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Original article

Design, synthesis and characterization of fluoro substituted novel pyrazolylpyrazolines scaffold and their pharmacological screening

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ABSTRACT

A novel series of fluoro substituted pyrazolylpyrazolines **7a**–**l** was synthesized in good to excellent yield (77–88%) from pyrazole chalcones **5a**–**d** and substituted phenyl hydrazine hydrochlorides **6a**–**c** under microwave irradiation. The newly synthesized compounds were screened for their preliminary *in vitro* antibacterial activity against a panel of pathogenic stains of bacteria and fungi, antituberculosis activity against *Mycobacterium tuberculosis* H37Rv and antimalarial activity against *Plasmodium falciparum*. Compounds **7a**, **7b**, **7g**, **7h**, **7j** and **7k** displayed excellent activity against *P. falciparum* stain as compared to quinine IC_{50} 0.268. Good antitubercular activity was exhibited by compounds **7a**, **7e**, **7h** and **7k**. Some of them also exhibited superior antibacterial activity as compared to the first line drugs.

1. Introduction

The most imperative vector-borne infectious diseases malaria is caused by the protozoan parasite *Plasmodium falciparum* [1]. The most recent report from the WHO states that malaria is responsible for the death of over 1 million persons every year including children under the age of five. It is most common in subtropical and tropical areas and 90% of the cases are originated in sub-Saharan Africa [2]. Tuberculosis is the second most common lethal infectious disease subsequent to AIDS and HIV [3]. About one-third of the world's population is infected by *Mycobacterium tuberculosis* every year and more than 2 million deaths are reported [4]. In this context, it was thought worth to synthesize novel compounds which may exhibit synergistic potency to be employed as antimicrobial, antituberculosis and antimalarial agents.

The substitution of fluorine in to a potential drug molecule can improve efficacy of drugs by extending pharmacokinetic and pharmacodynamics properties [5]. Trifluoromethyl group is a well-known substituent of unique qualities. Its high lipophilicity enables to improve pharmacological activities of the molecule [6,7]. Pyrazoles and their derivatives possess numerous medicinal applications because of their versatile biological activities [8–14]. They have occupied a distinct place due to a range of bioactivities such as antiproliferative [15], antimicrobial [16–18], antidepressant [19], antipyretic [20], anti-inflammatory [21] and anticonvulsant [22]. Pyrazoline is also an important nitrogenous heterocyclic moiety in many drugs. Literature survey revealed that various pyrazoline derivatives have displayed significant biological roles [23–29].

Microwave irradiation as a source of energy leads to environmentally benign protocols in terms of reduction in reaction time, energy saving with high efficiency, improved yields and selectivity [30]. In context of the above consequences and in continuation to our previous studies directed toward the synthesis of biologically active novel heterocyclic scaffolds [30–37], herein we report microwave assisted synthesis of some fluorinated novel pyrazolylpyrazoline derivatives. The synthesized compounds exhibited an interesting profile as antimalarial, antitubercular and antimicrobial agents.

2. Chemistry

The synthesis of novel series of pyrazolylpyrazolines **7a**–**l** was performed as outlined in Scheme 1. The starting material 5-chloro-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde **1** was prepared according to Vilsmeier–Haack reaction of 3-methyl-1-phenyl-1H-pyrazol-5(4H)-one [38]. 3-methyl-5-substituted aryloxy-1-phenyl-1H-pyrazole-4-carbaldehydes **3a**–**d** were prepared by refluxing compound **1** and substituted phenols **2a**–**d** in presence of anhydrous K_2CO_3 as basic catalyst in DMF as solvent. 3-methyl-5-

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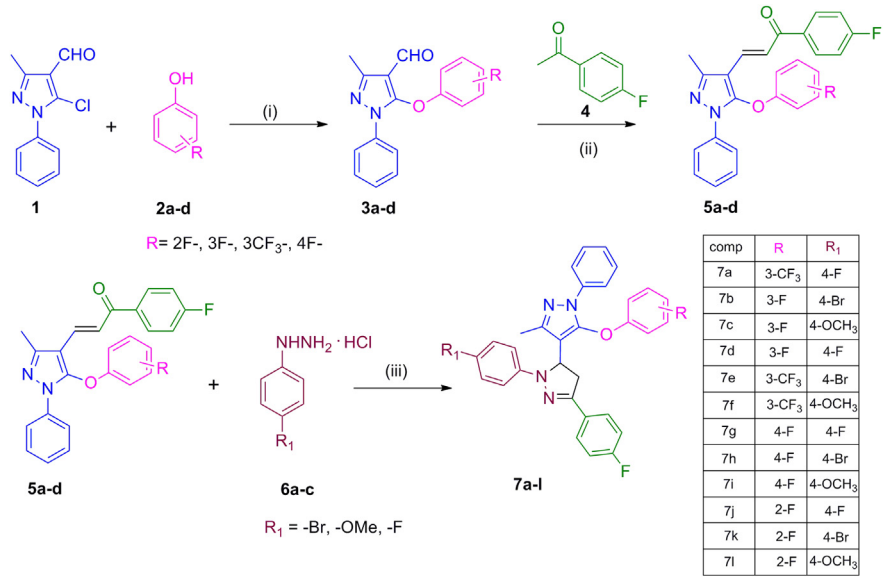

comp	R	R_1
7a	3-CF_3	4-F
7b	3-F	4-Br
7c	3-F	4-OCH_3
7d	3-F	4-F
7e	3-CF_3	4-Br
7f	3-CF_3	4-OCH_3
7g	4-F	4-F
7h	4-F	4-Br
7i	4-F	4-OCH_3
7j	2-F	4-F
7k	2-F	4-Br
7l	2-F	4-OCH_3

Scheme 1. Synthesis of 5-(4-fluorophenyl)-3′-methyl-5′-substituted aryloxy-1′,2-diphenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7a**–**l**) (i) DMF, K_2CO_3, Reflux 2 h. (ii) 20% ethanolic NaOH, room temperature. (iii) Ethanol, catalytic glacial acetic acid, MW, 8–10 min, 350 W.

substituted aryloxy-1-phenyl-1H-pyrazole-4-carbaldehydes **3a**–**d** were subjected to base catalysed Claisen–Schmidt condensation reaction with 4-Fluoro acetophenone **4** generating the required (*E*)-1-(4-fluorophenyl)-3-(3-methyl-5-substituted aryloxy-1-phenyl-1H-pyrazol-4-yl)prop-2-en-1-ones **5a**–**d**. Finally pyrazolyl-pyrazolines **7a**–**l** were obtained by the condensation of **5a**–**d** and substituted phenyl hydrazine hydrochlorides **6a**–**c** in ethanol containing catalytic amount of glacial acetic acid under microwave irradiation at 350 W power level for 8–10 min.

