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Original article

# Synthesis, biological assay *in vitro* and molecular docking studies of new Schiff base derivatives as potential urease inhibitors

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ABSTRACT

A series of new and novel Schiff base derivatives were synthesized and investigated as potential new inhibitors of Jack bean urease. The most potent compounds were **3f** with ($K_i$ = 0.09 μM) and **3k** ($K_i$ = 0.122 μM). A pure competitive mechanism of inhibition was observed. Molecular docking studies were also performed to illustrate the binding mode of the compounds. Docking studies were performed on both enzymes from Jack bean urease and *H. pylori* urease. It was observed that both share the same binding mode. The binding sites of the two urease structures also aligned very well indicating the similarity in binding sites of the enzymes.



## 1. Introduction

Urease (urea amidohydrolase EC3.5.1.5) is a large heteropolymeric enzyme which belongs to the super family of amidohydrolases and phosphotriestreases [1]. It catalyzes the hydrolysis of urea to ammonia and carbamate. The active site of urease possesses metal center i.e. two nickel (II) atoms [2]. It is widely distributed in a variety of bacteria, fungi, algae, and plants [3,4]. The order of amino acids and their enzymatic mechanism in all the ureases is preserved, Ciurli and his co-workers also proposed an efficient and workable enzymatic reaction mechanism [5]. The active center of Urease traps three water molecules and a hydroxide ion links between two nickel ions. Meanwhile, urea displaces the water molecules and links itself with the two nickel ions [2,6]. Urea being bi-dentate is held firmly by the network of hydrogen bonding. This state activates urea molecule and the result is attack of Ni bridging hydroxide ion developing a tetrahedral like transition state. Consequently, ammonia is discharged from the active site accompanied by negatively charged carbamate. The latter is further broken down instantly, generating another molecule of ammonia. This reaction mechanism is known to be the main cause of increase in pH and major contributing factor in pathologies caused by *Helicobacter pylori (H. pylori)* and permits bacteria to endure acidic pH of the stomach during colonization. The enzymatic activity of urease plays an effective role in pathogenesis of urolithiasis, pyelonephrities, ammonia and hepatic encephalopathy, hepatic coma and urinary catheter encrustation [2,7–9]. Urease inhibitors are known to lessen the environmental issues and augment the uptake of urea nitrogen by plants [10–14]. Urease inhibitors are known to be potent antiulcer drugs [15]. Strategies based on urease inhibition are the main treatment of diseases caused by urease producing bacteria.

Several classes of compounds are known to show significant inhibitory activities against urease enzyme, among them hydroxamic acids are the best recognized urease inhibitors [2,16,17]. Whilst, phosphoramidates being the most potent compounds [18,19]. However, because of teratogenicity of hydroxamic acid in rats [20] and degradation of phosphoramidates at low pH [21] prevented their use as a drug *in vivo*. Polyphenols, another class of compounds that showed enzyme inhibitory activities [22]. Gallocatechin [23], a polyphenol which is extracted from green tea and quercetin [24], a naturally occurring flavonoids have been reported as inhibitors of *H. pylori* urease. A few triazoles have also been reported as inhibitors of urease [25]. Recently, some complexes of Schiff bases with metal ions showed significant inhibitory activity against urease [26,27]

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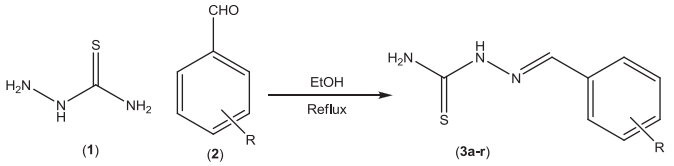



**Fig. 1.** Synthesis of 1-(substituted benzylidene) thiosemicarbazides derivatives (**3a**–**r**).

along with other metal complexes [28]. However, as these metal complexes have heavy metal atoms, therefore, they can be toxic to human body [29], hence such molecules cannot be used as drugs. Schiff base hydrazones are well known class of compounds, possess various activities like antimicrobial [30,31], antimycobacterial [32], antitumor [33], anti-inflammatory [34], trypanocidal [35], anti-HIV [36], antimalarial [37] and antidiabetic activities [38]. However, the present study was aimed at design, synthesis, and evaluation of novel Schiff base analogs as inhibitors of urease. The inhibitory constants of the novel Schiff based compounds were in micromolar range.

## 2. Results and discussion

### 2.1. Chemistry

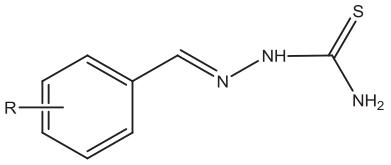
The synthesis of target semicarbazide hydrazones was carried out as outlined in Fig. 1. The reaction of semicarbazide in dry ethanol with suitably substituted aromatic aldehydes in the presence of catalytic amount of sulphuric acid afforded the hydrazones (**3a**–**p**) in case of (**3r**) the aliphatic hydrazone respectively. The target compounds were obtained in appreciable yields and were purified by recrystallization from ethanol. The structures were ascertained on the basis of their consistent IR, $^{1}$H NMR, $^{13}$C NMR, elemental analysis and mass spectroscopic studies. In a typical case **(3j)** the presence of C=S functional group was marked by the appearance of stretching bands at 1110, at 1602 for CN, at 1590 cm$^{-1}$ for Ar–CC and a stretching band around 3385 and 3229 cm$^{-1}$, indicated the presence of $NH_2$ and NH linkage of the hydrazide moiety. The aromatic nitro stretching around 1343 cm$^{-1}$ (symmetric $NO_2$ stretching) and 1538 cm$^{-1}$ (asymmetric $NO_2$ stretching) depicts the presence of nitro functional group in compound **(3f–g).** The characteristic azomethine protons were observed in the range of 7.5–8.1 ppm. In $^{1}$H NMR of **(3j)**, the appearance of a singlet at δ 8.11 for azomethine proton (CHN) and display of singlets at δ 10.4 and 7.50 were noticed for NH and $NH_2$ respectively. In $^{13}$C NMR signals at δ 179.0 (CS) and 153 (CN) confirmed formation of the semicarabazone.

