Synthesis, characterization, and biological activity of novel azole piperazine congeners

Priyanka R. Sadalge, Vrushabh Karnawadi, Lairikyengbam Deepti Roy*, Manikanda Prabu, Geeta Krishnamurthy, Pooja Gour, Shivanjali Esther Arland, Jyotsna Kumar*

Department of Chemistry, Faculty of Mathematical and Physical Sciences, M.S. Ramaiah University of Applied Sciences, Bangalore, India.

ARTICLE INFO
Received on: 10/10/2022
Accepted on: 09/02/2023
Available Online: 04/04/2023

Key words:
Antimicrobial, antifungal, imidazole, piperazine, triazole.

ABSTRACT
A novel series of piperazine analogs were synthesized comprising azole moieties with good yield. Structures of all four synthesized azole analogs were established by spectral characterization viz 'H-nuclear magnetic resonance, liquid chromatography-mass spectrometry, and FT-IR. Synthesized derivatives were further assessed for their antimicrobial and antioxidant studies. Antimicrobial activity was conducted against Pseudomonas aeruginosa, Staphylococcus aureus, and Candida albicans by well diffusion method. Antimicrobial activity reflects that synthesized piperazine derivatives containing imidazole moiety possess better antibacterial and antifungal activity than piperazine analogs comprising triazole moiety. Staphylococcus aureus exhibited strong resistance over all synthesized piperazine derivatives. Antioxidant activity of synthesized molecules were carried out by using 1, 1-diphenyl-2-picrylhydrazyl. In vitro antioxidant activity results revealed that IC50 values of synthesized piperazine analogs are comparative with control—gallic acid where piperazine derivatives with triazole unit ITZ-1 and ITZ-2 are exhibiting better antioxidant activities. Hence, synthesized novel molecules with better antimicrobial and antioxidant activities may be further used in the formulation of new antimicrobial and antioxidant drugs. Observed IC50, zone of inhibition values and drug resistance of S. aureus strain against all synthesized novel piperazine derivatives in the present investigation will be helpful to be used as a reference for auxiliary studies and further evaluation is worth to develop a new generation drug.

INTRODUCTION
Fungi, classified as kingdom fungi, are eukaryotic organisms whose functions are closely related to those of humans. Fungal infection, also known as mycosis, occurs when a fungus invades over an area of the human body and its immune system cannot handle it well (Kainz et al., 2020). Worldwide, around 150 million acute cases of fungal infections happen, resultant approximately 1.7 million deaths annually, which is much higher than malaria or tuberculosis (Bongomin et al., 2017). The mortality rate is alarmingly increasing, thus marking it as a global threat.

Although fungal infections are more common than bacterial infections, but it seems that the development of antifungal agents is far behind than that of antibacterial agents because of the slow growth of fungi in multicellular forms, hence making it more challenging to quantify than bacteria. Also, fungi are eukaryotes, and the maximum number of antifungal agents are found to be noxious to fungi and hosts as well, thereby making the evaluation of the biological activities of potent antifungal agents via in vitro or in vivo studies much more difficult (Dixon et al., 1996). Basic treatments of fungal infection still face many challenges, for example, drug resistance (Al-Wabli et al., 2018), alarming drug interactions, poor systemic availability, and low safety profiles (Sheng and Zhang, 2011). These challenges become a major hindrance, making the fungal infection very difficult to treat. Despite these drawbacks and difficulties, recent advancements in
technology and well-defined literature backup support researchers in coping with these challenges by understanding the existing antifungal drugs, modifying them and synthesizing novel antifungal agents with improved safety profiles.

Antifungal medications are topically or systemically administered as pills, creams, powders, etc., and work either by killing fungi directly or preventing them from growing. Antifungal drugs include azoles, polycenes, allylamines, echinocandins, and others (https://www.healthline.com/health/fungal-infection/antifungal). Among them, azoles are the most commonly used anti-fungal drugs. Azole antifungals contain anazole ring and inhibit fungi from growing by slowing down the process. Azole antifungal drugs are further classified into three groups—(i) Imidazoles are possessing two atoms of nitrogen in oneazole ring (e.g., miconazole, econazol, dapaconazole, and ketoconazole), (ii) Triazoles have three nitrogen atoms in anazole ring (e.g., fluconazole, fosfluconazole, and itraconazole), and (iii) Tetrazoles have four nitrogen atoms in oneazole ring (e.g., quilseonazole, oteseezonazole, etc.) (Fig. 1) (Shafiei et al., 2020).

