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AQUEOUS PHASE SYNTHESIS, CRYSTAL STRUCTURE AND ANTIMICROBIAL ACTIVITY OF 4-(SUBSTITUTED PHENYLAZO)-3-METHYL-4*H*-ISOXAZOL-5-ONE AZO DYES

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ABSTRACT. 3-Methyl-4*H*-isoxazol-5-one was synthesized at room temperature by simple stirring method from ethyl acetoacetate and hydroxylamine hydrochloride in aqueous medium and coupled with diazotized substituted amine to form series of 4-(substituted phenylazo)-3-methyl-4*H*-isoxazol-5-ones through green chemistry. All the compounds formed were characterized by IR, ^{1}H and ^{13}C NMR spectroscopy, MS and elemental analysis. Crystal structure of novel 4-(4-fluorophenylazo)-3-methyl-4*H*-isoxazol-5-one was determined by the X-ray diffraction. Antibacterial and antifungal activity was studied against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus pyogenus* and *Candida albicans*, *Aspergillus niger*, *Aspergillus clavatus*, respectively. All synthesized compounds were found to be active against a gram-positive bacterium *Staphylococcus aureus*. Two compounds showed antifungal activity against *Candida albicans* close to standard greseofulvin.

KEY WORDS: Azo dyes, Substituted amines, Antibacterial and antifungal activity, X-ray diffraction, Spectroscopy, Green chemistry

INTRODUCTION

Azo dyes are the most widely used class of coloring materials because of their fruitful applications in various fields of science and technology [1-3] and also for their interesting structural and physicochemical properties [4-6]. Azo dyes are synthesized by diazotization of aromatic amines and coupling reagent, which include one or more azo groups (–N=N–) attached to one or more aromatic moieties [7]. Currently, heteroarylazoisoxazol compounds have received the attention of many research groups [8] because of their wide spread potential applications in fields of catalysis [9], cancer treatment and as antibacterial and antiviral agents, agricultural fungicides as well as other biological uses [10-12]. Intramolecular N–H···O hydrogen bonding study assisted by resonance for 1-ketone-2-arylhydrazones derivatives suggest that heterocyclic five member ring introduces geometrical constraint which hinders the strengthening of hydrogen bond [13]. Tautomerism and spectroscopic properties of some heteroarylazoisoxazolone dyes were studied earlier [14]. With these objects in view we focus on the synthesis and antimicrobial activity of 4-(substituted phenylazo)-3-methyl-4*H*-isoxazol-5-one azo dyes.

EXPERIMENTAL

All the starting materials and solvents were purchased from commercial sources and were used without further purification. Melting points were determined in open capillaries using electro thermal melting point apparatus and are uncorrected. Progress of reactions was monitored by TLC. Infrared (IR) spectra (4000–600 cm^{-1}) of the samples were recorded using a Perkin–Elmer Spectrum 100, equipped with a Specac Golden Gate Diamond ATR as a solid sample support.

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^{1}H NMR spectra were recorded with a Bruker Avance III 500 nuclear magnetic resonance spectrometer with TMS as internal reference. ^{13}C NMR spectra were recorded with a Bruker Avance III 500 spectrometer at 125 MHz and were referenced against the central line of the solvent signal ($CDCl_3$ triplet at 77.0 ppm or DMSO-d_6 septet at 39.5 ppm). MS spectra were recorded with an Agilent 6624 Accurate Mass TOF LC/MS instrument (ESI ionization).

