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Research Article

Synthesis and Biological Evaluation of Pyrazolyl Isoxazolines as Antimicrobial Agents

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Abstract

Pyrazolyl isoxazolines were synthesized by 1,3-dipolar cycloaddition of pyrazolyl nitrile oxide with various activated alkene such as acrylonitrile, methyl acrylate and vinyl acetate in the presence of iodobenzene diacetate in methanol containing a catalytic amount of TFA at room temperature with an aim to explore their effect on *in vitro* growth of microorganism causing microbial infection. Eleven compounds were tested *in vitro* for their antibacterial activity against two Gram-positive bacteria namely, *Staphylococcus aureus*, *Bacillus subtilis* and two Gram-negative bacteria namely, *Escherichia coli* and *Pseudomonas aeruginosa*. All the synthesized compounds were also tested for their inhibitory action against two strains of fungus.
Keywords: Pyrazolyl aldoxime, Pyrazolyl isoxazoline, Iodobenzene diacetate, Antibacterial activity, Antifungal activity

1. Introduction

Among five-membered nitrogen heterocycles, isoxazolines have always been a hot topic for research in organic and pharmaceutical chemistry because of their broad spectrum bioactivities and synthetic applications. In recent years, a special attention has been given to isoxazolines owing to their promising biological activities such as antibacterial [1], antiplatelet [2], antiviral [3], anticonvulsant [4], immunostimulatory [5], antinociceptive [6], anti-diabetic [7], anti-inflammatory and analgesic [8]. A number of recently reported pharmaceutically active agents, such as glycoprotein IIb/IIIa receptor antagonists [9] and human leukocyte elastase inhibitors [10], contained isoxazoline ring, which played an important role in their biological activities. In particular, certain drugs contain a isoxazoline moiety are known to useful against cardiovascular diseases and as anti-coagulants [11]. Isoxazolines are versatile intermediates in the synthesis of various natural products [12-16].

The reductive cleavage of isoxazoline ring can lead to many synthetically important compounds, such as β-hydroxy ketones, α,β-unsaturated ketones or γ-amino alcohols. The general and most widely used method for the synthesis of isoxazolines is 1,3-dipolar cycloaddition reactions of activated alkenes with nitrile oxides, generated *in situ* from aldoximes [17-18]. Nitrile oxides are versatile intermediates in heterocyclic chemistry, taking part in variety of 1,3-dipolar cycloaddition reactions to give various five membered heterocycles. Aliphatic nitrile oxides are predominantly generated *in situ* from primary nitro compounds in a Mukaiyama reaction [19], while their aromatic counterparts are prepared by dehydrohalogenation of hydroximoyl chlorides [20-24].

The oxidation of aldoximes to nitrile oxides with hypervalent iodine reagents such as dichloroiodo benzene ($PhICl_2$) [25], hydroxy(tosyloxy)iodo benzene (HTIB) [26] and iodobenzene diacetate (IBD) [27-31] have been reported recently. Importance of pyrazole containing structures stems from their widespread occurrence in molecules that exhibit significant activity as cyclooxygenase-2 (COX-2) inhibitors [32], HIV-1 protease inhibitors [33] and ligands for estrogen receptors [34]. In addition, pyrazole nucleus has pronounced pharmacological applications as anti-anxiety [35], antipyretic, antinociceptive [36-37] and anti-inflammatory drugs [38-39].

These significant biological importance of isoxazoline and pyrazole derivatives, prompted us to undertake the synthesis of pyrazolyl isoxazolines derivatives containing a system which involves combination of these

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pharmacophores in one molecular framework to give the title structure for screening their antimicrobial activities. We herein report the screening results of the antibacterial and antifungal activities of pyrazolyl isoxazoline derivatives and their synthesis by 1,3-dipolar cycloaddition of pyrazolyl nitrile oxide with activated alkenes such as the acrylonitrile, methyl acrylate and vinyl acetate.