Triazole compounds are one of the most significant groups for medicinal as well as for organic chemistry. When combined with different molecules, the triazole moiety has demonstrated a variety of biological activities, including antimicrobial (Chen et al., 2000; Sheremeta et al., 2004), anticancer (Durao et al., 2002), antimalarial (Gujjar et al., 2009), analgesic (Hafez et al., 2008), anti-inflammatory (Banu et al., 1999), and anticonvulsant. (Guan et al., 2007) Triazoles possess specific structural and chemical features such as high aromatic stabilization, which provide them stability under different conditions.

Another important compound in theazole family is imidazole, a five-membered heterocyclic molecule with two nitrogen atoms, positioned at 1 and 3 places (Debus, 1858). As a very common scaffold, imidazole is present in many highly significant biomolecules such as essential amino acids, histamine, histidine, alkaloids (Kleeman et al., 1999; Verma et al., 2013) with extensive biological activities like antibacterial (Vijesh et al., 2011), analgesic (Ucucu et al., 2001), antifungal (Vita et al., 2012; Yang et al., 2012), anticancer (Yang et al., 2012), anti-HIV (Johns et al., 2009; Zhan et al., 2009), and antitubercul (Lu et al., 2012). Imidazole, a relatively simple molecule, is amenable to several structural modifications. Imidazole has several commendable properties like excellent tissue permeability and penetrability, good bioavailability, and comparatively less toxicity and adverse effects.

Etomidate, Cimetidine, Oxicónazole, Ketoconazole, Ornidazole, Metronidazole, Azomycin, Clondine, etc. members of theimidazole family have been shown to have in vitro antifungal activity against a wide range of pathogenic fungi. As per a recent study, derivatives of imidazole, e.g., ketoconazole and miconazole, can cause the amassing of toxic peroxide compounds inside the cells of microbes (Gupta, 2001; Wright et al., 1992), where Ketoconazole, a water-soluble imidazole, can be used orally to treat both superficial as well as deep-seated fungal infections (Terrell et al., 1992).

A comprehensive literature review indicated that in medicinal chemistry, currently, piperazine, a nitrogen-containing heterocyclic, and its congeners are considered to be the most significant building blocks for drug synthesis (Alang et al., 2010; Al-Harthy et al., 2016; Al-Talib et al., 2016). Piperazine and its derivatives have attained a unique position as imperative pharmacophores in several therapeutic areas. Its multipurpose structure has always motivated researchers to use piperazine molecules as a scaffold to design novel drug molecules with numerous biological activities. The literature demonstrates the subsistence of several piperazine compounds along with triazoles and imidazole pharmacophores with varied pharmacological activities, including their incredible potential for analgesic and anti-inflammatory activities (Fromling, 1988; Shao et al., 2007). These molecules contained a core structure of piperazine where the imidazole/triazole ring linked to a dihalophenyl ring via a chain of two carbons. Along with this, a hydroxyl group is present on the alpha carbon of the phenyl ring. These molecules possess three chiral carbons, two fixed chiral centers are present in the dioxolane ring and arylxymethylene dioxolane-ring substituents, and triazolomethylene are cis to each other (Fig. 2). Literature supports modifications to the head and tail parts of the core structure of ketoconazole and itraconazole molecules to improve their biological activity and bioavailability (Liu et al., 2011; Pinal, 2004; Ruiz-Casillo and Buchwald, 2016).

Thus, to magnify the scope of piperazine derivatives as privileged therapeutic scaffolds and a considerable requirement for the discovery of novel chemical entities with potential biological activity and interest, we pursued our interest in developing new anti-fungal agents and strive for the synthesis and evaluation of biological activities of newly synthesized piperazine derivatives comprising imidazole and triazole moieties. The present paper reports the synthesis, characterization, and antimicrobial activity of novel piperazine analogs as prospective antifungal agents.

