2H, CH_2), 2.16 (s, 3H, CH_3), 4.65 (s, 1H, CH), 5.75 (s, 2H, NH_2), 6.75 (s, 2H, NH_2),7.12–7.66 (m, 11H, Ar–H), 7.89 (s, 1H, imidazole), 7.99 (s, 1H, OH); ESI-MS (*m/z*): Calcd. 549.2, found 550 (M^+); Anal. Calcd. (%) for $C_{31}H_{31}N_7O_3$:C, 67.74; H, 5.69; N, 17.84. Found: C, 67.69; H, 5.51; N, 17.91.

*6.1.4.0. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-7,7-dimethyl-5-oxo-1-phenyl-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (**8j**).* Yield 85%; m.p. 289 °C; IR (KBr, ν_{max}, cm^{-1}): 3429 & 3378 (asym. & sym. stretching of $-NH_2$), 2958 (Ar C–H), 2923 (C–H asym. stretching of CH_2 group), 2184(C≡N stretching), 1639 (C=O stretching), 1371 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.3 (CH_3), 27.1, 28.3 (2C, CH_3), 28.6 (C_4), 32.2 ($C(CH_3)_2$), 41.4, 49.8 ($2(CH_2)_2$), 58.9 (C–CN), 108.1, 120.0, 120.7, 121.3, 122.3, 122.4, 127.40, 129.5, 129.7, 130.1, 130.5, 134.8, 138.3, 147.1, 150.7, 151.1 (16C, Ar–C), 195.6 (C=O); ^1H NMR (400 MHz, DMSO-d_6): δ 0.84 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 1.73 (d, J = 17.2 Hz, 1H, CH_2), 1.95 (d, J = 17.2 Hz, 1H, CH_2), 2.16 (s, 2H, CH_2), 2.33 (s, 3H, CH_3), 4.32 (s, 1H, CH), 5.46 (s, 2H, NH_2), 7.05–7.68 (m, 12H, Ar–H), 7.84 (s, 1H, imidazole); ESI-MS (*m/z*): Cacld. 515.2, found 515.3 (M^+); Anal. Calcd. (%) for $C_{31}H_{29}N_7O$:C, 72.21; H, 5.67; N, 19.02. Found: C, 72.42; H, 5.79; N, 18.81.

*6.1.4.1. Ethyl 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-7,7-dimethyl-5-oxo-1-phenyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**8k**).* Yield 82%; m.p. 275 °C; IR (KBr, ν_{max}, cm^{-1}): 3421 & 3373 (asym. & sym. stretching of $-NH_2$), 2949 (Ar C–H), 2927 (C–H asym. stretching of CH_2 group), 1645 (C=O stretching), 1373 (-CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.2 (CH_3), 15.2 (CH_3), 25.3, 27.9 (2C, CH_3), 28.9 (C_4), 32.4 ($C(CH_3)_2$), 41.8, 50.0 ($2(CH_2)_2$), 58.7 (OCH_2), 76.6 (C–COOEt), 110.1, 118.9, 122.3, 122.4, 122.8, 127.4, 128.9, 129.1, 129.4, 139.1, 139.2, 139.5, 148.8, 151.0, 152.9, (15C, Ar–C), 169.3 (COOEt), 195.9 (C=O); ^1H NMR (400 MHz, DMSO-d_6): δ 0.81 (s, 3H, CH_3), δ 0.89 (s, 3H, CH_3), δ 0.94 (t, J = 7.2 Hz, 3H, CH_3), 1.71 (d, J = 17.6 Hz, 1H, CH_2), 1.92 (d, J = 17.2 Hz, 1H, CH_2), 2.12 (s, 2H, CH_2), 2.39 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 3.46 (m, 2H, CH_2), 4.82 (s, 1H, CH), 6.72 (s, 2H, NH_2), 6.98–7.42 (m, 11H, Ar–H), 7.85 (s, 1H, imidazole); ESI-MS (*m/z*): Calcd. 562.3, found 563.3 (M^+); Anal. Calcd. (%) for $C_{33}H_{34}N_6O_3$:C, 70.44; H, 6.09; N, 14.94. Found: C, 70.21; H, 6.00; N, 15.06.

