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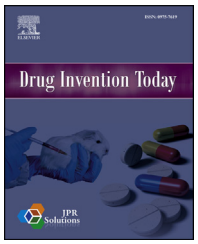

Original Article

Antifungal activities of novel 1,2,3-benzotriazole derivatives synthesized by ultrasonic and solvent-free conditions

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ABSTRACT

Objectives: To evaluate the antifungal activities of novel 1,2,3-benzotriazole derivatives synthesized by ultrasonic and solvent-free conditions.

Methods: Newer "1-(1H-benzo[d][1,2,3]triazole-1-carbonyl) derivatives" (5A–5P) were synthesized by using "1H–benzo[d][1,2,3]triazole" (1) as the starting material under ultrasonicated and solvent-free conditions. The resulting products were isolated and characterized by melting points and spectral studies. All the products were assayed for antifungal activity for various pathogenic fungi.

Results: Excellent antifungal activity was shown by derivative-5L against *Candida albicans* (MTCC – 3018) whereas other compounds have shown comparable activity. Except derivative-5P, all synthesized compounds have shown mild activity against *Candida glabrata* (MTCC – 3019). Towards *Aspergillus niger* (MTCC – 2638) and *Aspergillus flavus* (MTCC – 2737) most of the compounds were inactive and some were feebly active. All the synthesized derivatives were inactive against *Saccharomyces cerevisiae* (MTCC – 170). The Minimum Inhibitory Concentrations (MIC) of the most of the synthesized 1,2,3-benzotriazole derivatives for these fungi were found to be 62.5 μg/ml.

Conclusions: Some of the newer 1,2,3-benzotriazole derivatives synthesized under solvent-free and ultrasound irradiation with noteworthy advantages viz., shorter reaction times, operational simplicity, simple work-up, and eco-friendly nature, have shown antifungal activities against selected pathogenic strains. Attachment of phenyl or phenyl with electron withdrawing substituents to either nitrile or azo functional group can be attributed to the substantial antifungal activity of these benzotriazoles.

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1. Introduction

Although number of drugs are available in the market, but the need of discovering the new antimicrobial drugs with better pharmacokinetic profile and lesser toxicity has become the main objective in the field of medicinal chemistry, it is also due to the fast microbial resistance to the existing molecules.[1]

A large number of compounds containing benzotriazole system have been investigated because of their broad spectrum of biological activities which include analgesic, antibacterial, antifungal, antiparasitic, antiviral, anti-inflammatory, anticonvulsant, anti-nociceptive, DNA cleavage, herbicidal, antitubercular, antiemetic, protein kinase inhibition, respiratory syndrome protease inactivation, an active ester in the peptide synthesis and agonists of peroxisome proliferator activated receptors.[2,3] In addition to these considerable biological applications, benzotriazoles are important intermediates, protecting groups and final products in organic synthesis.[4]

A large number of organic reactions have been carried out in higher yield, shorter reaction time and milder condition under ultrasound irradiation. Large-scale use of organic solvents in synthesis causes environmental hazards. There were several advantages of performing syntheses in solvent-free media, such as, short reaction time, increased safety, and low cost.[2] In the present study, newer 1,2,3-benzotriazole derivatives were synthesized by ultrasonicated solvent-free conditions and their antifungal activities were evaluated.

2. Materials and methods

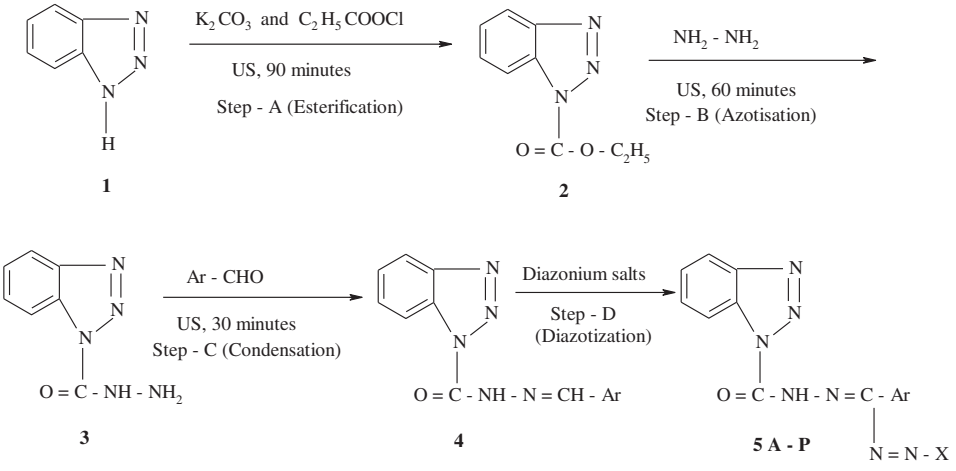
All organic solvents and chemicals were purchased from SD Fine Chemicals Ltd., Mumbai and were of analytical grade. For synthesis of benzotriazole derivatives, a 12 mm wide and 140 mm long probe (of a UP 400S ultrasonic processor) was immersed directly into the reaction mixture at room temperature. The operating frequency and the output power were 24 kHz and 240 W respectively. The synthesized compounds were characterized by spectral studies using Perkin Elmer 1600 series Fourier Transformer-Infrared Spectrophotometer in KBr-Pellet method; ^{1}H NMR spectra by Bruker 400 MHz NMR spectrometer (Bruker Bioscience, Billerica, MA, USA) in MeOD using TMS as internal standard.

