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SYNTHESIS AND *IN-VITRO* ANTIFUNGAL ACTIVITY OF 6-SUBSTITUTED-PHENYL-2-{[(4'-SUBSTITUTED PHENYL-5'-THIOXO)-1,2,4-TRIAZOL-3-YL]-METHYL}-2,3,4,5-TETRAHYDROPYRIDAZIN-3-ONE DERIVATIVES

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Abstract: The synthetic pathway for 6-substituted phenyl-2-[{(4'-substituted phenyl-5'-thioxo)-1,2,4-triazol-3-yl}-methyl]-2,3,4,5-tetrahydropyridazin-3-one compounds was achieved by a sequence of reactions starting from respective aryl hydrocarbons and is illustrated in Scheme1. All the compounds were tested for their *in vitro* antifungal activity on five fungal species, namely *Candida albicans, Trichophyton rubrum, Aspergillus flavus, Aspergillus niger* and *Penicillium citrinium.*
The chloro substituent derivative (compound **5g**) showed the highest activity against all the fungal species. The MIC of the standard drug voriconazole was between 0.10 – 0.40 μg/mL against all the fungal species except *A. fumigatus*. The two electronegative groups of Cl were increasing the activity of 1,2,4-triazole. As we increased the bulky group or aromatic group on benzene ring, there was a decrease of activity as in case of compound **l**.

Key words*:* 1,2,4-triazole; antifungal activity

In world up to 5% of all the infections are caused by fungi. Fungal infections in such a high risk patients progress rapidly and are difficult to diagnose and treat. Especially in the developed countries fungal infections have grown rapidly in last few decades. 1,2,4-triazole derivatives show diverse pharmacological properties such as antimicrobial (1-4), anti-inflammatory (5), analgesic (6), anticancer (7), antihypertensives (8), anticonvulsant and antiviral (9). Some of antifungal azole derivatives used as common antibiotics such as amphotericin B possess toxic effect on humans along with their antimicrobial effects (10). Although antimicrobial agents having different structures are frequently used in the treatment of fungal infections, there is an increasing resistance to these drugs. To overcome the development of drug resistance it is necessary to synthesize a new class of antifungal compounds possessing different chemical properties from those of used commonly. 5-Thioxo-1,2,4-triazole containing a pyridazinone side chain is an ideal heterocyclic system for antifungal activity. The following antifungal 1,2,4-triazole derivatives are applicable in medicine like fluconazole (11), itraconazole and terconazole.

A series of 1,2,4-triazole derivatives was synthesized and evaluated for their in vitro antifungal activity. The structures of the final triazoles were confirmed on the basis of their spectral data. The above triazoles were synthesized by five step reactions presented in Scheme1.

EXPERIMENTAL

Chemicals were supplied by E. Merck (Germany) and S.D Fine Chemicals (India). Melting points were determined by open tube capillary method and are uncorrected. Purity of the compounds was checked on thin layer chromatography (TLC) plates (silica gel G) in the solvent system toluene-ethyl formate-formic acid (5:4:1 v/v/v) and benzene-methanol (8:2 v/v), the spots were located under iodine vapors and UV light. The IR spectra were obtained on a Perkin-Elmer 1720 FT-IR spec-

