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Synthesis, spectral characterization, antibacterial, cytotoxic evaluation and docking studies of new urea and thiourea derivatives

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Isoniazid is one of the main API's used in the combination treatment of tuberculosis recommended by the WHO. Urea and its derivatives are an important class of heterocyclic compounds that possess a wide range of therapeutic and pharmacological properties, while thiourea is an organosulphur compound in that it resembles urea except that the atom oxygen has been replaced by a Sulphur atom, but the properties of urea and thiourea are significantly different. The current work concerns the synthesis of a new class of urea and thiourea derivatives of isoniazid with various isocyanates and isothiocyanates in the presence of trimethylamine. The IR and NMR spectral data were performed for the urea and thiourea derivatives of the compounds [(3c & 3f) & (3d & 3e)], respectively. Molecular docking studies of the compounds (3a-h) revealed the binding mode involved in the active site of DNA gyrase. The synthesized urea and thiourea derivatives of isoniazid with various isocyanates and isothiocyanates were tested for their antibacterial activity against gram-positive and gram-negative bacteria using the "disc diffusion method". Of all compounds tested, the urea derivatives (3a &3d), the thiourea derivatives (3e & 3g) showed more potent activity than the other compounds. The MTT assay revealed concentration dependent cytotoxic effects over a concentration range 25-200 µg/mL.

Keywords: Antimicrobial activity, Cytotoxicity studies, Disc diffusion method, Isoniazid, Molecular docking, Thiourea, Urea

Isoniazid, also known as isonicotinylhydrazide (INH), has the chemical formula C6H7N3O and is used to treat tuberculosis. It is also used in the treatment of active tuberculosis, along with rifampicin, pyrazinamide, and either streptomycin or ethambutol. Treatment with isoniazid is often associated with mild, transient and asymptomatic elevations in serum aminotransferase levels; however, isoniazid is a known cause of clinically evident liver damage, which can be serious and often fatal. Isoniazid is also indicated for the prevention of tuberculosis, especially in the elderly and others at high-risk, as well as for the treatment of leprosy in combination[1].

It has been observed that isoniazid is readily absorbed from the gut after oral administration[2]. Isoniazid has an apparent volume of distribution of 43 L after oral administration, which is compatible with organ penetration[3]. Large amounts of isoniazid have been found in cerebral fluid, lungs and skin. Isoniazid is not significantly bound to plasma proteins. The major metabolic pathway for isoniazid is acetylation by N-acetyltransferase, which is found in both the liver and small intestine[4,5]. Urinary excretion is the major route of elimination; more than 80% of the oral dose is excreted in the urine within 24 h, mainly as metabolites. Less than 10% of the oral dose is excreted in the faeces[6,7].

Urea was the first organic compound synthesized in the lab and ushered in a global green revolution. Friedrich Wohler, a German chemist, synthesized it in a synthetic laboratory in 1828. Later, thiourea was discovered, a structurally similar compound, which was also important in agriculture for improving yields. Urea is produced in the liver and is found in the urine of mammals, plants, birds, yeast, and many

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microorganisms. It has the highest nitrogen content of any solid nitrogenous fertilizer in use today and is the most widely used nitrogen fertilizer in the world. According to the literature, diaryl urea scaffolds possesses various biological activities such as antitumor activity, potential antiproliferative agents[8], hypocholesterolemia agents and act as kinase inhibitors[9]. Phenyl urea derivatives have been used to control plant growth[10], the diphenylurea moiety has been described for their serotonergic antagonistic properties and as inhibitors of Raf kinases[11].

Urea and its derivatives are an important class of heterocyclic compounds with different therapeutic and pharmacological properties, while thiourea is an organosulphur compound with formula SC(NH2)2 as the oxygen atom of Urea OC(NH2)2 has been replaced by a sulphur atom, but the properties of urea and thiourea are very different. Thiourea are precursors of pyrimidine derivatives. Thiourea are also used in the study of organocatalysis of thiourea. Thiourea derivatives have antibacterial, hypnotic, antitubercular, and anticonvulsant properties[12].

Antibiotic repositories around the world have become less effective in recent decades due to both antibiotic misuse and increased resistance to antimicrobial drugs. As a result, microbial drug resistance has become a serious public health problem worldwide. Accordingly, techniques are needed to develop new therapeutic chemicals that can either function as new drugs or make existing drug therapy more effective by acting as an adjuvant[13].

Infectious diseases are the common health problem despite many discoveries and extensivescientific research. Research is pursuing the development of new drugs that can overcome the emergence of infections[14].

The present study aimed to synthesize novel urea and thiourea derivatives using an environmentally friendly synthetic protocol with biological significance. The structure of the synthesized compounds was analyzed by spectral studies and evaluated for antimicrobial and cytotoxicity studies. In addition, molecular docking studies were performed to gain a better understanding of the binding mechanisms of the most active derivatives.