2.1. Scheme for the synthesis of the compounds

By suitable modifications to the classical synthesis carried out by other workers viz., Asati et al,[5] Chitre et al,[6] Sukla and Srivatsava,[7] sixteen compounds were synthesized under ultrasonication and solvent-free conditions in four steps (Step A – esterification, Step B – azotisation, Step C – condensation, Step D – diazotization) as shown in Fig. 1.

2.2. Antifungal activity by disc diffusion method and MIC for fungi

All the synthesized compounds of present study were screened for *in-vitro* antifungal activity against five different strains of fungi i.e., *Aspergillus niger* MTCC – 2638, *Aspergillus flavus* MTCC – 2737, *Candida albicans* MTCC – 3018, *Candida glabrata* MTCC – 3019 and *Saccharomyces cerevisiae* MTCC – 170 by paper disc diffusion method.[8] Whatmann filter paper grade-1 discs of 5 mm diameter were sterilized by autoclaving for 15 min at 121 °C. The synthesized compounds were dissolved in DMSO at the concentration level of 100 μg/ml and the sterile discs were impregnated with these synthesized compounds. The nutrient agar of 20 ml was placed in flat bottomed Petri dish. When solidified 4 ml of second nutrient solution seeded with test fungi was poured evenly on to the first layer [40–48 °C, 15 lb/in^2]. As soon as the second layer was solidified,



5A – D: Ar = C_6H_5 ; 5E – H: Ar = C_4H_3O ; 5 I – L: Ar = $C_6H_4NO_2$ and 5M – P: Ar = C_6H_4Cl

5A, E, I, M: X = C_6H_5 ; 5B, F, J, N: X = $C_6H_4NO_2$; 5C, G, K, O: X = C_6H_4Cl and 5D, H, L, P: X = C_6H_4Br

Note : US = Ultrasonication

Fig. 1 – Scheme of Synthesis.

the impregnated discs were placed on the medium suitably spaced apart and plates were transferred to an incubator at 37 °C for 48 h and zones of inhibition, if any, around the discs were recorded. Nystatin was used as reference drug for fungi. An additional control disc without any sample but impregnated with an equivalent amount of solvent (DMSO) was also used. The Minimum Inhibitory Concentration (MIC) study was carried out at different concentrations of the synthesized compounds such as 31.25, 62.5 and 125 μg/ml.

3. Results and discussion

3.1. *Characterization of newer 1,2,3-benzotriazole derivatives*

All newer "1-(1H-benzo[d][1,2,3]triazole-1-carbonyl) derivatives" (1,2,3-benzotriazole derivatives) synthesized by solvent-free ultrasound activation were characterized by melting points and spectral studies.

3.1.1. *Melting point*

Melting points of the synthesized derivatives were determined by an open-end capillary tube method and were uncorrected. Molecular formulae, molecular weights, melting points and yields of the synthesized derivatives were given in Table 1.

3.1.2. *Spectral analysis*

FTIR and ^{1}H NMR values were measured in cm^{-1} and δ (ppm) respectively. The data of FTIR and ^{1}H NMR were interpreted with reference to standard values[9] and interpreted data for some of compounds given below.