*6.1.4.2. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-7,7-dimethyl-5-oxo-1-phenyl-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**8l**).* Yield 81%; m.p. 235 °C; IR (KBr, ν_{max}, cm^{-1}): 3411 & 3389 (asym. & sym. stretching of $-NH_2$), 2948 (Ar C–H), 2932 (C–H asym. stretching of CH_2 group), 1639 (C=O stretching), 1372 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.6 (CH_3), 26.8, 27.7 (2C, CH_3), 28.9 (C_4), 32.2 ($C(CH_3)_2$), 41.8, 50.0 ($2(CH_2)_2$),78.4 (C–$CONH_2$), 109.7, 119.9, 121.6, 122.6, 127.7, 129.6, 129.8, 130.1, 130.5, 132.1, 136.5, 138.2, 148.1, 150.1, 150.9 (15C, Ar–C), 172.4 ($CONH_2$), 195.7 (C=O); ^1H NMR (400 MHz, DMSO-d_6): δ 0.82 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 1.71 (d, J = 17.2 Hz, 1H, CH_2), 1.95 (d, J = 17.2 Hz, 1H, CH_2), 2.15 (s, 2H, CH_2), 2.47 (s, 3H, CH_3), 4.72 (s, 1H, CH), 5.76 (s, 2H, NH_2), 6.78 (s, 2H, NH_2), 6.98–7.61 (m, 12H, Ar–H), 7.93 (s, 1H, imidazole); ESI-MS (*m/z*): Calcd. 533.3, found 534.0 (M^+); Anal. Calcd. (%) for $C_{31}H_{31}N_7O_2$:C, 69.77; H, 5.86; N, 18.37. Found: C, 70.03; H, 6.00; N, 18.18.

*6.1.4.3. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(2-hydroxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (**8m**).* Yield 84%; m.p. 264 °C; IR (KBr, ν_{max}, cm^{-1}): 3440 & 3352 (asym. & sym. stretching of $-NH_2$), 2953 (Ar C–H), 2915 (C–H asym. stretching of CH_2 group), 2180 (C≡N stretching), 1640(C=O stretching), 1370 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.1 (CH_3), 27.3, 28.1 (2C, CH_3), 28.4 (C_4), 32.5 ($C(CH_3)_2$), 41.5, 49.8 ($2(CH_2)_2$), 57.9 (C–CN), 108.4, 113.9, 118.3, 121.1, 121.2, 121.4, 122.3, 126.1, 127.3, 129.6, 129.9, 130.3, 134.8, 138.2, 147.7, 150.7, 151.1, 152.1 (18C, Ar–C), 196.0 (C=O); ^1H NMR (400 MHz, DMSO-d_6): δ 0.86 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 1.78 (d, J = 17.2 Hz, 1H, CH_2), 1.94 (d, J = 17.2 Hz, 1H, CH_2), 2.18 (s, 2H, CH_2), 2.35 (s, 3H, CH_3), 4.34 (s, 1H, CH), 5.46 (s, 2H, NH_2), 7.12–7.64 (m, 11H, Ar–H), 7.81 (s, 1H, imidazole), 8.12 (s, 1H, OH); ESI-MS (*m/z*): Calcd. 531.2, found, 532.2 (M^+); Anal. Calcd. (%) for $C_{31}H_{29}N_7O_2$:C, 70.04; H, 5.50; N, 18.44. Found: C, 69.89; H, 5.41; N, 18.29.