Materials and Methods

Chemicals

Chemicals used in this study were purchased from Sd. fine Chem. Ltd (SDFCL), Mumbai, Boisar, Qualigens, Gayathri industries private limited, Leo chemo plast chemical Limited, and Sigma – Aldrich Company, Inc. Thin layer chromatography (TLC) on silica gel 60 F 254 aluminum sheets, E-MERK, Germany, was used to monitor the reaction progress and the purity of the compounds. Melting points (MP) were calculated in open capillary tubes using a thermometer calibrated by the GUNA digital melting point apparatus, and are expressed in degrees Celsius (°C). On a Perkin-Elmer, FT-IR 100 spectrophotometer (Thermo-Fisher Scientific), infrared spectra (v in cm^{-1}) were recorded as KBr pellets and calibrated with a standard polystyrene film. On a Bruker AMX 400 MHz spectrometer operating at 400 MHz for 1h, 1 h was recorded as solutions in DMSO-d6. For tetramethyl silane, 1H chemical changes were expressed in parts per million (ppm) (TMS). The following abbreviations have been used in the presentation of NMR data, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. The compounds were subjected to a molecular docking study with a specific pharmacological target, such as the *E. Coli* DNA Gyrase A protein, which is a good target for antibacterial activity. The antibacterial activity of the compounds was tested.

Synthesis of novel urea derivatives with isocynate (3 a-d)

According to the study scheme (Fig. 1), a stirred solution of isoniazid (1) in dry tetrahydrofuran (THF) (10 mL) were added to different isocyanates (2) in the presence of triethylamine (TEA) at 10-15°C. The reaction mixture was stirred at 50°C for 3 h. Thin layer chromatography was used to monitor the development of the reaction's (TLC). After the reaction was

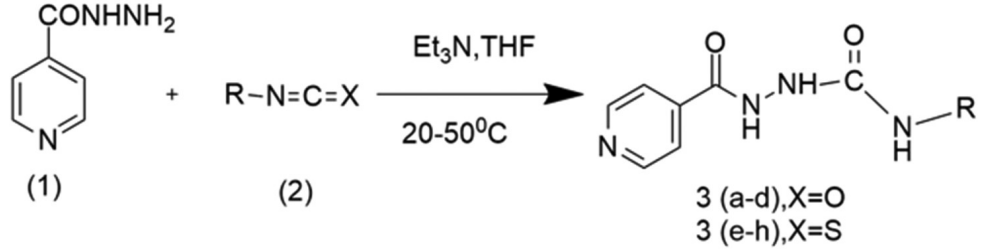


Fig. 1 — Synthesis of urea and thiourea derivatives 3(a-h) from isoniazid with varying isocyanates and isothiocyanates

completed, Et3N.HCl was extracted by filtration, and the solvent was removed in a rotary evaporator to obtain a crude product. The synthesized compounds 3(a-d) were purified by silica gel column chromatography and eluted with pure methanol. Yield and melting point were determined for all synthesized compounds.

Synthesis of novel urea derivatives with isothiocynate (3e-h).

The same procedure above was performed with different iso thiocyanates to synthesize a number of thiourea derivatives (3 e-h). Yield and melting point were determined for all synthesized compounds.

***In vitro* screening**

Antibacterial activity

Using a "disc diffusion technique," isoniazid urea and thiourea derivatives were evaluated for their antibacterial efficacy against Gram-positive bacteria such as *Bacillus subtilis* (MTCC-441), *Staphylococcus aureus* (MTCC-737), and Gram-negative bacteria such as *Escherichia coli* (MTCC-443), *Klebsiella pneumonia*. 2 mg of the synthesized compounds and the reference drug were dissolved in 2 mL of dimethylsulphoxide (DMSO) and further diluted to form test solutions at 250 g/mL and 500 g/mL respectively, and exactly 1 mL of these prepared samples were used for the tests. The nutrient agar medium was prepared by dissolving 20 g of nutrient agar in one litre of distilled water (pH-7.0), autoclaving and cooling to 400°C. The sterile nutrient agar medium was placed in a set of sterilized petri plates and allowed them to solidify for a few minutes. 20 µL of the corresponding bacterial culture was uniformly dispersed on the surface of the nutrient agar medium with sterile in ocula or a 6 mm diameter "L" shaped glass rod previously soaked in 250 and 500 g/mL test solutions. They were placed on petri plates and incubated for for 24 h at 37°C. The inhibitory zone surrounding the disc was measured. Streptomycin was used as positive control, while DMSO was used as negative control. Tests were performed in triplicate for each treatment and the mean zone of inhibition in mm was calculated.

