Original Article

Microwave-Assisted Synthesis And Antifungal Evaluation Of 1,2,4- Triazole Derivatives

Rani S. Kankate[1*], Vrushali Pawar[2], Pooja Abhang[3], Kamini Bhalerao[4], Yogesh Wankhede[5], Eknath D. Ahire[6], Sanjay J. Kshirsagar[7]

[1*]Assistant Professor, Department of Pharmaceutical Chemistry, MET's Institute of Pharmacy, Bhujbal Knowledge City, Adgaon, Nashik 422003, Maharashtra, India

[2,3,4,5]Department of Pharmaceutical Chemistry, MET's Institute of Pharmacy, Bhujbal Knowledge City, Adgoan, Nashik 422003, Maharashtra, India

[6,7]Department of Pharmaceutics, MET's Institute of Pharmacy, Bhujbal Knowledge City, Adgaon, Nashik 422003, Maharashtra, India

***Corresponding Authors:-** Dr. Rani S. Kankate, Assistant Professor
*Department of Pharmaceutical Chemistry, MET's Institute of Pharmacy, Bhujbal Knowledge City, Adgoan, Nashik 422003,
Maharashtra, India E-mail: ranipharmacy@gmail.com
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Abstract

A Pharmaceutical Fungicide Or Fungistatic Used To Treat And Prevent Mycoses Such As Athlete's Foot, Ringworm, Candidiasis (Thrush), Serious Systemic Infections Such As Cryptococcal Meningitis, And Others Is Known as An Antifungal Medicine, Sometimes Known as An Antimycotic Medication. A Series Of 1,2,4 Triazole Analogs Have Been Designed Synthesized Characterized And Screened For A Wide Variety Of Therapeutically Interesting Drug Candidates Including Antifungal Agents, Anti-Inflammatory, Central Nervous System Stimulants, Antianxiety And Antimicrobial Agents. Among Screened Compound N-((4-P-Toluidine Methyl) Piperazine -1-Yl) Methyl)-4H-1,2,,4-Triazole -3-Amine Mono, Di Or Tri-Substituted Aniline Derivative Were Synthesized By Microwave Irradiation And Characterized By Proton Nuclear Magnetic Resonance (^{1}H NMR), Infra-Red(IR), And Mass Spectroscopy (MS), And Biologically Screen For Antifungal Activity. The Novel Synthesized Derivative Triazole Show Antifungal Activity, Candida Albicans.

Keywords: Antifungal agent, Candida Albicans, Triazole, Microwave irradiation, Piperazine

INTRODUCTION

Microwave-assisted synthesis is a subfield of green chemistry. Microwave-assisted synthesis has received a lot of attention in recent years. Microwave irradiation-assisted chemical transformations are pollution-free, eco-friendly, and provide high yields while being simple to process and handle. Heating reactions with traditional equipment, such as oil baths, sand baths, and heating mantles, are not only slow, but also create a hot surface on the reaction vessel where products, substrates, and reagents frequently decompose over time [1].Microwave dielectric heating drives chemical reactions by taking advantage of the ability of some liquids and solids to convert electromagnetic radiation into heat, where chemical reactions are accelerated due to the selective absorption of microwave energy by polar molecules. The use of microwave energy instead of conventional heating often results in good yields in a short time as compared with reactions by classical synthetic methods [2].Nitrogen-containing heterocycles became a research hot spot because they displayed excellent activities. Due to resistance development, severe adverse effects, and excessive toxicity, several fungicidal treatments are no longer effective [3, 4]. As a result, several new antifungal drugs must be synthesized and developed. One of the most important Pharmacophore systems between five-membered heterocycles is 1,2,4-triazole. This nitrogen-containing heterocyclic compound's structure-activity relationship (SAR) revealed potential antifungal action [5]. A variety of antifungal drugs contain the 1,2,4-triazole core as the nucleus. Triazoles have been verified as pharmacologically important compounds due to their powerful and wide action [3, 6].

Advantages of the microwave method over the traditional method

- Microwave assisted organic synthesis is a simple, non-traditional technique for the synthesis of a wide range of compounds with medicinal, pharmaceutical, and commercial significance.
- The main advantage is the highly accelerated reaction rate, which allows chemists to complete a synthesis in much less time and with reasonable yields.
- It allows for quick, low-cost access to extremely high temperatures and pressures in a sealed container, similar to a Teflon bomb. Conventional methods currently available necessitate elaborate apparatus, longer heating times, large volumes of organic solvents, and virtually no control over energy input.

