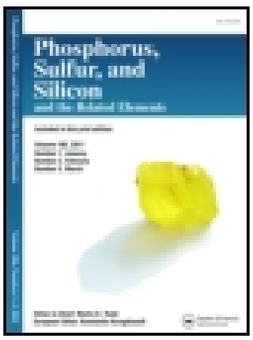

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Urea and thiourea derivatives of 3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine: Synthesis, characterization, antimicrobial activity and docking studies

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ABSTRACT

An efficient and robust synthetic procedure was developed primarily for the synthesis of a precursor compound; 3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-*a*]pyrazine **(11)**, from 2-chloropyrazine **(7)** through the chemical transformations such as hydrazine substitution, trifluoroacetyl group induction, cyclization and pyrazine ring reduction. A new series of urea derivatives **13a-e** and thiourea derivatives **13f-j** of compound **11** have been synthesized and the structures of all the compounds were confirmed using spectroscopic analyses such as IR, ^{1}H NMR, ^{13}C NMR, LC-MS and HRMS. The newly synthesized compounds were screened for their *in vitro* antimicrobial activity against five bacteria and two fungi, in which compounds **13d**, **13i** and **13j** displayed potential activity against bacterial strains and **13a**, **13d**, **13g** and **13j** against fungal strains with the MIC values in the range of 6.25–25.0 μg/mL. An overall comparison of the activity results revealed that thiourea derivatives contain better activity than that of urea compounds. Molecular docking studies on poly (ADP-ribose) polymerase 15 (ARTD7, BAL3) demonstrated that all the synthesized compounds possess significant binding energies (-8.1 to -9.8 kcal/mol) with no adverse effect in the active site of protein.

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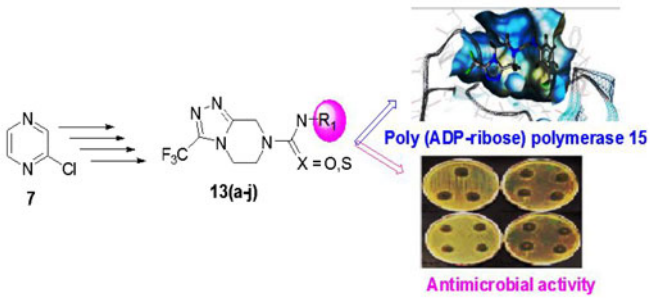
Urea and thiourea derivatives; sitagliptin intermediate; structural characterization; antimicrobial activity; molecular docking; adenosine-5-diphosphoribose enzyme

GRAPHICAL ABSTRACT



Introduction

The increased consumption of antibiotics and inaccurate diagnoses are major factors for antimicrobial resistance.[1] Many antimicrobial resistant human pathogenic microbes have been identified in past few decades such as penicillin-resistant *Streptococcus pneumonia*, vancomycin-resistant *Enterococcus* and methicillin-resistant *Staphylococcus aureus*.[2–4] Treatment of such drug-resistant bacterial infections is very difficult, especially in immunocompromised patients, and requires a high dose of drugs that are possibly more toxic and expensive,[5] and hence, it has emerged as one of the principal public health problems throughout the world.[6] Therefore, there is an urgent need to discover a new class of antimicrobial agents with broad activity-spectrum against multi-resistant pathogens. Structural modifications of well-known existing antimicrobial agents and novel classes of antimicrobials are two imperative strategies to overcome the resistant problems.[7–8]

Heterocycles represent a prominent class of compounds and have pharmaceutical importance due to their vital role

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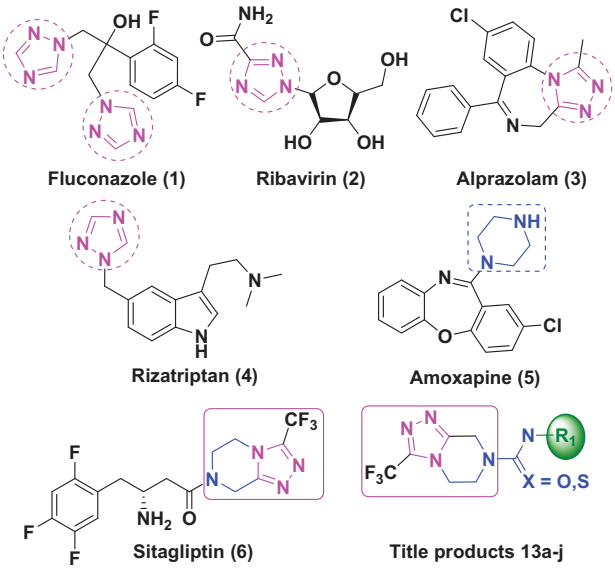


Figure 1. Biologically active 1,2,4-triazole and piperazine derivatives and title products.

in metabolism. They can be used as biomimetics as well as active pharmacophore in a wide variety of drugs, vitamins and many natural products.[9] Particularly, 1,2,4-triazole derivatives are recognized as one of the most promising chemotherapeutic agents in medicinal chemistry.[10] *For example*, fluconazole **(1)** is used in the treatment of fungal infections,[11] while ribavirin **(2)** as antiviral agents,[12] alprazolam **(3)** in anxiolytic treatment[13] and rizatriptan **(4)** as antimigraine.[14] 1,2,4-Triazole derivatives have also prevalent biological activities such as fungicidal,[15] insecticidal,[16] antimicrobial,[17] antidepressant[18] and plant growth regulators.[19] Similarly, the piperazine scaffold has considerable importance in biology, which is an important pharmacophore in numerous medicinal molecules such as amoxapine **(5)** (antidepressant).[20] In addition, unsymmetrical urea and thiourea derivatives have diverse biological activities including antibacterial, antifungal, antitubercular, anti-inflammatory, antithyroid, antihelminitic, rodenticidal, insecticidal and herbicidal properties.[21–24] Hence, we focused, as in the part of our medicinal chemistry programme, on the synthesis and biological evaluation of urea and thiourea derivatives. Recently, we synthesized many urea and thiourea compounds of valsartan[25] and lopinavir intermediates,[26] diazaphospholes,[27] and piperazine doped febuxostat[28] that exhibited promising antimicrobial activities.

Fused scaffolds, i.e., where two or more different pharmacophore units are combined together in one molecule, occupied a unique place in the realm of medicinal chemistry.[29] It has been recognized that 1,2,4-triazole fused compounds are important scaffolds in pharmacologically active compounds with significant antimicrobial and antitubercular activities.[30–31] The precursor compound, 3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-*a*]pyrazine **(11)** used in the present study is an interesting fused molecule of 1,2,4-triazole and piperazine, and is an active pharmacophore of Sitagliptin drug **(6)** (Figure 1). It is well-known in the field of drug discovery that the structural modification of either drug intermediates or target compounds with assimilation of functional groups like urea and thiourea can lead new drug-like compounds. Therefore, considering the biological importance of 1,2,4-triazole fused molecules as well as urea and thiourea derivatives, we expected that the structural modifications of fused molecule **11** with incorporation of urea and thiourea functionalities may produce promising chemotherapeutic agents.