*6.1.4.4. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(3-hydroxyphenyl)-7, 7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (**8n**).* Yield 71%; m.p. 270 °C; IR (KBr, ν_{max}, cm^{-1}): 3429 & 3364 (asym. & sym. stretching of $-NH_2$), 2961 (Ar C–H), 2925 (C–H asym. stretching of CH_2 group), 2169 (C≡N stretching), 1645(C=O stretching), 1373 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.4 (CH_3), 27.5, 28.2 (2C, CH_3), 28.6 (C_4), 32.2 ($C(CH_3)_2$), 41.4, 49.7 ($2(CH_2)_2$),58.2 (C–CN), 107.6, 108.3, 108.4, 114.7, 121.2, 122.5, 123.1, 127.8, 129.6, 129.9, 130.2, 130.5, 134.7, 138.2, 147.3, 150.7, 151.1, 160.7 (18C, Ar–C), 195.4 (C=O); ^1H NMR (400 MHz, DMSO-d_6): δ 0.87 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 1.76 (d, J = 17.2 Hz, 1H, CH_2), 1.96 (d, J = 17.2 Hz, 1H, CH_2), 2.19 (s, 2H, CH_2), 2.38 (s, 3H, CH_3), 4.36 (s, 1H, CH), 5.46 (s, 2H, NH_2), 7.09–7.66 (m, 11H, Ar–H), 7.84 (s, 1H, imidazole), 8.32 (s, 1H, OH); ESI-MS (*m/z*): Cacld. 531.2, found 532.1 (M^+); Anal. Calcd. (%) for $C_{31}H_{29}N_7O_2$:C, 70.04; H, 5.50; N, 18.44. Found: C, 69.93; H, 5.36; N, 18.25.

*6.1.4.5. Ethyl 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(3-hydroxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**8o**).* Yield 69%; m.p. 282 °C; IR (KBr, ν_{max}, cm^{-1}): 3417 & 3383 (asym. & sym. stretching of $-NH_2$), 2963 (Ar C–H), 2932 (C–H asym. stretching of CH_2 group), 1636 (C=O stretching), 1375 ($-CH_3$ stretching); ^{13}C NMR

(100 MHz, DMSO-d_6) δ: 13.8 (CH_3), 15.0 (CH_3), 25.4, 27.8 (2C, CH_3), 28.6 (C_4), 32.5 ($C(CH_3)_2$), 41.7, 50.1 ($2(CH_2)_2$), 58.6 (OCH_2), 76.5 (C–COOEt), 107.1, 108.2, 110.9, 116.2, 122.5, 123.0, 127.4, 127.6, 129.6, 129.7, 138.7, 138.9, 139.4, 148.5, 151.1, 153.01, 160.6 (17C, Ar–C), 169.3 (COOEt), 196.9 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.80 (s, 3H, CH_3), δ 0.87 (s, 3H, CH_3), δ 0.93 (t, J = 7.2 Hz, 3H, CH_3), 1.69 (d, J = 17.6 Hz, 1H, CH_2), 1.93 (d, J = 17.2 Hz, 1H, CH_2), 2.13 (s, 2H, CH_2), 2.40 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 3.43 (m, 2H, CH_2), 4.86 (s, 1H, CH), 6.69 (s, 2H, NH_2), 6.93–7.41 (m, 11H, Ar–H), 7.86 (s, 1H, imidazole), 8.26 (s, 1H, OH); ESI-MS (m/z): Calcd. 578.3, found 579.0 (M^+); Anal. Calcd. (%) for $C_{33}H_{34}N_6O_4$: C, 68.49; H, 5.92; N, 14.52. Found: C, 68.66; H, 6.11; N, 14.35.