### 2.2. Urease inhibition assays (in vitro)

Novel Schiff base compounds were evaluated against Jack bean urease *in vitro*. Initially, the compounds were screened at a concentration of 1 mM. The compounds which exhibited more than 50% inhibition were further selected for complete characterization. All of the Schiff base compounds were potent inhibitors of Jack bean urease as they were structurally similar with the substrate of urease i.e. urea. Thiourea was used as the reference compound for the assay, its affinity is also included in Table 1. The synthesized compounds bear various functional groups at phenyl ring. The obtained results were shown in Table 1. The screened compounds exhibited excellent inhibitory activity in micromolar range. Among the tested compounds **3f** was the most potent with $K_i$ being 0.09 μM. Other potent compounds were **3k** and **3h** having $K_i$, 0.122 and 0.127 μM respectively. Upon further modification, moderate activity was exhibited by **3a, 3n, 3m, 3p and 3q** ranging from 0.125 to 0.200 μM. Among the synthesized analogs, **3b, 3c, 3d, 3e, 3g, 3i, 3j, 3l, 3o and 3r** were least potent with $K_i$ values greater than 0.200 μM.

The activities of compounds were greatly varied by changing the substitution at *meta* and *para* position. In case of highly potent **3f**

**Table 1**
Inhibition of Jack bean urease by Studied Compounds.




| Entry | 2 | 3 | 4 | 5 | $IC_{50}$ (μM) | $K_i$ (μM) |
|---|---|---|---|---|---|---|
| **3a** | Cl | | | | 0.215 ± 0.09 | 0.170 ± 0.08 |
| **3b** | | Cl | | | 0.670 ± 0.04 | 0.625 ± 0.07 |
| **3c** | | | Cl | | 0.410 ± 0.07 | 0.398 ± 0.04 |
| **3d** | | Br | | | 0.767 ± 0.08 | 0.670 ± 0.06 |
| **3e** | | | Br | | 0.707 ± 0.09 | 0.770 ± 0.14 |
| **3f** | | $NO_2$ | | | 0.102 ± 0.50 | 0.090 ± 0.06 |
| **3g** | | | $NO_2$ | | 0.547 ± 0.06 | 0.497 ± 0.09 |
| **3h** | | O–$CH_3$ | | | 0.177 ± 0.03 | 0.127 ± 0.03 |
| **3i** | | | O–$CH_3$ | | 0.887 ± 0.01 | 0.807 ± 0.05 |
| **3j** | | O–$CH_3$ | O–$CH_3$ | O–$CH_3$ | 0.989 ± 0.09 | 0.910 ± 0.11 |
| **3k** | | | N–$(CH_3)_2$ | | 0.127 ± 0.01 | 0.122 ± 0.11 |
| **3l** | | | | | 0.457 ± 0.01 | 0.407 ± 0.11 |
| **3m** | O–$CH_2$-ph | | | | 0.263 ± 0.006 | 0.152 ± 0.004 |
| **3n** | | O–$CH_2$-ph | O–$CH_3$ | | 0.330 ± 0.15 | 0.191 ± 0.08 |
| **3o** | | | –OH | | 0.682 ± 0.04 | 0.391 ± 0.02 |
| **3p** | | O–$CH_3$ | –OH | O–$CH_3$ | 0.220 ± 0.02 | 0.127 ± 0.01 |
| **3q** | –OH | –OH | | | 0.233 ± 0.06 | 0.134 ± 0.03 |
| **3r** | | | | | 0.746 ± 0.01 | 0.430 ± 0.06 |
| Thiourea (Standard) | | | | | 21.0 ± 11 | 19.6 ± 0.45 |

which bears a $NO_2$ substituent at *meta* position that is most effective as compared to **3g** when it is present at *para* position. However, when the substituent was changed to activating group N-$(CH_3)_2$ in **3k,** the activity was reduced. Upon further modification in the substituent on aryl ring in **3h,** the activity further declined. Among the compounds containing halogens inductive effect plays its role, chloro at *ortho* (**3a**) exhibited good activity, $K_i$ being 0.170 μM, compared to **3b** and **3c** where it is present on *meta* and *para* positions respectively. The electron donating group show moderate activity.

### 2.3. Molecular docking studies

In the docking study, the X-ray crystal structure of *Jack bean* urease (entry 3LA4 in the Protein Data Bank) was used. In order to give an explanation and understanding of potent inhibitory activity observed, molecular docking of the most potent compound **3f** into binding site of *Jack bean* urease was carried out. The binding model of compound **3f** and *Jack bean* urease was shown in Fig. 2. In the docked conformation of inhibitor **3f** (Fig. 2) Ni atoms coordinate with the sulphur atom, similarly hydrogen bonding can be observed by Arg439 and Cme592 residues with oxygen of nitro group, whereas Ala440 with Hydrogen of inhibitor. In Fig. 3, another interaction diagram can be seen for inhibitor **3l.** The docking results for the compounds are given in Table 2, which are ranked on the basis of their FlexX docking scores.

Basically the scoring functions are physics base 'molecular mechanics force fields' that approximate the energy of the masquerade (pose) a low energy i.e. a negative value indicates a firm system hence a possible binding mode. The FlexX Docking score is tabulated in Table 2, along with the rank of each molecule. As **3f** gives the lowest score i.e. −29.52, it suggests that this is the strongest binding inhibitor. The binding score ranges from −10 to −30 proposing that the binding differs as the functional group is varied. Other molecules show a greater value than −29.52, giving an indication that no other molecule is as strong as **3f.**

The molecular docking studies showed that all the compounds interact with the bi-nickel center of the urease enzyme. The top 10 docking poses were retained for each compound to analyze the interactions of the docked conformations within the active site. Single atoms were included in the active site during receptor preparation, FlexX [39] offers an automated pharmacophore query generation through FlexX-Pharm [40] module to filter the docking solutions on the basis of pharmacophoric constraints. The two Nickel atoms were set to be used as essential part of the pharmacophore query that was desired to be present in the predicted docking solutions. When more pharmacophoric constraints were added together with the metal pharmacophores, it did not produce the docking solutions for all of the compounds. Therefore, interactions constraints with the other active site residues were set as optional. However, these interactions constraints can be very useful in filtering the good binders in the predicted conformational space. An interaction diagram for compound **3f** is shown in Fig. 2.