RESULTS AND DISCUSSION

Chemistry

Due to the varied antifungal properties of triazole and imidazole derivatives, we planned to synthesize new piperazine derivatives. By using the core structures of ketoconazole (KTZ) and ITZ as a platform, methodically, we altered the side chains to synthesize four effective systemic antifungal drug molecules comprising a broad spectrum of antifungal activity with less probability of developing resistance. All four novel derivatives were synthesized by coupling of Cis tolylate with synthesized piperazine derivatives comprising imidazole/triazole moieties to get an intermediate. This intermediate on further treatment with several phenyl derivatives produced the designed novel derivatives.

Biological activities of KTZ-1, KTZ-2, ITZ-1, and ITZ-2

The anti-oxidant activities and anti-microbial activities of all the synthesized novelazole piperazine congeners (KTZ-1, KTZ-2, ITZ-1, and ITZ-2) were evaluated using the 1, 1-diphenyl-2-picyrylhydrazyl (DPPH) method and the Well Diffusion method against microbial strains of Candida albicans, Staphylococcus aureus Gram-positive bacteria, and Pseudomonas aeruginosa, Gram-negative bacteria, respectively.
Antioxidant activity

When DPPH (2, 2-diphenyl-1-picrylhydrazyl, purple colored) free-radical interacts with odd electrons, absorption occurs at 517 nm. Free-radical scavengers react to DPPH to form DPPH-H, which exhibits lower absorbance due to the smaller number of hydrogens. As the number of electrons increases, DPPH gets decolorized (yellow hue).

Figure 3 reflects the relation between the absorbance and sample concentrations of KTZ-1, KTZ-2, ITZ-1, ITZ-2, and gallic acid (control). It can be observed from Figure 4 that the antioxidant activity of gallic acid as a control showed a linear relationship between concentration and percentage inhibition when absorbance is at 517 nm. The antioxidant activity of four synthesized novel molecules by the DPPH assay also shows that the antioxidant effect is proportional to the concentration. Comparative inhibition percentage of each synthesized derivative at different concentrations and gallic acid (control) are shown in Figure 5. In comparison to the IC50 of gallic acid (0.282 µg/ml), the IC50 of ITZ-1 and ITZ-2 is 0.070 and 0.224 µg/ml, indicating that these two molecules have very good antioxidant activity. For KTZ-1 and KTZ-2, IC50 values are 1.259 and 1.438 µg/ml, respectively; their antioxidant activity is relatively moderate. This demonstrated that synthesized molecules possessed antioxidant potential.

Antimicrobial activity

Values of observed antimicrobial activities of control and all synthesized derivatives are collated in Figures 5 and 6. Antimicrobial results showed that all synthesized compounds, excluding ITZ-2, have significant antimicrobial activity against the P. aeruginosa strain (Gram-negative bacteria) and C. albicans fungi.
Antimicrobial results demonstrate that ITZ-1, KTZ-1, and KTZ-2 had the maximum zone of inhibition against *P. aeruginosa* strain (11 ± 1.2 mm), (15 ± 1.1 mm) and (14 ± 1.4 mm), respectively, at 400 µg/ml.

It is evident that for the *P. aeruginosa* strain and *C. albicans* fungi, KTZ-1, and KTZ-2 inhibit pathogen progression even at low concentrations (100–400 µg/ml). However, in the case of ITZ-1 maximum zone of inhibition was observed at higher concentration only, there is no significant antimicrobial activity at 100 to 200 µg/ml concentrations. No zone of inhibition (ZOI) was observed when all the three strains were exposed to ITZ-2 (100 to 400 µg/ml). Hence, apart from ITZ-2, ITZ-1, KTZ-1, and KTZ-2 are competently subduing the growth of pathogens thus displaying good antimicrobial activity against the two pathogen microbial strains (Fig. 6). It is noteworthy that none of the synthesized molecules were active against *S. aureus* (Gram-positive bacteria).

Antimicrobial results demonstrate that piperazine derivatives with imidazole derivatives are more susceptible to antimicrobial activity than triazole piperazine derivatives. The Gram-positive organism, *S. aureus* strain, showed strong resistance to all synthesized piperazine derivatives. Among all four synthesized molecules, KTZ-1 was found to be the most active one.