Table 1
In vitro antimicrobial activity (MIC, μg/mL) of compounds **7a**–**l**.

Comp.	*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
7a	500	500	500	200	250	125	1000	250
7b	500	**250**	**250**	500	200	125	**250**	>1000
7c	**100**	**200**	**200**	500	500	200	1000	>1000
7d	200	**250**	500	200	200	250	**250**	1000
7e	500	**250**	**250**	250	200	250	1000	500
7f	200	**100**	**200**	**100**	**100**	250	**250**	500
7g	**100**	500	**250**	500	250	200	**200**	>1000
7h	500	500	**125**	200	200	500	**500**	>1000
7i	125	**62.5**	**200**	500	500	**100**	**500**	250
7j	250	500	**250**	200	250	250	1000	**100**
7k	250	**200**	500	**100**	200	500	**250**	1000
7l	500	**100**	500	500	500	500	**500**	250
A	100	250	250	100	100	100	n. t.[a]	n. t.
B	10	100	50	10	10	10	n. t.	n. t.
C	50	50	50	50	50	50	n. t.	n. t.
D	25	50	100	25	25	25	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: Norfloxacin, C: Chloramphenicol, D: Ciprofloxacin, E: Nystatin, F: Griseofulvin. The bold values indicate comparable/superior potency as compared to the reference drugs.

[a] n.t.: not tested.

3. Pharmacology

3.1. In vitro antimicrobial activity

The synthesized pyrazolylpyrazoline derivatives **7a**–**l** were evaluated for their antimicrobial activity by broth micro dilution method according to National Committee for Clinical Laboratory Standards (NCCLS) [39]. The compounds were screened for anti-bacterial activity employing three Gram positive (*Clostridium tetani* MTCC 449, *Bacillus subtilis* MTCC 441, and *Streptococcus pneumoniae* MTCC 1936) and three Gram negative (*Escherichia coli* MTCC 443, *Salmonella typhi* MTCC 98 and *Vibrio cholerae* MTCC 3906) bacteria against ampicillin, norfloxacin, ciprofloxacin and chloramphenicol as the reference 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 drugs. The result of the antimicrobial screening data is shown in Table 1.

3.2. In vitro antituberculosis activity

A primary *in vitro* antituberculosis activity of novel pyrazolylpyrazolines **7a**–**l** was conducted at 250 μg/mL against *M. tuberculosis* H37Rv stain by using Lowensteine–Jensen medium as described by Rattan [40]. The obtained result is presented in Table 2 in the form of % inhibition. Rifampicin and Isoniazid were employed as the standard drugs.

Table 2
In vitro antituberculosis activity (% inhibition) of compounds **7a**–**l** against *M. tuberculosis* H37Rv (at concentration 250 μg/mL).

Comp.	% Inhibition	Comp.	% Inhibition
7a	**90**	**7h**	**96**
7b	56	7i	74
7c	56	7j	10
7d	65	**7k**	**94**
7e	**91**	7l	22
7f	52	**Rifampicin**	98
7g	40	**Isoniazid**	99

The bold values indicate comparable/superior potency as compared to the reference drugs.

3.3. In vitro antimalarial activity

In vitro antimalarial activity of the novel pyrazolylpyrazolines derivatives **7a**–**l** against *P. falciparum* stain was performed using chloroquine and quinine as the reference drugs. The consequence of antimalarial screening is expressed as the drug concentration resulting in 50% inhibition of parasite growth (IC_{50}) and is listed in Table 3.

4. Results and discussion

4.1. Analytical results

The structures of the synthesized compounds were confirmed by ^{1}H and ^{13}C NMR, FT-IR, mass spectrometry and elemental analysis. The IR spectra of compounds **7a**–**l** exhibited characteristic absorption band in the range of 1260–1253 cm^{-1} due to the presence of ether linkage. The absorption band around 3062–3051 cm^{-1} is due to aromatic C–H stretching. The absorption band observed for all the compounds in between of 1626–1598 cm^{-1} corresponds to –C=N stretching. The strong absorption band is also observed in the range of 1368–1357 cm^{-1} due to $-CH_3$ rocking. The ^{1}H NMR spectra of the target pyrazolylpyrazolines displayed a typical ABX type pattern of doublet of doublet due to three pyrazoline protons. Methine proton of pyrazoline was found at around 5.39–5.17 ppm as a doublet of doublet with coupling constants of nearly 12.8 Hz and 5.6 Hz. Two methylene protons displayed two signals; a doublet of doublet at around 3.74–3.62 ppm with coupling constants of nearly 17 Hz and 12 Hz and a doublet of doublet at around 3.32–3.22 ppm merging with the water signal from DMSO-d_6. At around 3.67 ppm C_4–H pyrazoline proton got merged with the methoxy signal in most of the cases. The data from ^{13}C NMR spectral studies is also in accordance with the suggested structures.