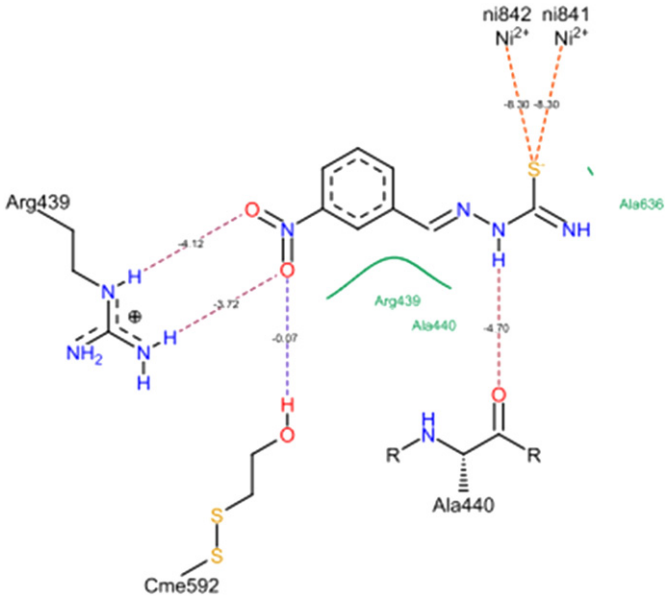



**Fig. 2.** Docked conformation of **3f** in Jack bean Urease.

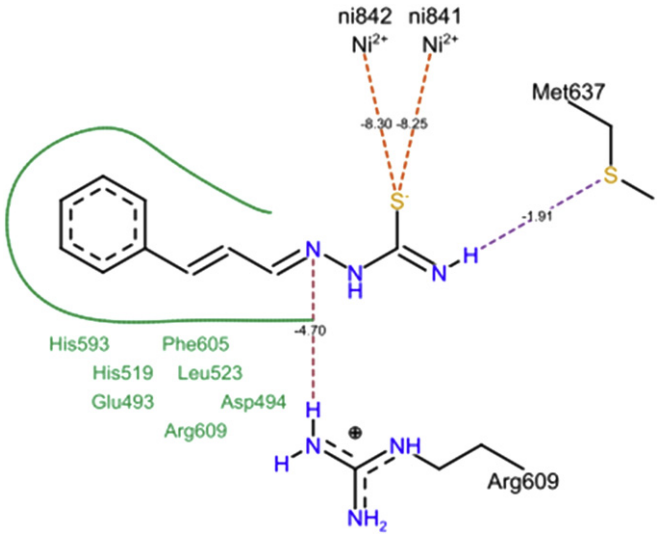



**Fig. 3.** Docked conformation of **3l** in Jack bean Urease.

Fig. 4 shows the binding modes of all compounds in the top 10 docked solutions. Out of the top 10 predictions, all compounds have a similar binding mode in the 1st ranked docked solution, except **3f** and **3q** whose ranks were 7 and 9 respectively. This also indicates that all the compounds fulfilled the metal pharmacophoric constraints and the thiourea moiety interacts with the two nickel atoms. The aromatic rings make similar stack of interactions with HIS593, PHE605 and LEU523 on one side and HIS593, ARG439, ALA440 and ALA636 on the other side in the opening of the active site pocket. The compounds also exhibit flexibility at the wide opening part of the urease binding pocket and adopt different

**Table 2**
FlexX Docking Score.

| S.No | ID | Rank | Docking Score |
|---|---|---|---|
| 1 | **3a** | 1 | −22.04 |
| 2 | **3b** | 1 | −14.41 |
| 3 | **3c** | 1 | −22.62 |
| 4 | **3d** | 1 | −14.78 |
| 5 | **3e** | 1 | −22.64 |
| 6 | **3f** | 7 | −29.52 |
| 7 | **3g** | 1 | −26.62 |
| 8 | **3h** | 1 | −24.68 |
| 9 | **3i** | 1 | −11.28 |
| 10 | **3j** | 1 | −23.28 |
| 11 | **3k** | 1 | −17.17 |
| 12 | **3l** | 1 | −28.54 |
| 13 | **3m** | 1 | −14.63 |
| 14 | **3n** | 1 | −18.76 |
| 15 | **3o** | 1 | −23.80 |
| 16 | **3p** | ND | – |
| 17 | **3q** | 9 | −19.32 |
| 18 | **3r** | 1 | −21.23 |

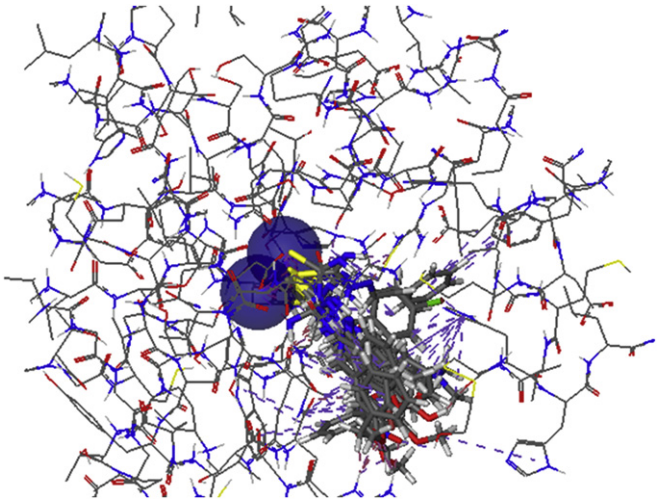


**Fig. 4.** Predicted docked conformations of all compounds inside the binding pocket of Jack bean urease. The blue spheres indicate the metal pharmacophores around the two nickels ($Ni^{+2}$). The dotted lines indicate various types of interactions of the compounds with the active site residues including hydrogen bonding and aromatic interactions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

conformations, but the aromatic interactions with histidine ring of HIS593 on one side and ARG439 and ALA440 on the other side is common to majority of the compounds. Similarly, most of the compounds make hydrogen bonding interactions with the side chains of CME592, ARG439 and ALA440 residues at the entrance of the binding pocket.