2. Experimental

2.1 General

Melting points were determined in open capillaries with electrical melting point apparatus and are uncorrected. The IR spectra were obtained with a Buck Scientific IR M-500 spectrophotometer. The ^{1}H NMR spectra were recorded on a Bruker (300 MHz) spectrometer using tetramethylsilane as an internal standard. All the reagents were purchased from commercial sources and used without further purification. Reaction progress was monitored by TLC using Rankem silica gel. Column chromatography was performed using Rankem silica gel (100-200) mesh size with freshly distilled solvents. The starting material formyl pyrazoles and pyrazolyl aldoximes were prepared by literature method [40].

2.2 General procedure for the synthesis of Pyrazolyl isoxazolines 4a-4k

2.2.1 Synthesis of Pyrazolyl aldoximes [40] 2a-2k

To a cold solution of dimethylformamide (10 ml) and phosphorus oxychloride (0.5 ml, 6 mmol), was added acetophenone phenylhydrazone (**1**, 4 mmol). The mixture was stirred at 50-60 ^{0}C for 5-6 hrs, cooled to room temperature and then poured into a cold saturated solution of hydroxylamine hydrochloride and sodium bicarbonate. The reaction mixture was stirred overnight. The solid product thus obtained was filtered, washed with water and recrystallised from ethanol to give pyrazolyl aldoximes in good yield.

2.2.2 Synthesis of Pyrazolyl isoxazolines [31] 4a-4k

IBD (708 mg, 2.2 mmol) was added to a stirred solution of pyrazolyl aldoxime **2a** (526 mg, 2 mmol) in 20 mL of methanol at room temperature, portion wise over a period of 10 minutes. During this period, a white precipitate was separate out. After the complete addition of IBD, the solution of acrylonitrile (117 mg, 2.2 mmol) in 20 mL methanol and 2 mL of TFA was added to this reaction mixture. The mixture was allowed to stir at room temperature for 30-40 minutes. The progress of reaction was monitored by TLC. Upon consumption of the pyrazoly aldoxime, the mixture was concentrated *in vacuo* and the residue was subjected to column chromatography over silica gel to obtain pure pyrazolyl isoxazoline **4a** (565 mg, 90% yield).

2.3 *In vitro* biological assay

2.3.1 Antibacterial activity

2.3.1.1 Medium

Two solid media, namely Muller–Hinton Agar (MHA; Beef infusion 300 g/L, casein acid hydrolysate 17.5 g/L, starch 1.5 g/L, agar-agar 17 g/L, and distilled water 1000 mL, adjusted to pH 7.4) and soyabean casein digest agar (SCDA; casein enzymatic hydrolysate 17.0 g/L, papain digest of soyabean 3.0 g/L, NaCl 5.0 g/L, dipotassium phosphate 2.5 g/L, dextrose 2.5 g/L, and distilled water 1000 mL, adjusted to pH 7.3), were used for the biological assays.

2.3.1.2 Test microorganisms

Total four microbial strains were selected on the basis of their clinical importance in causing diseases in humans. Two Gram-positive bacteria namely, *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 121), and two Gram-negative bacteria namely, *Escherichia coli* (MTCC 1652) and *Pseudomonas aeruginosa* (MTCC 741) the ear pathogens isolated from the patients of Kurukshetra [41] were used in the present study for evaluation of antimicrobial activity of the compounds. All the bacterial cultures were procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh. The bacteria were sub-cultured on Nutrient agar whereas fungi on Sabouraud dextrose agar.

2.3.1.3 Primary screening

The antibacterial activity of eleven compounds was evaluated by the agar well diffusion method. All the microbial cultures were adjusted to 0.5 McFarland Standard, which is visually comparable to a microbial suspension of approximately 1.5 x 10^{8} cfu/mL. 20 mL of Mueller Hinton agar medium was poured into each petri plate and plates were swabbed with 100 mL inocula of the test microorganisms and kept for 15 min for adsorption. Using sterile cork borer of 8 mm diameter, wells were bored into the seeded agar plates and these were loaded with a 100

mL volume with concentration of 2.0 mg/mL of each compound reconstituted in the dimethylsulphoxide (DMSO). All the plates were incubated at 37 °C for 24 h. Antibacterial activity of each synthetic compound was evaluated by measuring the zone of growth inhibition against the test organisms with zone reader (Hi Antibiotic zone scale). DMSO was used as a negative control whereas ciprofloxacin was used as positive control. This procedure was performed in three replicate plates for each organism [42-43].