❖1-(1H-benzo[d][1,2,3]triazole-1-carbonyl)-3,5-diphenylformazan (5A) IR (KBr, cm^{-1}):

1696.66 (Ar C═C, stretch); 1603.37 (N═N, stretch), 1542.28 (N–H, stretch), 1256.37 (Aryl C–N, stretch), 1007.57 (Aniline C–N, stretch) and 737.28 (CHO–deformation); ^{1}H NMR (400 MHz) (MeOD) δ (ppm): 7.0 (1H, s, N–H), 7.06 (1H, d, C–H), 7.33 (2H, d, C–H), 7.40 (2H, d, C–H), 7.45–7.48 (2H, m, C–H), 7.56–7.59 (3H, m, C–H), 7.83 (2H, d, C–H) and 7.96 (2H, d, C–H).

❖1-(1H-benzo[d][1,2,3]triazole-1-carbonyl)-5-(4-nitrophenyl)-3-phenyl formazan (5B) IR (KBr, cm^{-1}):

1698.47 (Ar C═C, stretch); 1593.99 (N═N, stretch), 1495.50 (Ar–NO_2, stretch), 1301.09 (Aryl C–N, stretch), 1206.78 (Aniline C–N, stretch), 843.07 (p – disubstitution, stretch) and 739.50 (CHO – deformation); ^{1}H NMR (400 MHz) (MeOD) δ (ppm): 7.0 (1H, s, N–H), 7.18 (2H, d, C–H), 7.40 (2H, d, C–H), 7.52–7.59 (3H, m, C–H), 7.83 (2H, d, C–H), 7.96 (2H, d, C–H) and 8.10 (2H, d, C–H).

❖1-(1H-benzo[d][1,2,3]triazole-1-carbonyl)-5-(4-chlorophenyl)-3-(furan-2-yl) formazan (5G) IR (KBr, cm^{-1}):

3649.31 (Amide–CONH, stretch), 1594.63 (N═N, stretch), 1485.68 (Furan Ring, C═C, stretch), 1206.43 (Aniline C–N, stretch), 1007.96 (C–O–C, stretch), 820.27 (p – disubstitution, stretch), 740.62 (CHO deformation, stretch) and 539.68 (C–Cl); ^{1}H NMR (400 MHz) (MeOD) δ (ppm): 6.52 (1H, m, Furan C–H), 6.54 (1H, d, Furan C–H), 7.0 (1H, s, N–H), 7.27 (2H, d, C–H), 7.40 (2H, d, C–H), 7.49 (2H, d, C–H), 7.75 (1H, d, Furan C–H) and 7.96 (2H, d, C–H).

❖1-(1H-benzo[d][1,2,3]triazole-1-carbonyl)-5-(4-bromophenyl)-3-(furan-2-yl) formazan (5H) IR (KBr, cm^{-1}):

3652.10 (Amide–CONH, stretch), 1722.17 (Furan Ring, stretch), 1622.31 (N═N, stretch), 1511.18 (Ar C═C, stretch), 1457.07 (Furan Ring C═C, stretch), 1202.93 (Aniline C–N, stretch), 1005.36 (C–O–C, stretch), 875.16 (p – disubstitution, stretch), 773.52 (CHO deformation, stretch) and 515.35 (C–Br, stretch); ^{1}H NMR (400 MHz) (MeOD) δ (ppm): 6.52 (1H, m, Furan C–H), 6.54 (1H, d, Furan C–H), 7.0 (1H, s, N–H), 7.22 (2H, d, C–H), 7.40 (2H, d, C–H), 7.75 (1H, d, Furan C–H), 7.76 (2H, d, C–H) and 7.96 (2H, d, C–H).

Table 1 – Physical data of synthesized 1,2,3-benzotriazole derivatives.

S. no.	Compound code	Molecular formula	Molecular weight	Melting point (°C)	Yield (%)
1.	5 A	$C_{20}H_{15}N_7O$	369.4	99	82
2.	5 B	$C_{20}H_{14}N_8O_3$	414.4	96	79
3.	5 C	$C_{20}H_{14}ClN_7O$	403.8	99	81
4.	5 D	$C_{20}H_{14}BrN_7O$	448.3	98	82
5.	5 E	$C_{18}H_{13}N_7O_2$	359.3	88	79
6.	5 F	$C_{18}H_{12}N_8O_4$	404.3	93	72
7.	5 G	$C_{18}H_{12}ClN_7O_2$	393.8	95	73
8.	5 H	$C_{18}H_{12}BrN_7O_2$	438.2	97	72
9.	5 I	$C_{20}H_{14}N_8O_3$	414.4	101	71
10.	5 J	$C_{20}H_{13}N_9O_5$	459.4	102	79
11.	5 K	$C_{20}H_{13}ClN_8O_3$	448.8	99	75
12.	5 L	$C_{20}H_{13}BrN_8O_3$	493.3	103	77
13.	5 M	$C_{20}H_{14}ClN_7O$	403.8	80	72
14.	5 N	$C_{20}H_{13}ClN_8O_3$	448.8	82	77
15.	5 O	$C_{20}H_{13}Cl_2N_7O$	438.3	100	82
16	5 P	$C_{20}H_{13}BrClN_7O$	482.7	95	81

