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SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL EVALUATION OF CERTAIN PYRAZOLINES

FARITHA A[1], NASSER AJA[2], AHAMED AP[3], THAJUDDIN N[3]

1. P.G. and Research Dept. of Chemistry, Periyar E.V.R. College, Tiruchirappalli-620023, Tamil Nadu, India
2. P.G. and Research Dept. of Chemistry, Jamal Mohamed College, Tiruchirappalli-620020, Tamil Nadu, India.
3. Department of Microbiology, Bharathidasan University, Tiruchirappalli-620024, Tamil Nadu, India.

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Abstract: Some 5-(1H-indol-3-yl)-3-phenyl-4,5-dihydropyrazole-1-carbothioamide derivatives were synthesized by reacting (E)-3-(1H-indol-3-yl)-1-substituted phenylprop-2-en-1-one with thiosemicarbazide and sodium hydroxide in ethanol. The structural elucidations of the compounds were performed by IR, ^{1}H NMR, ^{13}C NMR and Mass spectral data and elemental analyses. These compounds are screened for their in-vitro antibacterial and antifungal potentials against various strains of microbial organisms. Among the six compounds, the compound 4d showed good antibacterial and antifungal activities against all the organisms. Minimum inhibitory concentration (MIC) has been determined for the compound 4d.

Keywords: Pyrazoline, Chalcones, Thiocarbamoyl pyrazoline, Anti bacterial activity, Anti fungal activity

Corresponding Author: MS. A. FARITHA

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INTRODUCTION

Bacterial infections can be caused by a wide range of bacteria, resulting in mild to life-threatening illnesses (such as bacterial meningitis) that require immediate intervention. In the United States, bacterial infections are a leading cause of death in children and the elderly. Hospitalized patients and those with chronic diseases are at especially high risk of bacterial infection. Common bacterial infections include pneumonia, ear infections, diarrohea, urinary tract infections, and skin disorders. The fight against bacterial infection represents one of the high points of modern medicine. The development of antibiotics in the 1940s offered physicians a powerful tool against bacterial infections that has saved the lives of millions of people. However, because of the widespread, natural resistance of the bacteria to different classes of antibiotics or acquiring resistance from other bacteria through exchange of resistant genes, indiscriminate, inappropriate and prolonged use of antibiotics, strains of bacteria have begun to emerge that are antibiotic-resistant[1]. These new stronger bacteria pose a significant threat to general health and welfare and a challenge to researchers. Women who take systemic antibiotics for treating bacterial infections are more likely to experience opportunistic yeast infections.

The incidence of fungal infections has increased at an alarming rate in the past two decades. Most of this increase is due to opportunistic fungal infections related to the growing population of people with weakened immune systems due to HIV, cancer, and other diseases; and to modern medical practices such as the use of intensive chemotherapy and drugs that suppress the immune system. However, fungal infections like vaginal yeast infections and athlete's foot are common in healthy people, too. Within the limited data available, an increased incidence of invasive candidiasis, aspergillosis, and zygomycosis are reported. The emergence of fungal rhinosinusitis, penicilliosis marneffei and zygomycosis due to Apophysomyces elegans is unique in the Indian scenario[2].

Also the antimicrobial resistance (AMR), which is the resistance of a microorganism to an antimicrobial drug, threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi[3]. It is present in all parts of the world. In 2012, there were about 450 000 new cases of multidrug-resistant tuberculosis (MDR-TB). Extensively drug-resistant tuberculosis (XDR-TB) has been identified in 92 countries. MDR-TB requires treatment courses that are much longer and less effective than those for non-resistant TB. A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant Gram-negative bacteria. Treatment failures due to resistance to treatments of last resort for gonorrhoea (third-generation cephalosporins) have now been reported from 10 countries. Gonorrhoea may soon become untreatable as no vaccines or new drugs are in development.

Therefore, it is an increasingly serious threat to global public health that requires action across all government sectors and society.