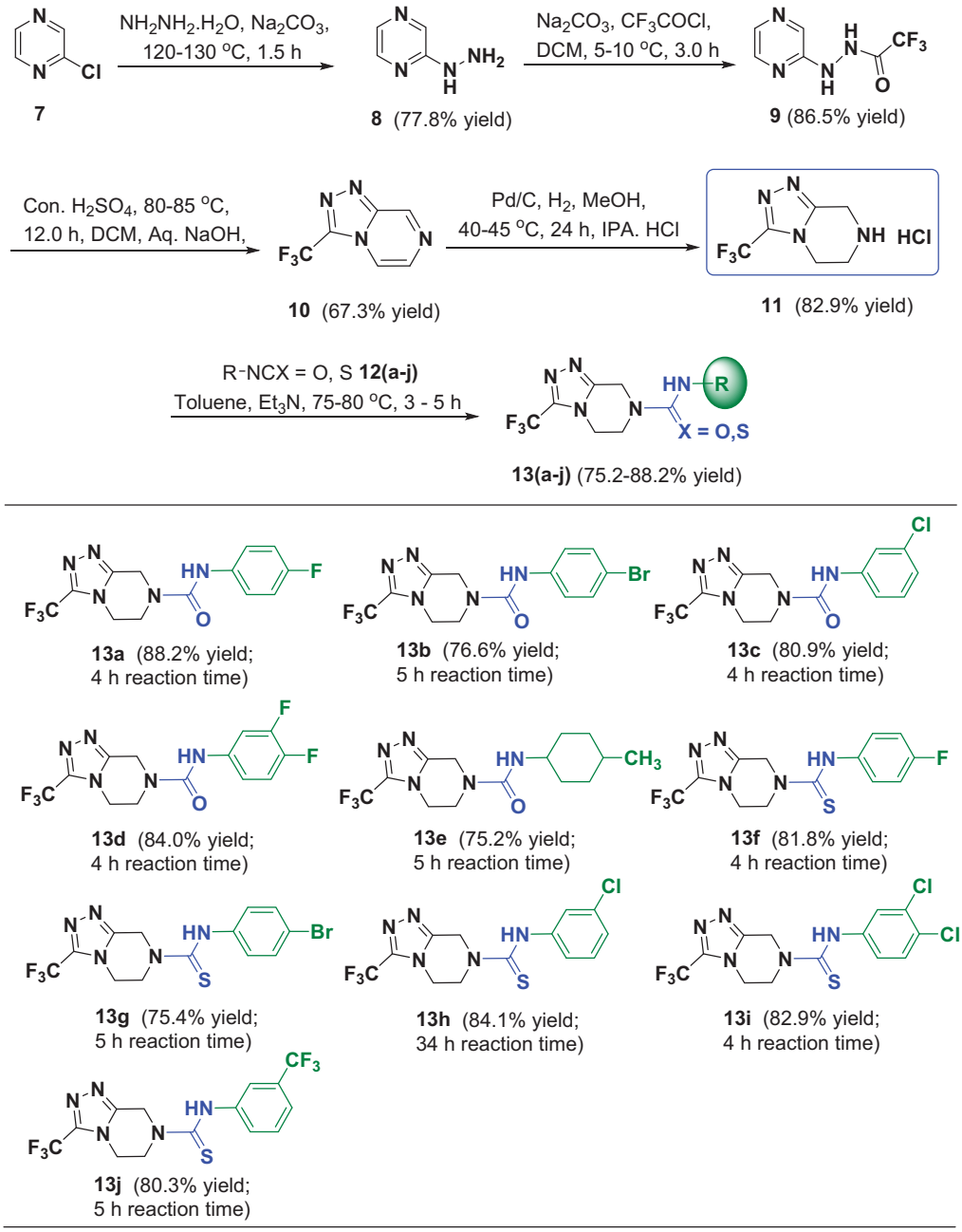
Based on the above considerations and as extension of our discovery for potential antimicrobial agents, we attempted to synthesize a new class of urea and thiourea derivatives of 3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-*a*]pyrazine **(11)**. All the compounds synthesized in this study were evaluated for their *in vitro* antimicrobial activity. Their molecular docking studies were also performed to know the binding orientation of the title products into the enzyme, poly (ADP-ribose) polymerase 15 (ARTD7, BAL3), macro domain 2 complex with adenosine-5-diphosphoribose enzyme (3V2B).

Results and discussion

Chemistry

The desired precursor intermediate **11** is not readily available, and hence attention has been focused initially to synthesize it, using the reported procedure[32] with minor modifications. The schematic of the whole procedure is illustrated in Scheme 1.

The key starting material, 2-chloropyrazine **(7)**, was treated with hydrazine mono-hydrate in the presence of base, sodium carbonate (Na_2CO_3) at harsh temperature (120–130°C) under optimized neat reaction conditions to obtain desired 2-hydrazinopyrazine **(8)** with high yield after isolation from mixture of water:isopropyl alcohol (IPA) (2.0:4.5 v/v). The above reaction in the presence of Na_2CO_3 resulted worthy reaction progress with improved yield (77.8%) when compared with the reaction conducted without base according to the previously reported procedure (yield 53.6%).[32] The hydrazine intermediate **8** was reacted with trifluoroacetyl chloride in the presence of Na_2CO_3 to afford 2,2,2-trifluoro-*N*′-(pyrazin-2-yl)acetohydrazide **(9)** in optimum yield (86.5%). Compound **9** was further cyclized in the presence of concentrated sulfuric acid (H_2SO_4) to give 3-(trifluoromethyl)-1,2,4-triazolo[4,3-*a*] pyrazine **(10)** as a pale brown solid. This cyclization reaction in the presence of concentrated H_2SO_4 was offered good yield of product **10** (67.3%) when compared with the reaction in the presence of polyphosphoric acid (PPA) (51.4%). During the isolation of product **10**, the sulfuric acid was quenched with aqueous sodium hydroxide (NaOH) solution and the product was extracted with dichloromethane (DCM) and then it was purified by recrystallization from the mixture of acetone:cyclohexane (1.5:6.5 v/v). The purification of product **10** using simple recrystallization method, instead of laborious chromatography technique used in the previous protocol, was an additional merit of the described protocol in the present



Scheme 1. Synthetic scheme to the preparation of title urea and thiourea derivatives.

study. Compound **10** was reduced with palladium-carbon (Pd/C) under hydrogen pressure to obtain crude 3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo-[4,3-*a*]pyrazine and then treated with isopropyl alcohol saturated with hydrogen chloride (IPA.HCl) to obtain the desired compound **11** in optimum yield (82.9%) as a white crystalline solid.