- Recent simplifications of this technique have increased the safety and practical utility of microwave ovens for use in organic laboratories without any risk of contamination.
- Furthermore, sealed vessels, reflux condensers, stirrers, and water separators are not required for routine synthesis.
- Organic synthesis solvents are major environmental pollutants, with many of them being proven carcinogenic and mutagenic.
- An eco-friendly method is an important feature of microwave assisted organic synthesis chemistry, as it requires no or very little solvent as an energy transfer medium (Dry media synthesis). Rapid synthesis also results in less solvent evaporation, reducing environmental pollution.As a result, it is a step toward Green Chemistry [3, 4, 6, 13, 14].

Triazoles

A triazole is a five-membered ring made up of two carbon atoms and three nitrogen atoms. It is a heterocyclic molecule with the chemical formula $C_2H_3N_3$[7].Depending on where the nitrogen atoms are positioned within the ring, triazoles display strong isomerism. As fungicides and plant retardants, several triazoles are bioactive substances with a wide range of applications. The huge amount of nitrogen atoms in triazoles, on the other hand, leads them to react similarly to azides, making them helpful in bioorthogonal chemistry. Finally, triazoles are effective coordination molecules even though they are not frequently used as haptic ligands due to their abundance of free lone pair (Freddy H. et al) [8,15].

History of Triazoles:

The intresting class of heterocyclic compounds known as triazoles is defined by the presence of three heteroatoms in a five-membered ring structure. According to Figure 1, they come in two varieties: 1, 2, 3-triazoles and 1, 2, 4-triazoles[9]. The carbon-nitrogen ring system $C_2N_3H_3$ was given the name triazole by Bladin, who identified its derivatives in the early 1885. Between 1925 and 1946, this field saw a small increase in attention. The most thorough research on the chemistry of 1, 2, and 4-triazoles was done by Andreocci's successor. After it was discovered that some triazoles could prevent fog from forming on photographic emulsion and that others were practical herbicides and convulsants, the chemical industry paid increased interest to the synthesis of both simple and fused triazole systems.There are no naturally occurring triazole systems; all triazoles are manufactured [8, 9].

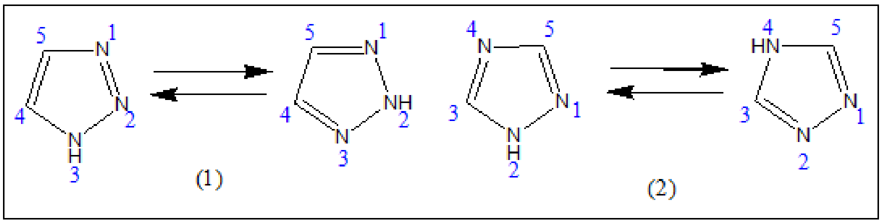


Fig. 1 Isomers of triazoles

Tautomerism In 1, 2, 4-Triazoles:

The most common form of 1, 2, 4-triazoles is the 1H-1, 2, 4-triazole tautomeric form (3); the 4H-1, 2, 4-triazole (4) form depicted in Figure 2 is not seen in either solid-state or solution [8, 16]. In tautomeric equilibrium, amino-substituted 1, 2, and 4-triazoles were generally favored electrically, with the 1H form being more preferred [18]. 1, 2, 4-triazoles have two proton transfer tautomeric forms shown in Fig. 2

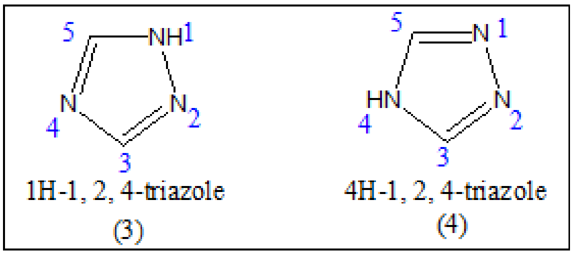


Figure 2: Tautomerism in 1, 2, 4-triazoles

Fungal Infection:

Human health and life are continuously and seriously threatened by fungus infections. These fungal infections in people can be divided into three categories: infections, toxic reactions to particular fungi's toxins, and allergic reactions to fungal proteins (mycoses). Healthy people are susceptible to a wide range of superficial, cutaneous, subcutaneous, and in some cases, systemic infections that result in a variety of illnesses ranging from serious, life-threatening disseminated disease to athlete's foot and nail infections (e.g., histoplasmosis). Numerous fungal infections are brought on by opportunistic pathogens, which may be internal (such as infections from Candida) or external (acquired from the environment) (Cryptococcus, Aspergillus infections).Aside from people with acquired immunodeficiency syndrome, the other fungal infections are invasive fungal infections and dermatomycoses caused by fungal organisms in people with increased vulnerability, such as newborns, cancer patients receiving chemotherapy, patients undergoing organ transplants, and people who have had burns (AIDS). The use of corticosteroids and antibiotics, diabetes, diseases of the epidermis and dermis, malnutrition, neutropenia, and surgery are additional risk factors. Fungal illnesses have become more common and severe recently, especially in people with weakened immune systems. A recurring pattern is a rise in fungus cases linked to sepsis. Concern over fungus sickness is widespread among medical professionals. Although seldom fatal, dermatophyte diseases like tinea pedis and candidiasis are ubiquitous worldwide. Immunocompromised patients have significant morbidity and death from pathogens such as Candida albicans, Cryptococcus neoformans, Pneumocystis carinii, and Aspergillus fumigatus. The majority of known infections are caused by Aspergillus and Candida species. Recent epidemiological patterns point to an increase in infections caused by Aspergillus spp., Candida spp. that aren't Albicans, and previously uncommon fungi that frequently have reduced sensitivity to modern antifungal medications. Clinically, between 80% and 90% of systemic fungal infections in immunocompromised patients are caused by candidiasis and aspergillosis. Although the antifungal drug repertoire has grown, the antifungal medications now on the market do not satisfy the expanding demands of controlling infection in complicated patient populations. Clinical therapy has consistently called for the creation of novel antifungal medications (Kathiravan M.K. et al, 2012) [9, 10].

Targets for Antifungal Therapy:

Different targets for antifungal therapy are shown in Figure 3.,

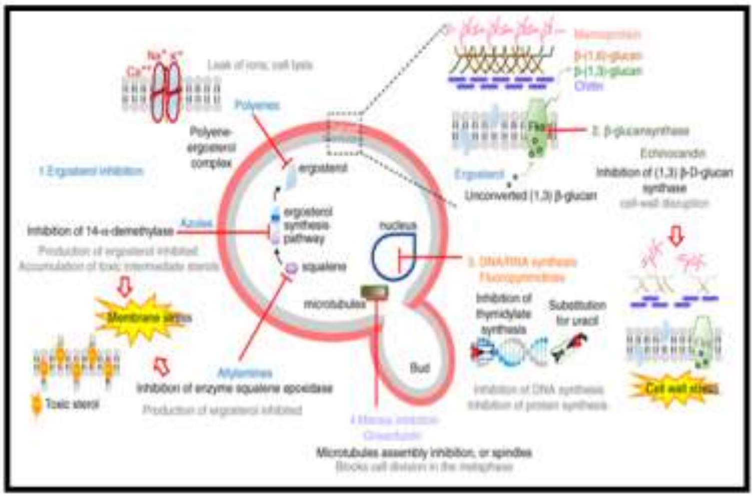


Figure 3: Different targets for antifungal therapy

Inhibitors of Fungus-Produced Ergosterol

The primary component of the fungal cell membrane is ergosterol. It controls the fluidity, asymmetry, and integrity of membranes biologically. Ergosterol synthesis will be reduced due to inhibition of the 14alpha-demethylase, and there will also be a corresponding buildup of 14-methylated sterols [11]. They block the ergosterol synthesis pathway's 14-alpha demethylation of lanosterol into ergosterol. Therefore, stopping the oxidosqualene cyclase methyltransferase and other post-squalene production components. Ergosterol functions similarly to hormones in fungus cells, stimulating growth; azoles ultimately hinder fungus growth [12].