*6.1.4.6. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(3-hydroxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**8p**).* Yield 64%; m.p. 240 °C; IR (KBr, ν_{max}, cm^{-1}): 3408 & 3369 (asym. & sym. stretching of $-NH_2$), 2949 (Ar C–H), 2933 (C–H asym. stretching of CH_2 group), 1642 (C=O stretching), 1372 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.6 (CH_3), 26.7, 27.8 (2C, CH_3), 28.9 (C_4), 32.3 ($C(CH_3)_2$), 42.0, 49.9 ($2(CH_2)_2$),78.5 (C–$CONH_2$), 102.5, 107.1, 109.8, 113.9, 120.1, 122.8, 127.6, 129.9, 130.2, 130.3, 131.5, 136.5, 138.4, 147.7, 150.2, 150.9, 156.9 (17C, Ar–C), 172.5 ($CONH_2$), 195.6 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.83 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 1.73 (d, J = 17.2 Hz, 1H, CH_2), 1.93 (d, J = 17.2 Hz, 1H, CH_2), 2.13 (s, 2H, CH_2), 2.17 (s, 3H, CH_3), 4.70 (s, 1H, CH), 5.77 (s, 2H, NH_2), 6.77 (s, 2H, NH_2),7.11–7.65 (m, 11H, Ar–H), 7.91 (s, 1H, imidazole), 8.29 (s, 1H, OH); ESI-MS (m/z): Cacld. 549.2, found 550.2 (M^+); Anal. Calcd. (%) for $C_{31}H_{31}N_7O_3$:C, 67.74; H, 5.69; N, 17.84. Found: C, 67.73; H, 5.81; N, 17.63.

*6.1.4.7. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-methoxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carbonitrile (**8q**).* Yield 82%; m.p. 278 °C; IR (KBr, ν_{max}, cm^{-1}): 3450 & 3360 (asym. & sym. stretching of $-NH_2$), 2949 (Ar C–H), 2931 (C–H asym. stretching of CH_2 group), 2175 (C≡N stretching), 1641(C=O stretching), 1372 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.3 (CH_3), 27.2, 28.2 (2C, CH_3), 28.7 (C_4), 32.3 ($C(CH_3)_2$), 41.6, 49.8 ($2(CH_2)_2$), 55.1 (OCH_3), 58.1 (C–CN), 107.9, 119.4, 121.2, 121.3, 122.2, 122.6, 127.5, 129.6, 130.3, 130.7, 134.9, 138.6, 147.4, 150.7, 151.2, 156.3 (16C, Ar–C), 195.6 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.85 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 1.75 (d, J = 17.2 Hz, 1H, CH_2), 1.93 (d, J = 17.2 Hz, 1H, CH_2), 2.15 (s, 2H, CH_2), 2.38 (s, 3H, CH_3), 3.58 (s, 3H, CH_3), 4.33 (s, 1H, CH), 5.52 (s, 2H, NH_2), 7.09–7.70 (m, 11H, Ar–H), 7.84 (s, 1H, imidazole); ESI-MS (m/z): Calcd. 545.3, found 546.1 (M^+); Anal. Calcd. (%) for $C_{32}H_{31}N_7O_2$:C, 70.44; H, 5.73; N, 17.97. Found: C, 70.23; H, 5.59; N, 18.07.

*6.1.4.8. Ethyl 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-methoxyphenyl)-7,7-dimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**8r**).* Yield 79%; m.p. 249 °C; IR (KBr, ν_{max}, cm^{-1}): 3413 & 3369 (asym. & sym. stretching of $-NH_2$), 2959 (Ar C–H), 2930 (C–H asym. stretching of CH_2 group), 1644 (C=O stretching), 1375 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.6 (CH_3), 14.9 (CH_3), 25.3, 27.9 (2C, CH_3), 28.9 (C_4), 32.2 ($C(CH_3)_2$), 41.9, 49.9 ($2(CH_2)_2$), 55.6 (OCH_3) 58.7 (OCH_2), 76.8 (C–COOEt), 110.8, 119.9, 120.1, 122.3, 122.9, 127.6, 129.4, 129.5, 138.5, 138.9, 139.4, 148.4, 150.6, 153.1, 157.9 (15C, Ar–C), 169.2 (COOEt), 196.8 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.82 (s, 3H, CH_3), δ 0.85 (s, 3H, CH_3), δ 0.94 (t, J = 7.2 Hz, 3H, CH_3), 1.72 (d, J = 17.6 Hz, 1H, CH_2), 1.96 (d, J = 17.2 Hz, 1H, CH_2), 2.14 (s, 2H, CH_2), 2.39 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 3.45 (m, 2H, CH_2), 3.55 (s, 3H, CH_3), 4.83 (s, 1H, CH), 6.72 (s, 2H, NH_2), 6.97–7.44 (m, 11H, Ar–H), 7.88 (s, 1H, imidazole); ESI-MS (m/z): Calcd. 592.3, found 593.0 (M^+); Anal. Calcd. (%) for $C_{34}H_{36}N_6O_4$:C, 68.90; H, 6.12; N, 14.18. Found: C, 68.81; H, 5.99; N, 14.32.