Single-crystal X-ray diffraction data for **6c**·0.3H_2O were collected on an Agilent Technologies SuperNova Dual diffractometer with the Atlas detector using monochromated Mo-Kα radiation (λ = 0.71073 Å) at room temperature. The data were processed using CrysAlis Pro. [15]. The structure was solved by direct methods using the program Superflip [16] and refined on F^2 using full-matrix least-squares procedures using SHELXL2014 [17]. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were readily located in a difference Fourier maps and were subsequently treated as riding atoms in geometrically idealized positions, with C–H = 0.93 (aromatic) or 0.96 Å (CH_3), N–H = 0.86 Å and with U_{iso}(H) = kU_{eq}(C or N), where k = 1.5 for methyl groups, which were permitted to rotate but not to tilt, and 1.2 for all other H atoms. The water H-atoms were refined with a distance restraint and with U_{iso}(H) = 1.5U_{eq}(O). Initially, the occupation factor for a water solvate molecule was refined, but was set as fixed at 0.30 in the final stage of the refinement. Structure was refined as a twin. Crystallographic data are listed in Table 1.

Determination of minimal inhibition concentrations by micro broth dilution for antibacterial and antifungal activity

All the synthesized drugs were used for antibacterial test procedures. All necessary controls like drug control, vehicle control, agar control, organism control, known antibacterial drugs control, all MTCC cultures were tested against above mentioned known and unknown drugs. Mueller Hinton broth was used as nutrient medium to grow and dilute the drug suspension for the test bacteria. Inoculum size for test strain was adjusted to 10^8Cfu [Colony Forming Unit] per milliliter by comparing the turbidity. Common standard strains were used for screening of antibacterial and antifungal activities: The strains were procured from Institute of Microbial Technology, Chandigarh: *E. coli* (MTCC443), *P. aeruginosa* (MTCC1688), *S. aureus* (MTCC96), *S. pyogenus* (MTCC442), *C. albicans* (MTCC227), *A. niger* (MTCC282), *A. clavatus* (MTCC1323). DMSO was used as diluents / vehicle to get desired concentration of drugs to test upon standard bacterial strains.

The main advantage of the 'Broth Dilution Method' for minimal inhibition concentration (MIC) determination lies in the fact that it can readily be converted to determine the MIC as well [18-22]. Serial dilutions were prepared in primary and secondary screening. The control tube containing no antibiotic is immediately sub cultured (before inoculation) by spreading a loopful evenly over a quarter of plate of medium suitable for the growth of the test organism and put for incubation at 37 ^{o}C overnight. The tubes are then incubated overnight. The MIC of the control organism is read to check the accuracy of the drug concentrations. The lowest concentration inhibiting growth of the organism is recorded as the MIC. The amount of growth from the control tube before incubation is compared [23].

Each synthesized drug was diluted obtaining 2000 µg/mL concentration, as a stock solution. In primary screening 1000, 500, and 250 µg/mL concentrations of the synthesized drugs were taken. The active synthesized drugs found in this primary screening were further tested in a second set of dilution against all microorganisms. The drugs found active in primary screening were similarly diluted to obtain 200, 100, 50, 25, 12.5, 6.250 µg/mL end concentrations. The highest dilution showing at least 99% inhibition zone is taken as MIC.

General procedure for synthesis of 3-methyl-4-H-isoxazol-5-one

Hydroxylamine hydrochloride (1.041 g, 15 mmol) was dissolved in 20 mL of water and cooled to about 10 ^{o}C, added ethyl acetoacetate (1.95 g, 15 mmol). The mixture was shaken thoroughly in absence of any catalyst till the appearance of pale yellow color solution of 3-methyl-4*H*-isoxazol-5-one [24].

General procedure for synthesis of 4-(substituted phenylazo)-3-methyl-4H-isoxazol-5-one azo dyes

Substituted amine **4a–e** (15 mmol) were dissolved in dilute HCl (1.5 mL in 15 mL water) and cooled in an ice bath to 0–5 ^{o}C. The reaction mixture was added to the solution of sodium nitrite (1.043 g, 15 mmol in 15 mL of water) and 0.01 M HCl (15 mL) at about 0–5 ^{o}C with continuous stirring to give yellow colored solution **5a–e.** To this solution, equimolar amount of cooled 3-methyl-4*H*-isoxazol-5-one solution was added in ice bath with continuous stirring for 30 min to get yellow orange colored precipitate. The pH of reaction mixture was maintained between 5 and 6. The reaction mixture was kept overnight at about 15 ^{o}C for the completion of the reaction. The precipitate obtained **6a–e** was washed with cold water, dried and recrystallized using ethanol. Crystals of **6c** suitable for single crystal XRD analysis were obtained by slow evaporation of ethanol and chloroform (1:1 v/v) solvent mixture.