Mechanism of Action:

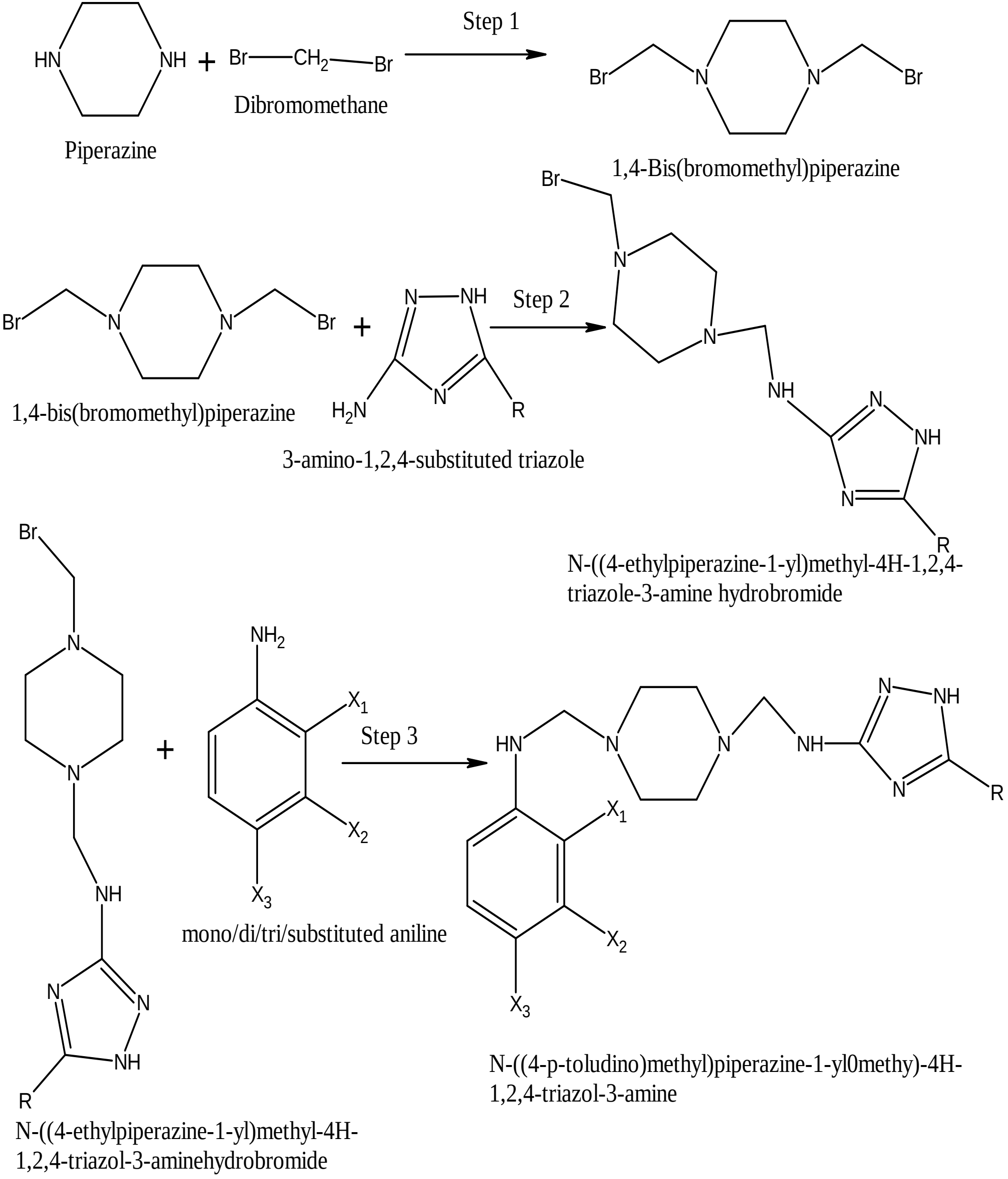
Fluconazole, itraconazole, voriconazole, and the two antifungal triazoles posaconazole and ravuconazole that are currently being researched are all synthetic compounds containing one or more azole rings that have three nitrogen atoms arranged in a five-membered ring [17]. They work by preventing the conversion of lanosterol to ergosterol, which is cytochrome P450-dependent[19].Cytochrome P450 14-demethylase is inhibited by triazoles. This enzyme aids in the conversion of lanosterol to ergosterol, which is necessary for the creation of cell walls [20].In this mechanism, the heme iron of the fungal cytochrome P450 is strongly linked to the basic nitrogen of the azole ring to prevent substrate and oxygen binding. Sterols accumulate as a result of inhibition of the 14-demethylase, which also alters the permeability of membrane proteins (Beale J.M., and Block J.H. (eds.) 2010) [10, 20, 21].