**CONCLUSION**

In this study, four new piperazine derivatives with triazole and imidazole moieties have been synthesized and characterized for the first time. Furthermore, they were evaluated for antimicrobial and antioxidant studies. All synthesized compounds except ITZ-2, have shown significant antibacterial activity for the *P. aeruginosa strain* (MTCC 2453) and antifungal activity against *Candida albicans* (MTCC 3958) fungi. *Staphylococcus aureus strain* (MTCC 96) has shown strong resistance to all synthesized piperazine derivatives. ITZ-1, KTZ-1, and KTZ-2 showed significant activity against *P. aeruginosa strains* and *Candida albicans* fungi. Among all these synthesized molecules, KTZ-1 exhibited excellent and promising antifungal and antibacterial activities, whereas ITZ-1 showed the weakest effect. The order of antimicrobial activity for the synthesized piperazine derivatives
against *P. aeruginosa* strain is as follows: Imidazole ≥ Triazole. An antioxidant study shows that the IC50 of ITZ-1 and ITZ-2 is 0.70 and 0.224 µg/ml, respectively, indicating their strong antioxidant activity. For KTZ-1 and KTZ-2 it is moderate. This demonstrated that synthesized molecules possessed antioxidant potential. Therefore, ITZ-1, KTZ-1, and KTZ-2 may be further used for the synthesis of new antimicrobial and antioxidant drug molecules. Observed ZOI values and drug resistance of *S. aureus* (Gram-positive bacteria) against all synthesized novel piperazine derivatives in the present investigation will be helpful to use as a reference for auxiliary studies and further evaluation is worthwhile. However, the in vivo activity of these synthesized drugs should be assessed in clinical trials.

**EXPERIMENTAL MATERIAL AND METHOD**

All required chemicals and reagents were procured from Sigma-Aldrich Chemical Company (Sigma-Aldrich Chemical Company, St. Louis, MO) and used without any further purification. To perform the thin-layer chromatography (TLC), F254 plates (silica gel coated with fluorescent indicator) were purchased from Fluka Company and column chromatography was carried out on Silica gel 60 (0.063–0.2 mm). Gallenkamp melting point apparatus was used to determine the Melting points and were uncorrected. By using KBr pellets IR spectra were measured on a Thermo Nicolet model 470 Fourier-transform spectrophotometer. Nuclear magnetic resonance (NMR) (400 MHz spectrophotometer. Chemical shifts were expressed in parts per million with tetramethylsilane as an internal standard. For the synthesis of KTZ-a, in the suspension of NaH (50% dispersion) in 10 ml of dimethylformamide (DMF), 0.484 g of 1-(bromo-methyl)-3-methyl-benzylpiperazine-(KTZ-b) was prepared by taking 0.5 g of KTZ-b in 10 ml of DMF. On adding 0.3 g of potassium carbonate and 0.5 g of 1-(bromo-methyl)-3-methylbenzene, mixture was refluxed with overnight stirring at 145°C. Reaction progress was mentioned by TLC using the solvent system ethyl acetate:methanol in 9:1 ratio. After the completion of the reaction, the reaction mixture, water was added. The mixture was subsequently extracted with chloroform (CHCl3). The organic layer was dried using MgSO4 and evaporated in a vacuum to obtain a chocolate-colored product (KTZ-1).

**Synthesis of KTZ-1 and KTZ-2**

A detailed synthetic scheme of KTZ-1 and KTZ-2 with key intermediates is shown in scheme 1. For that purpose, first, key intermediates KTZ-a and KTZ-b were synthesized as per the standard procedure (Heeres et al., 1984).

**Synthesis of 1-(4-(4-((2-((1H-imidazol-1-yl) methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl) methoxy) phenyl)piperazine-1-Carboxylate -(KTZ-2)**

For the synthesis of KTZ-a, in the suspension of NaH (50% dispersion) in 10 ml of dimethylformamide (DMF), 0.484 g of 1-(4-(4)-hydroxyphenylpiperazin-1-yl)ethanone was added and the solution was stirred for 70 minutes. Afterward, 1.0 g of (2 ((1H-imidazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl) methyl 4 methylbenzene sulfonate (Cis Tosylate) was added and stirring was continued for 5 hours at 80°C. Progress of the reaction was monitored by TLC using a solvent system of ethyl acetate: methanol (9:1). After completion of the reaction, the mixture was cooled and extracted with Dichloromethane (CH2Cl2). The obtained organic layer was dried up and evaporated to afford a solid residue of KTZ-a.