Finally, the synthesis of the title *N*-(substituted phenyl/cyclohexyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-*a*] pyrazine-7-carboxamides/carbothioamides **13a-j** were accomplished by the reaction of compound **11** with substituted isocyanate/thioisocyanate **12a-j** in the presence of excess triethylamine (TEA) under mild reaction conditions. The crude products obtained after the workup were purified by subjecting column chromatography using 10-50% ethyl acetate:hexane mixture as the mobile phase based on the elution of the desired compound.

Spectroscopic data

Structures of the newly synthesized compounds were elucidated using spectroscopic data such as IR and NMR (^{1}H and ^{13}C), mass and HRMS analyses. The precursor intermediate **11** was synthesized according to reported procedure with modifications and its spectroscopic data matched to that reported data.[32] The characteristic absorption bands in the region of 3200–3350 cm^{-1} in all the title products, 1630–1690 cm^{-1} in urea compounds and 1180–1300 cm^{-1} in thiourea derivatives of IR spectra are mainly attributed due to the presence of N-H, C = O and C = S linkages,

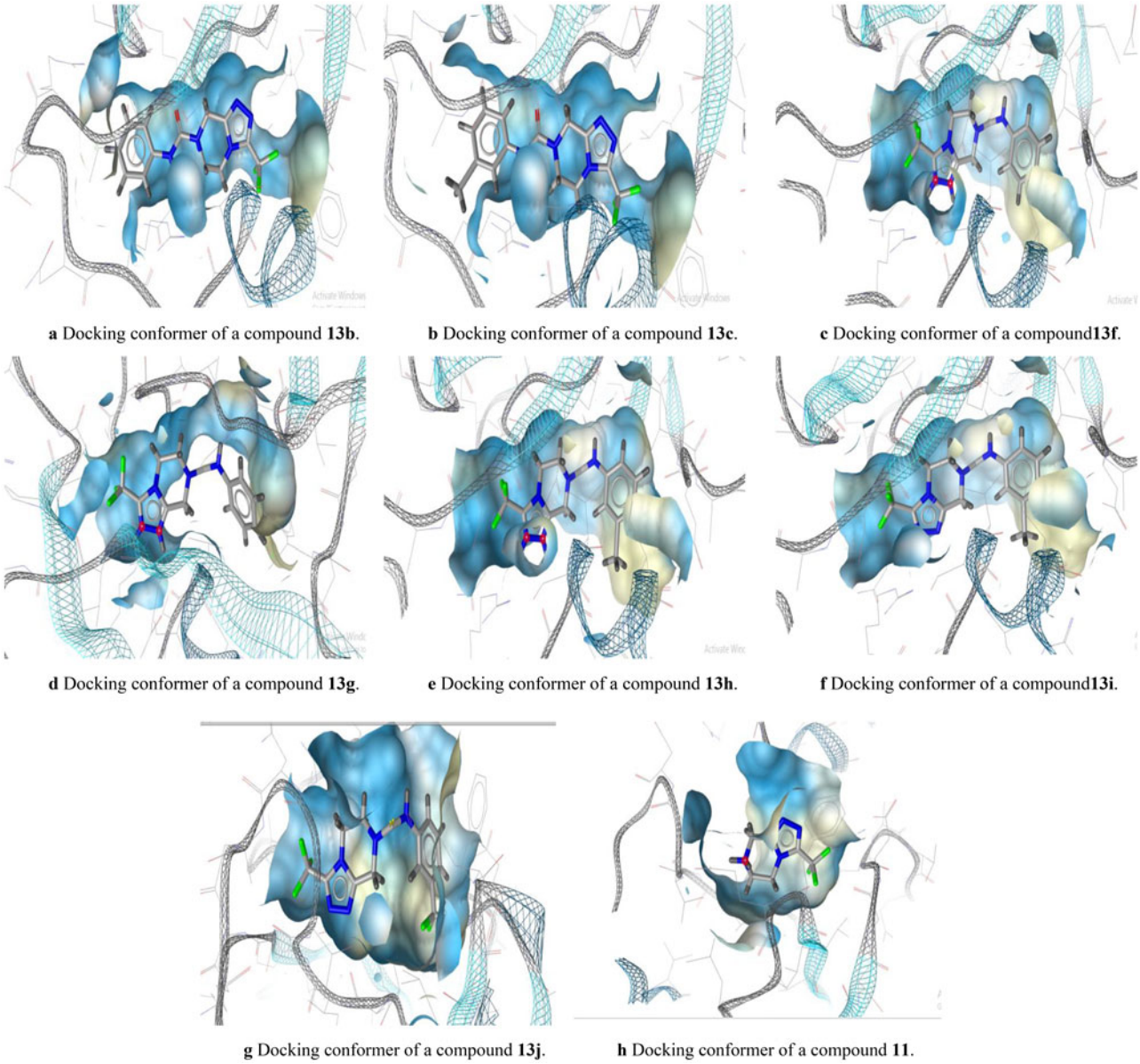


Figure 2. Docking conformers of the active title products and compound **11** in the active site of adenosine-5-diphosphoribose enzyme (3V2B).

respectively. Likewise, the absorption bands around 1045–1130 cm^{-1} confirms the presence of CF_3 group as well as absorption bands in the range of 1520–1620 cm^{-1} due to C = N linkage in triazole ring. The two protons resonated at the chemical shift 10.58 ppm corresponding to NHHCl of piperazine of precursor intermediate **11** was not appeared in the newly synthesized urea and thiourea products, suggesting that it involves in the formation of urea and thiourea derivatives by interacting with cyanate/isocyanate groups. In ^1H NMR spectra of title products, the chemical shift values in the region of 9.00–9.98 ppm as singlets indicated the presence of NH proton attached to urea and thiourea functionalities. The peaks at 3.90–4.70 ppm as triplets and 4.80–5.00 ppm as singlet confirmed the protons –N-CH_2-CH_2-N- and –N-CH_2-C- present in the piperazine ring, respectively. Moreover, the aromatic protons, based on the structural orientation, resonated in the range of 6.80–7.95 ppm. The signals between 152–155 ppm and 181–183 ppm in ^{13}C NMR spectra are attributed the carbons attached to carbonyl group in urea derivatives and thiocarbonyl group in thiourea compounds, respectively. The doublet peaks at 115–119 ppm with a coupling constant 260–270 Hz confirmed the presence of the –CF_3 group. Chemical shifts in the region of 40–46 ppm are correlated with the carbons in the piperazine ring and other aromatic carbons are observed in the range of 105–155 ppm. The mass values found in ESI-MS in positive mode are well coordinated with mass of the newly synthesized urea and thiourea derivatives **13a-j** and HRMS data provides additional evidence in the structural characterization of the products.