Experimental Section

Dibromomethane was added to piperazine 1 mol (1.06 g) in ethanol (25 mL) while being stirred at room temperature. TLC was used to track the reaction's development as it was stirred for a further 24 hours at the same temperature. TLC was used with chloroform:methanol (9:1) as the mobile phase to monitor the reaction's conclusion. A microwave flask with the condenser was used to combine 1,4-diethylpiperazinedihydrbromide (1 mol), substituted 3-amino-1,2,4-triazole (1 mol), para toluene sulphonic acid (PTSA) (0.24 g, 3 percent), and xylene or toluene (5 ml). For 15 minutes, the combination was exposed to microwave radiation at a power level of 350W.

1 mole of N-((4-ethyl piperazine-1-yl) methyl-4H- (substituted) Hydrobromides of 1,2,4-triazole-3-amine and (1 mol) Mono, di, or tri-substituted aniline weighing 1.21g was separately dissolved in dry DMF and combined in a flask with a

flat bottom. Triethylamine was added to this reaction mixture in a volume of 0.76 ml (0.005 mol), and it was microwaved at a power of 350 W for 20 minutes. TLC was used to observe the reaction. The precipitate was then drawn out by suction and dried after the reaction mixture was placed into ice-cold water. The solid was created by recrystallizing ethanol.



Antifungal Susceptibility Testing of Synthesized Antifungal Compounds:

Yeast Testing

CLSI document M27-A3 describes the most recent approach for antifungal susceptibility testing of yeast fungus. It's worth noting that only Candida spp. and Cryptococcus neoformans were tested. Despite this, these characteristics are commonly used to test other species [22]. RPMI-1640 as the test medium; an inoculum size of 0.5-2.5×10^3 CFU/ml prepared spectrophotometrically; incubation times of 24, 48, or 72 hours depending on species and/or drug; and endpoint determinations of optically clear for amphotericin B or 50% reduction in turbidity for the other drugs in the microtiter system are the current parameters for yeast testing [23]. When using the macro broth approach, the endpoints are slightly different.AMB endpoints are kept optically clear, but the remaining medications' endpoints are set at the lowest concentration which results in an 80% reduction in turbidity when compared to the drug-free control tube [24]. The yeast's breakpointsare divided into five groups. Susceptible, intermediate, susceptible-dose-dependent, resistant, and nonsusceptible are the different classifications. Isolates with MICs in the susceptible range are inhibited by antifungal concentrations that are commonly attained in patients being treated with a normal dose.AMB endpoints are kept optically clear, but the remaining medications' endpoints are set at the lowest concentration which results in an 80% reduction in turbidity when compared to the drug-free control tube [25].The yeast's breakpoints are divided into five groups. Susceptible, intermediate, susceptible-dose-dependent, resistant, and nonsusceptible are the different classifications. Isolates with MICs in the susceptible range are inhibited by antifungal concentrations that are commonly attained in patients being treated with a normal dose.A well's turbidity is graded from 0 to 4, with 0 denoting optical clarity and 4 denoting no reduction in turbidity from the control well's turbidity level. The remaining numbers are graded as follows: 1 for mildly foggy wells, 2 for wells with a noticeable reduction in turbidity (often around 50%), and 3 for wells with a slight reduction in turbidity. Since a true 50% reduction in turbidity as determined spectrophotometrically is generally significantly more turbid than the eye detects when rating a 50% reduction, this grading system may be challenging [26].

PRINCIPLE:

When an isolate is cultivated with an antifungal agent present, the MIC or MFC can be calculated using antifungal medication dilutions. Antifungal drugs are manufactured in series dilutions and placed in tubes with the necessary labels [27]. The testing fungus, Candida albicans (ATCC 10231), is then injected into each tube using a standardized Sabouraud Dextrose Broth solution. The main benefit of the broth dilution test is that it enables a quantitative estimation of the antifungal agent's inhibitory and fungicidal effects (REFF) [28].