Fig. 5 shows the electrostatic charge distribution of the predicted docked compound poses inside the bi-nickel center of the urease enzyme. The bi-nickel center of the enzyme forms a metal complex of the modified Lysine residue KCX490 (LYSINE NZ-Corboxylic acid), ASP633 and four histidine residues (HIS519, HIS545, HIS407 and HIS409) around the two positively charged $Ni^{+2}$ atoms. All the histidines are protonated at delta positions except HIS519 which is protonated at epsilon position. The negatively charged S atoms of the thiourea derivatives are interacting with the positively charged metal atoms in almost all of the predicted docked poses. The negatively charged S atoms and the O atoms of the two negatively charged KCX490 and ASP633 residues creates an overall negative charge distribution around the metal center in the urease enzyme which is shown from the electrostatic charge distribution around the compounds. Therefore, the electrostatic potential surface representation shows the favorable binding modes of the thiourea compound derivatives.

### 2.4. Kinetics of inhibition

The most potent compound, **3f** was further investigated to determine the kinetics of inhibition. Enzyme kinetics was determined in the absence and in the presence of various concentrations of inhibitor. It was found that the $V_{max}$ value did not significantly change in the presence of the inhibitor. However, $K_m$ value increased with increasing inhibitor concentrations indicating that **3f** exhibit a competitive mechanism of inhibition. Fig. 6, showed Lineweaver–Burk plots for compound **3f**. The both plots visualize the competitive mechanism of inhibition.

### 2.5. Conclusions

New series of Schiff base (**3a**–**r**) derivatives were synthesized and evaluated for Jack bean urease inhibition activity. All of the compounds were potent inhibitors of *Jack bean* urease due to the similarity of their basic skeleton with urease substrate. The compound (**3f**) showed excellent inhibitor activity with competitive mechanism of inhibition. Furthermore, molecular dockings of different inhibitors into Jack bean urease active site was performed. The binding pattern of this potential inhibitor could help to understand the inhibitory mechanism. Scaffold of Schiff base urease inhibitors can be utilized in further optimization to improve potency and selectivity by variations in the basic skeleton. Electrostatic potential was also determined.

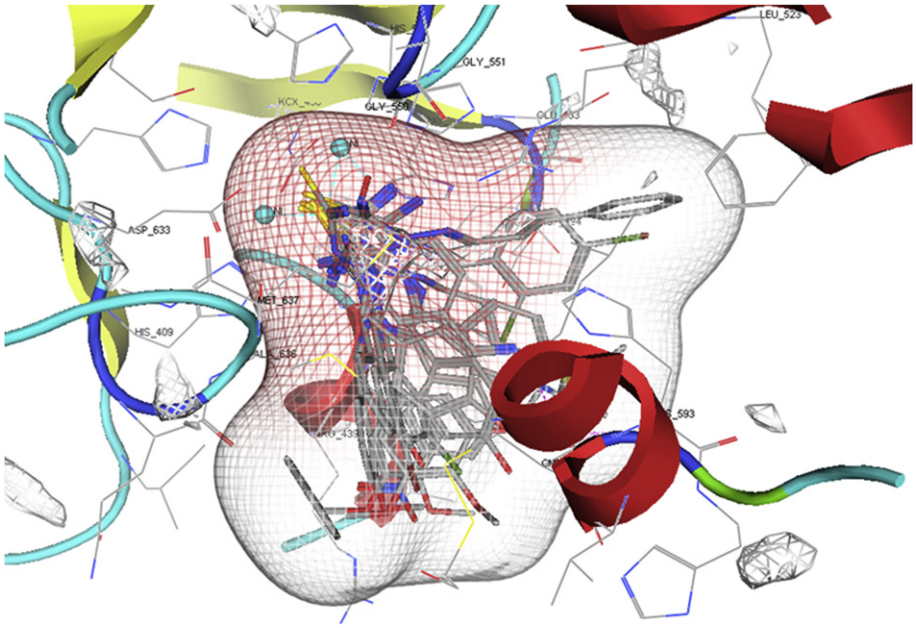



**Fig. 5.** Electrostatic potential surface of predicted docked poses of all 3-series compounds inside the Jack bean urease. The electrostatic charge distribution of functional group is shown clearly towards the bi-nickel center of the enzyme. The two nickels are represented by small spheres in light blue color. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

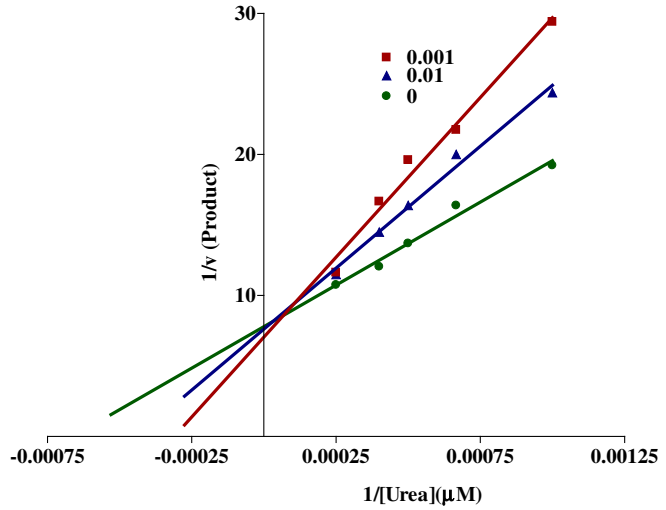



**Fig. 6.** Lineweaver–Burk plots of inhibition of Jack bean urease by compound **3f**; concentration of inhibitor **3f**: ● 0 μM, ▲ 0.01 μM, ■ 0.001 μM.