Cytotoxicity studies (MTT assay)

For cytotoxicity analysis, the MTT test is used to follow up in toxicology[16]. The MTT assay is one of the most effective methods for determining the viability of a cell line and determining a safe and effective dose of a drug using a DMEM media containing 10% FBS, the cell culture was centrifuged, and the cell number was adjusted to 1.0×10^ 5 cells/mL. J774 macrophage cell lines are the murine cell lines used for the study. It is derived from BALB/c mice and was immortalized by transformation with Abelson murine leukemia virus. Each well of a 96 well flat bottom micro titre plate received 100 µL of the diluted cell solution. After 24 h, when the cell population was sufficient, the cells were centrifuged, and the pellets were suspended in 100 µL of various aliquots of test samples prepared in maintenance medium. The plates were then incubated for 48 h at 37°C of 5% CO2 atmosphere, with microscopic examination and observations were recorded every 24 h. After 48 h, 20 µL of MTT was added to MEM-PR. The plates were shaken gently before being incubated for 2 h at 37°C in an atmosphere of 5% CO2. After addition of 100 µL of DMSO, the plates were gently shaken to dissolve the formed formazan. A microplate reader was used to measure the absorbance at a wavelength of 540nm. The formula below was used to calculate % cell viability, and dose response curves were used to determine the quantities of drug or test substance needed to inhibit cell growth by 50%

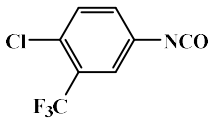
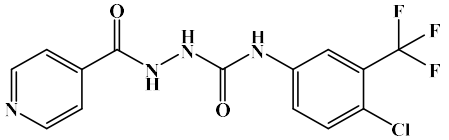
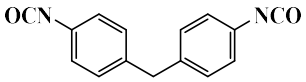
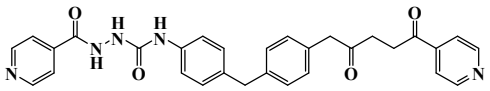
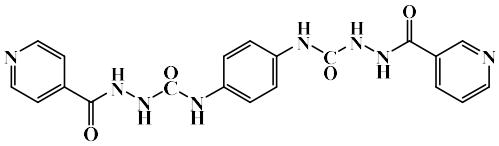
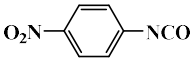
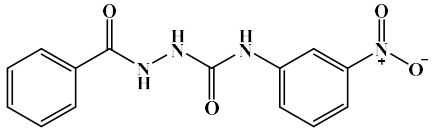
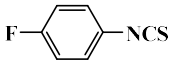
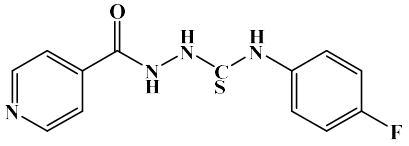
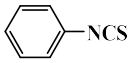
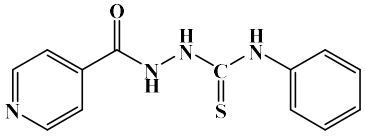
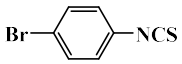
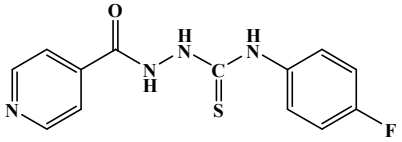
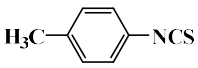
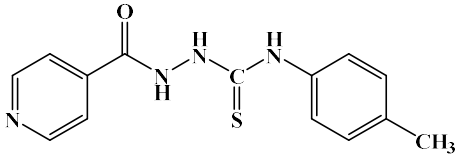
$$\%\,\text{Cell viability} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

Studies were performed in triplicate, and the inhibitory concentration (IC50), defined as the concentration required to suppress cell growth by 50% relative to control values, was obtained using a dose response curve. The results were based on the mean values of three separate trials, each containing at least four micro-cultures per concentration level.

Molecular docking

Docking helps predict the predominant binding of ligand to protein. Docking is performed to find the actual binding site[15]. The structure of DNA Gyrase A (PDB: 3LPX) and the reference drug Streptomycin were obtained from the RCSB Protein Data Bank (Pub Chem ID 19649). The enzyme 3LPX (A), as well as the reference drug (B), were shown in (Fig. 1). The chemical structures of the compounds were made with Chem Bio Draw, and ligands were converted to Pdbqt file format. The Pyrx 2010.12's docking module was used to perform molecular docking studies against the DNA Gyrase A protein using compounds 3(a-h) and the reference drug Streptomycin. The dimensions of the grid are as follows: X: 28.27, Y:

Table 1 — List of novel synthesized compounds 3(a-h) with their structure, yield and melting point

S. No	R-X	Product	Yield (%)	M.P (C)
3(a)			65	120
3(b)			75	180
3(c)			62	100
3(d)			78	175
3(e)			67	210
3(f)			64	109
3(g)			76	195
3(h)			72	170

27.13, and Z: 28.51. Docking was done using the default placement settings: triangle matcher, recording 1: London dG, refinement: force field, and a maximum of 10 conformations of each compound can be stored in a separate.mdb database file. The binding energy and affinity of protein–ligand complexes were assessed using the PyMol viewer approach (www.pymol.org).

Results and Discussion

Chemistry

Compounds 3a-3h were synthesized by the addition reaction of isoniazid with isocyanates and isothiocyanates. Scheme 1 depicted the overall synthetic technique followed in this work. The physical data of new synthesized urea and thiourea derivatives were tabulated in (Table 1 & TLC was shown in Fig. 2).

Characterization

The synthesized derivatives were characterized by IR and 1H NMR. The IR spectra of compounds 3c & 3f are shown in (Figs 3 & 4), respectively.