Spectral data

*3-Methyl-4-phenylazo-4H-isoxazol-5-one (**6a**)*. Yellow solid, yield: 2.45 g (85%), m.p.: 170 ^{o}C, R_f 0.214 (EtOH). Elemental analysis: found %, C 59.26; H 4.42; N 20.73; $C_{10}H_9N_3O_2$ (203.20 g/mol). Calculated, % C 59.11; H 4.46; N 20.68;MS (ESI+) *m/z*: 204.0767 (MH^+), HRMS: found, *m/z*: 204.0768; $C_{10}H_9N_3O_2$ calculated, *m/z*: 204.0767. IR (cm^{-1}): 3209 (NH), 1708 (C=O), 1570 (CH=N), 1482 (C=C), 1277 (C–O); ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 2.26 (s, 3H, 3-CH_3), 7.37–7.19 (m, 5H, Ar-H), 12.60 (s, 1H, -NH); ^{13}C NMR, δ ppm, $CDCl_3$: 10.3, 116.1, 121.1, 126.7, 129.8, 140.4, 159.4, 164.9.

*4-(4-Chloro-phenylazo)-3-methyl-4H-isoxazol-5-one (**6b**)*. Yellow solid, yield: 2.79 g (82%), m.p.: 155 ^{o}C, R_f 0.224 (EtOH). Elemental analysis: found %, C 50.57; H 3.47; N 17.31; $C_{10}H_8ClN_3O_2$ (237.64 g/mol). Calculated, % C 50.54; H 3.39; N 17.68. MS (ESI+) *m/z*: 238.0377 (MH^+), HRMS: found, *m/z*: 238.0378; $C_{10}H_9N_3O_2$ calculated, *m/z*: 238.0377. IR (cm^{-1}): 3204 (NH), 1739 (C=O), 1554 (CH=N), 1477 (C=C), 1220 (C–O). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 2.26 (s, 3H, -CH_3 isoxazol), 7.35–7.28 (m, 4H, Ar-H), 12.57 (s, 1H, -NH), ^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 10.3, 117.2, 130.0, 132.0, 139.0, 159.3, 164.8.

*4-(4-Fluorophenylazo)-3-methyl-4H-isoxazol-5-one (**6c**)*. Orange yellow solid, yield: 2.60 g (83%), m.p.: 160 ^{o}C, R_f 0.217 (EtOH). Elemental analysis: found %, C 54.60; H 3.25; N 18.80; $C_{10}H_8FN_3O_2$ (221.19 g/mol). Calculated, % C 54.30; H 3.65; N 19.00. MS, (ESI+) *m/z*: 222.0671 (MH^+), HRMS: found, *m/z*: 222.0673; $C_{10}H_8FN_3O_2$ calculated, *m/z*: 222.0671. IR (cm^{-1}): 3199 (NH), 1714 (C=O), 1565 (CH=N), 1493 (C=C), 1215 (C–O). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 2.26 (s, 3H, -CH_3isoxazol), 7.09–7.06 (m, 2H, Ar-H), 7.35–7.32 (m, 2H, Ar-H), 12.62 (s, 1H, -NH). ^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 10.2, 116.7, 136.7, 159.3, 160.1, 162.1, 164.0.