**Synthesis of 1-(4-(4-((2-((1H-imidazol-1-yl) methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl) methoxy) phenyl)piperazine-(KTZ-b)**

A solution of 1-(4-(4-(2-(1H-imidazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl) methoxy phenylpiperazin-1-yl) ethanone, containing 1.0 g of KTZ-a in 10 ml of MeOH and 0.1 g of NaOH pellets, was refluxed with overnight stirring at 60°C. Progress of reaction was monitored by TLC using a solvent system of ethyl acetate: methanol in 9:1 ratio. After completion of the reaction, the reaction mixture was cooled and subsequently extracted with chloroform. The organic layer was dried and evaporated in vacuo to obtain KTZ-b.

**Synthesis of 1-(4-((2-(1H-imidazol-1-yl) methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-ylmethoxy) phenyl)-4-3-methyl-benzylpiperazine-(KTZ-1)**

A solution of 1-(4-((2-(1H-imidazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)methoxy phenyl piperazin is prepared by taking 0.5 g of KTZ-b in 10 ml of DMF. On adding 0.3 g of potassium carbonate and 0.5 g of 1-(bromo-methyl)-3-methylbenzene, mixture was refluxed with overnight stirring at 145°C. Reaction progress was mentioned by TLC using the solvent system ethyl acetate:methanol in 9:1 ratio. After the completion of the reaction, the reaction mixture, water was added. The mixture was subsequently extracted with chloroform (CHCl3). The organic layer was dried using MgSO4 and evaporated in a vacuum to obtain a chocolate-colored product (KTZ-1).

1H NMR: δ-7.63 (1H, J = 8), δ-7.48 (s, 1H, J = 12), δ-7.47 (1H, J = 9), δ-7.44 (1H, J = 9), δ-7.28 (1H, J = 7), δ-7.24 (1H, J = 8), δ-7.14 (1H, J = 16), δ-4.50 (1H, J = 14), δ-4.43 (1H, J = 30), δ-4.23 (2H, J = 20), δ-4.23 (1H, J = 20), δ-3.73 (2H, J = 8), δ-3.56–3.33 (2H), 2.51 (s, 3H). IR (KBr, cm-1): 3,871.2 and 3,673.8 cm-1 (N-H stretching of imidazole and piperazine), 2,916.8 cm-1 (C-H stretching of C-CH3), 1,632 cm-1 (C=C aromatic). 1,225.6 cm-1 (C-O stretching of the ether linkage), 1,102.9 and 1,025.7 cm-1 (C-O stretching), 817.6 cm-1 (C-Cl linkage) (Kujawski et al., 2017). Further confirmation was done by finding the mass by liquid chromatography-mass spectrometry (LC-MS) and comparing it with the calculated one. Calculated LC/MS (m/z): 593.4 while from the graph, it is 591.14 g/mol.

**Synthesis of phenyl 4-(4-((2-(1H-imidazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-ylmethoxy) phenyl) piperazine-1-Carboxylate -(KTZ-2)**

A 0.5 g solution of 1-(4-(1H-imidazol-1-yl) methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl methoxy) phenyl piperazine (De acetylation ketoconazole) in 10 ml of DMF, 0.202 g of triethylamine (TEA), and 0.18 g of Phenyl Chloroformate was refluxed with overnight stirring at 145°C. Reaction progress was mentioned by TLC using the solvent system ethyl acetate:methanol in 9:1 ratio. After completion of the reaction, the reaction mixture was cooled and subsequently extracted with chloroform. The organic layer was dried using MgSO4 and evaporated in vacuo to obtain a chocolate-colored product (KTZ-2).
SYNTHESIS OF ITZ-1 AND ITZ-2

Furthermore, the key intermediate ITZ-(a–e) was synthesized as stated in the literature (Heeres et al., 1984). By esterification and acylation of ITZ-e, final molecules, ITZ-1 and ITZ-2 were synthesized. Synthesis of ITZ-1 and ITZ-2 is shown in Scheme 2.