Progress in the development of new antimicrobial agents with new structure along with mode of action remains the main purpose of scientists for the solution of growing microbial resistance gained by micro-organisms to classical antimicrobial agents[4]. A wide variety of hetero cyclic compounds [5,6] have been explored for developing pharmaceutically important molecules like pyrazolines. Literature survey revealed that pyrazolines have gained great interest due to their broad spectrum of biological activities such as antibacterial[7], antifungal[8], antioxidant[9] , anti-inflammatory[9], cytotoxic[10], respiratory[11], cardiovascular[12] properties and etc., Also a wide spectrum of pharmacological activities are associated with pyrazole derivatives when present along with indole nucleus[13]. The present report describes the synthesis of a series of 5-(1H-indol-3-yl)-3-substituted phenyl-4, 5-dihydropyrazole-1-carbothioamide (4a-4f) by the reaction of substituted (E)-3-(1H-indol-3-yl)-1-phenylprop-2-en-1-one (3a - 3f) and thiosemicarbazide in the presence of a base. The structures of the various synthesized compounds are assigned on the basis of elemental analyses, IR, ^{1}H NMR, ^{13}C NMR and mass spectral data[14]. Antimicrobial evaluation is carried out for the synthesized pyrazolines.

MATERIALS AND METHODS

All chemicals and reagents used in the present study were purchased from Alfa Aesar and MERCK. Melting points were determined on a capillary melting point apparatus and are uncorrected. The NMR spectra were recorded on BRUKER AVANCE III 500 MHz multi nuclei solution NMR spectrometer and Bruker WM 400 MHz spectrometer with TMS as internal standard. Infrared spectra were recorded in KBr on a Schimatzu 8201 pc (4000-400cm^{-1}). The mass spectra (EI) were recorded on a JEOL GCMATE II GC-MS Spectrometer operating at 70 eV. Elemental analyses were performed for C, H, N and S and were found to be within ± 0.5% of the theoretical values. TLC was used to monitor the reaction and to check the purity.

General procedure for the synthesis of derivatives of *(E)-3-(1H-indol-3-yl)-1-phenylprop-2-en-1-one (*3a – 3f):

A mixture of indole-3-carboxaldehyde **1** (0.01 mol) and various substituted acetophenones(1a-1f) (0.01mol) was refluxed in the presence of methanolic NaOH for 6 to 25 hrs. The reaction mixture was poured into crushed ice and neutralized with dil. HCl. The solid obtained was filtered and recrystallised from ethanol to obtain pure chalcones. The purity of the product was checked on TLC by using the mixture of toluene and ethyl acetate as mobile phase (Scheme-1). The IR and ^{1}H NMR spectral data of these compounds are given below.

(E)-1-(4-chlorophenyl)-3-(1H-indol-3-yl)prop-2-en-1-one (3a)

Yellow crystalline solid. M.Pt.: 184± 2°C. IR(KBr, cm^{-1}): 3217(NH), 2924(Ar-C-H), 1631(C=O), 1581 (-CH=CH), 738; ^{1}H NMR (DMSO-d6, δ (ppm)): 7.24-8.10(m,9H,Ar), 7.52(d,1H, J=15Hz, Hα), 8.03(d,1H,J=15Hz, Hβ), 11.77(s,1H,NH)

(E)-3-(1H-indol-3-yl)-1-(4-methoxyphenyl)prop-2-en-1-one (3b)

Orange crystalline solid. M.Pt.: 169 ± 2°C. IR (KBr, cm^{-1}): 3190(NH), 2924(Ar-C-H), 1633 (C=O), 1585 (-CH=CH), 1173; ^{1}H NMR (DMSO-d6, δ (ppm)): 7.08-8.14(m,9H,Ar), 7.66(d,1H,J=15.6Hz, Hα), 8.03(d,1H,J=15.6Hz, Hβ), 11.86(s,1H,NH), 3.86 (s,3H,OCH_3).

(E)-3-(1H-indol-3-yl)-1-(4-nitrophenyl)prop-2-en-1-one (3c)

Red crystalline solid. M.Pt.: 210±2°C. IR (KBr, cm^{-1}): 3437(NH), 2924(Ar-C-H), 1653(C=O), 1560 (-CH=CH), 1340; ^{1}H NMR (DMSO-d6, δ (ppm)): 7.22-8.37(m,9H,Ar), 7.51(d,1H,J=15Hz, Hα), 8.14(d,1H,J=15Hz, Hβ), 12.06(s,1H,NH)

(E)-1-(4-hydroxyphenyl)-3-(1H-indol-3-yl)prop-2-en-1-one (3d)

Orange crystalline solid. M.Pt.: 147 ± 2°C. IR (KBr, cm^{-1}): 3430(OH), 3288(NH), 2927(Ar-C-H), 1639 (C=O), 1556 (-CH=CH); ^{1}H NMR (DMSO-d6, δ (ppm)): 6.89-8.28 (m,9H,Ar), 7.64(d,1H,J=15Hz, Hα), 7.99(d,1H,J=15Hz, Hβ),11.83(s,1H,NH), 3.39(s,1H,OH).