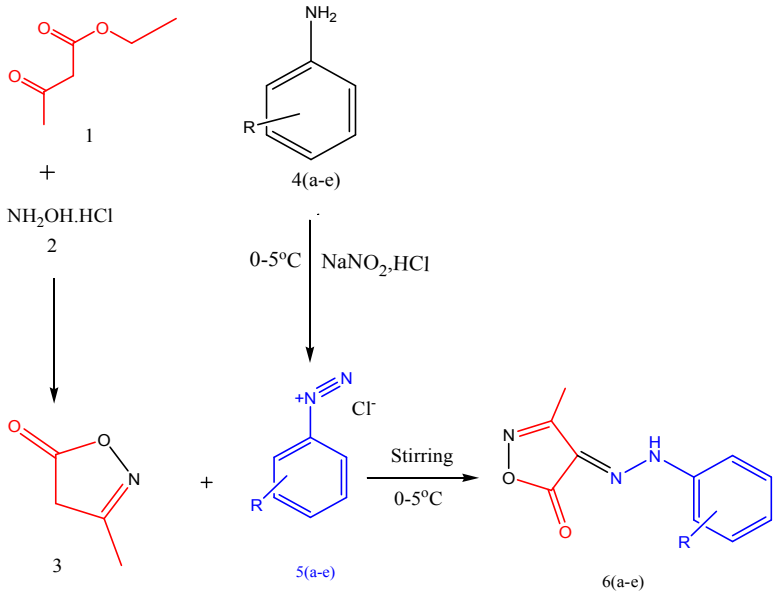
*4-(3-Methyl-5-oxo-4,5-dihydroisoxazol-4-ylazo)benzenesulfonamide (**6d**)*. Orange yellow solid, yield 3.20 g (79%), m.p. 240 ^{o}C, R_f 0.260 (EtOH). Elemental analysis found, %: C 42.40; H 3.41; N 19.40; $C_{10}H_{10}N_4O_4S$ (282.28 g/mol). Calculated, % C 42.55; H 3.57; N, 19.85.MS, (ESI+) *m/z*: 283.0491 (MH^+), HRMS: found, *m/z*: 283.0495; $C_{10}H_{10}N_4O_4S$ calculated, *m/z*:

283.0491. IR (cm^{-1}): 3317 (NH), 1719 (C=O), 1570 (CH=N), 1482 (C=C), 1246 (C–O). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 2.29 (s, 3H, -CH_3 isoxazol), 4.71 (s, 2H, -NH_2), 7.46–7.44 (d, 2H, Ar-H), 7.94–7.92 (d, 2H, Ar-H), 12.56 (s, 1H, -NH). ^{13}C NMR (125 MHz, DMSO, δ ppm): 10.6, 117.2, 122.7, 127.5, 141.2, 144.4, 160.5, 162.4.

4-(3-Acetylphenylazo)-3-methyl-4H-isoxazol-5-one ***(6e)***. Orange solid, yield: 2.76 g (79%), m.p.: 210 °C, R_f 0.251 (EtOH). Elemental analysis found, %: C 59.42; H 3.20; N 16.77; $C_{12}H_{11}N_3O_3$ (245.23 g/mol). Calculated, % C 58.77; H 4.52; N 17.17, MS, (ESI+) *m/z*: 246.0871 (MH^+), HRMS: found, *m/z*: 246.0873; $C_{12}H_{11}N_3O_3$ calculated, *m/z:* 246.0871. IR (cm^{-1}): 3204 (NH), 1734 (C=O), 1549 (CH=N), 1431 (C=C), 1272 (C–O). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm): 2.29 (s, 3H, -CH_3 isoxazol), 2.51 (s, 3H, -$COCH_3$), 7.92–7.47 (m, 4H, Ar-H), 12.63 (s, 1H, -NH). ^{13}C NMR (125 MHz, $CDCl_3$, δ ppm): 10.3, 26.7, 115.3, 120.2, 126.3, 130.2, 138.6, 140.9, 159.4, 164.7, 196.9.