1. Synthesized antifungal compounds used for *in vitro* testing of antifungal susceptibility.

Based on early antifungal screening investigations, a range of 2-64 µg/ml (2, 4, 8, 16, 32, 64 µg/ml) was choosen for antifungal testing.

i. Stock Solution Preparation

to create workable dilutions within the chosen range of 2-64 µg/ml. By dissolving 10 mg of synthetic chemicals in 100 ml of dimethyl formamide, the stock solution of 100 µg/mlwas created (DMF)

ii. Stock Dilution Preparation

The first batch of six test tubes was made and labeled with concentration ranges starting at 2, 4, 8, 16, 32, and 64 µg/ml (2, 4, 8, 16, 32, and 64 ppm).
0.2 ml of the stock solution was taken out and diluted with DMF up to 10 ml to create a 2 ppm solution.

iii. Inoculum Preparation

A loopful of previously maintained Candida albicans colonies were streaked on an SDA plate, which was then incubated for the entire night at 35 ^{0} C in an ambient-air incubator. Five milliliters of sterile, 0.85 percent NaCl were added to five separate colonies with similar colony morphology and at least 1 mm in diameter. Lasted about 15 to 20 seconds. Using a spectrophotometer, the suspension was modified to achieve sterile 0.85 percent NaCl transmittance at 540 nm. The suspension that resulted ranged from 1 x 10^6 to 5 x 10^6 CFU/ml. 9 ml of nutrient broth were mixed with 1 ml of the aforementioned solution.As a result, the suspension increased from 1 x 10^5 to 5 x 10^5 CFU/ml.
Before being used, tubes of this suspension were kept at 2^0 Cto 8^0 C for up to 3 hours.

iv. Preparation of the Working Dilution (Inoculation and incubation)

1) Six test tubes, or Set II, were constructed and labeled with /ml (2, 4, 8, 16, or 32 µg/ml)
2) To each test tube 9ml of broth solution and 0.9 ml of inoculum was added.
3) From a stock dilution tube containing 2 µg/ml, 0.1 ml of the drug solution was removed and put in a test tube labeled with 2 µg/ml that also contained 0.9 ml of inoculums and 9 ml of broth solution (this step involves diluting the inoculums from 1 x 10^5 to 5 x 10^5 CFU/ml suspension to 1 x 10^4 to 5 x 10^4 Repeat the procedure until 0.1 ml of the desired concentration (between 2 and 64 µg/ml) has been added to each of the five test tubes.
4) The two empty tubes on the far right were chosen to serve as the positive and negative growth controls.

v. Controls on growth

1)To control positive growth, 0.1 ml of broth and 0.9 ml of final inoculums were combined.
2) One millilitre of broth was added to the tube to control negative growth.

2. ASSAY FOR DISC DIFFUSION.

A. Making Standard Solutions: Ketoconazole, which was made in DMF, served as the reference standard medication.

B. Candida Albicans ATCC 10231 was the test organism used.

C. The culture medium was prepared using Sabouraud Dextrose Agar, which was utilized to test the antifungal activity.

D. Inoculum preparation: This was sterilized for 15 minutes in an autoclave using a 15 lb/sq inch pressure. A loopful of organisms were transferred from the mother culture to this sterilized SDA medium after sterilization. In this case, the Pour Plate Technique was used.

E. Equipment sterilization: Petri dishes, glass syringes, test tubes, and conical flaskswere all sterilized inan autoclave for 15 minutes at a pressure of 15 pounds per square inch.

F. Petri dish preparation: The infected media was transferred to the Petri plates and allowed to harden. The sterile filter disc that had been dipped in drug solution was placed on the media after it had solidified. The control utilised was

DMF. For proper media diffusion, the plates were stored in the refrigerator at 8–10°C. The plates were then moved to the BOD incubator, where they were kept at 37°C for 24 to 48 hours. The Petri plates were examined for the zone of inhibition following incubation. The zone of inhibition's diameter was given in millimetres

RESULT AND DISCUSSION

Spectral Analysis of Derivatives

Total 4 Derivatives were synthesized. % Yield, Melting point and Rf value (TLC) were calculated for all 4 Derivatives. The Derivatives were characterized by IR, 1H NMR and MS.