4.2. Biological section

4.2.1. In vitro antibacterial activity

Upon investigation of antimicrobial activity data (Table 1), it has been observed that against *Bacillus subtillis*, compound **7i** (R = 4-F, R_1 = 4-OCH_3) was found to possess excellent potency i.e. 62.5 μg/mL as compared to ampicillin i.e. 250 μg/mL as well as norfloxacin i.e. 100 μg/mL. Compounds **7f** (R = 3-CF_3, R_1 = 4-OCH_3) and **7l** (R = 2-F, R_1 = 4-OCH_3) were found to be more potent against *B. subtillis* i.e. 100 μg/mL as compared to ampicillin. Compounds **7c** (R = 3-F, R_1 = 4-OCH_3) and **7k** (R = 2-F, R_1 = 4-Br) were found to be more effective (MIC = 200 μg/mL) against *B. subtillis* as compared to ampicillin. Compounds **7b** (R = 3-F, R_1 = 4-Br), **7d** (R = 3-F, R_1 = 4-F), **7e** (R = 3-CF_3, R_1 = 4-Br) were equipotent as that of ampicillin against *B. subtillis*. Against *S. pneumonia* compounds **7c** (R = 3-F, R_1 = 4-OCH_3) and **7g** (R = 4-F, R_1 = 4-F) showed comparable activity to that of ampicillin. Against *C. tetani*, compounds **7b** (R = 3-F, R_1 = 4-Br), **7e** (R = 3-CF_3, R_1 = 4-Br), **7g** (R = 4-F, R_1 = 4-F), and **7j** (R = 2-F, R_1 = 4-F) displayed same influence as that of ampicillin. Compounds **7h** (R = 4-F, R_1 = 4-Br), **7c** (R = 3-F, R_1 = 4-OCH_3), **7f** (R = 3-CF_3, R_1 = 4-OCH_3), **7i** (R = 4-F, R_1 = 4-OCH_3) were found to exhibit superior activity as compared to ampicillin against *C. tetani*. In case of inhibiting gram negative bacteria compounds **7f** (R = 3-CF_3, R_1 = 4-OCH_3) and **7k** (R = 2-F, R_1 = 4-Br) were found equipotent against *E. coli* as compared to ampicillin. Compounds **7f** (R = 3-CF_3, R_1 = 4-OCH_3) and **7i** (R = 4-F, R_1 = 4-OCH_3) also exhibited the same power as that of ampicillin i.e. 100 μg/mL against *S. typhi* and *V. cholera* respectively.

Table 3
In vitro antimalarial activity of compounds **7a**–**l**.

Comp.	IC_{50} (μg/mL)	Comp.	IC_{50} (μg/mL)
7a	**0.034**	**7h**	**0.025**
7b	**0.060**	**7i**	0.25
7c	0.57	**7j**	**0.025**
7d	0.82	**7k**	**0.088**
7e	1.46	**7l**	0.65
7f	0.30	**Chloroquine**	0.020
7g	**0.022**	**Quinine**	0.268

The bold values indicate comparable/superior potency as compared to the reference drugs.

4.2.2. In vitro antifungal activity

The antifungal screening data (Table 1) revealed that, against *C. albicans*, compounds **7g** (R = 4-F, R_1 = 4-F), **7b** (R = 3-F, R_1 = 4-Br), **7d** (R = 3-F, R_1 = 4-F), **7f** (R = 3-CF_3, R_1 = 4-OCH_3) and **7k** (R = 2-F, R_1 = 4-Br) were found to possess significant activity as compared to griseofulvin. Compounds **7h** (R = 4-F, R_1 = 4-Br), **7i** (R = 4-F, R_1 = 4-OCH_3) and **7l** (R = 2-F, R_1 = 4-OCH_3) exhibited equivalent potency against *C. albicans* as compared to griseofulvin. Compound **7j** (R = 2-F, R_1 = 4-F) showed equal potency against *A. fumigates* as compared to griseofulvin as well as nystatin.

4.2.3. In vitro antituberculosis activity

Antituberculosis screening of all the synthesized compounds **7a**–**l** was conducted at 250 μg/mL concentrations against tuberculosis H37Rv stains. Compounds **7a** (R = 3-CF_3, R_1 = 4-F), **7e** (R = 3-CF_3, R_1 = 4-Br), **7h** (R = 4-F, R_1 = 4-Br) and **7k** (R = 2-F, R_1 = 4-Br) found to possess brilliant activity (i.e. 90%, 91%, 96 and 94% at 250 μg/mL) against *M. tuberculosis* H37Rv (Table 2). Remaining all other compounds showed poor inhibition against *M. tuberculosis* growth.

4.2.4. In vitro antimalarial activity

Compounds **7a**–**l** were also evaluated for their antimalarial screening against chloroquine and quinine sensitive stains of *P. falciparum*. All experiments were performed in duplicate and a mean value of IC_{50} is mentioned in Table 3. As shown in Table 3, they were found to have IC_{50} between 0.022 and 0.088 upon *P. falciparum* stain. It is important to note that compounds **7a** (R = 3-CF_3, R_1 = 4-F), **7b** (R = 3-F, R_1 = 4-Br), **7g** (R = 4-F, R_1 = 4-F), **7h** (R = 4-F, R_1 = 4-Br), **7j** (R = 2-F, R_1 = 4-F) and **7k** (R = 2-F, R_1 = 4-Br) displayed excellent activity against *P. falciparum* stain as compared to quinine IC_{50} 0.268. From the above results, it can be concluded that compound **7g**, **7h** and **7j** may prove themselves as new antimalarial agents in future.

4.3. Structure–activity relationship (SAR)

The results of the biological evaluation revealed that the activity was significantly affected by introducing fluorinated aryloxy nucleus at the C-5′ position in pyrazoline scaffold (Scheme 1). It has been observed that electron donating group (4-OCH_3) existing at R_1 position and 3-CF_3 group on aryloxy ring on pyrazoline induced the greatest antibacterial activity against *B. subtilis* and *C. tetani* (Fig. 1).