Synthesis of 1-(4-methoxy phenyl) piperazine-(ITZ-a)

In a 500 ml round bottom flask fitted with a stirrer and reflux condenser, 20 ml of isopropyl alcohol (IPA) was taken. Then, 2 g of each potassium carbonate and Bis (2-Chloro Ethyl) Amine hydrochloride were added. After stirring for 10 minutes, 2 g of P-Anisidine was added and refluxed at 85°C overnight. The reaction was supervised by checking TLC. Once the reaction was completed, the mixture was allowed to attain the room temperature, further followed by filtration and distillation of the mother liquor. Solid 1-(4-methoxy phenyl) piperazine was obtained by precipitation using hexane.

Synthesis of 1-(4-methoxy phenyl)-4-(4-nitrophenyl)-(ITZ-b)

In a dried and clean round bottom flask fitted with a reflux condenser, 1 g of 1-(4-methoxy phenyl) piperazine (ITZ-a), 10 ml of DMF, and 0.56 g of potassium carbonate were added. Stirring was carried out at room temperature for 15 minutes. After the addition of 0.86 g of 1- chloro-4-nitro benzene, the reaction mixture was refluxed at 125°C for 8 hours and the reaction was monitored by checking TLC. Once the reaction was completed, the reaction mixture was quenched with distilled water and extracted using Dichloromethane (DCM). In the DCM layer, sodium sulfate was added to remove any traces of water. Complete distillation of the organic layer gives the solid ITZ-b.

Synthesis of 4-[4-(4-Nitrophenyl)-1-piperazinyl] phenol-(ITZ-c)

In a 50 ml two-neck round bottom flask (fitted with a reflux condenser), 30 ml of HBr (63%) was taken. In this, 5 g of 1-(4-methoxy phenyl)-4-(4-nitrophenyl) (ITZb) was added portion-wise over a period of 10 minutes. The reaction mixture was refluxed for 16 hours at 150°C and it was monitored by checking TLC. After the completion of the reaction, the reaction mixture was quenched with water. A solid product was made into an amorphous powder using pestle and mortar.

Synthesis of ((2S, 4S)-2-((1H-1,2,4-triazol-1-yl) methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl) methyl 4-methylbenzenesulfonate (Cis Tosylate) to 4-[4-(4-Nitrophenyl)-1-piperazinyl] phenol-(ITZ-d)

In a 50 ml two-neck round bottom flask containing 10 ml of DMF, 1 g of ITZ-c was added. The solution was stirred while the reaction mixture was heated for 1 hours at 500°C. To dissolve it in the DMF solution, 1 g of (2S, 4S)-2-((1H-1,2,4-triazol-1-yl) methyl)-2-(2,4- dichlorophenyl)-1,3-dioxolan-4-yl) methyl 4-methylbenzenesulfonate (Cis Tosylate) was added slowly. The reaction was kept at 950–1,000°C overnight. After cooling to room temperature, the mixture was quenched with sodium chloride (aqueous solution) and extracted with ethyl acetate for three times. The organic layer was dried over Na₂SO₄ and distilled to get a solid ITZ-d.

Reduction of the nitro group of ITZ-d to amine group to get ITZ-e

In a two-neck round bottom flask, 2.5 g of ITZ-d and 10 ml of methanol were taken and for 30 minutes the reaction mixture was stirred at room temperature. Then zinc powder and ammonium formate in the 1:1 ratio were added. In the beginning, the color of the reaction mixture was yellow, but after 3 hours the yellowish color changed to a brown color. The reaction was constantly monitored by TLC. Once, the reaction was completed, the mixture was filtered using methanol. The collected filtrate was distilled to get a solid product. Further filtration and distillation were carried out to get a solid, pure product (ITZ-e). The obtained ITZ-e was further subjected to acylation and esterification to get two new derivatives of itraconazole—ITZ-1 and ITZ-2.