❖**1-(1H-benzo[d][1,2,3]triazole-1-carbonyl)-3-(4-chlorophenyl)-5-phenyl formazan (5M)** IR (KBr, cm^{-1}):

3650.62 (Amide–CONH), 1706.94 (Ar, C=C, stretch), 1593.76 (N=N, stretch), 1513.71 (N–H, stretch), 1264.35 (Aryl C–N, stretch), 1204.85 (Aniline C–N, stretch), 820.56 (p–disubstitution, stretch), 772.75 (CHO – deformation, stretch) and 605.30 (C–Cl, stretch); ^{1}H NMR (400 MHz) (MeOD) δ (ppm): 7.0 (1H, s, N–H), 7.06 (1H, d, C–H), 7.33 (2H, d, C–H), 7.40 (2H, d, C–H), 7.45 (2H, d, C–H), 7.52 (2H, d, C–H), 7.77 (2H, d, C–H) and 7.96 (2H, d, C–H).

❖**1-(1H-benzo[d][1,2,3]triazole-1-carbonyl)-3,5-bis(4-chlorophenyl) formazan (5O)** IR (KBr, cm^{-1}):

1710.45 (Ar, C=C, stretch), 1593.50 (N=N, stretch), 1486.45 (N–H, stretch), 1256.86 (Aryl C–N, stretch), 1143.95 (Aniline C–N, stretch), 827.70 (p – disubstitution, stretch), 773.01 (CHO – deformation, stretch) and 620.81 (C–Cl, stretch); ^{1}H NMR (400 MHz) (MeOD) δ (ppm): 7.0 (1H, s, N–H), 7.27 (2H, d, C–H), 7.40 (2H, d, C–H), 7.49 (2H, d, C–H), 7.52 (2H, d, C–H), 7.77 (2H, d, C–H) and 7.96 (2H, d, C–H).

3.2. *Antifungal assay and MIC of synthesized derivatives*

The zones of inhibitions (mm) and Minimum Inhibitory Concentrations of tested compounds against pathogenic strains were shown in Tables 2 and 3 respectively. The experimental result indicated variable degree of efficacy of the compounds against different strains of fungi (Fig. 2).

4. Discussion

The heterocyclic system containing benzotriazole moieties system is of wide interest because of their diverse biological activities including pharmacological properties and clinical applications.[2] Recently, the expansion of antifungal drug research has occurred because there is a critical need for new antifungal agents to treat these life threatening invasive fungal infections.

The frequency of invasive and systemic fungal infections during the past two decades has increased dramatically due to mainly *Candida* species.[10] *C. albicans* is the main causative fungi of the systemic mycosis and symptomatic vaginal candidiasis in about 75% of the sexually active women.[11] It infects the mucous membranes leading to thrush (an infection of the throat and mouth) and exhibits commensalism by colonizing skin, the gastrointestinal and the reproductive tracts.[12] Against such a pathogenic fungal strain *C. albicans* (MTCC – 3018), exceptionally high antifungal activity i.e., 1.8 fold activity was shown by derivative-5L (zone diameter-24.9 mm) compared to the reference compound Nystatin (zone diameter – 13.9 mm). Other compounds except 5E, have shown 1.5 to 1.1 fold activity compared to the reference compound Nystatin.

Considerable antifungal activity of these benzotriazoles can be contributed to phenyl or electron withdrawing pharmacophore (nitro/chloro/bromo) substituted phenyl on either nitrile or azo functional group. The best activity of the molecules containing a phenyl group on each of the pendant arms of the conjugated chain can be explained based on large and rigid

Table 2 – Antifungal activity of 1,2,3-benzotriazole derivatives.