2.3.1.4 Determination of Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration of the antimicrobial agent that prevents the development of visible growth after overnight incubation. MIC of the various compounds against bacterial strains was tested through a modified agar well diffusion method [44]. In this method, a two-fold serial dilution of each compound was prepared by first reconstituting the compound in DMSO followed by dilution in sterile distilled water to achieve a decreasing concentration range of 256 to 0.5 mg/mL. A 100 mL volume of each dilution was introduced into wells (in triplicate) in the agar plates already seeded with 100 mL of standardized inoculum (10^6 cfu/mL) of the test microbial strain. All test plates were incubated aerobically at 37 °C for 24 h and observed for the inhibition zones. MIC, taken as the lowest concentration of the chemical compound that completely inhibited the growth of the microbe, showed by a clear zone of inhibition, was recorded for each test organism. Ciprofloxacin was used as positive control while DMSO as negative control.

2.3.2 Antifungal activity

2.3.2.1 Medium

Potato dextrose agar (PDA) medium was used for biological assays. PDA medium was prepared by boiling 200 gm potato chips in 1 l of distilled water, filtering the extracts and making the final volume 1 l. To this 1% dextrose was added (pH adjusted at 5.5), after this 2% agar-agar was added and autoclaved at 121 ˚C for 20 min.

2.3.2.2 Test phytopathogenic fungi

Two phytopathogenic fungi, namely *Aspergillus niger* and *Aspergillus flavus* were used for biological assays. The synthesized compounds **4** and commercial antifungal compound Fluconazole were screened *in vitro* for their antifungal activity against these fungi by Poisoned Food Technique.

2.3.2.3 Biological procedure

The antifungal activity of chemical compounds was evaluated by poison food technique. The moulds were grown on Sabouraud dextrose agar (SDA) at 25 ^{0}C for 7 days and used as inocula. The 15 mL of molten SDA (45 ^{0}C) was poisoned by the addition of 100 mL volume of each compound having concentration of 4.0 mg/mL reconstituted in the DMSO, poured into a sterile Petri plate and allowed it to solidify at room temperature. The solidified poisoned agar plates were inoculated at the centre with fungal plugs (8 mm diameter) obtained from the colony margins and incubated at 25 ^{0}C for 7 days. DMSO was used as the negative control whereas fluconazole was used as the positive control. The experiments were performed in triplicates. Diameter of fungal colonies was measured and expressed as percent mycelial inhibition by applying the formula [45].

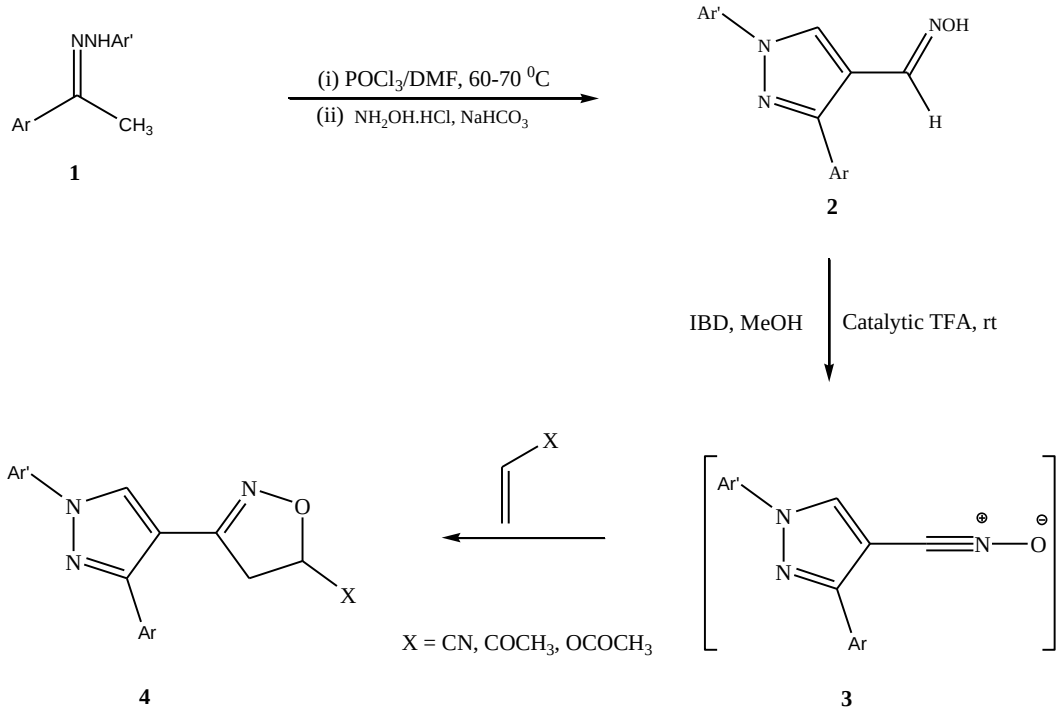
Percentage inhibition of myelial growth = (dc-dt) / dc × 100