(E)- 3-(1H-indol-3-yl)-1-(4-methylphenyl)prop-2-en-1-one (3e)

Yellow crystalline solid. M.Pt.: 174±2°C. IR (KBr, cm^{-1}): 3174(NH), 2924(Ar-C-H), 1600 (C=O), 1514(-CH=CH); 1H NMR (DMSO-d6, δ (ppm)): 7.22-8.10(m,9H,Ar), 7.64(d,1H,J=15.5Hz, Hα), 8.05(d,1H,J=15.5Hz, Hβ), 11.87(s,1H,NH), 2.40(s,3H, CH_3)

(E)-1-(2-chlorophenyl)-3-(1H-indol-3-yl)prop-2-en-1-one (3f)

Yellow crystalline solid. M.Pt.: 178± 2°C. IR(KBr, cm^{-1}): 3217(NH), 2924(Ar-C-H), 1633(C=O), 1581 (-CH=CH), 738; ^{1}H NMR (DMSO-d6, δ (ppm)): 7.22-8.13(m,9H,Ar), 7.54(d,1H, J=15Hz, Hα), 8.06(d,1H,J=15Hz, Hβ), 11.85(s,1H,NH)

General procedure for the synthesis of derivatives of 5-(1H-indol-3-yl)-3-phenyl-4, 5-dihydropyrazole-1-carbothioamide (4a-4f)

A mixture of appropriate indolyl chalcone (0.01mol) (3**a-3f**), thiosemicarbazide (0.015mol) and NaOH (0.02mol) in dry ethanol was refluxed for 16 hrs. The progress of the reaction was monitored by TLC. The excess of solvent was removed under reduced pressure and the reaction mixture was poured into ice cold water. The product obtained was filtered, washed with water and recrystallised with ethanol.

In-vitro Antibacterial Assay

The synthesized compounds were screened for antibacterial activity against the Gram-negative bacteria *Klebsiella pneumoniae*, *Psuedomonas aeruginosa, Salmonella typhi & Escherichia coli*. All the microorganisms were maintained at 4˚C on nutrient agar slants.

Media Preparation and Antibacterial Activity:

The antimicrobial assay was performed by agar well diffusion method[15] for synthesized compounds. The test microorganisms were seeded into Mueller Hinton agar by spread plate 10µl (10^6). For agar well diffusion method, the well (0.7 cm) was loaded with 50µl of the test compound on the seeded agar plate. The plates were incubated overnight at 37˚C. Microbial growth was determined by measuring the diameter of zone of inhibition. The result was obtained by measuring the zone diameter (Table 4).

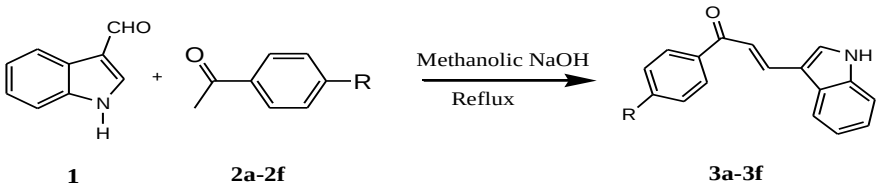
Minimum Inhibitory Concentration (MIC) of Compound-4d:

The minimum inhibitory concentration (MIC) was performed by the serial dilution technique using 96-well microplates[16]. The 12 wells of each row were filled with 0.5 ml sterilized Mueller Hinton broth. Sequentially, wells 3–12 received an additional 100µl of a mixture of culture medium and Compound-4d. The active compound-4d dissolved in Dimethyl Sulfoxide (DMSO) against *K. pneumoniae, P. aeruginosa,* S. *typhi & E. coli* serially diluted to create a concentration sequence from 50 to 1000µg/ml. Well 1 served as growth control and well 2 as solvent control. The deep-wells were incubated for 24h at 37°C. The resulting turbidity was observed, and after 24h MIC was determined to be where growth was no longer visible by assessment of turbidity by optical density readings at 600nm with a micro plate reader (Table. 5, Fig-1).