## 3. Experimental work

### 3.1. Materials

Melting points were recorded using a digital Gallenkamp (SANYO) model MPD BM 3.5 apparatus and are uncorrected. $^1$H NMR and $^{13}$C NMR spectra were determined in $CDCl_3$ or acetone-$d_6$ solutions at 300 MHz and 75.4 MHz respectively using Bruker AM-300 spectrophotometer. FT IR spectra were recorded using an FTS 3000 MX spectrophotometer; Mass spectra (EI, 70 eV) on a GC–MS instrument and elemental analyses with a LECO-183 CHNS analyzer.

### 3.2. Chemistry

#### 3.2.1. General procedure for synthesis of 1-(substituted benzylidene) thiosemicarbazides (Schiff bases)

The thiosemicarbazide (1.0 mmol) was added in a solution of suitably substituted benzaldehyde (1.0 mmol) in absolute ethanol (10 ml) using conc. sulphuric acid in catalytic amount. The reaction mixture was refluxed for 3–6 h and completion was monitored by TLC. The reaction mixture was concentrated and resulted solid product was separated and recrystallized from ethanol.

*3.2.1.1. (E)-1-(2-chlorobenzylidene)thiosemicarbazide (**3a**).* (65%): m.p 195 °C; $R_f$: 0.6; IR (Pure) υ: 3365 ($NH_2$), 3220 (N–H),1600 (CN), 1585 (Ar–C=C), 1080 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.4 (s, 1H, NH), 7.91 (s, 1H, CH=N), 7.7–7.62 (m, 4H, Ar–H), 7.1 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 180.0 (C=S), 141 (C=N), 127 (Ar), 129 (Ar), 130 (Ar), 132 (Ar), 134 (Ar); Anal. Calcd. For $C_8H_8N_3SCl$: C, 44.95; H, 3.74; N, 19.66; S, 14.98; Found: C, 44.97; H, 3.72; N, 19.64; S, 14.94; GC–MS m/z: 213 (M·+, 100).

*3.2.1.2. (E)-1(-(3-chlorobenzylidene)thiosemicarbazide (**3b**).* (62%): m.p 199 °C; $R_f$: 0.5; IR (Pure) υ: 3385 ($NH_2$), 3229 (N–H), 1602 (C=N), 1590 (Ar–C=C),1073 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.7 (s, 1H, NH), 7.92 (s, 1H, CH=N), 7.9–7.66 (m, 4H, Ar–H), 7.4 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 179.0 (1C, C=S), 140.99 (1C, C=N), 127 (Ar), 129 (Ar), 131 (Ar), 134 (Ar), 135 (Ar); Anal. Calcd. For $C_8H_8N_3SCl$: C, 44.95; H, 3.74; N, 19.66; S, 14.98; Found: C, 44.94; H, 3.73; N, 19.65; S, 14.96; GC–MS m/z: 213 (M·+, 100).

*3.2.1.3. (E)-1-(4-chlorobenzylidene)thiosemicarbazide (**3c**).* (64%): m.p 210 °C; $R_f$: 0.46; IR (Pure) υ: 3375 ($NH_2$), 3230 (N–H), 1603 (C=N), 1587 (Ar–C=C), 1090 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.6 (s, 1H, NH), 7.93 (s, 1H, CH=N), 7.6–7.3 (m, 4H, Ar-H), 7.1 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 181.0 (1C, C=S), 143 (1C, C=INS–>N), 129 (2C, Ar), 130 (2C, Ar), 131 (Ar), 136 (Ar),; Anal. Calcd. For $C_8H_8N_3SCl$: C, 44.95; H, 3.74; N, 19.66; S, 14.98; Found: C, 44.94; H, 3.73; N, 19.63; S, 14.97; GC–MS m/z: 213 (M·+, 100).

*3.2.1.4. (E)-1-(3-bromobenzylidene)thiosemicarbazide (**3d**).* (86%): m.p 212 °C; $R_f$: 0.45; IR (Pure) υ: 3370 ($NH_2$), 3235 (N–H), 1608 (C=N), 1585 (Ar–C=C), 1100 (C=INS–>S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.6 (s, 1H, NH), 7.96 (s, 1H, CH=N), 7.8–7.2(m, 4H, Ar–H), 7.2 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 182.0 (1C, C=S), 144 (1C, C=N), 123.1 (Ar), 128.4 (Ar), 131 (Ar), 133.0 (Ar), 134.2 (Ar), 136.0 (Ar); Anal. Calcd. For $C_8H_8BrN_3S$: C, 37.22; H, 3.12; N, 16.28; S, 12.42; Found: C, 37.21; H, 3.11; N, 19.26; S, 12.41; GC–MS m/z: 258 (M·+, 100).

*3.2.1.5. (E)-1-(4-bromobenzylidene)thiosemicarbazide (**3e**).* (75%): m.p 197 °C; $R_f$: 0.18; IR (Pure) υ: 3380 ($NH_2$), 3240 (N–H), 1605 (C=N), 1585 (Ar–C=C),1105 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.4 (s, 1H, NH), 7.93 (s, 1H, CH=N), 7.8–7.6 (m, 4H, Ar–H), 7.3 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 181.0 (1C, C=S), 143 (1C, C=N), 131.4 (2C, Ar), 131.9 (2C, Ar), 125 (Ar), 132.7 (Ar); Anal. Calcd. For $C_8H_8BrN_3S$: C, 37.22; H, 3.12; N, 16.28; S, 12.42; Found: C, 37.20; H, 3.10; N, 19.27; S, 12.40; GC–MS m/z: 258 (M·+, 100).