where, dc = average fungal colony diameter in negative control sets; dt = average fungal colony diameter in experimental sets

3. Results and Discussion

3.1 Chemistry

Synthesis of pyrazolyl isoxazolines **4a-4k** is outlined in **scheme 1** [31]. Starting compounds, pyrazolyl aldoximes **2a-2k** were prepared by Vilsmeier-Haack reaction on acetophenone arylhydrazones, generating 1,3-diaryl-4-formylpyrazoles *in situ*, followed by modified workup with NH_2OH and $NaHCO_3$ in one-pot [40] (**Scheme 1**). The reactions of various substituted pyrazolyl aldoximes **2a-2k** was carried out with 1.1 equivalent of IBD in methanol by stirring at room temperature for 10 minutes.



Scheme 1: Synthesis of pyrazolyl isoxazolines 4a-4k

After the complete addition of IBD, the solution of activated alkenes in methanol and catalytic amount of TFA was added to this reaction mixture and allowed to stir at room temperature for 30-40 minutes. The usual work-up of the reaction afforded single product pyrazolyl isoxazolines **4a-4k** in good yields and the results are summarized in Table 1. All the isolated products **4** were completely characterized by their spectral (IR, ^{1}H NMR) and elemental analytical data [31]. The IR spectra of **4a-4k** exhibited characteristic absorption band at 2242-2254 cm^{-1} and 1739-1749 cm^{-1} due to –CN stretch and C=O stretch respectively. The ^{1}H spectra of all the products **4a-4k** showed three characteristic doublet of doublet (dd) at δ ~ 5.2-5.4, 3.32-3.40 and 3.42-3.51. The C(5)-H in pyrazole ring appeared as singlet at δ ~ 8.31. Other protons appeared in aromatic regions.

Table 1: Preparation of Pyrazolyl isoxazoline derivatives [31]

Product	Ar	Ar'	X	Mp. (°C) [31]	Yield [31]
4a	C_6H_5	C_6H_5	CN	138-140	90
4b	4-$CH_3C_6H_4$	C_6H_5	CN	116-118	86
4c	4-ClC_6H_4	C_6H_5	CN	146-148	87
4d	4-BrC_6H_4	C_6H_5	CN	168-170	92
4e	4-FC_6H_4	C_6H_5	CN	140-142	82
4f	4-$NO_2C_6H_4$	C_6H_5	CN	218-220	84
4g	C_6H_5	4-$NO_2C_6H_4$	CN	210-212	89
4h	C_6H_5	C_6H_5	$COOCH_3$	151-152	88
4i	4-$CH_3C_6H_4$	C_6H_5	$COOCH_3$	132-133	91
4j	C_6H_5	C_6H_5	$OCOCH_3$	125-126	85
4k	4-$CH_3C_6H_4$	C_6H_5	$OCOCH_3$	120-121	87

3.2 Biological Results and Discussion

3.2.1 Antibacterial activity

Eleven compounds **4a-4k** were tested *in vitro* for their antibacterial activity against two Gram-positive bacteria namely, *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 121), and two Gram-negative bacteria namely, *Escherichia coli* (MTCC 1652) and *Pseudomonas aeruginosa* (MTCC 741) by agar well diffusion method (**Table 2**). Standard antibiotic namely Ciprofloxacin was used for comparison of antibacterial activity shown by compounds **4a-4k**. The results were recorded for each tested compound as average diameter of zone of inhibition of bacterial growth adjoining the well in millimeters (**Table 2, Fig. 1**). Minimum inhibitory concentration (MIC)

measurements were performed using a macro dilution tube method (**Table 3, Fig. 2**). MIC of these compounds was determined which are showing activity in primary screening.