The presence of electron withdrawing group (4-F, 4-Br) at R_1 position and $-CF_3$ group at *meta* position of aryloxy ring illustrated superior antimalarial activity as well as antituberculosis activity. The presence of bromo group at R_1 position and fluoro group at *ortho*, *meta*, and *para* positions of aryloxy ring showed enhanced antituberculosis activity as well as remarkable antimalarial activity. The presence of fluoro group at R_1 position and the *ortho*, *meta*, and *para* positions of aryloxy ring displayed excellent antimalarial activity. It can be concluded that the presence of electron

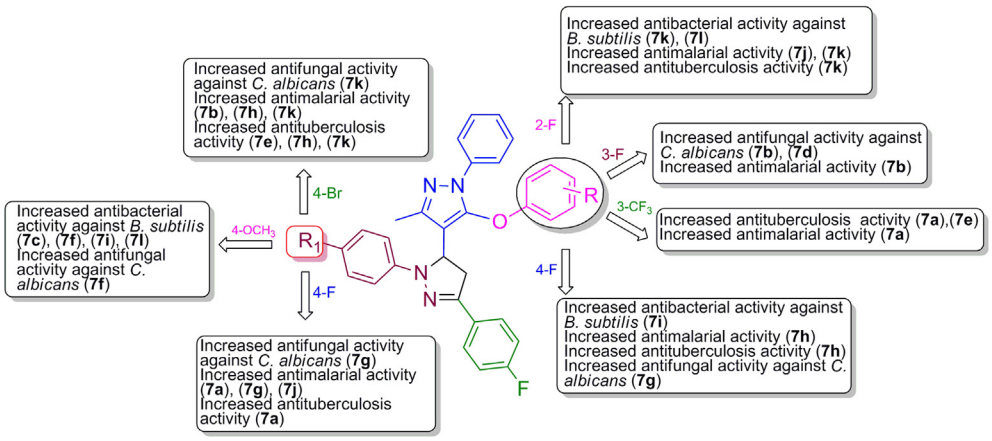

Fig. 1. Structure–activity relationships for antimicrobial, antituberculosis and antimalarial activity of the synthesized compounds **7a**–**l**.

withdrawing groups (4-F, 4-Br) at R_1 position and fluoro group at different positions of aryloxy ring are responsible for increasing antimalarial activity.

5. Conclusion

The novel series of fluoro substituted pyrazolylpyrazoline derivatives have been synthesized in excellent yield using microwave irradiation and examined for their pharmacological screening. The results demonstrated that some analogues of this series were found to have more potency against *C. tetani* and *B. subtilis*. The compounds **7b**, **7d**, **7f**, **7g** and **7k** illustrated remarkable antifungal activity as compared to griseofulvin. Amongst the tested compounds, **7a**, **7e**, **7h**, and **7k** showed pronounced antituberculosis activity against *M. tuberculosis* H37Rv. Whereas, compounds **7a**, **7b**, **7g**, **7h**, **7j** and **7k** displayed superior antimalarial activity against *P. falciparum* stain as compared to quinine. Compound **7h** with an excellent dual profile as antimalarial and antituberculosis agent was recognized as the most active member among the prepared series.

6. Experimental section

6.1. Chemistry

Melting points in °C were determined in open capillaries using μThermoCal$_{10}$ melting point apparatus (Analab Scientific Pvt. Ltd, India) and are uncorrected. Precoated silica gel plates (silica gel 0.25 mm, 60 G F 254; Merck, Germany) were used for thin layer chromatography. The IR spectra were recorded on Shimadzu FTIR 8401 spectrophotometer using potassium bromide pellets in the range 4000–400 cm^{-1} and frequencies of only characteristic peaks are expressed in cm^{-1}. Electron impact Mass Spectra were recorded on Shimadzu LCMS 2010 spectrometer (Shimadzu, Tokyo, Japan) purchased under PURSE program of DST at Sardar Patel University, Vallabh Vidyanagar, India. NMR spectra (in DMSO-d$_6$) were recorded on Bruker Avance 400F NMR Spectrometer at 400 MHz using TMS as the internal standard. The elemental analysis was performed on Perkin–Elmer 2400 series-II elemental analyzer (Perkin–Elmer, USA) at Sophisticated Instrumentation Centre for Applied Research & Training (SICART), Vallabh Vidyanagar, India. All compounds were found within ±0.4% of their theoretical values. All the reactions were carried out at atmospheric pressure using a multimode microwave reactor (Microwave Synthesis System, Model: Cata-R, Catalyst Systems, Pune-India) with an individual sensor for temperature control through attachment of reflux condenser with constant stirring. Microwaves are generated by magnetron at a frequency of 2450 MHz having an adjustable output power levels (i.e. 1 to 10 levels from 140 to 700 Watts).

6.1.1. General procedure for synthesis of 3-methyl-5-substituted aryloxy-1-phenyl-1H-pyrazole-4-carbaldehyde (**3a**–**d**)

5-chloro-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde **1** (1 mmol), substituted phenols **2a**–**d** (1 mmol) and anhydrous potassium carbonate (2 mmol) in dimethylformamide (10 mL) were charged in a 100 mL round bottom flask equipped with a mechanical stirrer and a condenser. The reaction mixture was heated at 90 °C for 2 h. The progress of the reaction was monitored by TLC. After the completion of reaction as confirmed by TLC, the reaction mixture was poured in to 100 mL ice-water, filtered, washed thoroughly with water, dried and recrystallized from ethanol to obtain a white solid.

*6.1.1.1. 5-(2-Fluorophenoxy)-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde (**3a**).* Yield 85%; m.p. 225–227 °C; ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 2.46 (s, 3H, CH$_3$), 7.13–7.64 (m, 9H, Ar–H), 9.56 (s, 1H, CHO).

*6.1.1.2. 5-(3-fluorophenoxy)-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde (**3b**).* Yield 78%; m.p. 210–212 °C; ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 2.47 (s, 3H, CH$_3$), 6.92–7.63 (m, 9H, Ar–H), 9.61 (s, 1H, CHO).

*6.1.1.3. 5-(4-Fluorophenoxy)-3-methyl-1-phenyl-1H-pyrazole-4-carbaldehyde (**3c**).* Yield 82%; m.p. 245–247 °C; ^{1}H NMR (400 MHz, DMSO-d$_6$): δ 2.46 (s, 3H, CH$_3$), 7.17–7.64 (m, 9H, Ar–H), 9.54 (s, 1H, CHO).

6.1.2. General procedure for synthesis of (E)-1-(4-fluorophenyl)-3-(3-methyl-5-substituted aryloxy-1-phenyl-1H-pyrazol-4-yl)prop-2-en-1-ones(**5a**–**d**)

To a mixture of 3-methyl-5-substituted aryloxy-1-phenyl-1H-pyrazole-4-carbaldehydes **3a**–**d** (5.0 mmol) and 4-fluoro acetophenone **4** (5.0 mmol), 20% ethanolic NaOH (5 mL) was added. The reaction mixture was stirred at room temperature until formation of precipitate. The solid obtained was isolated by filtration, washed with cold ethanol and recrystallized from CHCl$_3$.