*3.2.1.6. (E)-1-(3-nitrobenzylidene)thiosemicarbazide (**3f**).* (64%): m.p 218 °C; $R_f$: 0.26; IR (Pure) υ: 3375 ($NH_2$), 3245 (N–H), 1606 (C=N), 1590 (Ar–C=C), 1090 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.3 (s, 1H, NH), 7.99 (s, 1H, CH=N), 8.5–7.6 (m, 4H, Ar–H), 7.1 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 180.0 (1C, C=S), 143 (1C, C=N), 123.1 (Ar), 124.4 (Ar), 129.1 (Ar), 134.3 (Ar), 135.2 (Ar), 148.2 (Ar); Anal. Calcd. For $C_8H_8N_4O_2S$: C, 42.85; H, 3.60; N, 24.99; S, 14.30; Found: C, 42.84; H, 3.58; N, 24.97; S, 14.28; GC–MS m/z: 224 (M·+, 100).

*3.2.1.7. (E)-1-(4-nitrobenzylidene)thiosemicarbazide (**3g**).* (87%): m.p 225 °C; $R_f$: 0.22; IR (Pure) υ: 3379 ($NH_2$), 3243 (N–H), 1608 (C=N), 1580 (Ar–C=C), 1110 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.5 (s, 1H, NH), 8.00 (s, 1H, CH=N), 8.6–7.6 (m, 4H, Ar–H), 7.2 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 181.0 (1C, C=S), 143 (1C, C=N), 121.2 (2C, Ar), 130.1 (2C, Ar), 139.5 (Ar), 150.5 (Ar); Anal. Calcd. For $C_8H_8N_4O_2S$: C, 42.85; H, 3.60; N, 24.99; S, 14.30; Found: C, 42.86; H, 3.57; N, 24.98; S, 14.27; GC–MS m/z: 224 (M·+, 100).

*3.2.1.8. (E)-1-(3-methoxybenzylidene)thiosemicarbazide (**3h**).* (73%): m.p 187 °C; $R_f$: 0.53; IR (Pure) υ: 3365 ($NH_2$), 3225 (N–H), 1600 (C=N), 1575 (Ar–C=C), 1095 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.1 (s, 1H, NH), 8.1 (s, 1H, CH=N), 7.9–7.1 (m, 4H, Ar–H), 7.1 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 179.0 (1C, C=S), 150 (1C, C=N), 113.1 (Ar), 116.1 (Ar), 121.1 (Ar), 129.3 (Ar), 135.2 (Ar), 140.2 (Ar); Anal. Calcd. For $C_9H_{11}N_3OS$: C, 51.65; H, 5.30; N, 20.08; S, 15.32; Found: C, 51.66; H, 5.29; N, 20.07; S, 15.30; GC–MS m/z: 209 (M·+, 100).

*3.2.1.9. (E)-1-(4-methoxybenzylidene)thiosemicarbazide (**3i**).* (61%): m.p 180 °C; $R_f$: 0.37; IR (Pure) υ: 3380 ($NH_2$), 3230 (N–H), 1606 (C=N), 1580 (Ar–C=C), 1105 (C=S), $cm^{-1}$; $^1$H NMR (Acetone-$d_6$, 300 MHz) δ: 10.2 (s, 1H, NH), 8.0 (s, 1H, CH=N), 7.9–7.1 (m, 4H, Ar–H), 7.1 (s, 2H, $-NH_2$); $^{13}$C NMR (75 MHz) δ: 179.0 (1C, C=S), 149 (1C, C=N), 114.2 (2C, Ar), 129.4 (2C, Ar), 125 (Ar), 140.1 (Ar); Anal. Calcd. For $C_9H_{11}N_3OS$: C, 51.65; H, 5.30; N, 20.08; S, 15.32; Found: C, 51.64; H, 5.28; N, 20.06; S, 15.31; GC–MS m/z: 209 (M·+, 100).

*3.2.1.10. (E)-1-(3,4,5-trimethoxybenzylidene)thiosemicarbazide (**3j**).* (64%): m.p 198 °C; $R_f$: 0.25; IR (Pure) υ: 3370 ($NH_2$), 3235 (N–H), 1610 (C=N), 1590 (Ar–C=C), 1110 (C=S), $cm^{-1}$; $^1H$ NMR (Acetone-$d_6$, 300 MHz) δ: 10.4 (s, 1H, NH), 8.11 (s, 1H, CH=N), 7.5 (s, 2H, $-NH_2$),7.1 (s, 2H, Ar–H), 3.86(s,6H,O–CH),3.6(s,3H,O–CH); $^{13}C$ NMR (75 MHz) δ: 179.0 (1C, C=S), 153 (1C, C=N), 110.2 (2C, Ar), 140.4 (2C, Ar), 141 (Ar), 130 (Ar), 59.3 (O–Me), 55.65 (2C, OMe); Anal. Calcd. For $C_{11}H_{15}N_3O_3S$: C, 49.06; H, 5.61; N, 15.60; S, 11.91; Found: C, 49.07; H, 5.60; N, 15.59; S, 11.90; GC–MS m/z: 269 (M·+, 100).

*3.2.1.11. (E)-1-(4–(N,N-dimethylamino)benzylidene)thiosemicarbazide (**3k**).* (79%): m.p 205 °C; $R_f$: 0.38; IR (Pure) υ: 3413 ($NH_2$), 3265 (N–H), 1588 (C=N), 1575 (Ar–C=C), 1168 (C=S), $cm^{-1}$; $^1H$ NMR (Acetone-$d_6$, 300 MHz) δ: 10.2 (s, 1H, NH), 8.10 (s, 1H, CH=N), 7.4–7.1 (m, 4H, Ar–H), 7.0 (s, 2H, $-NH_2$) 3.1 (s, 6H, N-$CH_3$); $^{13}C$ NMR (75 MHz) δ: 180.0 (1C, C=S), 152 (1C, C=N), 120.2 (2C, Ar), 129.4 (2C, Ar), 124 (Ar), 140.1 (Ar), 40.1 (2C, N–C); Anal. Calcd. For $C_{10}H_{14}N_4S$: C, 54.03; H, 6.35; N, 25.20; S, 14.42; Found: C, 54.00; H, 6.34; N, 25.18; S, 14.41; GC–MS m/z: 222 (M·+, 100).