Results revealed that in general, all the tested compounds possessed variable antibacterial activity against Gram-positive (*Staphylococcus aureus, Bacillus subtilis*) bacteria. However all the compounds in this series did not show activity against any Gram-negative bacteria (*E. coli* and *P. aeruginosa*). On the basis of zone of inhibition produced against the test bacterium, four compounds **4a**, **4e**, **4h** and **4j** showed good activity against *B. subtilis* showing maximum zone of inhibition >21.0 mm as compared to standard drug ciprofloxacin which showed the zone of inhibition of 24.0 mm against *B. subtilis*. Similarly, on the basis of zone of inhibition four compounds **4a**, **4e**, **4h** and **4j** were found to be most effective against *S. aureus* showing the maximum zone of inhibition ranging between 19.6 and 21.6 as compared with standard drug ciprofloxacin which showed the zone of inhibition of 26.6 mm against *S. aureus*. Furthermore, seven compounds **4b**, **4c**, **4d**, **4f**, **4g**, **4i** and **4k** showed the maximum zone of inhibition ranging between 14.1 and 18.6 mm.

In the whole series, MIC of the synthesized compounds ranged between 16 and 256 mg/mL against the Gram-positive bacteria (**Table 3, Fig. 2**). In case of *S. aureus*, compound **4h** is most potent member having MIC of 16 mg/mL in comparison to standard drug having MIC of 5 mg/mL. Other compounds showing moderate activity are **4a**, **4e**, **4g**, and **4j** having MIC of 64 mg/mL. In case of *B. subtilis*, compound **4j** is most potent members having MIC of 16 mg/mL. Other compounds showing moderate activity having MIC ranged between 32 and 64 mg/mL are **4a**, **4c**, **4d**, **4e** and **4g**. All other compounds showed reasonable activity against Gram-positive bacteria.

Table 2: Antibacterial activity of compounds 4a-4k by using agar well diffusion assay method

Compounds	Diameter of growth of inhibition zone (mm)[a]			
	Staphylococcus aureus	***Bacillus subtilis***	***Escherichia coli***	***Pseudomonas aeruginosa***
4a	20.3	21.6	-	-
4b	15.6	17.6	-	-
4c	16.3	18.3	-	-
4d	15.6	18.6	-	-
4e	19.6	21.6	-	-
4f	15.3	17.3	-	-
4g	14.3	16.3	-	-
4h	21.6	20.8	-	-
4i	13.6	15.6	-	-
4j	20.3	21.8	-	-
4k	14.1	17.6	-	-
Ciprofloxacin	26.6	24.0	25.0	22.0

-No activity, [a]Values, including diameter of the well (8mm), are means of three replicates.

Table 3: Minimum inhibitory concentration (MIC) (in µg/mL) of compounds 4a-4k by using macrodilution method

Compounds	MIC (µg/mL)[b]			
	Staphylococcus aureus	***Bacillus Subtilis***	***Escherichia coli***	***Pseudomonas aeruginosa***
4a	64	32	-	-
4b	128	128	-	-
4c	128	64	-	-
4d	128	64	-	-
4e	64	32	-	-
4f	128	128	-	-
4g	64	64	-	-
4h	16	128	-	-
4i	256	128	-	-
4j	64	16	-	-
4k	128	128	-	-
Ciprofloxacin	5	5	5	5

[b]Mean of three replicates

3.2.2 Antifungal activity

All the eleven compounds **4a-4k** were also tested *in vitro* for their antifungal activity against two fungi, namely *Aspergillus niger* and *Aspergillus flavus* through poisoned food method.