In the regions 1696(C=O), 3613 (NH), 1054(C-N), 3279(N-H), and 1526(C=S), 3617 (NH), 1050(C-N), 3279(N-H), distinct IR stretching absorptions have been observed. The functional groups present in

compounds 3(c) and 3(f) are confirmed by IR spectral data. Proton NMR chemical shifts for compounds 3(d) and 3(e) was shown in (Figs 5 & 6), respectively. All the title compounds had aromatic protons in the range of 8.65-7.68 ppm for 3d and 8.70-7.11 ppm for 3e. For 3d and 3e, the NH protons attached to –C=O /-C=S appeared as singlets in the range of 2.46-2.02 ppm.

Biological evaluation

Antibacterial activity

The newly synthesized compounds were evaluated for their antibacterial activity using the disc diffusion method and showed potent to moderate antibacterial activity. The antimicrobial activity of the compounds was evaluated against bacteria Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*) (Fig. 7). Among the tested bacterial strains, urea derivatives 3a and 3d carrying chloro-trifluoro group, nitro functionality, and thiourea derivatives 3e with the fluoro functionality, and 3g with the bromo group showed promising inhibition of bacterial growth. Compound 3e showed significant activity for Gram-negative bacteria (*Klebsiella pneumoniae*) as well as other compounds as well as the standard drug streptomycin. The results are tabulated in (Table. 2).

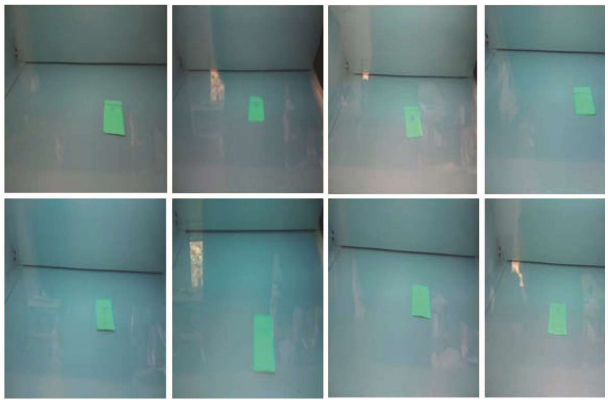


Fig. 2 — TLC of synthesized urea and thiourea compounds 3(a-h)

Cytotoxicity studies

The MTT test was used to assess the *in vitro* cytotoxicity of compounds synthesized on the J774 macrophage cell lines. Cytotoxic activities were evaluated at four different concentrations and presented as a percentage of cell survival. As a positive control, Urea was used against the J774 macrophage cell lines. Figure 1, Tables 3 and 4 shows the cytotoxicity screening of compounds 3c & 3d. The MTT assay revealed concentration dependent cytotoxic effects over a concentration range 25-200 µg/mL. Data reported showed a reduction in cell viability after treatment with all samples at a concentration range 25-200 µg/mL. Cell viability based on MTT assay was demonstrated, with the control viability of untreated cells considered to be 100%.

Molecular docking

The 3(a-h) Compounds were subjected to a molecular docking study with a specific pharmacological target,

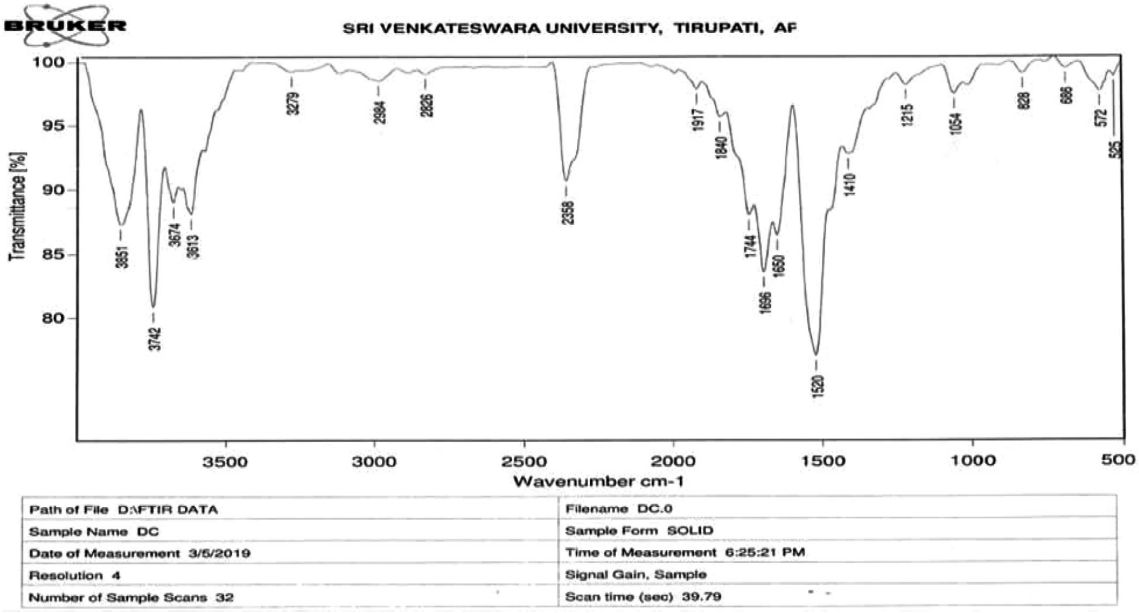


Fig. 3 — IR spectral data for the title compounds - N, N-(I,4-phenylene)bis2-isonicotinoyl hydrazine carboxamide) 3(c)