RESULTS AND DISCUSSION

A series of 4-(substituted phenylazo)-3-methyl-4-*H*-isoxazol-5-one azo dyes were obtained from easily available, economically feasible chemicals like ethyl acetoacetate, hydroxylamine hydrochloride and some substituted amines by environmentally benign, green route in aqueous medium without using any catalyst. In the first step the reaction of ethyl acetoacetate (**1**) and hydroxylamine hydrochloride (**2**) resulted in the formation of oxime, which on further ring closure formed 3-methyl-4*H*-isoxazol-5-one (**3**) (Scheme 1). Electron withdrawing effect of N atom in 3-methyl-4*H*-isoxazol-5-one along with electron donating effect of O atom and presence of electron releasing methyl and keto groups makes it more reactive [15]. In the second step diazotization of substituted amine **4a–e** was carried out in the presence of sodium nitrite and hydrochloric acidwhich then coupled with active hydrogen atom of isoxazol nucleus under cold condition to give desired product (Scheme 1).



a: R = H, b: R = 4-Cl, c: R = 4-F, d: R = 4-SO_2NH_2, e: R = 3-$COCH_3$

Scheme 1: Synthesis of 4-(substituted phenylazo)-3-methyl-4*H*-isoxazol-5-one azo dyes.

All compounds **6a–e** show singlet for NH proton in the range of 12.50–12.60 ppm. Multiplets for aromatic ring were observed between 7–8 ppm. A singlet for three protons in the range of 2.26–2.29 is attributed to CH_3 group of isoxazol. Compound **6d** shows a singlet for two protons of NH_2 group related to sulphanilamide moiety at 4.71 ppm and compound **6e** show a singlet for three protons related to $COCH_3$ group at 2.51 ppm. ^{13}C NMR study of **6a–e** shows a singlet at about 10.24–10.56 ppm for CH_3 group of isoxazol, about 160–165 ppm for C=O group of isoxazol, compound **6e** shows singlet at 197 ppm for C=O group of acetophenone and at 26 ppm for CH_3 group of acetophenone. The IR spectrum of compound **6a–e** showed characteristic ν(-NH) absorption band in the region 3024-3317 cm^{-1}, ν(C=O) absorption band in the region 1708-1739 cm^{-1} and ν(CH=N) absorption band in the region 1550-1570 cm^{-1} (Figure 1).

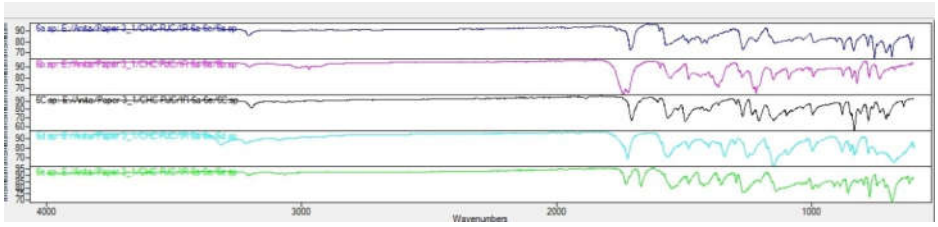


Figure 1. IR spectra of **6a–e**.

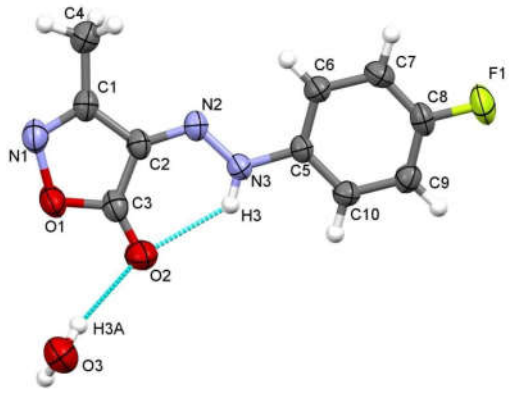


Figure 2. Molecular structure and atom numbering scheme for **6c**. Elipsoid Probability are drawn at the 50% level and hydrogen bonding of C4–H4A···O2 and C10–H10···O1 is indicated by blue dashed lines.