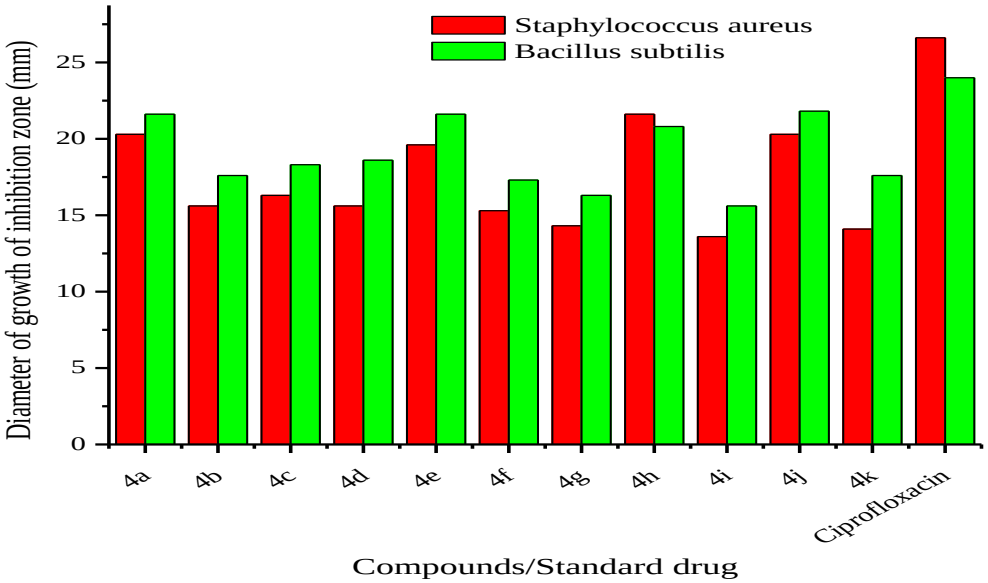


Fig.1. Comparison of diameter of growth of inhibition zone (mm) of compounds 4a-4k with standard drug ciprofloxacin