*6.1.2.1. (E)-3-(5-(2-fluorophenoxy)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-1-(4-fluoro phenyl)prop-2-en-1-one (**5a**).* Yield 78%; m.p.

229–231 °C; IR (KBr, ν_{max}, cm^{-1}): 1362 (−CH_3 rocking.), 1257 (C–O–C ether str.), 1660 (C=O and −C=N), 1582 (C=C), 3060 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.52 (s, 3H, CH_3), 6.94–7.86 (m, 15H, Ar–H + −CH=CH−). ESI-MS (*m*/*z*): 417.3 (M^+); Anal. Calcd (%) for $C_{25}H_{18}F_2N_2O_2$: C, 72.11; H, 4.36; N, 6.73. Found: C, 71.90; H, 4.14; N, 6.49.

*6.1.2.2. (E)-3-(5-(3-fluorophenoxy)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-1-(4-fluoro phenyl)prop-2-en-1-one (**5b**).* Yield 83%; m.p. 201–203 °C; IR (KBr, ν_{max}, cm^{-1}): 1358 (−CH_3 rocking.), 1254 (C–O–C ether str.), 1659 (C=O and −C=N), 1580 (C=C), 3062 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.51 (s, 3H, CH_3), 6.87–7.85 (m, 15H, Ar–H + −CH=CH−). ESI-MS (*m*/*z*): 417.3 (M^+); Anal. Calcd (%) for $C_{25}H_{18}F_2N_2O_2$: C, 72.11; H, 4.36; N, 6.73. Found: C, 71.91; H, 4.12; N, 6.48.

*6.1.2.3. (E)-1-(4-fluorophenyl)-3-(3-methyl-1-phenyl-5-(3-(trifluoromethyl)phenoxy)-1H-pyrazol-4-yl)prop-2-en-1-one (**5c**).* Yield 80%; m.p. 258–260 °C; IR (KBr, ν_{max}, cm^{-1}): 1362 (−CH_3 rocking.), 1257(C–O–C ether str.), 1660 (C=O and −C=N), 1576 (C=C), 3060 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.52 (s, 3H, CH_3), 7.11–7.86 (m, 15H, Ar–H + −CH=CH−). ESI-MS (*m*/*z*): 467.3 (M^+); Anal. Calcd (%) for $C_{26}H_{18}F_4N_2O_2$: C, 66.95; H, 3.89; N, 6.01. Found: C, 66.74; H, 3.69; N, 5.79.

*6.1.2.4. (E)-3-(5-(4-fluorophenoxy)-3-methyl-1-phenyl-1H-pyrazol-4-yl)-1-(4-fluoro phenyl)prop-2-en-1-one (**5d**).* Yield 75%; m.p. 238–240 °C; IR (KBr, ν_{max}, cm^{-1}): 1358 (−CH_3 rocking.), 1255 (C–O–C ether str.), 1662 (C=O and −C=N), 1578 (C=C), 3060 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.51 (s, 3H, CH_3), 7.09–7.90 (m, 15H, Ar–H + −CH=CH−). ESI-MS (*m*/*z*): 417.3 (M^+); Anal. Calcd (%) for $C_{25}H_{18}F_2N_2O_2$: C, 72.11; H, 4.36; N, 6.73. Found: C, 71.91; H, 4.10; N, 6.47.

*6.1.3. General procedure for synthesis of 5-(4-fluorophenyl)-3′-methyl-5′-substituted aryloxy-1′,2-diphenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazoles (**7a**–**l**)*

Pyrazole chalcones **5a**–**d** (1.0 mmol) and substituted phenyl hydrazine hydrochlorides **6a**–**c** (1.0 mmol) were thoroughly mixed in ethanol (5 mL) with catalytic amount of glacial acetic acid (2–3 drops) in a 50 mL round bottom flask. The reaction mixture was subjected to microwave irradiation at 350 W (50% of output power) for 8–10 min. After the completion of the reaction as monitored by TLC (ethyl acetate:hexane: 1:1), the reaction mixture was cooled to room temperature. The solid separated was filtered, washed with cold ethanol (10 mL), dried and recrystallized from ethanol, affording compounds **7a**–**l**.

*6.1.3.1. 2,5-Bis(4-fluorophenyl)-3′-methyl-1′-phenyl-5′-(3-(trifluoromethyl)phenoxy)-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7a**).* Yield 82%; m.p. 146–148 °C; IR (KBr, ν_{max}, cm^{-1}): 1368 (−CH_3 rocking.), 1260 (C–O–C ether str.), 1608 (−C=N), 3059 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.63–6.84 (17H, m, ArH), 5.34 (1H, dd, *J* = 6.4, 12.8 Hz, C_5–H pyrazoline), 3.71 (1H, dd, *J* = 12.8, 17.6 Hz, C_4–H pyrazoline), 3.27 (1H, dd, *J* = 6.4, 17.6 Hz, C_4–H pyrazoline), 2.27 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.7 (−CH_3 of pyrazole), δ, 54.4 (−CH_2 of pyrazoline), δ, 109.0, 112.3, 114.3, 114.4, 115.6, 115.8, 115.9, 116.0, 119.3, 120.4, 122.0, 127.5, 127.9, 128.0, 129.1, 129.7, 137.5, 137.7, 141.1, 144.6, 147.0, 147.3, 156.5, 164.3 (24 signals, aromatic carbons, pyrazole carbons, C_3, C_4 of pyrazoline and −CF_3 of aryloxy ring of pyrazole); ESI-MS (*m*/*z*): 575.5 (M^+); Anal. Calcd (%) for $C_{32}H_{23}F_5N_4O$: C, 66.90; H, 4.03; N, 9.75. Found: C, 66.69; H, 3.80; N, 9.50.