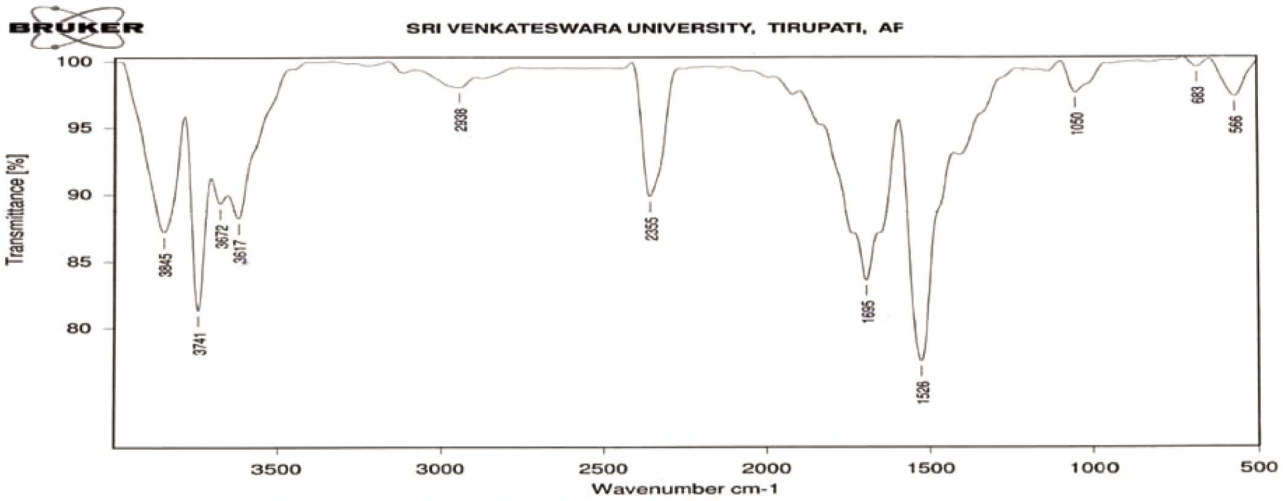


Fig. 4 — IR spectral data for the title compounds – isonicotinoyl-N-phenyl hydrazine carbothiamide 3(f)