*3.2.1.12. (E)-1-((E)-3-phenylallylidene)thiosemicarbazide (**3l**).* (46%): m.p 190 °C; $R_f$: 0.57; IR (Pure) υ: 3413 ($NH_2$), 3265 (N–H), 1588 (C=N), 1575 (Ar–C=C), 1168 (C=S), $cm^{-1}$; $^1H$ NMR (Acetone-$d_6$, 300 MHz) δ: 10.2 (s, 1H, NH), 8.10 (s, 1H, CH=N), 7.64 (d, 1H, -$J$ = 16.0 Hz, CH=CH) 7.30–7.14 (br m, 5H, Ar), 7.0 (s, 2H, $-NH_2$), 6.39 (d, 1H, $J$ = 16.0 Hz, (CH–C=N); $^{13}C$ NMR (75 MHz) δ: 182.0 (C=S), 152 (C=N), 126.2 (2C, Ar), 128.4 (2C, Ar), 127 (Ar), 134.9 (Ar), 142.8 (CH=C), 117.6 (C–C=N), 40.1 (2C, C–N); Anal. Calcd. For $C_{10}H_{14}N_4S$: C, 54.03; H, 6.35; N, 25.20; S, 14.42; Found: C, 54.00; H, 6.34; N, 25.18; S, 14.41; GC–MS m/z: 222 (M·+, 100).

*3.2.1.13. (E)-2-(2-(benzyloxy)benzylidene)hydrazinecarbothioamide (**3m**).* (49%): m.p 163 °C; Rf: 0.62; IR (Pure) υ: 3425 ($NH_2$), 3248 (N–H), 1593 (C=N), 1525 (Ar–C=C), 1089 (C=S), $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) δ: 11.50 (s, 1H, NH), 8.56 (s, 1H, CH=N), 8.13 (s, 2H, $-NH_2$), 7.9–6.9 (m, 9H, Ar–H), 5.18 (s, 2H, $CH_2$); $^{13}C$ NMR (75 MHz) δ: 180.0 (C=S), 159 (Ar), 145 (C=N), 135 (Ar), 132 (Ar), 128 (Ar), 127 (Ar), 121 (Ar), 120 (Ar), 112 (Ar), 70.3 (C-O); Anal. Calcd. For $C_{15}H_{15}N_3OS$: C, 63.13; H, 5.30; N, 14.73; O, 5.61; S, 11.24; Found: C, 63.10; H, 5.29; N, 14.72; O, 5.60; S, 11.22; GC–MS m/z: 285.09 (M +, 100).

*3.2.1.14. (E)-2-(3-(benzyloxy)-4-methoxybenzylidene)hydrazinecarbothioamide (**3n**).* Oil; (45%); Rf: 0.5; IR (Pure) υ: 3420 ($NH_2$), 3244 (N–H), 1590 (C=N), 1522 (Ar–C=C), 1084 (C=S), $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) δ: 11.40 (s, 1H, NH), 8.46 (s, 1H, CH=N), 8.03 (s, 2H, $-NH_2$), 7.9–7.0 (m, 8H, Ar–H), 5.16 (s, 2H, $CH_2$), 3.80 (s, 3H, $CH_3$); $^{13}C$ NMR (75 MHz) δ: 179.0 (C=S), 150 (Ar), 148 (Ar), 146 (C=N), 136 (Ar), 130 (Ar), 128 (Ar), 126 (Ar), 120 (Ar), 119(Ar), 112 (Ar), 109 (Ar), 70.3 (C–O), 55 (C–H); Anal. Calcd. For $C_{16}H_{17}N_3O_2S$: C, 60.93; H, 5.43; N, 13.32; O, 10.15; S, 10.17; Found: C, 60.92; H, 5.42; N, 13.31; O, 10.13; S, 10.16; GC–MS m/z: 315.10 (M +, 100).

*3.2.1.15. (E)-2-(4-hydroxybenzylidene)hydrazinecarbothioamide (**3o**).* (40%): m.p 230 °C; Rf: 0.36; IR (Pure) υ: 3466 ($NH_2$), 3357 (N–H), 1600 (C=N), 1580 (Ar–C=C), 1163 (C=S), $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) δ: 10.7 (s, 1H, NH), 8.30 (s, 1H, CH=N), 8.05 (s, 2H, $-NH_2$), 7.8–6.8 (m, 4H, Ar-H), 5.25 (s, 1H, O–H); $^{13}C$ NMR (75 MHz) δ: 180.0 (C=S), 159 (Ar), 146 (C=N), 130 (Ar), 126 (Ar), 116 (Ar); Anal. Calcd. For $C_8H_9N_3OS$: C, 49.21; H, 4.65; N, 21.52; O, 8.19; S, 16.42; Found: C, 49.20; H, 4.64; N, 21.51; O, 8.20; S, 16.41; GC–MS m/z: 195.05 (M +, 100).

*3.2.1.16. (E)-2-(2,3-dihydroxybenzylidene)hydrazinecarbothioamide (**3p**).* (45%): m.p 220 °C; Rf: 0.07; IR (Pure) υ: 3378 ($NH_2$), 3300 (N–H), 1613 (C=N), 1599 (Ar–C=C), 1200 (C=S), $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) δ: 11.2 (s, 1H, NH), 8.31 (s, 1H, CH=N), 8.10 (s, 2H, $-NH_2$), 7.2–6.8 (m, 3H, Ar–H), 5.30 (s, 2H, O–H); $^{13}C$ NMR (75 MHz) δ: 179.0 (C=S), 150 (Ar), 145 (C=N), 144 (Ar), 124 (Ar), 122 (Ar), 119 (Ar); Anal. Calcd. For $C_8H_9N_3O_2S$: C, 45.49; H, 4.29; N, 19.89; O, 15.15; S, 15.18; Found: C, 45.48; H, 4.28; N, 19.88; O, 15.14; S, 15.16; GC–MS m/z: 211.04 (M +, 100).