*6.1.3.2. 2-(4-Bromophenyl)-5′-(3-fluorophenoxy)-5-(4-fluorophenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7b**).* Yield 79%; m.p. 176–178 °C; IR (KBr, ν_{max}, cm^{-1}): 1366 (−CH_3 rocking.), 1259 (C–O–C ether str.), 1618 (−C=N), 3061 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.68–6.56 (17H, m, ArH), 5.35 (1H, dd, *J* = 5.6, 12.4 Hz, C_5–H pyrazoline), 3.72 (1H, dd, *J* = 12.8, 17.6 Hz, C_4–H pyrazoline), 3.32 (1H, dd C_4–H pyrazoline merged with peak of H_2O), 2.19 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.5 (−CH_3 of pyrazole), δ, 53.4 (−CH_2 of pyrazoline), δ, 108.3, 110.3, 115.2, 116.4, 117.3, 122.2, 124.8, 125.4, 127.3, 128.3, 128.7, 129.7, 132.0, 137.5, 143.5, 143.6, 144.8, 147.0, 147.6, 150.1, 152.4, 161.2, 164.8 (23 signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (*m*/*z*): 585.4 (M^+); 587.4 (M+2); Anal. Calcd (%) for $C_{31}H_{23}BrF_2N_4O$: C, 63.60; H, 3.96; N, 9.57. Found: C, 63.36; H, 3.73; N, 9.31.

*6.1.3.3. 5′-(3-Fluorophenoxy)-5-(4-fluorophenyl)-2-(4-methoxyphenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7c**).* Yield 88%; m.p. 166–168 °C; IR (KBr, ν_{max}, cm^{-1}): 1367 (−CH_3 rocking.), 1253 (C–O–C ether str.), 1616 (−C=N), 3060 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.66–6.61 (17H, m, ArH), 5.22 (1H, dd, *J* = 7.2, 12.8 Hz, C_5–H pyrazoline), 3.64 (4H, s, C_4–H pyrazoline merged with −OCH_3), 3.27 (1H, dd, *J* = 7.2, 17.6 Hz, C_4–H pyrazoline), 2.18 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.9 (−CH_3 of pyrazole), δ, 54.9 (−CH_2 of pyrazoline), δ, 55.6 (−OCH_3), δ, 109.1, 110.7, 110.9, 111.5, 114.7, 115.0, 115.8, 116.0, 122.0, 127.4, 127.8, 129.8, 131.6, 137.9, 138.8, 144.9, 146.1, 147.2, 153.4, 157.6, 164.3 (23 signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (*m*/*z*): 537.5 (M^+); Anal. Calcd (%) for $C_{32}H_{26}F_2N_4O_2$: C, 71.63; H, 4.88; N, 10.44. Found: C, 71.36; H, 4.66; N, 10.21.

*6.1.3.4. 5′-(3-fluorophenoxy)-2,5-bis(4-fluorophenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7d**).* Yield 80%; m.p. 144–146 °C; IR (KBr, ν_{max}, cm^{-1}): 1361 (−CH_3 rocking.), 1256 (C–O–C ether str.), 1616 (−C=N), 3062 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.68–6.57 (17H, m, ArH), 5.30 (1H, dd, *J* = 6.4, 12.4 Hz, C_5–H pyrazoline), 3.69 (1H, dd, *J* = 12.8, 17.6 Hz, C_4–H pyrazoline), 3.28 (1H, dd, C_4–H pyrazoline merged with peak of H_2O), 2.20 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.5 (−CH_3 of pyrazole), δ, 54.7 (−CH_2 of pyrazoline), δ, 109.3, 112.8, 114.4, 114.6, 115.2, 115.4, 115.6, 116.0, 119.4, 120.5, 122.0, 127.7, 127.9, 128.3, 129.4, 129.9, 137.6, 137.8, 141.4, 144.8, 147.5, 147.8, 156.7, 164.6 (23 signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (*m*/*z*): 525.4 (M^+); Anal. Calcd (%) for $C_{31}H_{23}F_3N_4O$: C, 70.98; H, 4.42; N, 10.68. Found: C, 70.73; H, 4.20; N, 10.41.

*6.1.3.5. 2-(4-Bromophenyl)-5-(4-fluorophenyl)-3′-methyl-1′-phenyl-5′-(3-(trifluoromethyl) phenoxy)-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7e**).* Yield 81%; m.p. 138–140 °C; IR (KBr, ν_{max}, cm^{-1}): 1361 (−CH_3 rocking.), 1259 (C–O–C ether str.), 1620 (−C=N), 3059 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.63–6.80 (17H, m, ArH), 5.39 (1H, dd, *J* = 6.0, 12.8 Hz C_5–H pyrazoline), 3.74 (1H, dd, *J* = 12.4, 17.6 Hz, C_4–H pyrazoline) 3.27 (1H, dd, C_4–H pyrazoline merged with peak of H_2O), 2.27 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 14.0 (−CH_3 of pyrazole), δ, 55.1 (−CH_2 of pyrazoline), δ, 108.9, 111.9, 114.1, 114.2, 115.1, 115.3, 115.8, 116.8, 119.5, 120.6, 122.2, 127.6, 127.9, 128.7, 129.4, 129.9, 136.9, 137.2, 141.0, 144.3, 146.8, 147.3, 156.7, 164.7 (24 signals, aromatic carbons, pyrazole carbons, C_3, C_4 of pyrazoline and −CF_3 of aryloxy ring of pyrazole); ESI-MS (*m*/*z*): 635.5 (M^+); 637.4 (M+2); Anal. Calcd (%) for $C_{32}H_{23}BrF_4N_4O$: C, 60.48; H, 3.65; N, 8.82. Found: C, 60.25; H, 3.44; N, 8.57.