Single-crystal X-ray structures of 4-(4-fluorophenylazo)-3-methyl-4*H*-isoxazol-5-one (**6c**·0.3H_2O) was determined (Figure 2, Table 1). Table 3 and 4 describes significant bond distances and bond angles. All the bond lengths and bond angles of compounds are within normal ranges for this type of structure as reported earlier [13]. Molecule **6c** is almost planar, maximum deviation from the mean plane described by all atoms of **6c** is –0.132(3) Å for atom C10. The dihedral angle between the isoxazolone and phenyl rings is 7.81(18)°. In the crystal structure bond distances indicates that the isoxazolone ring contains one double bond, i.e. C1-N1. Also, atoms C2 and N2 are connected through double bond and intramolecular N3–H3···O2 hydrogen bonding is present between NH group and the carbonyl group of the isoxazole ring. A water solvate molecule present in the crystal lattice with 30% occupancy

forms O3–H3A···O2 hydrogen bond with two adjacent molecules of **6c**. Furthermore, molecules are linked into chains v*ia* C4–H4A···O2 interactions between methyl group of isoxazole ring and carbonyl oxygen atom of isoxazole moiety of the adjacent molecule and C10–H10···O1 interactions between the phenyl moiety and the oxygen atom of isoxazole moiety of the adjacent molecule (Figure 3).

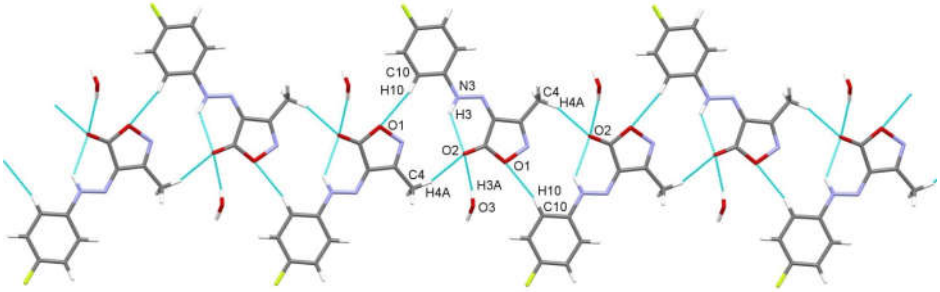


Figure 3. Chain formation generated by C4–H4A···O2 and C10–H10···O1 interactions. Hydrogen-bonds are indicated by blue dashed lines.

Table 1. Crystallographic data for 4-(4-fluorophenylazo)-3-methyl-4*H*-isoxazol-5-one (**6c**·0.3H_2O).

Compound code	**6c**·0.3H_2O
CCDC number	1561281
Molecular formula	$C_{10}H_{8.6}FN_3O_{2.3}$
Molecular weight	226.60
Crystal System	Orthorhombic
Space group	*Fdd*2
Unit cell dimensions (Å)	
a	24.2754(17)
b	24.5121(17)
c	6.8469(5)
Z	16
V (Å^3)	4074.2(5)
D_{calc} (g cm^{-3})	1.478
μ (mm^{-1})	0.120
F(000)	1872
Reflections collected	8741
Parameters	155
Goodness-of-fit on F^2	1.069
R_1, wR_2 [$I > 2\sigma(I)$]^a	0.0407, 0.0939
R_1, wR_2 (all data)^b	0.0560, 0.1023
$\Delta\rho_{min}$, $\Delta\rho_{max}$ (e Å^{-3})	–0.172, 0.13

Table 2. Hydrogen bond geometry of **6c** (Å and °).

D–H···A	D–H (Å)	H···A (Å)	D···A (Å)	D–H···A (°)	Symmetry code
N3–H3···O2	0.86	2.23	2.882(3)	132.4	*x*, *y*, *z*
O3–H3A···O2	0.82(2)	2.20(3)	3.007(4)	169(8)	*x*, *y*, *z*
C4–H4A···O2	0.96	2.56	3.371(4)	142.8	–*x* + 3/4, *y* – 1/4, *z* + 1/4
C10–H10···O1	0.93	2.55	3.379(3)	149.4	–*x* + 3/4, *y* + 1/4, *z* – 1/4

Table 3. Significant bond distances.