*3.2.1.17. (E)-2-(4-hydroxy-3,5-dimethoxybenzylidene)hydrazinecarbothioamide (**3q**).* (71%): m.p 104 °C; Rf: 0.17; IR (Pure) υ: 3449 ($NH_2$), 3311 (N–H), 1619 (C=N), 1585 (Ar–C=C), 1114 (C=S), $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) δ: 11.0 (s, 1H, NH), 8.21 (s, 1H, CH=N), 8.0 (s, 2H, $-NH_2$), 7.1 (s, 2H, Ar–H), 5.25 (s, 1H, O–H), 3.80 (s, 6H, $CH_3$); $^{13}C$ NMR (75 MHz) δ: 180.0 (C=S), 148 (Ar), 146 (C=N), 139 (Ar), 128 (Ar), 104 (Ar), 55.0 (C–O); Anal. Calcd. For $C_{10}H_{13}N_3O_3S$: C, 47.05; H, 5.13; N, 16.46; O, 18.80; S, 12.56; Found: C, 47.02; H, 5.11; N, 16.44; O, 18.79; S, 12.55; GC–MS m/z: 255.07 (M +, 100).

*3.2.1.18. (E)-2-((E)-but-2-enylidene) hydrazinecarbothioamide (**3r**).* (48%): m.p 164 °C; Rf: 0.44; IR (Pure) υ: 3378 ($NH_2$), 3160 (N–H), 1600 (C=N), 1584 (C=C), 1105 (C=S), $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$, 300 MHz) δ: 11.14 (s, 1H, NH), 8.04 (s, 1H, CH=N), 7.6 (s, 2H, $-NH_2$), 6.13 (m, 2H, CH=CH), 1.82 (d, 3H, $CH_3$); $^{13}C$ NMR (75 MHz) δ: 178.0 (C=S), 145 (C=N), 138 (C=C), 128 (C=C), 18.89 (C–H); Anal. Calcd. For $C_5H_9N_3S$: C, 41.93; H, 6.33; N, 29.34; S, 22.39; Found: C, 41.92; H, 6.32; N, 29.33; S, 22.38; GC–MS m/z: 143.05 (M +, 100).

Solvent system for $R_f$ (petroleum ether: ethyl acetate 1:1)

### 3.3. Assay for urease inhibition

The urease activity was determined by measuring amount of ammonia being produced using indophenol method described by Weatherburn [41]. The assay mixture, containing 10 μL of enzyme (5 U/mL) and 10 μL of test compound in 40 μL buffer (100 mM urea, 0.01 M $K_2HPO_4$, 1 mM EDTA and 0.01 M $LiCl_2$, pH 8.2), were incubated for 30 min at 37 °C in 96-well plates. Briefly, 40 μL each of phenol reagents (1%, w/v phenol and 0.005%, w/v sodium nitroprusside) and 40 μL of alkali reagent (0.5%, w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The absorbance at 625 nm was measured after 30 min, using a microplate reader (Bio-Tek ELx 800™, Instruments, Inc. USA). All reactions were performed in triplicate. Percentage inhibition was calculated by using the formula $100 - (OD_{testwell}/OD_{control}) \times 100$. Thiourea was used as the standard inhibitor of urease. The Cheng–Prusoff equation was used to calculate the $K_i$ values from the $IC_{50}$ values, determined by the non-linear curve fitting program PRISM 4.0 (GraphPad, San Diego, California, USA). At 37 °C, one μmol of ammonia being produced per minute by enzyme is known as one unit of enzyme at pH 8.2.

### 3.4. Protocol of docking study

In the docking study, the X-ray crystal structure of Jackbean urease (entry 3LA4 in the Protein Data Bank) was used. The protein structure was prepared prior to be used in docking study. The structure preparation includes the addition of hydrogens and force-field based parameterization of atoms followed by energy minimization. Protonate3D [42] algorithm implemented in MOE [43] was applied to protonate the protein structures and AMBER99 force-field was selected for energy minimization. It was made sure to set up the correct metal states for the two Ni atoms in the binding site of the enzyme. By using Protonate3D, the four surrounding histidine residues in the active site pocket were protonated according to the bound state of the two Nickel ions. After protonation, the crystal structures were energy minimized. Protein heavy atoms were restrained during the minimization to

allow the flexibility of protein side chains and hydrogen atoms only. After minimization, the water molecules and co-crystallized bound compounds were removed. The structure was also aligned with the HP urease structure (PDB code 4UBP) using UCSF Chimera. The RMSD value was 0.9 indicating the goodness of alignment. The binding sites of the two ureases also aligned very well indicating the similarity in binding sites. Re-docking of the bound ligands was performed with both structures to test the docking parameters and check for the correct preparation of crystal structures for use in screening by molecular docking.

### 3.5. Compounds library preparation

Before proceeding to perform docking molecules inside the protein structures, it is also of much importance to prepare the small molecules for docking. Preparation of small molecule includes generation of 3D confirmation, ionization and protonation states were designed. The ligand molecules were also prepared with MOE, by implementing MMFF94x force-field and using the 'wash' module by setting up the parameters for ionization and protonation states. The resulting molecule structures were energy minimized.

### 3.6. Docking

FlexX program was used in the docking study. Before carrying out dockings of small molecules into the protein structures, the non-standard protein residues (KCX and CME) and the metal ions were included in the binding site specification. The binding site for Jack bean urease was defined by 10 Å spacing of the residues surrounding the bound $PO_4$ and similarly, HAE (the co-crystallized ligand in bacterial urease structure) was used to define the binding site in the *H. Pylori* urease. The geometry parameters of the metal atoms were set to auto. The default docking parameters were chosen for scoring function and other chemical parameters relevant for the chemistry of docking except defining the metal pharmacophore constraints.

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