*6.1.3.6. 5-(4-Fluorophenyl)-2-(4-methoxyphenyl)-3′-methyl-1′-phenyl-5′-(3-(trifluoromethyl) phenoxy)-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7f**).* Yield 85%; m.p. 134–136 °C; IR (KBr, ν_{max}, cm^{-1}): 1363 (−CH_3 rocking.), 1259 (C–O–C ether str.), 1598 (−C═N), 3062 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.61–6.75 (17H, m, ArH), 5.27 (1H, dd, J = 7.2, 12.8 Hz, C_5–H pyrazoline), 3.66 (4H, s, C_4–H pyrazoline merged with −OCH_3), 3.24 (1H, dd, J = 6.8, 17.2 Hz, C_4–H pyrazoline), 2.24 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.9 (−CH_3 of pyrazole), δ, 54.0 (−CH_2 of pyrazoline), δ, 55.5 (−OCH_3), δ, 109.8, 112.8, 114.1, 114.9, 115.2, 115.6, 115.9, 116.0, 119.7, 120.4, 122.5, 127.8, 127.9, 128.5, 129.5, 129.9, 137.3, 137.9, 141.7, 144.5, 147.6, 147.9, 156.8, 164.4 (24 signals, aromatic carbons, pyrazole carbons, C_3, C_4 of pyrazoline and −CF_3 of aryloxy ring of pyrazole); ESI-MS (m/z): 587.4 (M^+); Anal. Calcd (%) for $C_{33}H_{26}F_4N_4O_2$: C, 67.57; H, 4.47; N, 9.55. Found: C, 67.34; H, 4.24; N, 9.27.

*6.1.3.7. 5′-(4-Fluorophenoxy)-2,5-bis(4-fluorophenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7g**).* Yield 83%; m.p. 148–150 °C; IR (KBr, ν_{max}, cm^{-1}): 1357 (−CH_3 rocking.), 1254 (C–O–C ether str.), 1610 (−C═N), 3059 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.82–6.84 (17H, m, ArH), 5.34 (1H, dd, J = 6.0, 12.4 Hz, C_5–H pyrazoline), 3.72 (1H, dd, J = 12.4, 17.2 Hz, C_4–H pyrazoline), 3.27 (1H, dd, J = 6.0, 17.2 Hz, C_4–H pyrazoline), 2.27 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.5 (−CH_3 of pyrazole), δ, 54.9 (−CH_2 of pyrazoline), δ, 109.7, 113.7, 114.4, 115.3, 116.9, 117.2, 122.2, 126.3, 127.8, 129.2, 136.9, 138.9, 145.4, 146.2, 147.5, 151.9, 152.3, 157.4, 159.9, 162.3, 164.8, (21signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (m/z): 525.4 (M^+); Anal. Calcd (%) for $C_{31}H_{23}F_3N_4O$: C, 70.98; H, 4.42; N, 10.68. Found: C, 70.73; H, 4.21; N, 10.43.

*6.1.3.8. 2-(4-Bromophenyl)-5′-(4-fluorophenoxy)-5-(4-fluorophenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7h**).* Yield 79%; m.p. 179–181 °C; IR (KBr, ν_{max}, cm^{-1}): 1361 (−CH_3 rocking.), 1254 (C–O–C ether str.), 1615 (−C═N), 3051 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.68–6.79 (17H, m, ArH), 5.30 (1H, dd, J = 6.0, 12.4 Hz, C_5–H pyrazoline), 3.69 (1H, dd, J = 12.8, 17.6 Hz, C_4–H pyrazoline), 3.27 (1H, dd, J = 6.0, 17.6 Hz, C_4–H pyrazoline), 2.16 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.8 (−CH_3 of pyrazole), δ, 53.8 (−CH_2 of pyrazoline), δ, 108.3, 110.2, 115.9, 116.5, 117.1, 121.9, 127.4, 128.4, 128.9, 129.7, 131.9, 137.8, 143.4, 145.5, 147.1, 147.8, 152.8, 157.1, 159.5, 161.3, 164.0 (21 signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (m/z): 585.4 (M^+); 587.4 (M+2); Anal. Calcd (%) for $C_{31}H_{23}BrF_2N_4O$: C, 63.60; H, 3.96; N, 9.57. Found: C, 63.33; H, 3.72; N, 9.35.

*6.1.3.9. 5′-(4-Fluorophenoxy)-5-(4-fluorophenyl)-2-(4-methoxyphenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7i**).* Yield 84%; m.p. 158–160 °C; IR (KBr, ν_{max}, cm^{-1}): 1361 (−CH_3 rocking.), 1258 (C–O–C ether str.), 1619 (−C═N), 3053 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.67–6.78 (17H, m, ArH), 5.17 (1H, dd, J = 7.2, 12.4 Hz, C_5–H pyrazoline), 3.63 (4H, s, C_4–H pyrazoline merged with −OCH_3), 3.22 (1H, dd, J = 7.2, 17.6 Hz, C_4–H pyrazoline), 2.15 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.9 (−CH_3 of pyrazole), δ, 55.0 (−CH_2 of pyrazoline), δ, 55.6 (−OCH_3), δ, 108.7, 114.7, 115.4, 116.3, 116.9, 117.2, 121, 127.3, 127.8, 129.5, 137.9, 138.9, 145.6, 146.1, 147.1, 152.9, 153.3, 157.2, 159.5, 161.3, 163.8, (21signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (m/z): 537.5 (M^+); Anal. Calcd (%) for $C_{32}H_{26}F_2N_4O_2$: C, 71.63; H, 4.88; N, 10.44. Found: C, 71.37; H, 4.60; N, 10.17.

*6.1.3.10. 5′-(2-Fluorophenoxy)-2,5-bis(4-fluorophenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7j**).* Yield 85%; m.p. 162–164 °C; IR (KBr, ν_{max}, cm^{-1}): 1361 (−CH_3 rocking.), 1260 (C–O–C ether str.), 1622 (−C═N), 3052 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.66–6.84 (17H, m, ArH), 5.32 (1H, dd, J = 6.4, 12.8 Hz, C_5–H pyrazoline), 3.71 (1H, dd, J = 12.4, 17.2 Hz, C_4–H pyrazoline), 3.27 (1H, dd, J = 6.4, 17.6 Hz, C_4–H pyrazoline), 2.27 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 14.0 (−CH_3 of pyrazole), δ, 54.1 (−CH_2 of pyrazoline), δ, 109.5, 109.9, 115.7, 116.5, 117.9, 123.5, 124.9, 125.8, 127.7, 128.5, 128.9, 129.0, 132.9, 137.7, 143.2, 143.9, 144.8, 147.9, 148.9, 150.3, 152.6, 162.6, 164.7 (23 signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (m/z): 525.4 (M^+); Anal. Calcd (%) for $C_{31}H_{23}F_3N_4O$: C, 70.98; H, 4.42; N, 10.68. Found: C, 70.73; H, 4.19; N, 10.45.