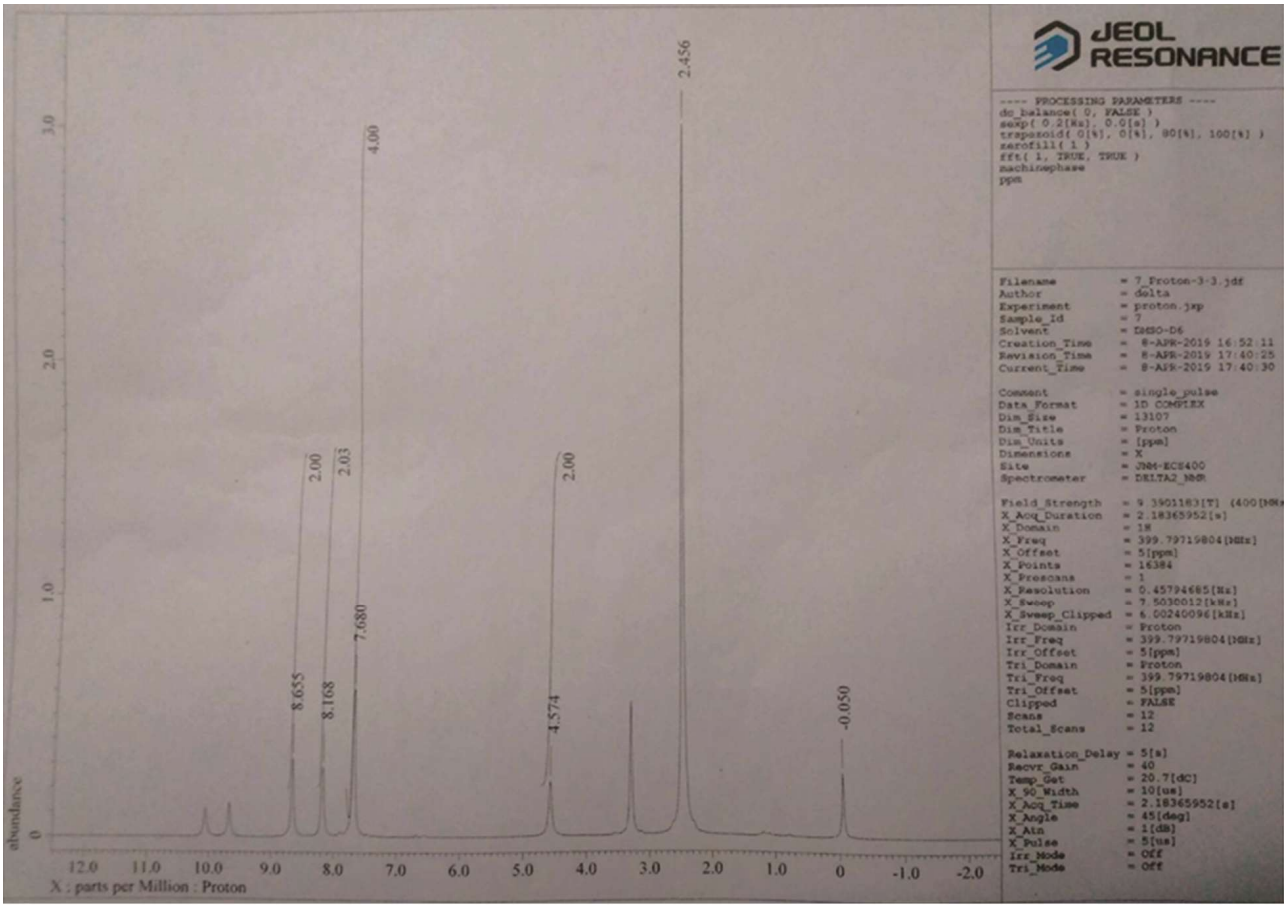


Fig. 5 — NMR spectral data for the title compounds - 2-isonicotionyl-N-(4-nitro phenyl) hydrazine carboxamide 3(d)

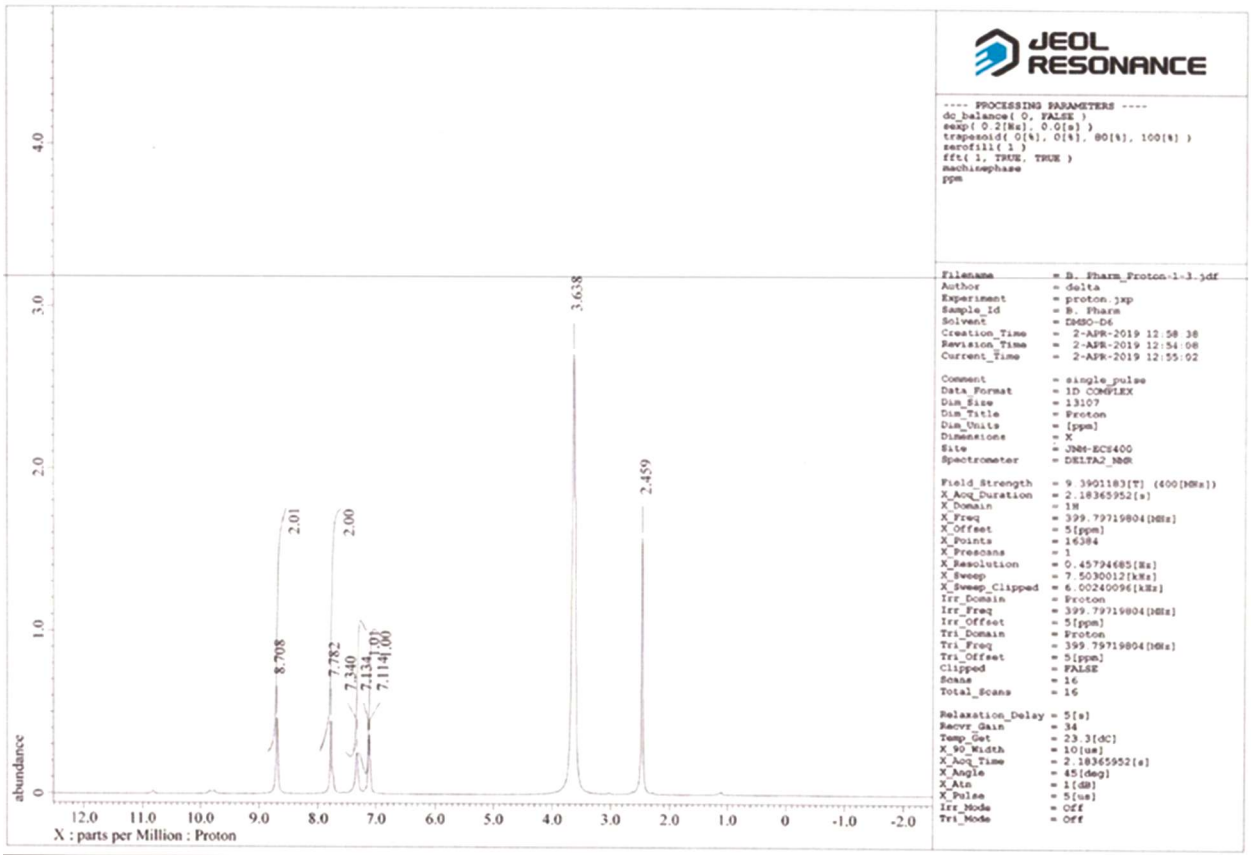


Fig. 6 — NMR spectral data for the title compounds -N-(4-fluoro phenyl)-2-isonicotionyl hydrazine carbothioamide 3(e)