*6.1.4.9. Ethyl 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-hydroxyphenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (**8s**).* Yield 78%; m.p. 281 °C; IR (KBr, ν_{max}, cm^{-1}): 3422 & 3362 (asym. & sym. stretching of $-NH_2$), 2964 (Ar C–H), 2926 (C–H asym. stretching of CH_2 group), 1643 (C=O stretching), 1369 (-CH_3 stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.6 (CH_3), 15.2 (CH_3), 21.3 (CH_2), 28.9 (C_4), 30.1, 38.5 ($2(CH_2)_2$), 58.6 (OCH_2), 76.3 (C–COOEt), 110.7, 117.2, 118.9, 122.3, 122.6, 127.8, 129.3, 129.5, 138.7, 138.9, 139.8, 147.9, 150.7, 152.8, 156.4 (15C, Ar–C), 169.1 (COOEt), 196.0 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 0.91 (t, J = 7.2 Hz, 3H, CH_3), 1.71 (d, J = 17.6 Hz, 1H, CH_2), 1.94 (d, J = 17.2 Hz, 1H, CH_2), 2.15 (s, 2H, CH_2), 2.42 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 3.47 (m, 2H, CH_2), 4.85 (s, 1H, CH), 6.71 (s, 2H, NH_2), 6.94–7.43 (m, 11H, Ar–H), 7.90 (s, 1H, imidazole), 7.99 (s, 1H, OH); ESI-MS (m/z): Calcd. 550.2, found 550.9 (M^+); Anal. Calcd. (%) for $C_{31}H_{30}N_6O_4$:C, 67.62; H, 5.49; N, 15.26. Found: C, 67.49; H, 5.62; N, 15.20.

*6.1.5. 4-(5-(1H-imidazol-1-yl)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-2-amino-1-(4-hydroxyphenyl)-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxamide (**8t**)*

Yield 73%; m.p. 240 °C; IR (KBr, ν_{max}, cm^{-1}): 3415 & 3385 (asym. & sym. stretching of $-NH_2$), 2960 (Ar C–H), 2923 (C–H asym. stretching of CH_2 group), 1641 (C=O stretching), 1374 ($-CH_3$ stretching); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.6 (CH_3), 21.2 (CH_2), 28.9 (C_4), 30.3, 38.2 ($2(CH_2)_2$), 78.5 (C–$CONH_2$), 109.9, 120.0, 112.7, 122.7, 115.1, 130.0, 130.1, 130.3, 155.4, 132.0, 136.7, 138.4, 148.5, 150.9, 151.3 (15C, Ar–C), 172.6 ($CONH_2$), 195.9 (C=O); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.67 (m, 2H, CH_2), 1.92 (s, 3H, CH_3), 1.98 (t, J = 7.2 Hz, 2H, CH_2), 2.95 (t, J = 17.6 Hz, 2H, CH_2), 4.76 (s, 1H, CH), 5.82 (s, 2H, NH_2), 6.78 (s, 2H, NH_2),7.04–7.62 (m, 11H, Ar–H), 7.95 (s, 1H, imidazole), 7.96 (s, 1H, OH); ESI-MS (m/z): Calcd. 521.2, found 522.0 (M^+); Anal. Calcd. (%) for $C_{29}H_{27}N_7O_3$:C, 66.78; H, 5.22; N, 18.80. Found: C, 66.94; H, 5.06; N, 18.71.