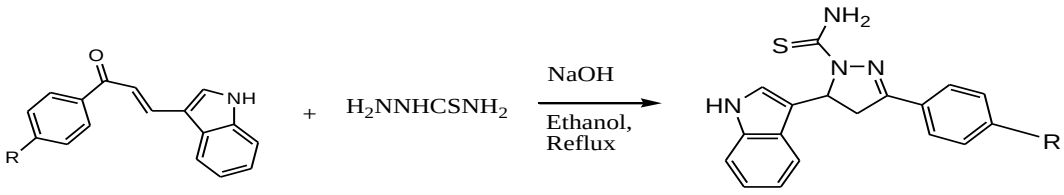
In-vitro Antifungal screening

The synthesized compounds were evaluated for their in-vitro antifungal activity against *Candida albicans* using the same cup plate method with PDA medium. The PDA medium were purchased form high media Laboratories Ltd., Mumbai, India. Preparation of nutrient broth, subculture, base layer medium and PDA medium was done as per the standard procedure. Each test compound (5mg) was dissolved in 5 mL of dimethyl sulfoxide (1000µg/ml). Zone of inhibition produced by each compound was measured in mm and results are presented in Table-4.

Scheme-1: Synthesis of pyrazolines



1 **2a-2f** **3a-3f**



4a-4f

	a	b	c	d	e	f
R =	Cl	OCH_3	NO_2	OH	CH_3	o-Cl

Results

Table-1 Physico-chemical characteristics of the synthesized compounds

Comp.	R	M.P. °C ±2	M.W.	Yield	M.F.	Elemental Calculated(found) %	Analysis
4a	Cl	246	355	65	$C_{18}N_4H_{15}SCl$	C60.85(60.78);H4.22(4.18); N15.77(15.35)	
4b	OCH_3	236	350	60	$C_{19}N_4H_{18}OS$	C65.14(65.05);H5.14(5.09); N16.00(15.85)	
4c	NO_2	225	365	45	$C_{18}N_5H_{15}SO_2$	C59.18(59.23);H4.11(4.05); N19.18(19.15)	
4d	OH	180	336	52	$C_{18}N_4H_{16}OS$	C64.29(64.25);H4.76(4.70); N16.67(16.62)	
4e	CH_3	220	334	58	$C_{19}N_4H_{18}S$	C68.26 (68.12);H5.39 (5.18); N16.77(16.43)	
4f	o-Cl	245	335	60	$C_{18}N_4H_{15}SCl$	C60.85(60.78);H4.22(4.14); N15.77(15.62)	

Spectral characteristics of compounds 4a-4f

Table-2: IR and Mass spectral data of compounds 4a-4f

Comp.	IR data, υ cm^{-1}							Mass m/z(M^+)
	Ar-C-H	C=S	C=N	C-N	N-N	NH	NH_2	
4a	2920	1348	1570	1085	1475	3360	3485	353.72
4b	2924	1346	1562	1080	1479	3365	3485	349.82
4c	2924	1363	1597	1072	1462	3362	3483	364.64
4d	2924	1348	1600	1109	1487	3422 (broad)		335.83
4e	2918	1346	1560	1080	1479	3365	3487	333.92
4f	2919	1346	1565	1082	1472	3360	3483	353.71