Biological activity

Because of the potential antimicrobial tendency of 1,2,4-triazole fused molecules,[30] urea and thiourea derivatives,[24] and our continuing efforts in the development of

new class of antimicrobials,[25–28] we planned to investigate the antimicrobial potency of the newly synthesized compounds **13a-j**. American Type Culture Collection (ATCC) bacterial strains such as *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 43300), and two fungi like *Candida albicans* (ATCC 90028) and *Cryptococcus neoformans* (ATCC 208821) were used as test organisms. The preliminary screening of the antimicrobial activity has been tested following the agar well diffusion method[33] and using the antibiotics; Ciprofloxacin and fluconazole, as reference drugs for bacterial and fungal activities, respectively. The results were recorded for each tested compound as the average diameter of zone of inhibition (ZOI) of microbial growth around the disks in mm. The experimental results obtained against bacteria and fungi are provided in Table S1 and Table S2 (Supplemental Materials), respectively. A few of the synthesized products exhibited potential growth of zone of inhibitions. Hence, the minimum inhibitory concentration (MICs) of the compounds, that is, the lowest concentration showing no growth of inhibition was evaluated. A Twofold serial dilution technique[34] was used to screen MICs and experimental results are shown in Table S3.

As depicted in Table S1 and Table S3, all the title products showed better antimicrobial activity than precursor compound **11**, demonstrating that the substituents and functional groups such as urea and thiourea are enriching the biological activity. In fact, urea compound **13d** substituted with 2,4-difluorophenyl ring, and the thiourea derivatives, **13i** bearing 3,4-dichlorophenyl ring and **13j** bound with 3-trifluoromethylphenyl ring exhibited potential bacterial activity against all the tested bacteria in the range MIC values of 6.25–25.0 μg/mL, although, the rest of the compounds showed lower activity when compared with the reference drug, Ciprofloxacin (MIC 1.562–3.125 μg/mL). The compounds such as **13a** linked with 4-fluorophenyl ring against *P. aeruginosa* (ATCC 27853) (MIC 6.25 μg/mL), *K. pneumonia* (ATCC 700603) (MIC 12.50 μg/mL) and *A. baumannii* (ATCC 19606) (MIC 12.50 μg/mL), **13b** bearing with 4-bromophenyl ring (MIC 12.50 μg/mL) and **13f** bound with 4-fluorophenyl ring (MIC 12.50 μg/mL) against *K. pneumonia* (ATCC 700603), 4-bromophenyl ring substituted thiourea **13g** against *A. baumannii* (ATCC 19606) (MIC 12.50 μg/mL) and *E. coli* (ATCC 25922) (MIC 25.0 μg/mL), and 3-chlorophenyl ring bound thiourea **13h** against *A. baumannii* (ATCC 19606) (MIC 12.50 μg/mL) showed good antibacterial activity independently. These results suggest that thiourea derivatives possess good antibacterial activity than urea compounds.

The antifungal activity (Table S2 and Table S3) revealed that most of title products possess good growth of inhibition against *C. neoformans* (ATCC 208821) than *C. albicans* (ATCC 90028). All the compounds except compound **13f** showed good activity against *C. neoformans* (ATCC 208821) at both the concentrations, 50 and 100 μg/mL, and only few compounds against *C. albicans* (ATCC 90028). The urea compounds **13a** bearing with 4-fluorophenyl ring and **13d** bound with 2,4-difluorophenyl ring, and thiourea derivatives such as **13g** connected with 4-bromophenyl ring and **13j** associated with 3-trifluoromethylphenyl ring displayed promising activity against both fungal strains, *C neoformans* (ATCC 208821) and *C. albicans* (ATCC 90028) in the range MIC values of 6.25-25.0 μg/mL. Thiourea derivative **13h** (MIC 12.50 μg/mL) and urea derivative **13c** (MIC 25.0 μg/mL) connected with 3-chlorophenyl ring showed good and moderate activities against *C. neoformans* (ATCC 208821), respectively.

Based on the overall biological activity observations, we found that only a few compounds possess potential antibacterial and antifungal activities as well with MIC range 6.25–12.50 μg/mL, while most of the title products activity against the pathogens such as *E. coli* (ATCC 25922), *S. aureus* (ATCC 43300) and *C. albicans* (ATCC 90028) was poor, and against *P. aeruginosa* (ATCC 27853), *K. pneumonia* (ATCC 700603), *A. baumannii* (ATCC 19606) and *C. neoformans* (ATCC 208821) was moderate to very good. The potential activity can be attributed to the presence of active pharmacophore groups such as fluoro- and chloro- attached to the phenyl ring. On the other hand, thiourea derivatives, based on the activity results comparison of the same substituted set of compounds **13a/f**, **13b/g** and **13c/h**, disclosed better activity than that of urea compounds, suggesting the thiourea functionality could also be a reason for the potential activity. Therefore, further investigation is required to better understand the structural-activity relationship (SAR) including altering the substituents on the phenyl ring, bonding with other active pharmacophore unit like acyclic, cyclic and heterocyclic, and structural modifications in basic pharmacophore unit.

Molecular docking study

ADP-ribosylation is a protein modification process that acts in pathogenic mechanisms, intracellular signaling systems, DNA repair, and cell division and that has been studied in animals, plants, and bacteria.[35–36] Numerous bacterial toxins are ADP-ribosyltransferases, which can add poly-ADP-ribose (PARylation) and mono-ADP-ribose (MARylation) to proteins as well as catalyze ADP-ribosylation by transferring the ADP-ribose moiety of NAD to target protein. Poly(ADP-ribose) polymerase (PARP) is a glutamate of a target protein (initiation) like the other bacterial ADP-ribosyltransferases distinctly from mono-ADP-ribosylation.[37] PARP is located in the nucleus of most eukaryotes and helps to maintain genomic integrity in base excision repair, in DNA recombination, amino acids modifications and in cellular differentiation.[36,38] Therefore, the topics of PARP proteins have attracted a new wave of interest. Recently, urea and thiourea derivatives of 2-nitro-5-[thiophene-2yl]benzenamine are reported as good antimicrobial agents and showed high binding energies with poly (adp-ribose) polymerase 15 (3V2B) enzyme.[39]

In order to interpret the binding mode of the synthesized urea and thiourea derivatives **13a-j**, the molecular docking study was performed against three-dimensional structure of poly(ADP-ribose) polymerase 15 (ARTD7, BAL3), macro domain 2 in complex with adenosine-5-diphosphoribose enzyme (3V2B), which retrieved from Protein Data Bank, using the AutoDockVina Tools.[40–41] The protein was refined with MD simulation which was carried out with the Visual Molecular Dynamics (VMD) tool.[42] The CHARMM 27 field was used for parameterization, and the program NAMD was used for energy minimization and molecular dynamics (MD) simulations.[43] The precursor intermediate **11** and the title ligands were redocked at the crystal enzyme structure of the protein and the best energy conformations of poly(ADP-ribose) polymerase 15 (ARTD7, BAL3)-ligand were studied. The analysis of the binding conformation has furnished using a scoring function based on the free energy of bindings.[44] The individual energies of the poly (ADP-ribose) polymerase 15 (ARTD7, BAL3) with the ligands are tabulated in Table S 4, and the active conformers are shown in Figure 2 and Figure S1.