Derivatives

T1-N-((4-((3,4-dimethylphenylamino) methyl) piperazine-1-yl) methyl)-5-methyl-1-H-1,2,4-Triazole-3-amine dihydrochloride hydrosulfide

Brown Color solid, Yield 85%;M.P.: 190-195℃, IR(KBr): 640(C-Cl), 1126(C-N), 3325(N-H), 2800(C-SH), 817/856(C-H out of plane), 1597(C=C); 1H NMRδppm: 2.52(t, 2H), 3.41(s, 1H), 3.09(t, 1H), 6.50(d, 2H), 6.74(d, 2H), 3.11(s, N-H)

ESI-MS m/z:385,351,328,171,81

T2- N-((4-((2,4-dimethylphenylamino)methyl)piperazine-1-yl)methyl)-5-methyl -1-H-1,2,4-triazole-3-amine dihydrochloride hydrosulfide

Reddish-Brownsolid,yield 65%; M.P- 190-195℃, IR (KBr):686/802 (c-cl) ,1087 (C-N), 3309 (N-H), 1504/1635(C=C),817/856 (C-H out of plane), 1400 (CH2); 1H NMR δppm: 7.15(t ,2H), 7.81 (t, 2H), 6., (t, 2H), 4.0 (t, 2H), 13.5 (5, 1H)

ESI- MS m/z: 384,356,328,171,81.

T3- N-((4-((p-toluidino) methyl) piperazine-1-yl) methyl)-1-h_1,2,4-triazole-3,5-diamine hydrochloride

Grey Solid, Yield 75%, M.P- 195-200℃, IR(KBr): 632(C-Cl), 1280(C-N), 3379(N-H), 3471(N-H),1612(C=C), 1489(CH_2); 1H NMR δ ppm: 2.52(t, 2H), 2.53(t, 2H), 3.39(s, 1H), 4.88(t, 1H), 6.50(d, 2H), 6.49(d, 2H), 3.11(s,1H)

ESI-MS m/z:339,142,97,82

T4- N- ((4-((-2-fluoro-4-methylphenylamino) methyl) piperazine-yl) methyl)-5-methyl-1-H-1,2,4-triazole-3amine hydrofluoride hydrosulfide

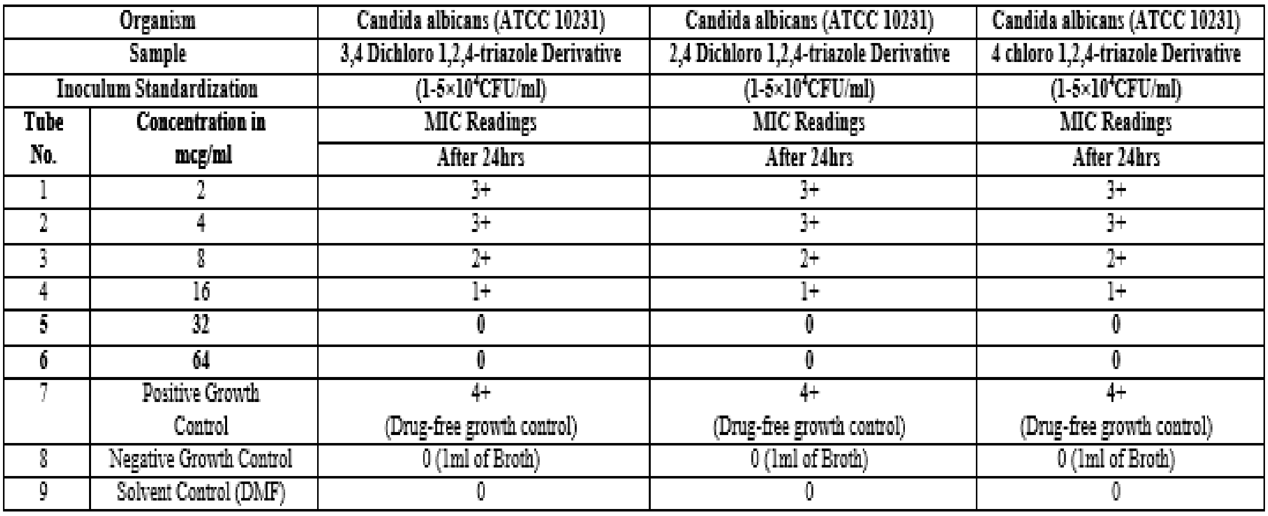
Black colour, yield 65%, M.P.;200 − 205℃ ; TLC: Rf value:0.80, IR(KBr): 980(C-F),1126(C-N), 3325(N-H),2775(C-SH), 1465(CH2) stretching

1H NMR δ ppm: 6.46(s, 1H), 6.79(s,2H), 7.10(s, 2H), 2.35(s, 2H), 4.13 (d, 1H), 4.0(d, 2H), 13.05(s, 1H), 13.5(s, 1H)

ESI-MS m/z:351,318,282,140,81.