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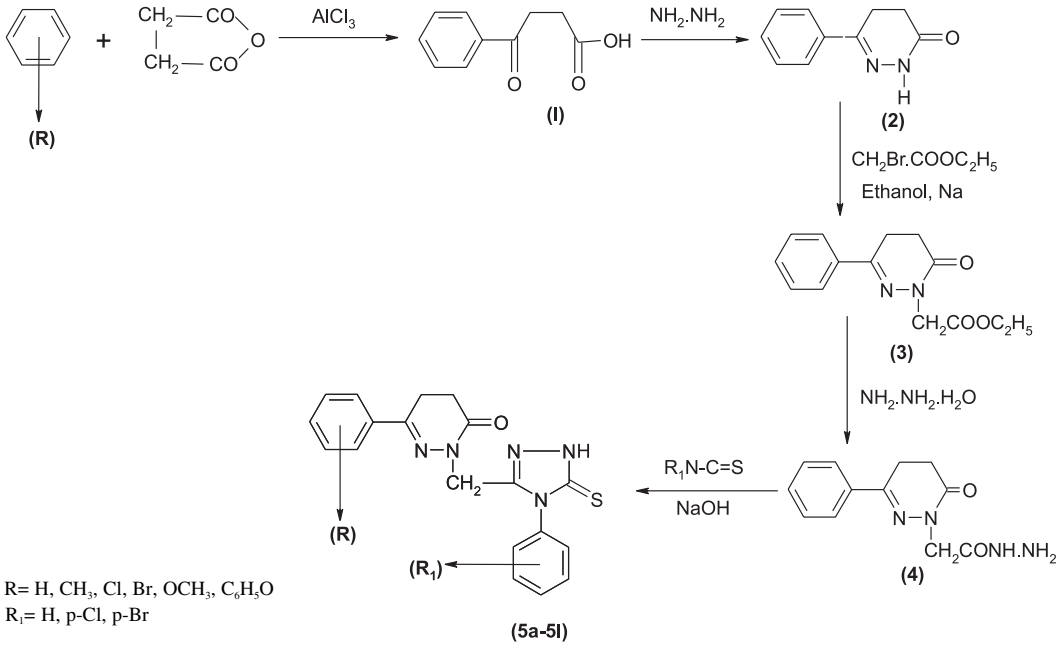

Scheme 1**.** Synthesis of 6-substituted-2-{[4'-(substituted phenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-ones.

trometer (KBr pellets). ^{1}H-NMR spectra were recorded or a Bruker AC 300 MHz spectrometer using TMS as internal standard in DMSO-d_6/$CDCl_3$ and mass spectra under electron impact conditions (EI) were recorded at 70 ev ionizing voltage with a VG Prospec instrument and are presented as m/z.

General procedure for synthesis of 6-substituted-2{[4'-(substituted phenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one derivatives

To the dry anhydrous aluminium chloride (24.5 g) dry benzene (50 mL) was added and the content was refluxed on a water bath under anhydrous condition. Succinic anhydride (10 g) was then added in small portions through the three-neck flask. After complete addition, the reaction mixture was refluxed for 3 h and then allowed to stand for 24 h. A cold solution of conc. hydrochloric acid (2.5% v/v) was then added to the reaction mixture and the content was subjected to distillation to remove the unreacted benzene. The content was concentrated on water bath and cooled to separate out the crystalline compound which was filtered and crystallized from hot water yielding compound **(1)**.

The β-benzoyl propionic acid **(1)** (1g) was dissolved in absolute ethanol (15 mL). Hydrazine hydrate (99%, 2 mL) was added and the content was refluxed for 8-10 h. After the completion of reaction, ethanol was distilled off and the residue was poured into ice-cold water. A solid, which separated out was filtered and crystallized from ethanol to give pure needle shape compound **(2)**.

Appropriate pyridazinone **(2)** (0.02M) was added to ethanolic solution (50 mL) of sodium (0.46 g). The mixture was refluxed for 30 min. Then ethyl bromoacetate (3.34 g, 0.02 M) was added dropwise to the cooled solution, which was refluxed for 24 h. The solvent was evaporated under vacuum and the residue was triturated with diisopropyl ether and the solid which was formed was collected by filtration and dried **(3)**.

To compound **(3)** (1 g) dissolved in absolute ethanol (50 mL) hydrazine hydrate (99%, 4 mL) was added and the reaction mixture was refluxed for 8-10 h under anhydrous condition using guard tube. After evaporation, the resulting solution was poured on the crushed ice and the precipitate which was separated was filtered off and crystallized from ethanol yielding **(4)**.

Ethanolic solution of hydrazide **(4)** (0.01 M) and respective phenyl isothiocynate (0.01 M) were refluxed for 5 h. The content was concentrated and poured into crushed ice to get intermediate thiosemicarbazide. Thiosemicarbazide (0.05 M) was refluxed in sodium hydroxide solution for 5 h, cooled and poured into excess of water containing crushed ice. Acidification with glacial acetic acid yielded a solid which was crystallized from ethanol to give compounds **(5)**.