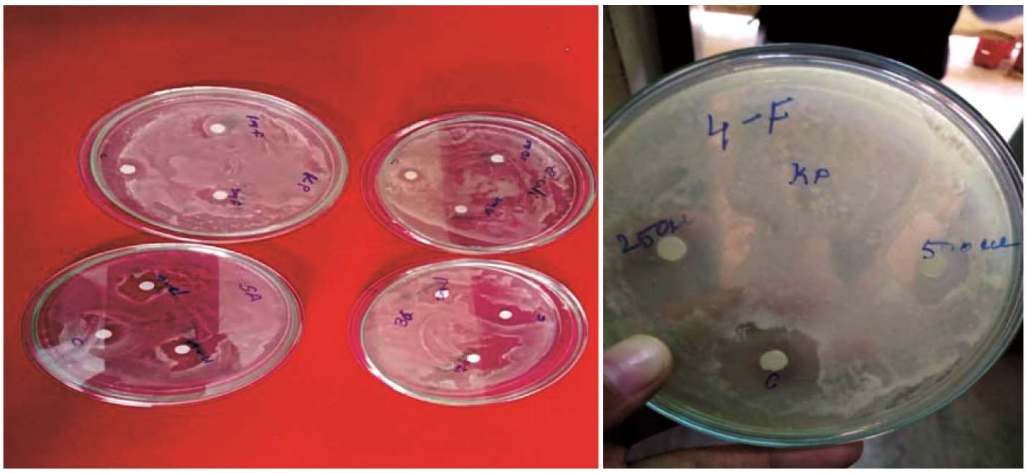


Fig. 7 — Anti-bacterial activity of various synthesized urea and thiourea compounds against Gram positive and Gram-negative bacteria and Anti-bacterial activity of compound (3e) against Gram negative bacteria (*Klebsiella pneumoniae*)

such as the *E. Coli* DNA Gyrase A protein, which is a good target for antibacterial activity. Docking studies of the synthesized compounds showed that all of them exhibited higher binding modes than the control drug, Streptomycin. The binding affinities and energy profiles of the compounds 3(a-h), as well as the reference drug, were summarized in (Table. 5). The findings of this study indicated that the synthesized compounds will be prospective next-generation antimicrobial drugs capable of killing germs and other related diseases. Figure 8 showed 3D bonding images of the strongest lead compounds.

Table 2 — Anti-bacterial activity of various synthesized urea and thiourea compounds

Compound	Diameter of inhibition zone (mm)							
	Bacillus subtilis		*Klebsiella pneumonia*		*Staphylococcus aureus*		*Escherichia coli*	
	250 µL	500 µL	250 µL	500 µL	250 µL	500 µL	250 µL	500 µL
3(a)	8.5	10.0	10.0	15.0	5.0	7.0	8.0	11.0
3(d)	9.0	10.0	-	-	3.0	10.0	2.0	3.0
3(e)	9.0	25.0	23.50	20.0	-	3.0	5.0	15.0
3(g)	-	-	8.0	11.0	-	-	-	4.0
3(h)	12.0	3.0	-	2.0	-	-	8.0	11.0
Streptomycin	18.0		20.0		20.0		20.0	

Table 3 — Cytotoxic results of the synthesized compound (3c) on the J774 cell lines

S. No	Concentration (µg/mL)	% Viability
1.	200	23.1
2.	100	45.2
3.	50	62.8
4.	25	78.4

Table 4 — Cytotoxic results of the synthesized compound (3d) on the J774 cell lines

S. No	Concentration (µg/mL)	% Viability
1.	200	26.8
2.	100	58.7
3.	50	75.4
4.	25	95.8

Table 5 — Bonding characterization of synthesized compounds (3a-h) and STM, (1 Reference drug) against *E. coli* DNA Gyrase A protein

Compound	Binding Energy (K cal mol^{-})	Binding Interaction	Bond Length (A°)	Bond Angle (°)	Bond Type
Streptomycin	−6.9	Asg 139 CG…HN	2.2 2.7	124.4 125.7	H-don H-don
		Leu 135 CD…HN	2.5 3.4	125.0 116.7	H-acc H-acc
		His 132 CB….OH	2.9 2.0	118.9 118.6	H-acc H-acc
		A sp 53 CG…OC	2.5	116.4	H-don
		A sp 53 CG…OC	2.8	126.2	H-acc
		A sp 58 OD…OH	2.7 2.5	120.0 119.8	H-acc H-acc
		A sp 58 OD…HN			
		His 132 ND…OC			
		His 132 ND…OC			
		His 132 OC…OH			
3a	−7.0	Va1 112 …OC	CA 2.6 2.2	114.3 112.2	H-acc H-acc
		Lue 264 …ON	CB		
3c	−9.8	Thr 219 …ON	CB 2.6	118.7	H-acc
3d	−7.6	Ala 220 …ON	CZ 2.8	126.7	H-acc
3e	−9.7	Ser 116 …HN	CZ 2.7 2.7	147.0 120.2	H-don H-acc
		Arg 91 …OC	CA		