NMR data of compounds 4a-4e

Comp.	^{1}H NMR data δ (ppm)	^{13}C NMR data δ (ppm)
4a	3.27(dd,1H, H_A, J_{AB}=18.0Hz, J_{AX}=3.5Hz), 3.80 (dd,1H, H_B, J_{AB}=18.0Hz, J_{BX}=11.5Hz), 6.19 (dd,1H, H_X, J_{AX}=3.5Hz, J_{BX}=11.5Hz), 6.83-7.96 (m,9H,Ar-H),7.97 (d,2H,NH_2), 10.96(d,1H,NH)	176.12(C=S), 154.13(C=N), 136.5,135.0,130.1,128.8,128.7,124.3, 123.9,120.9,118.7,118.2,115.4, 111.8(Ar-C), 57.2(CH), 40.8(CH_2)
4b	3.25(dd,1H, H_A, J_{AB}=17.5Hz, J_{AX}=3.5Hz), 3.85 (dd,1H, H_B, J_{AB}=18.0Hz, J_{BX}=11.5Hz), 6.15 (dd,1H, H_X, J_{AX}=3.5Hz, J_{BX}=11.5Hz), 6.88-7.80 (m,9H,Ar-H),3.81(s,3H,OCH_3) 7.88 (d,2H,NH_2), 10.94(d,1H,NH)	175.57(C=S), 161.13(C=N),155.2,136.5,128.8,124.3, 123.9,123.5,120.9,118.6,118.3,115.5,114.2,111.7(Ar-C), 56.8(CH), 40.9(CH_2)
4c	3.36(dd,1H, H_A, J_{AB}=17.2Hz, J_{AX}=3.5Hz), 4.27 (dd,1H, H_B, J_{AB}=18.0Hz, J_{BX}=11.5Hz), 6.65 (dd,1H, H_X, J_{AX}=3.5Hz, J_{BX}=11.5Hz), 7.6-8.04 (m,9H,Ar-H),8.06 (d,2H,NH_2), 11.76(d,1H,NH)	186.10(C=S), 166.9(C=N), 153.2,137.4,136.3,135.6,131.7,131.5,130.5,129.9,128.6,126.1,125.2,122.4 (Ar-C),61.3(CH), 54.2(CH_2)
4d	3.37(dd,1H, H_A, J_{AB}=18.0Hz, J_{AX}=3.5Hz), 3.82 (dd,1H, H_B, J_{AB}=18.0Hz, J_{BX}=11.5Hz), 6.15 (dd,1H, H_X, J_{AX}=3.5Hz, J_{BX}=11.5Hz), 6.83-8.05 (m,9H,Ar-H), 8.07 (d,2H,NH_2), 10.03(s,1H,NH), 10.28(s,1H,OH)	187.03(C=S), 161.54(C=N),159.8,137.7,137.5,132.5, 130.6,129.9,125.2,123.9,122.6,121.0,120.3,118.6(Ar-C),57.2(CH), 40.9(CH_2)
4e	3.39(dd,1H, H_A, J_{AB}=16.0Hz, J_{AX}=3.8Hz), 3.76 (dd,1H, H_B, J_{AB}=16.0Hz, J_{BX}=11.2Hz), 6.22 (dd,1H, H_X, J_{AX}=3.8Hz, J_{BX}=11.2Hz),6.92-7.62 (m,9H,Ar-H),8.20 (d,2H,NH_2), 2.34(s,3H,CH_3)	176.32(C=S), 155.86(C=N), 140.9,137.1,129.8,128.8,127.5,124.8, 124.5,121.4,119.1,118.8,115.9,112.3, (Ar-C), 57.4(CH), 41.4(CH_2)

DISCUSSION:

Synthesis of a series of pyrazoline derivatives(4a-4f), their characterization and anti microbial evaluation are described in this study and their preparation is outlined in

Scheme-1. A series of chalcones 3a-3f were prepared by the base catalysed Claisen-Schmidt condensation of appropriate substituted acetophenone and indole aldehydes in good yield (70-75%). They were characterized by IR and ^{1}H NMR spectroscopic techniques. The cyclization of these chalcones with thiosemicarbazide in the presence of base led to the formation of pyrazoline derivatives (4a-4f).

IR spectral studies:

Assignment of selected characterized IR bands [17, 18] provides significant indication for the formation of chalcones (3a-3f) and cyclized pyrazoline analogues of thiosemicarbazide (4a-4f). The bands due to υ(c=o) and υ(c=c) stretch at 1600 - 1653cm^{-1} and 1514 - 1581cm^{-1} respectively favours the formation of indolyl chalcones (3a-3f). The absence of υ(c=o) and

υ(c=c) bands in the IR spectra of compounds 4a-4f shows the formation of pyrazoline derivatives. These compounds showed $\upsilon_{C=N}$ stretching at 1560-1600cm^{-1} and υ_{C-N} stretching vibration at 1072 - 1109 cm^{-1}. In addition, the absorbtion band at 1462 -1487cm^{-1} due to

υ_{N-N} stretching vibration and the intense bands in the region 1346 - 1363cm^{-1} due to the $\upsilon_{C=S}$ stretch of thiocarbamoyl group confirm the formation of pyrazoline derivatives.

NMR and Mass spectral studies:

Further evidence for the formation of compounds (3a-3f) and 4a-4f was obtained by NMR spectroscopy. Two doublets in the ^{1}H NMR spectra of chalcones 3a-3f in the region 7.54-7.66 and 8.03-8.14ppm due to –CO-CH= and =CH-Ar protons confirms the formations of chalcones. In the ^{1}H NMR spectra of pyrazolines, the three hydrogen atoms attached to the C-4 and C-5 carbon atoms of the pyrazoline ring gave an ABX spin system[19]. The CH_2 protons of the pyrazoline ring resonated as a pair of doublet of doublets around δ 3.25 to 3.39ppm (Ha) and δ 3.76 to 4.27ppm(Hb). The CH(Hx) protons appeared as a doublet of doublets around δ 6.15 to 6.65ppm due to vicinal coupling with the two magnetically non-equivalent protons of the C-4 carbon of pyrazoline ring. The J values were calculated for these signals and found to be around 18.0Hz and 3.5Hz for Ha and 18.0Hz and 11.5Hz for Hb. The 'dd' pattern of Hx proton (5-H) of pyrazoline ring showed J value around 11.5 and 3.5 Hz. The $-NH_2$ proton of these compounds showed a doublet at δ 7.9ppm and other aromatic protons were observed at the expected regions. The ^{13}C NMR spectra of all the compounds were recorded in DMSO and spectral signals are in good agreement with the given structure. The C-5 and C-4 carbons of pyrazolines 4a-4f resonated at δ 56.8 to 61.3ppm and 40.8 to 54.2ppm respectively. Thiocarbamoyl carbon (C=S) displayed a signal at 175.57 - 187.03ppm. The characteristics M^+

peaks were observed in the mass spectra of all the compounds which followed the similar fragmentation pattern.

Antibacterial activity

The results of antibacterial screening showed that compound-4d was active against all the four gram negative bacteria *E.Coli*, *K.pneumoniae*, *P.aeruginosa* and *S.typhi* (Table-4). The compound 4e was found to be active against K.pneumoniae and the compounds 4b and 4c were found to be moderately active against *E.Coli* and *P.aeruginosa* while 4a did not show any activity against all the four bacteria. The graph shows the MIC of the compound 4d (Fig1 Table-5) which is 150µg/ml. The antibacterial results of present study indicated that presence of electron releasing groups (-OH & $-CH_3$ in compounds 4d & 4e) on phenyl ring increased the antibacterial activity of the compounds. This is in accordance with the results obtained by Nandagokula et al[20].

Antifungal activity

The antifungal screening data of the compounds revealed a good response of compound 4d to the tested fungi, *Candida albicans*. None of the other compounds was found to be active against *C. albicans*.

Table 4 Antibacterial and Anti-candidal activity of synthesized compounds:

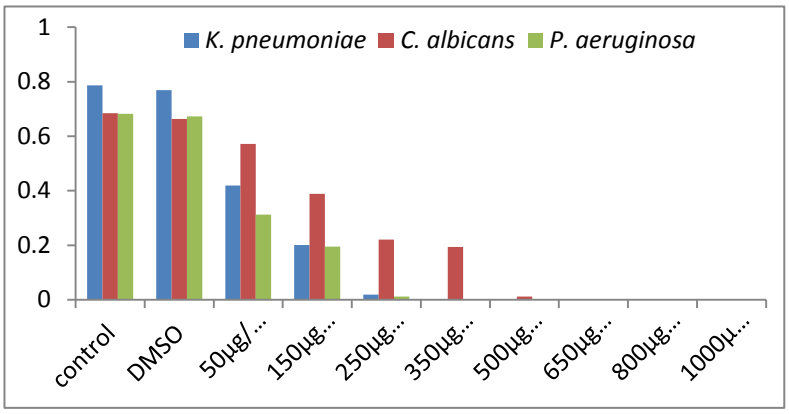
Clinical isolates	Zone of inhibition by series of compound in diameter					
	4a	4b	4c	4d	4e	4f
E. coli	-	8	9	17	-	-
S.typhi	-	-	-	16	10	-
K. pneumoniae	-	-	-	17	12	-
P. aeruginosa	-	10	8	14	-	-
C. albicans	-	-	-	19	-	-

(-) No zone of inhibition

Table 5 MIC value of compound-4d against clinical isolates

Clinical isolates	MIC value (4d)
Klebsiella pneumoniae	150 µg ml^{-1}
Pseudomonas aeruginosa	150 µg ml^{-1}
C. albicans	350 µg ml^{-1}

Fig. 1 MIC determination of compound 4d



CONCLUSION

The series of the compounds 4a-4f thiocarbamoyl pyrazoline were synthesized from indolyl chalcones. These compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR and mass spectral methods and the results support the structures of the compounds. All the synthesized compounds were screened for their antimicrobial activities. Compounds 4d exhibited highest degree of antibacterial and antifungal activity while compounds 4b, 4c, and 4e showed moderate activity. This study thus provides a lead for the syntheses of more pyrazole derivatives with specific substituent to increase the anti microbial activity.

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