Table 1. MIC of the tested compounds against the fungal isolates using microdilution method.

MIC in μg/mL					
Compound	***Candida albicans***	***Trichophyton rubrum***	***Aspergillus niger***	***Aspergillus flavus***	***Aspergillus fumigatus***
5a	4.0	5.0	5.0	5.0	4.0
5b	6.0	6.0	6.0	5.0	4.0
5c	5.0	5.0	5.0	5.0	6.0
5d	7.0	6.0	7.0	7.0	8.0
5e	6.0	7.0	5.0	7.0	8.0
5f	10.0	9.0	8.0	7.0	8.0
5g	3.0	3.0	4.0	4.0	3.0
5h	4.0	4.0	4.0	4.0	5.0
5i	8.0	8.0	9.0	10.0	9.0
5j	5.0	6.0	7.0	8.0	7.0
5k	10.0	12.0	12.0	13.0	10.0
5l	12.0	12.0	15.0	16.0	14.0
Voriconazole	0.20	0.15	0.20	0.30	0.50

β-Benzoyl propionic acid (**1**)

IR: (KBr, cm^{-1}): 3428 (COOH), 1679 (C=O); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.59 (t, 2H, CH_2), 3.23 (t, 2H, CH_2), 7.53-7.62 (m, 3H, H-3'-H-5'), 7.97 (d, 2H, H-2'-H-6'), 12.17 (s, 1H, COOH).

6-Phenyl-2,3,4,5-tetrahydropyridazin-3-one (**2**)

IR: (KBr, cm^{-1}): 3206 (NH), 1678 (C=O); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.45 (t, 2H, CH_2), 2.93 (t, 2H, CH_3), 7.41 (m, 3H, H-3'-H-5'), 7.74 (d, 2H, H-2'-H-6'), 10.94 (s, 1H, CONH); MS (m/z): 174 (M^+), 159, 147, 130, 115, 109.

Ethyl-3-oxo-6-phenyl-2,3,4,5-tetrahydropyridazin-2-acetate (**3**)

IR: (KBr, cm^{-1}): 3085 (NH), 1626 (C=O), 1375 (CH_3), 1219 (C-O), 743 (CH_2); ^{1}H-NMR (DMSO-d_6, δ ppm): 1.2 (t, 3H, CH_3), 2.63 (t, 2H, CH_2), 3.0 (t, 2H, CH_2), 4.2 (q, 2H, $COOCH_2$), 4.59 (s, 2H, N-CH_2-COO), 7.28-7.79 (m, 5H, Ar-H); MS (m/z): 260 (M^+), 247, 244, 218, 186, 173.

3-Oxo-6-phenyl-2,3,4,5-tetrahydropyridazinyl acetohydrazides (**4**)

IR: (KBr, cm^{-1}): 3439 (NH_2 stretching), 2925, 1619 (NH bending), 1612 (C=O); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.34 (s, 2H, NH_2), 2.61 (t, 2H, CH_2), 2.79 (s, 2H, CH_2), 2.96 (t, 2H, CH_2), 7.25-7.66 (m, 5H, Ar-H), 8.52 (s, 1H, CONH).

6-Phenyl-2-{[(4'-phenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5a**)

IR: (KBr, cm^{-1}): 3400 (NH), 1658(C=O), 1560(C-N), 1416(C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.053 (t, 2H, CH_2), 2.46 (s, 2H, CH_2), 2.95 (t, 2H, CH_2CO), 7.16-7.75 (m, 10H, Ar-H), 10.717 (s, 1H, CSNH).

6-Phenyl-2-{[(4'-p-chlorophenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5b**)

IR: (KBr, cm^{-1}): 3439.45 (NH), 1679 (C=O), 1642 (C-N), 1415 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.4 (t, 2H, CH_2), 2.44 (s, 2H, CH_2), 2.94 (t, 2H, CH_2), 7.41-7.75 (m, 9H, Ar-H), 10.96 (s, 1H, CSNH).