As seen in Table S4, the synthesized compounds are bind well into the active site of enzyme with the best binding energies (scores) in order of **13j** (−9.8 kcal/mol)>**13h** (−9.4 kcal/mol)>**13i** (−9.3 kcal/mol)>**13f** (−9.2 kcal/mol)>**13g** (−8.9 kcal/mol)=**13b** (−8.9 kcal/mol)>**13c** (−8.8 kcal/mol)>**13e** (−8.6 kcal/mol)>**13a** (−8.3 kcal/mol), **13d** (−8.1 kcal/mol) and compound **11** (−6.1 kcal/mol). Interestingly, all the synthesized urea and thiourea molecules **13a-j** showed the better binding energies with enzyme than a precursor intermediate **11**. Thiourea derivatives, among the same substituted set of compounds **13a/f**, **13b/g** and **13c/h**, were bound well (> −9.0 kcal/mol) with enzyme than that of urea derivatives (< −9.0 kcal/mol), and it is provided a good agreement to *in vitro* antimicrobial activity. It demonstrated that thiourea functionality perform as an active pharmacophore unit to explore potential activity than urea group. The active site residues such as Ser302, Ala385, Val424, Gln427, Tnr388, Gly389 and Ala391 are play vital role in the ligands interactions and could be involved in antimicrobial activity.

ADME calculations

A computational study has been carried out on the synthesized products for the prediction of ADME properties. The obtained results are summarized in Table S5 and Table S6 (Supplemental Materials). "Lipinski Rule of 5" stated that a compound can more likely to be membrane permeable and easily absorbed by the body if its molecular weight is less than 500, lipophilicity would be expressed as a quantity known as logP is less than 5, the number of groups in the molecule that can donate hydrogen atoms to hydrogen bonds is less than 5, the number of groups that can accept hydrogen atoms to form hydrogen bonds is less than 10 and the number of rotatable bonds is less than 10.[45] The data displayed that all the synthesized compounds having logP ≤ 5, molecular weight ≤500, number of hydrogen bond acceptors ≤10, number of hydrogen bond donors ≤5, and number of rotatable bonds ≤10 and obeyed Lipinski Rule 5 without any violation, suggesting the synthesized title products are orally active chemotherapeutic agents with good adsorption.

The pharmacokinetic properties and toxicities were predicted for all the synthesized compounds using OSIRIS property explorer. LogS indicating the parameter of aqueous solubility, and other properties like mutagenicity, tumorigenicity, irritation effect, and risk of reproductive effect are predicted for toxicity study. The drug score combines drug likeness, cLogP, LogS, and toxicity risks in one value can show overall potential to qualify drug. All the synthesized compounds showed more aqueous solubility (-3.81 to -5.50 mol./L), drug likeness (-10.4 to -19.8) and drug score (0.18 to 0.31), and did not explored toxicity properties such as mutagenicity, tumorigenicity, irritation effect, and risk of reproductive effect which demonstrating the title products having high bio-availability and possess good drug-like properties. However, urea compounds such as **13b** and **13c** and thiourea derivatives like **13h**, **13i** and **13j** showed good binding energies than other title compounds which provide a good agreement to *in vitro* antimicrobial activity.

Experimental

Materials and methods

Chemicals, solvents and reagents used in this study were purchased from Sigma-Aldrich and sd Fine-Chem. Limited and used directly without further purification. The progress of reactions was monitored by thin layer chromatography (TLC) (Merck silica plates), using visualization by UV light or in iodine vapor. The column chromatography packed with Merck 120 mesh silica gel as a stationary phase and a mixture of different ratios of ethyl acetate (EtOAc) and hexane, based on the elution of compounds, as a mobile phase were used to purify the title crude products. Melting range of the title products was recorded using open capillary tube on Guna melting point apparatus with increasing temperature rate 3 °C/min and is uncorrected. IR spectra were recorded on Shimadzu IR Affinity-I FT-IR spectrophotometer, using KBr dispersion method, over the range of 4000–400 cm^{-1}. One dimensional nuclear magnetic resonance spectroscopy (1D NMR) (^{1}H and ^{13}C) was performed on Ascend™ Bruker 400 instrument which operated at 400 MHz frequency for ^{1}H NMR and 100 MHz frequency for ^{13}C NMR in DMSO-d_6 solvent, and tetramethylsilane (TMS) was used as the internal standard. NMR results are reported as chemical shifts (δ) in ppm, multiplicity, coupling constant values (J) in Hertz (Hz), number of protons and proton's position. Multiplicities are shown as the abbreviations: s (singlet), brs (broad singlet), d (doublet), t (triplet) and m (multiplet). High-resolution mass spectra were obtained by using a QTOF mass spectrometer. International principles and regulations are followed during the screening of biological activity. The Supplemental Materials contains sample ^{1}H and $_{13}C$ NMR and mass spectra for products 13a-j (Figures S2 – S48).

Preparation of 2-hydrazinopyrazine (8)

The mixture of 2-chloropyrazine **(7)** (20.0 g, 0.175 mol), hydrazine hydrate (60 mL) and sodium carbonate (27.84 g, 0.264 mol) was stirred for 2 h at 120-130 °C. After completion of the reaction that was checked by TLC, the reaction mass was allowed to cool at 50-60 °C and then water (60.0 mL) followed by isopropyl alcohol (IPA) (40.0 mL) were added. The reaction mass was maintained for 16 h at 0-3 °C and then the precipitated white solid was separated by filtration. The wet solid was dried under vacuum at 45-50 °C to obtain desired 2-hydrazinopyrazine (14.8 g, 77.8%). ^{1}H NMR (400 MHz; CD_3OD), δ, ppm (J, Hz): 7.94-8.14 (2H, m, Ar-H); 7.69 (1H, s, Ar-H). ESI-MS *m/z* (*rel*, %): 111.07 ($M+H^+$) (100). HRMS (FAB) Calc.: $C_4H_6N_4$: 110.059246; Found: 111.0592 [$M+H^+$].

Preparation of 2,2,2-trifluoro-N′-(pyrazin-2-yl) acetohydrazide (9)

To a stirred reaction mixture of 2-hydrazinopyrazine **(8)** (14.0 g, 0.127 mol) and sodium carbonate (16.2 g, 0.153 mol) in dichloromethane (DCM) (112 mL), the solution of trifluoroacetyl chloride (18.5 g, 0.14 mol) in DCM (28 mL) was added slowly at 0-5 °C. The reaction mixture was agitated at 5-10 °C until completion of the reaction that was judged by TLC. Water (140 mL) was added to the reaction mass to remove water soluble components and then it was allowed to stand to attain the partition between DCM and water. The organic layer was concentrated under atmospheric conditions at below 45 °C. The crude residue was recrystallized from mixture of EtOAc:hexane to obtain 2,2,2-trifluoro-*N*′-(pyrazin-2-yl)acetohydrazide **(9)** as off-white solid (22.5 g, 86.5%). ^{1}H NMR (400 MHz; CD_3OD), δ, ppm (J, Hz): 7.94-8.22 (2H, m, Ar-H); 7.66 (1H, s, Ar-H). ESI-MS *m/z* (*rel*, %): 207.12 ($M+H^+$) (100).