7. Biological evaluation

7.1. In vitro antimicrobial assay

The *in vitro* antimicrobial activity of all synthesized compounds was carried out by broth microdilution method. 2% DMSO in water was used as the diluent to get the desired concentration of compounds to test upon standard bacterial strains. Mueller–Hinton broth was used as nutrient medium to grow and dilute the compound suspension for the test bacteria. Sabouraud Dextrose broth was used for fungal nutrition. Inoculum size for test strain was adjusted to 10^8 CFU mL^{-1} by comparing the turbidity. Serial dilutions were prepared in primary and secondary screening. Each synthesized compounds and the standard drugs were diluted obtaining 2000 μg/mL concentration as a stock solution. The drugs which were found to be active in primary screening (i.e. 500, 250 and 200 μg/mL concentrations) were further screened in their second set of dilution at 100, 50, 25 and 12.5 μg/mL concentration against all microorganisms. 10 micro litre suspensions were further inoculated on appropriate media and growth was noted after 24 and 48 h. The control tube containing no antibiotic was instantaneously subcultured (before inoculation) by spreading a loopful evenly over an area of plate of medium suitable for the growth of the test organism. The tubes were then put for incubation at 37 °C overnight. The highest dilution preventing appearance of turbidity after spot subculture was considered as minimal inhibitory concentration (MIC, μ/L). All the tubes showing no visible growth

(same as control tube) were subcultured and incubated overnight at 37 °C. The amount of growth from the control tube before incubation was compared. In this study Ampicillin, Norfloxacin and Chloramphenicol were used as the standard antibacterial drugs. Nystatin and Griseofulvin were used as standard antifungal drugs. The results are summarized in Table 1.

7.2. In vitro antituberculosis assay

The antitubercular activity of all synthesized compound against *M. tuberculosis* H37Rv was performed by Lowensteine–Jensen method [26] with minor modification where 250 μg/mL and 100 μg/mL dilution of each compound was added to Lowensteine–Jensen medium and then media was uncontaminated by inspissation method. A culture of *M. tuberculosis* H37Rv growing on Lowensteine–Jensen medium was harvested in 0.85% saline in bijou bottle. The stock solutions of the title compounds were prepared in DMSO i.e. 250 μg/mL and 100 μg/mL. These tubes were then incubated at 37 °C for 24 h followed by streaking of *M. tuberculosis* H37Rv (5 _ 104 bacilli per tube). The growth of bacilli was seen after 2 weeks, 3 weeks and finally after 4 week of incubation. The tubes having the compounds were compared with control tubes where medium alone was incubated with *M. tuberculosis* H37Rv. The concentration at which complete inhibition of colonies occurred was taken as active concentration of the tested compound. The standard strain *M. tuberculosis* H37Rv was tested with known drug isoniazid and rifampicin for comparison purpose. The results were summarized in Table 2.

7.3. In vitro antimalarial assay

All the synthesized compounds were screened for their antimalarial activity against the *P. falciparum* strain. The *P. falciparum* strain was acquired from Shree R. B Shah Mahavir Super-speciality hospital, Surat, Gujarat, India, and was used in *in vitro* tests. The *P. falciparum* strains were cultivated by a modified method described by Trager and Jensen [28]. Compounds were dissolved in DMSO. The final concentration of DMSO used was not toxic and did not interfere with the assay. The antiparasitic effect of the compounds was measured by growth inhibition percentage as described by Carvalho and Krettli [29]. For experimental purposes, the cultures were synchronized with 5% D-sorbitol when the parasites were in the ring stage [30]. The parasitic suspension, consisting of predominately the ring stage parasites, was adjusted to a 1–2% parasitaemia and 2.5% haematocrit in hypoxanthine-free RPMI-1640 culture medium with 10% human plasma and was exposed to 7 concentrations of each compound for a single cycle of parasite growth for 48 h at 37 °C. A positive control with reference to standard antimalarial drugs, chloroquine and quinine, in standard concentrations were used in each experiment. The stock solutions were additionally diluted in whole medium (RPMI 1640 plus 10% human serum) to each of the used concentrations. The concentration that inhibited 50% of parasite growth (IC_{50} value) was determined by interpolation using Microcal Origin software. The blood smears used were read blind and each duplicate experiment was repeated three times.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.02.015.

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