6-Phenyl-2-{[(4'-p-bromophenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5c**)

IR: (KBr, cm^{-1}): 3415 (NH), 1683 (C=O), 1565 (C-N), 1432 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.44 (t, 2H, CH_2), 2.5 (s, 2H, CH_2), 2.94 (t, 2H, CH_2), 7.41-7.75 (m, 9H, Ar-H), 10.96 (s, 1H, CSNH).

6-Tolyl-2-{[(4'-phenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5d**)

IR: (KBr, cm^{-1}): 3504 (NH), 1670 (C=O), 1548 (C-N), 1420 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm):

Table 2. Physical constants of the synthesized compounds.

Compound	R	R'	Formula Molecular weight	M.P.	% Yield
5a	H	H	$C_{19}H_{18}N_5OS$, 364.0	185°C	75
5b	H	Cl	$C_{19}H_{17}ClN_5OS$, 398.5	172°C	78
5c	H	Br	$C_{19}H_{17}BrN_5OS$, 442.5	165°C	69
5d	CH_3	H	$C_{20}H_{19}N_5OS$, 380.0	175°C	72
5e	CH_3	Cl	$C_{20}H_{19}ClN_5OS$, 412.5	186°C	79
5f	CH_3	Br	$C_{20}H_{19}BrN_5OS$, 456.8	190°C	64
5g	Cl	Cl	$C_{19}H_{16}Cl_2N_5OS$, 432.0	175°C	76
5h	Cl	Br	$C_{19}H_{17}BrClN_5OS$, 467.5	185°C	72
5i	Br	H	$C_{19}H_{17}BrN_5OS$, 473.0	170°C	84
5j	Br	Cl	$C_{19}H_{16}BrClN_5OS$, 467.5	183°C	83
5k	OCH_3	Br	$C_{20}H_{19}BrN_5O_2S$, 473.0	188°C	80
5l	C_6H_5O	Br	$C_{25}H_{21}BrN_5O_2S$, 531.0	178°C	62

2.08 (s, 3H, CH_3), 2.43 (t, 2H, CH_2), 2.5 (t, 2H, CH_2), 2.92 (t, 2H, CH_2CO), 7.21-7.23 (dd, *J* = 7.8, H-3', H-5'), 7.34-7.40 (m, 5H, Ar-H), 7.61-7.64 (dd, *J* = 7.8, H-2', H-6'), 10.66 (s, 1H, CSNH).

6-Tolyl-2-{[(4'-p-chlorophenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5e**)

IR: (KBr, cm^{-1}): 3392 (NH), 1638 (C=O), 1543 (C-N), 1410 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.32 (m, 2H, CH_2), 2.42 (s, 3H, CH_3), 2.98 (m, 2H, CH_2CO), 7.01-7.86 (m, 8H, Ar-H), 10.90 (s, 1H, CSNH); MS (m/z): 411(M^+).

6-Tolyl-2-[{(4'-p-bromophenyl-5'-thioxo)-1,2,4-triazol-3-yl}-methyl]-2,3,4,5-tetrahydropyridazin-3-one (**5f**)

IR: (KBr, cm^{-1}): 3410 (NH), 1634 (C=O), 1555 (C-N), 1450 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.32 (t, 2H, CH_2), 2.37 (s, 2H, CH_2), 2.5 (s, 3H, CH_3), 2.96 (t, 2H, CH_2CO), 7.13-7.85 (m, 8H, Ar-H), 10.89 (s, 1H, CSNH), 2.32 (t , 2H , CH_2), 2.37 (s, 2H, CH_2), 2.5 (s, 3H, CH_3), 2.96 (t, 2H, CH_2CO), 7.13-7.85 (m, 8H, Ar-H), 10.89 (s, 1H, CSNH).