Preparation of 3-(trifluoromethyl)-1,2,4-triazolo[4,3-a] pyrazine (10)

To the solution of concentrated H_2SO_4 (20.0 mL), compound **9** (20.0 g, 0.097 mol) was added in a portion-wise at 0-5 °C and then the reaction mass was stirred for 12 h at 80-85 °C. After completion of the reaction that was checked by TLC, DCM (120 mL) was added to the reaction mass at ambient temperature and then the reaction mass was quenched with 30% aqueous NaOH solution by maintaining temperature at -5 to 5 °C (Highly exothermic) up to attain the mass pH to 8.0-10.0. The inorganic salts were separated by filtration, and the solvent was removed from the organic layer under atmospheric conditions at below 45 °C. The crude residue product was recrystallized from acetone:cyclohexane (1.5:6.5 v/v) to obtain, 3-(trifluoromethyl)-1,2,4-triazolo[4,3-*a*]pyrazine **(10)** as a pale brown solid (12.4 g, 67.3%). ^{1}H NMR (400 MHz; $CDCl_3$) δ, ppm (J, Hz): 8.12-8.22 (2H, m, Ar-H); 9.43 (1H, s, Ar-H). ESI-MS *m/z* (*rel*, %): 189.03 $(M+H)^+$ (100). HRMS (FAB) Calc.: $C_6H_3F_3N4$: 188.0309816199; Found: 189.0309 [$M+H^+$].

Preparation of 3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo-[4,3-a]pyrazine hydrochloride (11)

A mixture of compound **10** (12.0 g, 0.064 mol) and the catalyst, Pd/C (10% w/w, 120.0 mg) in methanol (MeOH) (120 mL) was charged into an autoclave (500 mL) at ambient temperature. The reaction mass was agitated for 24 h by maintaining 3.5-4.5 Kg/cm^2 hydrogen pressure at 40-45 °C. The completion of reaction was judged by TLC. The reaction mass was concentrated under reduced pressure after remove the Pd/C catalyst by passing through celite under nitrogen atmosphere. The residue was dissolved in IPA (24.0 mL) and then IPA.HCl (24.0 mL) was added slowly at 0-5 °C. The precipitated solid was separated by filtration, after one hour agitation of the reaction mass, to obtain 3-trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo-[4,3-*a*]pyrazine hydrochloride **(11)** as a white crystalline solid (12.1 g, 82.9%). m.p.: 237-249 °C (Lit. 236-246 °C reported in chemical book). ^{1}H NMR (400 MHz; DMSO-d_6), δ, ppm (J, Hz): 3.65 (2H, t, $J=5.6$, Piperazine); 4.46 (2H, t, $J=5.6$, Piperazine); 4.61 (2H, s, Piperazine); 10.58 (2H, br, NH_2^+). ^{13}C NMR (100 MHz; DMSO-d_6) δ, ppm (J, Hz): 39.09 (-**C**H$_2$-CH$_2$-), 40.14 (-CH$_2$-**C**H$_2$-), 40.90 (-**C**H$_2$-), 117.17 (d, $J=268.4$, **C**F$_3$), 142.29 (**C** triazole), 148.73 (**C** triazole). LC-MS *m/z* (*rel*, %): 193.08 $(M+H)^+$ (100).

General procedure for the synthesis of title urea and thiourea derivatives (13a-j)

To the stirred reaction mixture of 3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo-[4,3-*a*]pyrazine hydrochloride **(11)** (300 mg, 1.32 mmol), TEA (0.55 mL, 3.95 mmol) and toluene (10 mL), substituted cyanates **12(a-e)**/isocyanates **12(f-j)** (1.32 mmol) were added at ambient temperature. The reaction mass was agitated at 75-80 °C until the completion of the reaction that was monitored by TLC. The reaction mass was allowed to cool at ambient temperature and it was washed sequentially with 3% aqueous HCl (5.0 mL) and then water (5.0 mL). The organic fraction was concentrated under vacuum at 50-55 °C to obtain crude product. It was purified by column chromatography using 10-50% of EtOAc:hexane mixture as a mobile phase.

N-(4-Fluorophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo [4,3-a]pyrazine-7-carboxamide (13a)

White crystalline solid; m.r.: 206-208 °C. IR (KBr, $\tilde{\nu}$/cm^{-1}): 3360 (N-H, str); 1674 (C=O, str); 1544 (C=N, str); 1163 (-CF$_3$, str). ^{1}H NMR (400 MHz; DMSO-d_6), δ, ppm (J, Hz): 3.95-3.98 (2H, t, $J=5.2$, Piperazine), 4.22-4.24 (2H, t, $J=5.2$, Piperazine), 4.95 (2H, s, Piperazine), 7.08-7.13 (2H, m, Ar-H), 7.43-7.79 (2H, m, Ar-H), 8.9 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm (J, Hz): 39.98 (-**C**H$_2$-CH$_2$-); 40.87 (-CH$_2$-**C**H$_2$-); 43.30 (**C**H$_2$-C-), 114.79 (d, $J=22.0$, **C**F$_3$), 117.12 (d, $J=268.0$, **C**-F Ar), 121.68 (d, $J=8.0$, **C** Ar), 136.14, 151.09 (-**C**=O), 154.59 (**C** Ar), 156.50 (**C** Ar), 158.87 (**C** Ar). LC-MS, *m/z*, (*rel*, %): 330.09

$[M+H]^+$ (100). HRMS (FAB) Calc.: $C_{13}H_{11}F_4N_5O$: 329.08997; Found: 330.0967 $[M+H^+]$.

N-(4-Bromophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carboxamide (13b)

Off-white solid; m.r.: 218-221 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3407 (N-H, str); 1668 (-C=O, str); 1533 (C=N, str); 1147 (CF_3, str); 835 (C-Br, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 3.96-3.98 (2H, t, $J=5.2$, Piperazine); 4.22-4.24 (2H, t, $J=5.2$, Piperazine); 4.96 (2H, s, Piperazine); 7.42-7.48 (4H, m, Ar-H); 8.99 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 40.03 (-**C**H_2-CH_2-); 40.92 (-CH_2-**C**H_2-); 43.32 (-**C**H_2-); 113.88 (**C** Ar); 117.14 (d, $J=268.0$, **C**F_3); 121.70 (**C** Ar); 131.19 (**C** Ar); 139.41 (**C** Ar); 142.33 (**C** Ar); 151.07 (**C**=O); 154.34 (**C** Ar). LC-MS m/z (*rel*, %): 390.00 $[M+H]^+$ (100); 392.00 $[M+H+2]^+$ (97). HRMS (FAB) Calc.: $C_{13}H_{11}BrF_3N_5O$: 389.00990; Found: 390.0154 $[M+H^+]$.