*6.1.3.11. 2-(4-Bromophenyl)-5′-(2-fluorophenoxy)-5-(4-fluorophenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7k**).* Yield 77%; m.p. 165–167 °C; IR (KBr, ν_{max}, cm^{-1}): 1357 (−CH_3 rocking.), 1258 (C–O–C ether str.), 1623 (−C═N), 3054 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.67–6.62 (17H, m, ArH), 5.32 (1H, dd, J = 6.4, 12.8 Hz, C_5–H pyrazoline), 3.70 (1H, dd, J = 12.8, 17.6 Hz, C_4–H pyrazoline) 3.27 (1H, dd, C_4–H pyrazoline merged with peak of H_2O), 2.18 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 13.7 (−CH_3 of pyrazole), δ, 53.7 (−CH_2 of pyrazoline), δ, 108.5, 110.3, 115.0, 116.0, 117.4, 122.0, 124.8, 125.3, 127.6, 128.1, 128.9, 129.7, 131.9, 137.7, 143.4, 143.9, 144.7, 147.0, 147.9, 150.0, 152.5, 161.6, 164.9 (23 signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (m/z): 585.4 (M^+); 587.4 (M+2); Anal. Calcd (%) for $C_{31}H_{23}BrF_2N_4O$: C, 63.60; H, 3.96; N, 9.57. Found: C, 63.32; H, 3.73; N, 9.36.

*6.1.3.12. 5′-(2-Fluorophenoxy)-5-(4-fluorophenyl)-2-(4-methoxyphenyl)-3′-methyl-1′-phenyl-3,4-dihydro-1′H,2H-3,4′-bipyrazole (**7l**).* Yield 87%; m.p. 141–143 °C; IR (KBr, ν_{max}, cm^{-1}): 1359 (−CH_3 rocking.), 1259 (C–O–C ether str.), 1626 (−C═N), 3055 (−CH aromatic); ^{1}H NMR (400 MHz, DMSO-d_6): 7.66–6.68 (17H, m, ArH), 5.18 (1H, dd, J = 7.2, 12.4 Hz C_5–H pyrazoline), 3.67 (4H, s, C_4–H pyrazoline merged with −OCH_3), 3.62 (1H, dd, J = 12.8, 17.6 Hz, C_4–H pyrazoline), 3.23 (1H, dd, J = 7.2, 17.6 Hz C_4–H pyrazoline), 2.17 (3H, s, CH_3); ^{13}C NMR: (400 MHz, DMSO-d_6): δ, 14 (−CH_3 of pyrazole), δ, 55 (−CH_2 of pyrazoline), δ, 55.6 (−OCH_3), δ, 108.8, 114.7, 115.1, 115.8, 116.0, 116.8, 117.3, 117.5, 122, 124.9, 125, 125.4, 127.5, 127.8, 127.9, 129.7, 137.8, 138.9, 144.9, 146.2, 147.2, 153.4, 164.7 (23 signals, aromatic carbons, pyrazole carbons, C_3 and C_4 of pyrazoline); ESI-MS (m/z): 537.5 (M^+); Anal. Calcd (%) for $C_{32}H_{26}F_2N_4O_2$: C, 71.63; H, 4.88; N, 10.44. Found: C, 71.38; H, 4.65; N, 10.19.

7. Biological evaluation

7.1. In vitro antimicrobial assay

The antimicrobial activity of pyrazolylpyrazolines derivatives **7a**–**l** was carried out by broth micro dilution method. DMSO was used as the diluent to get the desired concentration of compounds to test upon standard bacterial stains. 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 stain was adjusted to 10^8 CFU mL^{-1} by comparing the turbidity. Serial dilutions were prepared in primary and secondary screening. Each synthesized compound 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 μL suspensions were further inoculated on appropriate media and growth was noted after 24 and 48 h. The control tube containing no antibiotic was instantaneously subculture (before inoculation) by evenly spreading a loopful over an area of plate of medium suitable for the growth of the test organism. The tubes were then put overnight for incubation at 37 °C. The highest dilution preventing appearance of turbidity after spot subculture was considered as minimal inhibitory concentration (MIC, μg/mL). All the tubes showing no visible growth (same as the 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, chloramphenicol and ciprofloxacin were used as the standard antibacterial drugs. Nystatin and Griseofulvin were used as the standard antifungal drugs. The results are summarized in Table 1.

7.2. In vitro antituberculosis assay

All pyrazolylpyrazolines derivatives **7a–l** were screened for their antitubercular activity against *M. tuberculosis* H37Rv performed by Lowensteine–Jensen method with minor modification where 250 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 grown on Lowensteine–Jensen medium was harvested in 0.85% saline in bijou bottle. The stock solutions of title compounds (250 μg/mL) were prepared in DMSO. These tubes were then incubated at 37 °C for 24 h followed by streaking of *M. tuberculosis* H37Rv (5×10^4 bacilli per tube). The growth of bacilli was observed after 2 weeks, 3 weeks and finally after 4 weeks 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 stain *M. tuberculosis* H37Rv was also tested with the known drugs isoniazid and rifampicin for comparison. The results are summarized in Table 2.

7.3. In vitro antimalarial assay

In vitro antimalarial activity of the pyrazolylpyrazolines derivatives **7a–l** was screened against *P. falciparum* stain. *P. falciparum* stain was acquired from Shree R. B Shah Mahavir Super-speciality hospital, Surat, Gujarat, India and was used in *in vitro* tests. *P. falciparum* stain was cultivated by a modified method described by Trager and Jensen [41]. 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 [42]. For experimental purpose, the cultures were synchronized with 5% D-sorbitol when the parasites were in the ring stage [43]. The parasite suspension, consisting of predominately the ring stage, 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 parasitic growth for 48 h at 37 °C. A positive control with reference to antimalarial drugs in standard concentrations was used for 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 method using Microcal Origin software. The standard drugs chloroquine and quinine were used as the reference antimalarial agents, blood smears were read blind and each duplicate experiment was repeated thrice. The results are summarized in Table 3.

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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.2014.07.008.

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