6-(4'-Chlorobenzene)-2-[{(4'-p-chlorophenyl-5'-thioxo)-1,2,4-triazol-3-yl}-methyl]-2,3,4,5 tetrahydropyridazin-3-one (**5g**)

IR: (KBr, cm^{-1}): 3400 (NH), 1684 (C=O), 1600 (C-N), 1454 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.42 (t, 2H, CH_2), 2.50 (s, 2H, N-CH_2), 2.50 (s, 2H, N-CH_2), 2.939 (t, 2H, CH_2CO), 7.32-7.83 (dd, 4H, Ar-H), 10.98 (s, 1H, CSNH).

6-(4'-Chlorobenzene)-2-{[(4'-p-bromophenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5h**)

IR: (KBr, cm^{-1}): 3400 (NH), 1679 (C=O), 1591 (C-N), 1492 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.45 (t, 2H, CH_2), 2.50 (s, 2H, CH_2), 2.939 (t, 2H, CH_2CO), 7.46-8.18 (m, 8H, Ar-H), 10.98 (s, 1H, CSNH).

6-(4'-Bromobenzene)-2-{[(4'-phenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5i**)

IR: (KBr, cm^{-1}): 3425 (NH), 1672 (C=O), 1554 (C-N), 1424 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.4 (t, 2H, CH_2), 2.507 (s, 2H, N-CH_2), 2.959 (t, 2H, CH_2CO), 7.12-7.73 (m, 9H, Ar-H), 10.73 (s, 1H, CSNH).

6-(4'-Bromobenzene)-2-{[(4'-p-chlorophenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5 tetrahydropyridazin-3-one (**5j**)

IR: (KBr, cm^{-1}): 3430 (NH), 1 665 (C=O), 1534 (C-N), 1398 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.46 (t, 2H, CH_2), 2.506 (s, 2H, N-CH_2), 2.96 (t, 2H, CH_2CO), 7.34-7.76 (m, 8H, Ar-H), 10.72 (s, 1H, CSNH).

6-(4'-Anisyl)-2-{[(4'-p-bromophenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5k**)

IR: (KBr, cm^{-1}): 3400 (NH), 1684 (C=O), 1600 (C-N), 1554 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.43 (t, 2H, CH_2),2.507 (s, 2H, N-CH_2), 2.86 (m, 2H, CH_2), 3.67 (s, 3H, OCH_3), 7.0-7.9 (m, 8H, Ar-H), 10.86 (s, 1H, CSNH).

6-(4'-Phenoxyphenyl)-2-{[(4'-p-bromophenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl}-2,3,4,5-tetrahydropyridazin-3-one (**5l**)

IR: (KBr, cm^{-1}): 3400 (NH), 1691 (C=O), 1588 (C-N), 1490 (C=S); ^{1}H-NMR (DMSO-d_6, δ ppm): 2.40 (t, 2H, CH_2), 2.506 (s, 2H, N-CH_2), 2.92 (t, 2H, CH_2-CO), 7.01-7.98 (m, 13H, Ar-H), 10.90 (s, 1H, CSNH).

Biological evaluation

The determination of *in vitro* antifungal activity of the compounds tested was performed using the microdilution method, according to the National Committee for Clinical Laboratory Standards (NCCLS) (12-14).

The micro organisms used were: *Candida albicans, Trichophyton rubrum, Aspergillus flavus, Aspergillus niger* and *Penicillium citrinium*. The majority of strains under study were clinical isolates. The MIC of the compounds was determined by broth microdilution method and was compared with that of the standard drug voriconazole.

Microdilution assays

The minimal inhibitory concentration (MIC) values for all compounds was tested and defined as the lowest concentration of the compound preventing the visible growth. The inocula of fungal species were prepared prior to the experiment. The test compounds dissolved in dimethyl sulfoxide (DMSO) were first diluted to the highest concentration of 20 µg/mL. The serial fold dilutions were made in concentration ranges from 0.10 µg/mL to 20 µg/mL in 10 mL sterile test tubes. 100 µL of inoculum were inoculated in the different concentration of test compounds. After keeping in the incubator for three days 50 µL medium of the test drug were spread on the Petri plates containing solidified medium and again kept in the incubator for three days. Growth (or its lack) of microorganism was determined visually after incubation for three days at 37°C. The lowest concentration at which there was no visible growth was taken as MIC.