S. no.	Compound code	Antifungal activity (in mm)[a] For disks soaked in 100 μg/ml solutions of compounds				
		Candida albicans MTCC – 3018	*Aspergillus niger* MTCC – 2638	*Aspergillus flavus* MTCC – 2737	*Candida glabrata* MTCC – 3019	*Saccharomyces cerevisiae* MTCC – 170
1.	5 A	17.5	–	–	8.0	–
2.	5 B	16.8	–	–	8.0	–
3.	5 C	17.1	–	9.6	10.2	–
4.	5 D	18.2	–	8.5	10.4	–
5.	5 E	–	18.7	–	10.9	–
6.	5 F	16.2	15.5	–	10.5	–
7.	5 G	20.0	–	–	11.2	–
8.	5 H	19.0	–	–	9.5	–
9.	5 I	20.7	–	7.7	10.4	–
10.	5 J	17.5	–	–	9.2	–
11.	5 K	15.4	–	12.4	11.9	–
12.	5 L	24.9	–	10.9	9.7	–
13.	5 M	16.5	–	–	9.9	–
14.	5 N	18.4	–	–	6.3	–
15.	5 O	20.2	–	–	6.3	–
16.	5 P	18.2	–	–	–	–
	Nystatin	13.9	22.4	21.2	20.3	15.2
	Control (10%DMSO In Methanol)	–	–	–	–	–

(–) Indicates no zone of inhibition.
a Indicates average of triplicate.

Table 3 – Minimum Inhibitory Concentrations for fungi (μg/ml).

S. no.	Compound code	*Aspergillus niger* MTCC – 2638	*Aspergillus flavus* MTCC – 2737	*Candida albicans* MTCC – 3018	*Candida glabrata* MTCC – 3019	*Saccharomyces cerevisiae* MTCC – 170
1.	5 A	–	–	62.5	62.5	–
2.	5 B	–	–	62.5	62.5	–
3.	5 C	–	62.5	62.5	31.25	–
4.	5 D	–	62.5	31.25	31.25	–
5.	5 E	62.5	–	–	62.5	–
6.	5 F	62.5	–	31.25	62.5	–
7.	5 G	–	–	62.5	62.5	–
8.	5 H	–	–	15.62	31.25	–
9.	5 I	–	62.5	62.5	62.5	–
10.	5 J	–	–	31.25	62.5	–
11.	5 K	–	31.25	62.5	62.5	–
12.	5 L	–	31.25	31.25	62.5	–
13.	5 M	–	–	125	125	–
14.	5 N	–	–	62.5	125	–
15	5 O	–	–	31.25	125	–
16	5 P	–	–	62.5	–	–

character that the phenyl groups confer on the molecule. Hydrophobic molecules with rigid, planar structures such as aromatic rings, have been shown to have the ability to insert into membranes and induce localized permeability changes leading to leakage out of the membrane.[13] Subtle alteration by addition of a nitro group affecting the charge distribution confers significant improvements due to in biological effects. The enhanced inhibition observed in the presence of nitro group is then more likely its interaction with some intracellular target. The presence of a strong electron withdrawing group must alter the nature of the compound in such a way as to promote binding to the target(s).[14] The electron density from the σ space of benzene ring is removed by electron withdrawing substituents like halogens, which leads to a decrease in the energy of HOMO and hence, the presence of electron withdrawing substituents on the benzene ring should increase the activity.[15] Halogen/nitrogroup on substituents of benzotriazole derivatives form hydrogen bonds with azomethine group with the active centres of the cell constituents.[16] Good activity against *C. albicans* was reported by Suma et al[17] for the chloro substituted benzotriazole containing nitrophenyl group on pyrazolidine dione moieties. Literature survey shows that halo and nitro groups at ortho and/or para positions of the phenyl ring exhibit significant antifungal activity.[18]

C. glabrata has recently emerged as an important nosocomial pathogen. It may present as a limbal keratitis in the setting of systemic infection.[19] Among the synthesized derivatives only 5P was ineffective against *C. glabrata* (MTCC – 3019) whereas others have shown mild activity compared to the reference compound – Nystatin.

Food borne pathogens such as *A. niger* and *A. flavus* are widely distributed in nature, causing considerable mortality and morbidity in the population.[20] *A. niger*, if inhaled with large amounts of spores, causes a serious lung disease, aspergillosis and it is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss and, in severe cases, damage to the ear canal and tympanic membrane.[21] In the present study, against such a pathogenic *A. niger* (MTCC – 2638), moderate activity was shown by derivatives-5E and 5F (zone diameters 18.7 and 15.5 mm respectively) compared to the reference compound – Nystatin (zone diameter 22.4 mm), whereas others were ineffective. In both 5E and 5F, furfuryl was attached to nitrile group of benzotriazole derivatives. Furfuryl substitution in indolyl pyrimidine carbohydrazides also improved the activity against *A. niger*.[22]