Sr. No.	Bond	Distance (Å)
1	F1 C8	1.363(3)
2	O1 C3	1.359(3)
3	O1 N1	1.470(3)
4	O2 C3	1.213(4)
5	N1 C1	1.292(3)
6	N2 N3	1.306(3)
7	N2 C2	1.311(3)
8	N3 C5	1.412(3)

Table 4. Significant bond angles.

Sr. No.	Bond	Angle (°)
1	C3 O1 N1	109.2(2)
2	C1 N1 O1	106.6(2)
3	N3 N2 C2	118.4(2)
4	N2 N3 C5	120.0(2)
5	N1 C1 C2	111.9(3)
6	N1 C1 C4	120.9(3)
7	C2 C1 C4	127.2(2)
8	N2 C2 C1	125.2(2)
9	N2 C2 C3	129.1(2)
10	C1 C2 C3	105.6(2)
11	O2 C3 O1	122.1(3)
12	O2 C3 C2	131.1(3)
13	O1 C3 C2	106.7(2)

All synthesized compounds **6a–e** were screened for antibacterial and antifungal study against *E. coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenus* as bacterial strain and *C. albicans*, *A. niger*, *A. clavatus* as fungal strain. All synthesized compounds **6a–e** showed antibacterial activity against *S. aureus*. Compound **6c** and **6e** showed antibacterial activity against *E. coli* whereas compound **6b** showed antibacterial activity against *S. pyogenus* close to activity of standard ampicillin (Table 5). It was found that compounds **6b** and **6e** show antifungal activity against *C. albicans* close to standard greseofulvin (Table 6).

Table 5. Antibacterial activity study of **6a–e** with standard drugs.

Minimal inhibition concentration (µg/mL)					
Entry	Compound	*E. coli* (MTCC443)	*P. aeruginosa* (MTCC1688)	*S. aureus* (MTCC96)	*S. pyogenus* (MTCC442)
1	**6a**	125	200	100	125
2	**6b**	200	500	200	100
3	**6c**	62.5	250	200	200
4	**6d**	200	250	100	200
5	**6e**	100	250	250	250
9	Gentamycin	0.05	1	0.25	0.5
10	Ampicillin	100	-	250	100
11	Chloramphenicol	50	50	50	50
12	Ciprofloxacin	25	25	50	50
13	Norfloxain	10	10	10	10

Table 6. Antifungal activity study of **6a–e** with standard drugs.

Minimal inhibition concentration (μg/mL)				
Entry	Compound	*C. albicans* (MTCC227)	*A. niger* (MTCC282)	*A. clavatus* (MTCC1323)
1	**6a**	1000	>1000	>1000
2	**6b**	500	>1000	>1000
3	**6c**	1000	1000	1000
4	**6d**	1000	500	500
5	**6e**	500	>1000	>1000
9	Nystatin	100	100	100
10	Griseofulvin	500	100	100

CONCLUSION

The method of synthesis of **6a–e** is aqueous phased, environmentally friendly and gives product easily in good yield without using any hazardous chemicals. Spectroscopic study of **6a–e** and X-ray crystallographic study of **6c** shows azo dye formation with tautemerism to retain aromaticity of isoxazole ring. All synthesized compounds showed antibacterial activity against *S. aureus* close to standard ampicillin. Compounds **6c** and **6e** showed antibacterial activity against *E. coli* close to ampicillin and **6b** and **6e** showed antifungal activity against *C. albicans* close to gresiofulvin. The crystal structure of **6c** shows hydrogen bonding interaction with water molecule. Oxygen atom attached to isoxazole ring shows intermolecular as well as intramolecular hydrogen bonding.

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