N-(3-Chlorophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carboxamide (13c)

Off-white powder; m.r.: 166-168 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3315 (N-H, str); 1664 (C=O, str); 1527 (C=N, str); 1143 (CF_3, str), 777 (C-Cl, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 3.96-3.99 (2H, t, $J=5.2$, Piperazine); 4.22-4.25 (2H, t, $J=5.2$, Piperazine); 4.96 (2H, s, Piperazine); 7.01-7.04 (1H, m, Ar-H); 7.27-7.31 (1H, t, $J=8.0$, Ar-H); 7.40-7-42 (1H, d, $J=8.4$, Ar-H); 7.66 (1H, s, Ar-H); 9.04 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 40.03 (-**C**H_2-CH_2-); 40.9 (-CH_2-**C**H_2-); 43.25 (-**C**H_2-); 117.11 (**C** Ar); 117.92 (d, $J=268.0$, -**C**F_3); 119.09 (**C** Ar); 121.84 (**C** Ar); 130.04 (**C** Ar); 132.75 (**C** Ar); 141.53 (**C** Ar); 142.31 (**C** Ar); 151.01 (**C**=O); 154.26 (**C** Ar). LC-MS m/z (*rel*, %): 346.11 $[M+H]^+$ (100); 348.06 $[M+H^++2]$ (34). HRMS (FAB) Calc.: $C_{13}H_{11}ClF_3N_5O$: 345.06042; Found: 346.0668 $[M+H^+]$, 348.0628 $[M+H^++2]$.

N-(2,4-Difluorophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carboxamide (13d)

Pale brown solid; m.r.: 156-158 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3242 (N-H, str); 1645 (C=O, str); 1516 (C=N, str); 1141 (CF_3, str); 1097 (C-F, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 3.95-3.98 (2H, t, $J=5.2$, Piperazine); 4.2-2-4.25 (2H, t, $J=5.2$, Piperazine); 4.93 (2H, s, Piperazine); 7.02-7.06 (1H, m, Ar-H); 7.25-7.30 (1H, m, Ar-H); 7.38-7.44 (1H, m, Ar-H); 8.75 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 40.12 (-**C**H_2-CH_2-); 40.89 (-CH_2-**C**H_2-); 43.27 (-**C**H_2-); 103.83 (**C** Ar); 110.77 (dd, $J=18.0$ Hz, 4.0, C, Ar); 117.11 (d, $J=269.0$, **C**F_3); 123.24 (**C** Ar); 128.03 (**C** Ar); 142.32 (**C** Ar); 151.02 (**C**=O); 154.52 (**C** Ar); 156.99 (**C** Ar); 160.14 (**C** Ar). LC-MS m/z (*rel*, %): 348.13 $[M+H]^+$ (100). HRMS (FAB) Calc.: $C_{13}H_{10}F_5N_5O$: 347.08055; Found: 348.0952 $[M+H^+]$.

N-(4-Methylcyclohexyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carboxamide (13e)

Pale brown powder; m.r.: 216-219 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3302 (N-H, str); 1637 (C=O, str); 1550 (C=N, str); 1113 (CF_3, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 0.85-0.86 (3H, m, Cyclohexyl); 0.88- 0.99 (2H, m, Cyclohexyl); 1.16-1.32 (3H, m, Cyclohexyl); 1.63-1.66 (2H, m, Cyclohexyl); 1.75- 1.77 (2H, m, Cyclohexyl); 3.34-3.42 (1H, m, Cyclohexyl); 3.80-3.83 (2H, t, $J=5.2$, Piperazine); 4.12-4.14 (2H, t, $J=5.2$, Piperazine); 4.77 (2H, s, Piperazine); 6.60 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 22.17 (**C**-Cyclohexane); 31.59 (**C**-Cyclohexane); 32.71 (**C**-Cyclohexane); 33.81 (**C**-Cyclohexane); 40.12 (-**C**H_2-CH_2-); 40.60 (-CH_2-**C**H_2-); 43.25 (-**C**H_2-); 49.48 (**C**-Cyclohexane); 117.10 (d, $J=269.0$, **C**F_3); 142.25 (**C**, Ar); 151.33 (**C**=O); 156.07 (**C**, Ar). LC-MS m/z (*rel*, %): 332.25 $[M+H]^+$ (100). HRMS (FAB) Calc.: $C_{14}H_{20}F_3N_5O$: 331.16199; Found: 332.1682 $[M+H^+]$.

N-(4-Fluorophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carbothioamide (13f)

White solid; m.r.: 204-206 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3232 (N-H, str); 1552 (C=N, str); 1274 (C=S, str); 1122 (CF_3, str); 1020 (C-F, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 4.31-4.33 (2H, t, $J=5.2$, Piperazine); 4.41-4.44 (2H, t, $J=5.2$, Piperazine); 5.38 (2H, s, Piperazine); 7.15-7.19 (2H, m, Ar-H); 7.31-7.34 (2H, m, Ar-H); 9.73 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 42.94 (-CH_2-**C**H_2-); 44.04 (-**C**H_2-CH_2-); 44.82 (-**C**H_2-); 114.70 (d, $J=22.0$, **C** Ar); 117.08 (d, $J=268$, CF_3); 128.10 (d, $J=9.0$, **C** Ar); 136.74 (d, $J=3.0$, **C** Ar); 142.28 (d, $J=38.0$, **C** Ar); 150.95 (**C** Ar); 158.37 (d, $J=240.0$, **C** Ar); 182.42 (**C**=S). LC-MS m/z (*rel*, %): 346.05 $[M+H]^+$ (100). HRMS (FAB) Calc.: $C_{13}H_{11}F_4N_5S$: 345.06712; Found: 346.0735 $[M+H^+]$.

N-(4-bromophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carbothioamide (13g)

Pale brown solid; m.r.: 153-155 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3240 (N-H, str); 1528 (C=N, str); 1274 (C=S, str); 1159 (CF_3, str); 783 (C-Br, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 4.33-4.34 (2H, t, $J=4.8$, Piperazine); 4.40-4.43 (2H, t, $J=4.8$, Piperazine); 5.38 (2H, s, Piperazine); 7-28-7.60 (4H, m, Ar-H); 9.82 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 42.93 (-CH_2-**C**H_2-); 44.18 (-**C**H_2-CH_2-); 44.99 (-**C**H_2-); 117.10 (d, $J=269.0$, **C**F_3); 120.52 (**C** Ar); 124.49 (**C** Ar); 127.58 (**C** Ar); 128.21 (**C** Ar); 142.16 (**C** Ar); 150.91 (**C** Ar); 182.14 (**C**=S). LC-MS m/z (*rel*, %): 405.97 $[M-H]^+$ (100); 407.95 $[M-H+2]^+$ (97).