A. flavus produce aflatoxin, which can cause acute hepatitis, immunosuppression, and hepatocellular carcinoma. The fungus is also an opportunistic animal and human pathogen

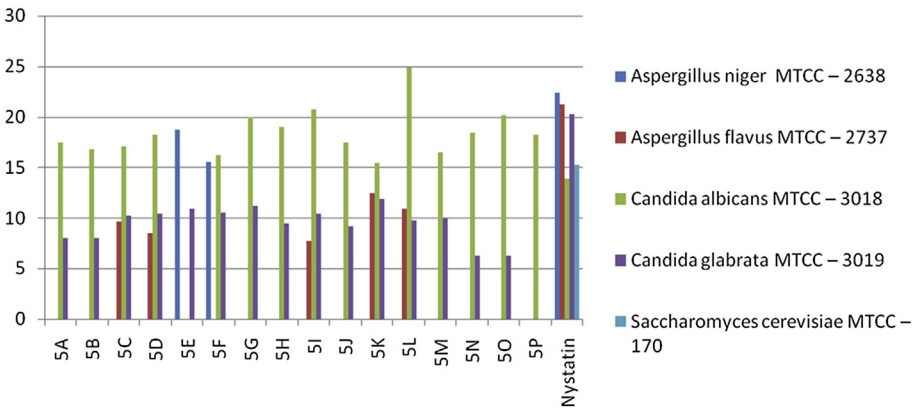


Fig. 2 – Antifungal activity of synthesized 1,2,3-benzotriazoles.

causing aspergillosis diseases with incidence increasing in the immunocompromised population.[23] Against *A. flavus* (MTCC – 2737), mild activity was shown by benzotriazole derivatives (5K, 5L, 5C, 5D and 5I) in which nitrophenyl and halogen substituted phenyl group were attached to nitrile and azo functional groups respectively, whereas others were ineffective. Inhibition zone diameters of derivatives - 5K, 5L, 5C, 5D and 5I were found to be 12.4, 10.9, 9.6, 8.5 and 7.7 mm respectively, whereas that of reference compound (Nystatin) was 21.2 mm. These results show that the synthesized derivatives were not competitive to the commercial antifungal agent – Nystatin.

Though, *S. cerevisiae* has been a very useful fungus for humans viz., bread making and spirit-making, it has now been demonstrated that this yeast can cause different forms of invasive infection in particularly, after administration as a probiotic for the treatment of antibiotic-related diarrhoea[24]and hence, this organism has been noted to be a human pathogen in immunocompromised patients.[25] The present experimental studies shows that all synthetic derivatives (5A–5P) exhibited lower zones of inhibition against *S. cerevisiae* (MTCC – 170) compared to the reference compound – Nystatin which conclude that these synthesized derivatives were inactive against *S. cerevisiae*.

The Minimum Inhibitory Concentrations (MIC) of the synthesized 1,2,3-benzotriazole derivatives for fungi were found to be 62.5 μg/ml for most of the synthesized compounds.

5. Conclusions

Except 5E, all the synthesized 1,2,3-benzotriazole derivatives were found to be effective antifungal agents against *C. albicans* (MTCC – 3018) and 5L can be concluded as an excellent antifungal agent compared to the reference compound Nystatin. All synthesized compounds except derivative-5P have shown mild activity against *C. glabrata* (MTCC – 3019). Moderate activity was shown by derivatives-5E and 5F against *A. niger* (MTCC – 2638), whereas others were ineffective. In view of either mild activity or ineffectiveness of the synthesized derivatives against *A. flavus* (MTCC – 2737), they can be considered as non-competitive to the commercial antifungal agent–Nystatin. Against *S. cerevisiae* (MTCC – 170), all the synthesized derivatives were inactive. The Minimum Inhibitory Concentrations (MIC) of the most of the synthesized 1,2,3-benzotriazole derivatives for these fungi were found to be 62.5 μg/ml.

Finally in conclusion, 1,2,3-benzotriazole derivatives synthesized under solvent-free and ultrasound irradiation with noteworthy advantages viz., shorter reaction times, operational simplicity, simple work-up, and eco-friendly nature, have shown antifungal activities against selected pathogenic strains. Attachment of phenyl or phenyl with electron withdrawing substituents to either nitrile or azo functional group can be attributed to the substantial antifungal activity of these benzotriazoles.

Conflicts of interest

All authors have none to declare.

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