1. Pharmacological Evaluation :

Table 1: Determination of Minimum Inhibitory Concentration (MIC):



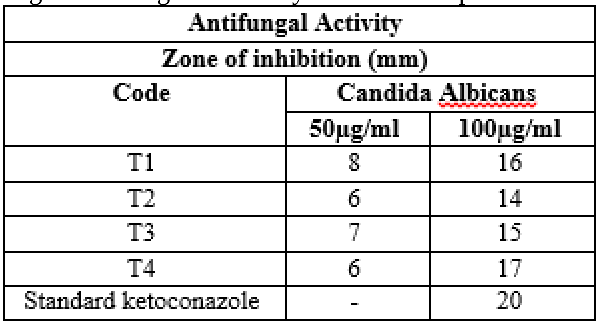
Organism		Candida albicans (ATCC 10231)	Candida albicans (ATCC 10231)	Candida albicans (ATCC 10231)
Sample		3,4 Dichloro 1,2,4-triazole Derivative	2,4 Dichloro 1,2,4-triazole Derivative	4 chloro 1,2,4-triazole Derivative
Inoculum Standardization		(1-5×10^4CFU/ml)	(1-5×10^4CFU/ml)	(1-5×10^4CFU/ml)
Tube No.	Concentration in mcg/ml	MIC Readings	MIC Readings	MIC Readings
		After 24hrs	After 24hrs	After 24hrs
1	2	3+	3+	3+
2	4	3+	3+	3+
3	8	2+	2+	2+
4	16	1+	1+	1+
5	**32**	**0**	**0**	**0**
6	**64**	**0**	**0**	**0**
7	Positive Growth Control	4+ (Drug-free growth control)	4+ (Drug-free growth control)	4+ (Drug-free growth control)
8	Negative Growth Control	0 (1ml of Broth)	0 (1ml of Broth)	0 (1ml of Broth)
9	Solvent Control (DMF)	0	0	0

2. DISC DIFFUSION ASSAY:

In Vitro Antifungal Activity

Agars Then, sterile glass Petri dishes were then filled with agar medium, and they were let to cool. The antifungal activity of compounds has been tested in vitro at concentrations of 50 µg/ml and 100 µg/ml against Candida albicans since the sensitivity was not seen at concentrations below 50 µg/mlfor the antifungal test, ketoconazole was employed as a common fungicide. Test fungi were grown on Sabourasud Dextrose Agar as a base media. Freshly seeded Petri dishes and melting Sabouraud Dextrose Agar were placed in the autoclave at 121^{0}C for 15 minutes to sterilize them. Glass lightly with sterile forceps on the hardened agar plate. One loopful of C. Albicans mycelium was gently dissolved in the Sabouraud Dextrose refrigerator after the medium had been sterilized [29-31].

Table 2: Antifungal screening results of synthesized compounds T1-T3 and standard



Antifungal Activity		
Zone of inhibition (mm)		
Code	**Candida Albicans**	
	50µg/ml	**100µg/ml**
T1	8	16
T2	6	14
T3	7	15
T4	6	17
Standard ketoconazole	-	20

CONCLUSION

All physicochemical metrics, including MP, Rf value, and spectroscopic techniques, including IR, NMR, and mass spectrometry, showed good correlation with the reference molecule ketoconazole when used to characterize the final synthesized product and intermediate. In vitro antifungal activity was evaluated using the serial dilution method and disc diffusion assay method against candida albicans. Compound T1(16mm), T2(14mm) and T3(15mm) showed good antifungal activity which is compared to the standard ketoconazole drug by using disc diffusion assay method. Among all synthesized compound T1 was found to be most active antifungal compound. The present investigation opens a new lead for antifungal agents and there is a wide scope for future investigation.

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Conflict of Interest:

The author declares that they do not have any competing interests.

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