Acylation reaction to obtain ITZ-1

In a two neck round bottom flask, ITZ-e and DCM were taken in a 1:20 ratio after the dissolution of ITZ-e, TEA and benzoyl chloride (1:1) were added and the reaction was allowed to proceed for 5 hours at room temperature. Reaction was supervised by checking TLC. After the quenching, separation, and distillation, ITZ-1 was obtained as a cream-colored amorphous powder.

Acidification of ITZ-1 was performed in LC-MS. The LCMS result revealed that the calculated LC/MS (m/z) is 685.9 while from the LC/MS graph, it is found to be 686.53 g/mol.

Esterification reaction to obtain ITZ-2

Similar to the above procedure, in a two-neck round bottom flask, ITZ-e and DCM were taken in a 1:25 ratio. After the dissolution of ITZ-e, 3-4 drops of each TEA and Phenyl chloroformate were added. The reaction was continued for 3 hours at room temperature. Reaction was constantly monitored by checking TLC. After the quenching, separation, and distillation, ITZ-2 was obtained as a light-yellow colored powder.

Acidification of ITZ-2 was performed in LC-MS. The LCMS result revealed that the calculated LC/MS (m/z) is 701.59 and, graphically, the LC/MS value is found to be 702.66 g/mol.
The physical properties of all synthesized piperazine derivatives are summarized in Table 1.

### ANTIOXIDANT ACTIVITY

#### DPPH assay

The free radical scavenging capacity of all four synthesized molecules was estimated using the stable DPPH radical (Benzie and Strain, 1999; Katalinic et al., 2006). Different concentrations (100–500 µg/ml) of the synthesized molecules were taken in separate test tubes. By using methanol, the volume of each test tube was made up to 0.1. 3 ml of DPPH solution (whose absorbance was pre-set to 1) was added in all the test tubes containing sample and methanol. Further all the test tubes incubated in dark conditions for 15 min. After incubation, by keeping methanol as a blank, the absorbance was read at 517 nm spectrophotometrically. Percentage inhibition was calculated using the formula:

\[
\text{Percentage inhibition} = \frac{[\text{Control Ab} - \text{Sample Ab}]}{\text{Control Ab}} \times 100
\]

### ANTI-MICROBIAL ACTIVITY

#### Bacteria, fungi, and culture media.

In the present work, *C. albicans*, *S. aureus* (Gram positive), and *P. aeruginosa* (Gram negative) were used to investigate the antifungal and antibacterial activity of all four synthesized compounds. Tetracycline is used as a control against these organisms (Magaldi et al., 2004; Valgas et al., 2007).

#### The well diffusion method

The samples ITZ-1, ITZ-2, KTZ-1, and KTZ-2 were tested in three replicates for their ZOI measurement against organisms (*C. albicans*, *S. aureus*, and *P. aeruginosa*).

#### Culture preparation

##### Culture and media preparation for fungus

30 ml of Dextrose Broth (PDB: Potato-200 g, Dextrose-20 g, and Distilled water-1,000 ml) was prepared in Erlenmeyer flasks by boiling 6 g of potato in 30 ml of distilled water and filtering. After adding 0.6 g of dextrose to the above filtrate, the final volume was made up to 30 ml using distilled water. 15 minutes in an autoclave at 121°C. *Candida albicans* (MTCC 3958) was inoculated and incubation was done at 25°C for 72 hours.

##### Bacterial culture media preparation

In two Erlenmeyer flasks, LB broth (Sodium chloride 10 g, Tryptone 10 g, Yeast extract 6 g, 1,000 ml Distilled water) was prepared by adding Tryptone 0.3 g, Sodium chloride 0.3 g, Yeast extract 0.18 g, and distilled water 30 ml and autoclaved at 121°C for 15 minutes. *Pseudomonas aeruginosa* (MTCC 2453) and *S. aureus* (MTCC 96) strains were inoculated in 30 ml sterilized LB broth flasks and incubation was done at 37°C for 24 hours.

#### Sample preparation

10 mg of samples (ITZ-1, ITZ-2, KTZ-1, and KTZ-2) were dissolved in 1 ml of dimethyl sulfoxide (DMSO),
respective, to prepare different aliquots of samples of 100, 200, 300, and 400 µg/ml concentrations.