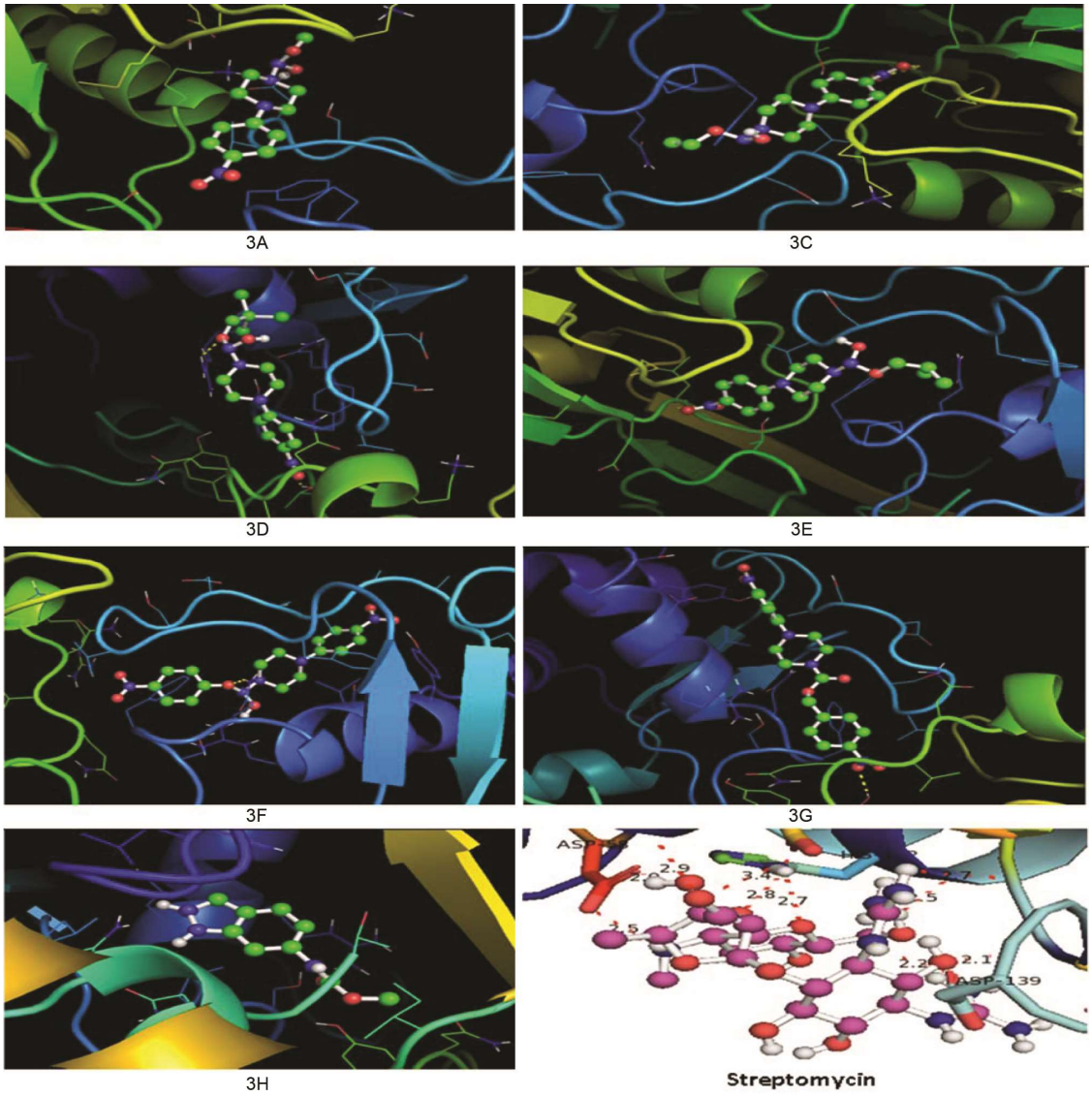


Fig. 8 — Bonding interactions of the synthesized compounds (3a-h) & standard with DNA Gyrase –A

Conclusion

Isoniazid is an antibiotic used to treat tuberculosis. Urea was the first organic compound synthesized in the lab, sparking a green revolution around the world. By adopting an environment friendly synthetic protocol, we have synthesized new compounds of urea and thiourea. Finally, the antibacterial and cytotoxic properties of several new hybrids were investigated. The antibacterial activity data showed that the produced Urea hybrid compounds were more active against Gram-positive bacteria, while the thiourea derivatives showed greater activity in Gram-negative bacteria, especially *Klebsiella pneumoniae*. In conclusion, compounds with electronegative atoms such as Cl, F, Br influenced bacterial growth. The 3d compounds showed significant activity. Molecular docking studies of newly synthesized drugs have better binding affinity. Current research on the molecular docking of synthesized compounds has been conducted and the promise of antimicrobial drugs for the future generation has been realized.

Conflict of interest

All authors declare no conflict of interest.

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