HRMS (FAB) Calc.: $C_{13}H_{11}BrF_3N_5S$: 404.98706; Found: 405.9857 $[M+H^+]$.

N-(3-Chlorophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carbothioamide (13h)

Off-white solid; m.r.: 137-139 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3219 (N-H, str); 1546 (C = N, str); 1273 (C = S, str), 1161 (CF_3, str); 715 (C-Cl, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 4.33-4.34 (2H, t, $J = 5.2$, Piperazine); 4.41-4.43 (2H, t, $J = 5.2$, Piperazine); 5.38 (2H, s, Piperazine); 7.21-7.22 (1H, d, $J = 7.6$, Ar-H); 7.30-7.38 (2H, m, Ar-H); 7.47 (1H, s, Ar-H); 9.82 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 42.92 (-CH_2-**C**H_2-); 44.19 (-**C**H_2-CH_2-); 44.98 (-**C**H_2-); 114.41 (**C** Ar); 117.10 (d, $J = 268.0$, CF_3); 122.46 (**C** Ar); 124.05 (**C** Ar); 125.37 (**C** Ar); 129.73 (**C** Ar); 132.19 (**C** Ar); 141.90 (**C** Ar); 150.90 (**C** Ar); 182.16 (**C**=S). LC-MS m/z (*rel*, %): 361.98 $[M+H]^+$ (100); 363.95 $[M+H^++2]^+$ (35). HRMS (FAB) Calc.: $C_{13}H_{11}ClF_3N_5S$: 361.03758; Found: 362.0314 $[M+H]^+$, 364.0297 $[M+H^++2]$.

N-(3,4-Dichlorophenyl)-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-a]pyrazine-7-carbothioamide (13i)

Off-white solid; m.r.: 187-188 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3215 (N-H, str); 1537 (C = N, str); 1273 (C = S, str); 1159 (CF_3, str); 717 (C-Cl, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 4.33-4.34 (2H, d, $J = 4.8$, Piperazine); 4.41-4.42 (2H, d, $J = 5.2$, Piperazine); 5.38 (2H, s, Piperazine); 7.35-7.37 (1H, m, Ar-H); 7.58-7.60 (1H, d, $J = 8.8$, Ar-H); 7.67 (1H, m, Ar-H); 9.86 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 42.87 (-CH_2-**C**H_2-); 44.21 (-**C**H_2-CH_2-); 45.0 (-**C**H_2-); 117.07 (d, $J = 268.0$, **C**F_3); 125.58 (**C** Ar); 126.78 (**C** Ar); 127.11 (**C** Ar); 129.91 (**C** Ar); 130.14 (**C** Ar); 140.70 (**C** Ar); 142.27 (d, $J = 39.0$, **C** Ar); 150.84 (**C** Ar); 182.08 (**C**=S). LC-MS m/z (*rel*, %): 396.00 $[M+H]^+$ (100); 398.01 $[M+H^++2]$ (65); 400.04 $[M+H^++4]$ (15). HRMS (FAB) Calc.: $C_{13}H_{10}Cl_2F_3N_5S$: 394.99860; Found: 396.0059 $[M+H]^+$, 398.0017 $[M+H^++2]$, 399.9978 $[M+H^++4]$.

3 -(Trifluoromethyl)-N-[3-(trifluoromethyl)phenyl]-5,6,7,8-tetrahydro[1, 2, 4]triazolo[4,3-a]pyrazine-7-carbothioamide (13j)

Pale brown solid; m.r.: 171-173 °C. IR (KBr, $\tilde{\nu}/cm^{-1}$): 3246 (N-H, str); 1562 (C = N, str), 1217 (C = S, str); 1124 (CF_3, str); 1072 (CF_3, str). ^1H NMR (400 MHz; DMSO-d_6), δ, ppm, (J, Hz): 4.35-4.36 (2H, d, $J = 4.8$, Piperazine); 4.43-4.46 (2H, d, $J = 5.2$, Piperazine); 5.51 (2H, s, Piperazine); 7.49-7.51 (1H, d, $J = 7.6$, Ar-H); 7.55-7.59 (1H, t, $J = 8.0$, Ar-H); 7.67-7.69 (1H, d, $J = 8.0$, Ar-H); 7.74 (1H, s, Ar-H), 9.92 (1H, s, NH). ^{13}C NMR (100 MHz; DMSO-d_6), δ, ppm, (J, Hz): 42.92 (-CH_2-**C**H_2-); 44.16 (-**C**H_2-CH_2-); 44.97 (-**C**H_2-); 117.09 (d, $J = 268.0$, CF_3); 121.24 (**C** Ar); 122.72 (**C** Ar); 125.43 (**C** Ar); 128.30 (**C** Ar); 128.62 (d, $J = 32.0$, **C** Ar); 128.94 (**C** Ar); 129.23 (**C** Ar); 141.34 (**C** Ar); 141.90 (**C** Ar); 150.89 (**C** Ar); 182.16 (**C**=S). LC-MS m/z (*rel*, %): 396.07 $[M+H]^+$ (100). HRMS (FAB) Calc.: $C_{14}H_{11}F_6N_5S$: 395.06393; Found: 396.0711 $[M+H^+]$.

Conclusion

We have synthesized a new series of urea **13a-e** and thiourea **13f-j** derivatives of a precursor compound, 3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1, 2, 4]triazolo[4,3-*a*]pyrazine **(11)**. An efficient and robust procedure has been developed for the preparation of a precursor compound **11** with high yield (overall yield 37.3%) from 2-chloropyrazine through the chemical transformations such as hydrazine substitution, trifluoroacetyl group induction, cyclization and pyrazine ring reduction. The improved yield, simple work-up procedure and purification of intermediates from simple recrystallization processes are the advantageous of the present protocol. The antimicrobial screening data displayed that the derivatives **13d**, **13i** and **13j** against bacteria and **13a**, **13d**, **13g** and **13j** against fungi possess significant potential activity with MIC range 6.25-25.0 µg/mL. The electronegative functional groups such as fluoro, trifluoromethyl and chloro substituents on phenyl ring might be a reason to provide promising antimicrobial activity. The activity assessment of the same substituted set of compounds **13a/f**, **13b/g** and **13c/h** demonstrated that thiourea derivatives possess better activity than urea compounds, suggesting the thiourea functionality could be a reason for high potential activity. Molecular docking studies showed good binding energies (-8.1 to -9.8 kcal/mol) of the synthesized compounds with in the active site of poly (ADP-ribose) polymerase 15 (ARTD7, BAL3), macro domain 2 in complex with adenosine-5-diphosphoribose enzyme (3V2B). Also, the compounds possess good solubility, drug likeness, drug score and followed the Lipinski's rule of five without any violation which demonstrate the synthesized products are orally active chemotherapeutic agents with good adsorption and bio-availability. The preliminary bioassay data and modeling studies described in the present study, confirms these class of molecules might be used as antimicrobial lead compounds on further developments with structural modifications.

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