Mediation preparation for ZOI

For fungal plate—Potato Dextrose Agar (PDA: Potato-200 g, Dextrose-20 g, Agar-20 g, distilled water-1,000 ml). 30 g of potatoes were boiled in 100 ml of distilled water and filtered and by using distilled water final volume was made up to 150 ml. 3 g of Dextrose and 3 g of Agar were added and further autoclaved for 15 minutes at 121°C.

For bacterial plate—Luria Bertani (LB) agar media (Tryptone 10 g, Sodium chloride 10 g, Yeast extract 6 g, Agar 20 g, Distilled water 1,000 ml), 400 ml was prepared in Erlenmeyer flasks by adding Tryptone 4 g, Sodium chloride 4 g, Yeast extract 2.4 g, Agar 8 g. Distilled water 400 ml and further autoclaved at 121°C for 15 minutes.

Plating to measure ZOI against all three organisms

About 25 ml of the media (LB agar and PDA) was poured on the sterilized petri-plates and then it was allowed to solidify. A 200 µl inoculum (S. aureus, P. aeruginosa and C. albicans) was poured on agar plates. By using a plate spreader it was spread thoroughly. Five wells of 0.6 cm were made in each plate by using the borer. 50 µl of prepared sample containing 100, 200, 300, and 400 µg were loaded into the respective wells, and as a control blank, 50 µl of DMSO was loaded in the middle well. Further all plates were kept for the incubation. Incubation period for the bacterial plates was 24 hours at 37°C and for fungal plates it was 72 hours at 25°C. Afterwards, the zone of inhibition was measured in Supplementary Figure S13a–e.

CONFLICT OF INTEREST

There are no conflicts to declare.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

REFERENCES


Benzine IFF and Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol, 1999; 299: 15–27.


Duran A, Dogan HN, Rollas H. Synthesis and preliminary anticancer activity of new 1,4-Dihydro-3-(3-hydroxy-2-naphtyl)-4-substituted-5H-1,2,4-triazoline-5-thiones. Farmaco, 2002; 57(7):559–64.


Guan LP, Jin QH, Tian GR, Chai KY, Quan ZS. Synthesis of some quinoline-2(1H)-one and 1, 2, 4-triazole [3, 4-a] quinoline derivatives as potent anticonvulsants. J Pharm Sci, 2007; 10(3):254–62.


Kujawski J, Czaja K, Jodłowska-Siewert E, Dettlaff K, Żwawiak AK. Fourier transform infrared spectra and normal mode analysis of 1-

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.

Khan AS, Khan MS, Malik ZA. Synthesis and antifungal activity of some quinoline-2(1H)-one and 1, 2, 4-triazole-5-thiones. Farmaco, 2002; 57(7):559–64.
SUPPLEMENTARY MATERIAL

For all four synthesized molecules (KTZ-1, KTZ-2, ITZ-1, and ITZ-2), a separate file is attached containing their respective:

1. $^1$HNMR,
2. FTIR,
3. LCMS graphs and
4. ZOI (Zone of Inhibition) plate images against all three pathogens and control.

Supplementary Figure S1. $^1$NMR data of KTZ-1 in DMSO.

Supplementary Figure S2. FTIR data of KTZ-1.
Supplementary Figure S3. LCMS data of KTZ-1.

Supplementary Figure S4. \(^1\)NMR data of KTZ-2 in DMSO.
Supplementary Figure S5. FTIR data of KTZ-2.

Supplementary Figure S6. LCMS data of KTZ-2.
Supplementary Figure S7. ¹H NMR data of ITZ-1 in CDCl₃.

Supplementary Figure S8. FTIR data of ITZ-1.
Supplementary Figure S9. LCMS data of ITZ-1.

Supplementary Figure S10. 1H NMR data of ITZ-2 in DMSO.
Supplementary Figure S11. FTIR data of ITZ-2.

Supplementary Figure S12. LCMS data of ITZ-2.
Supplementary Figure S13. MIC plate images of (a) tetracycline (control group), (b) KTZ-1, (c) KTZ-2, (d) ITZ-1, and (e) ITZ-2 against pathogens.