The MIC values in µg/mL are given in Table 1. All investigations were carried out in the Microbiology Laboratory, Faculty of Pharmacy, Jamia Hamdard, New Delhi.

RESULTS AND DISCUSSION

Chemistry

6-Substituted-2-{[(4'-substituted-phenyl-5'-thioxo)-1,2,4-triazol-3-yl]-methyl]-2,3,4,5-tetrahydropyridazin-3-one derivatives **(5a-5l)** described in this study are shown in Table 2**,** and the reaction sequence for the preparation is outlined in Scheme 1. In case of β-substituted benzoyl propionic acids the hydrogen of COOH group was present at 12.17 and these from two CH_2 groups were present at 2.59 and 3.23 ppm, respectively. The aromatic region describes at 7.53-7.62 a multiplet and a doublet at 7.97. The IR spectra confirm the position of COOH at 3428 cm^{-1} and C=O at 1679 cm^{-1}, respectively. The purity of compounds was checked by TLC. Both analytical and spectral data (^{1}H-NMR, IR) of all the compounds were in full agreement with the proposed structures. In the ^{1}H-NMR spectra the signals of the respective protons of the prepared compounds were verified on the basis of their chemical shifts, multiplicities and coupling constant.

The acid (**1**) reacted with hydrazine hydrate to give pyridazinone and the presence of C=O group at 1619 cm^{-1} and NH group at 3206 cm^{-1} confirms the structure of pyridazinone formed in step 2. Futher, the pyridazinones reacted with ethylbromoacetate in ethanolic solution of Na to give bromoester derivative of pyridazinone. The IR spectra showed the bands at 1375 cm^{-1} and 743 cm^{-1} due to the presence of CH_3 and CH_2 group, respectively. At 1219 cm^{-1} C-O stretching and at 1626 cm^{-1} C=O stretching are the confirmation of ester derivative. This ester derivative was further hydrazinolyzed to give respective acetohydrazides. The NH bending at 1619 cm^{-1} and NH_2 streching at 3439 cm^{-1} confirms the presence of NH and NH_2 group, respectively. The above final acetohydrazide was treated with semicarbazide to give intermediate compound which in the presence of aqueous NaOH and after acidification with glacial acetic acid yielded respective 1,2,4-triazoles.

The pyridazinone containing 1,2,4-triazoles was confirmed by NMR and IR spectral data. The triplets of CH_2-CO were observed at 2.95 ppm and singlet of C=S-NH at 10.17 pm. It is interesting to note that the C=S group was confirmed by the appearance of band at position 1416 cm^{-1} and the absence of absorption in the region 2500-2650 cm^{-1} for SH stretching. The C=N absorption band was present at 1560 cm^{-1} region. The presence of absorption band at 1658 cm^{-1} confirms the position of C=O group.

***In vitro* antifungal activity**

The antifungal activities of the tested compounds against different fungal species were carried out by microdilution method. The results were compared with the standard drug – voriconazole. The minimum inhibitory concentration of voriconazole

for all the fungal species were lower than 0.5 μg/mL. MIC values of the tested compounds are listed in Table1**.**

Twelve compounds tested in the present study were found to have significant antifungal activities against all the fungal species. The chloro substituent derivative (compound **5g)** showed the highest activity against all the fungal species. The MIC of the standard drug voriconazole was between 0.10 and 0.50 μg/mL against all the fungal species. The two electronegative groups of Cl were increasing the activity of 1,2,4-triazole. As we increased the bulky group or aromatic group on benzene ring, the activity was decreased as in case of compound **5l**.

Conclusion

It is an easy method to synthesize antifungal agents having 1,2,4-triazole group attached to pyridazinone nucleus. The tested compounds showed comparable results with standard drug voriconazole. Further optimization of the chemical synthesis can possibly lead to more active molecules against fungal infections. Since all twelve compounds showed promising results, studies to establish their *in vivo* efficacy will be carried in the future.

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