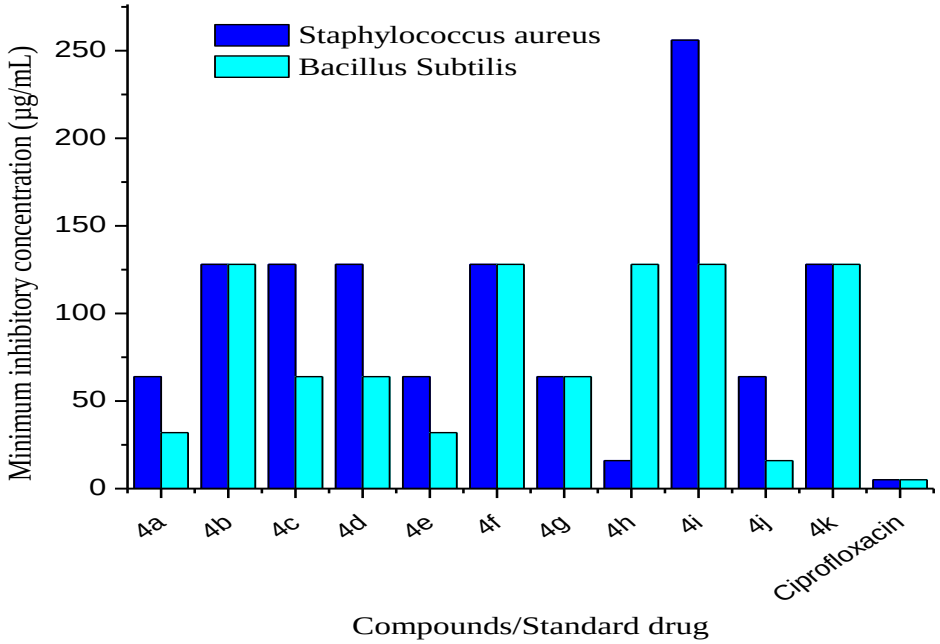


Fig.2. Comparison of MIC of compounds 4a-4k with standard drug ciprofloxacin

Standard antibiotic namely Fluconazole was used for comparison with antifungal activity shown by compounds **4a-4k** and results were recorded as percentage (%) of mycelial growth inhibition. A careful analysis of percentage mycelial growth inhibition revealed that almost all of the compounds **4a-4k** showed variable antifungal activity against two pathogens as shown in **Table 4**. From the careful comparison of the results, it has been revealed that mainly the four compounds **4a**, **4e**, **4h** and **4j** showed excellent antifungal activity with >55% inhibition of mycelial growth against *Aspergillus niger* in comparison with the standard drug (81.1%). In addition to these, two more compounds which showed >55% inhibition are **4a** and **4h** against *Aspergillus flavus* (77.7%). There are many other compounds which showed good and fair antifungal activities are summarized in **Table 4**. Comparisons of antifungal activity of all the synthesized compounds with reference drug in terms of % mycelial growth inhibition are also shown in **Fig. 3**.

Table 4: Antifungal activity of synthetic 4a-4k compounds through poisoned food method

Compounds	Mycelial growth inhibition (%)[c]	
	Aspergillus niger	***Aspergillus flavus***
4a	61.1	56.6
4b	51.1	48.8
4c	48.8	50
4d	45.5	44.4
4e	57.7	52.5
4f	48.8	45.5
4g	53.3	51.1
4h	56.6	58.8
4i	51.1	53.3
4j	58.9	54.2
4k	47.3	46.2
Fluconazole	81.1	77.7

[c]Mean of three replicates

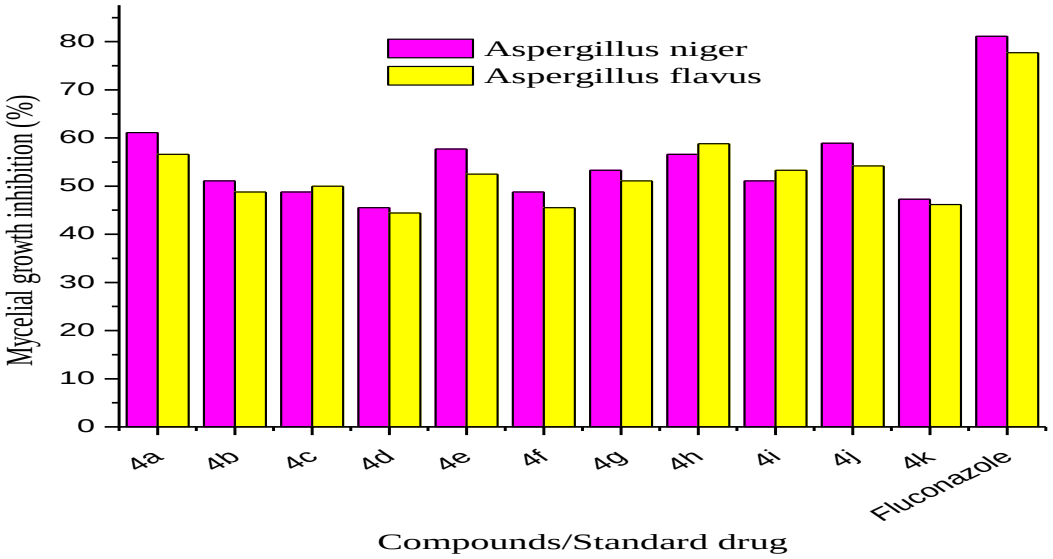


Fig.3. Comparison of antifungal activity of compounds 4a-4k with standard drug fluconazole

4. Conclusion

In conclusion, we have synthesised pyrazolyl isoxazoline derivatives **4a-4k** using IBD under mild reaction conditions in high yields. The antibacterial and antifungal activities of these compounds have proved them potent antimicrobial agents. Of all the compounds screened for activity some of the compounds were associated with considerably comparable antibacterial and antifungal activity with commercial antibiotics. Thus, the present work will have a good impact on chemist and bio-chemist and can be further used in pharmaceutical industry for mankind, as an antimicrobial agent, after testing its toxicity to human beings.

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