Bioorganic Chemistry 82 (2019) 163–177

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Synthesis and *in vitro* urease inhibitory activity of benzohydrazide derivatives, *in silico* and kinetic studies

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ARTICLE INFO

Keywords:
In vitro urease inhibitory activity
Benzohydrazide
In silico studies
Kinetic studies

ABSTRACT

Benzohydrazide derivatives **1–43** were synthesized *via* "one-pot" reaction and structural characterization of these synthetic derivatives was carried out by different spectroscopic techniques such as ^{1}H NMR and EI-MS. The synthetic molecules were evaluated for their *in vitro* urease inhibitory activity. All synthetic derivatives showed good inhibitory activities in the range of (IC_{50} = 0.87 ± 0.31–19.0 ± 0.25 μM) as compared to the standard thiourea (IC_{50} = 21.25 ± 0.15 μM), except seven compounds **17**, **18**, **23**, **24**, **29**, **30**, and **41** which were found to be inactive. The most active compound of the series was compound **36** (IC_{50} = 0.87 ± 0.31 μM) having two chloro groups at *meta* positions of ring A and methoxy group at *para* position of ring B. The structure–activity relationship (SAR) of the active compounds was established on the basis of different substituents and their positions in the molecules. Kinetic studies of the active compounds revealed that compounds can inhibit enzyme *via* competitive and noncompetitive modes. *In silico* study was also performed to understand the binding interactions of the molecules (ligand) with the active site of enzyme.

1. Introduction

Urease (EC 3.5.1.5) is a member of family of amidohydrolase enzymes, it possesses two nickel atoms in its core structure. The conversion of urea into ammonia and carbamate is catalyzed by the action of this enzyme. The excessive amount of ammonia released due to hyperactivity of urease enzyme leads to alkalinity of stomach which in turn increase the gastric mucosa permeability [1]. The nitrogen metabolism of cattle and various other animals is controlled by the action of urease enzyme [2]. The elevated levels of these enzymes leads to several pathogenic conditions, mostly it helps in the survival of some bacterial pathogens thus leading to some severe side effects [3]. In humans, the low pH of stomach facilitates the survival of *Helicobacter pylori* (HP) which leads to the development of gastric and peptic ulcer which may eventually cause cancer [4]. The increased level of ammonia is also responsible for several metabolic disorders and destructs the GIT epithelium. A number of compounds belonging to different classes are reported as urease inhibitors such as thiolates that binds with nickel atom of the enzyme, hydroxamic acid, its derivatives which acts as competitive inhibitor and competes with urea, phosphodiamidates, and few peptides chains having a ligand which may chelate with nickel of urease. Unfortunately, these molecules have adverse side effects associated with them. Therefore, it is of crucial importance to identify more urease inhibitors having significant stability, bioavailability, and low toxicity [5,6].

Hydrazides are acylated derivatives of hydrazine and constitute a class of organic compounds which had attained the attention of medicinal as well as synthetic chemists due to the fact that they contain azomethine group (−NH−N=CH−) connected with carbonyl group. They are well known for possessing a number of pharmacological activities, *i.e.* anti-inflammatory, antitumoral, and antitubercular [7,8]. Hydrazides commonly serves as intermediates and also the starting materials for the synthesis of surfactants as well as pharmaceutical products [9,10].

Our research group has contributed in identifying a number of urease inhibitors that belongs to different classes. In this regard, we had

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https://doi.org/10.1016/j.bioorg.2018.09.036
Received 7 August 2018; Received in revised form 27 September 2018; Accepted 28 September 2018
Available online 10 October 2018
0045-2068/

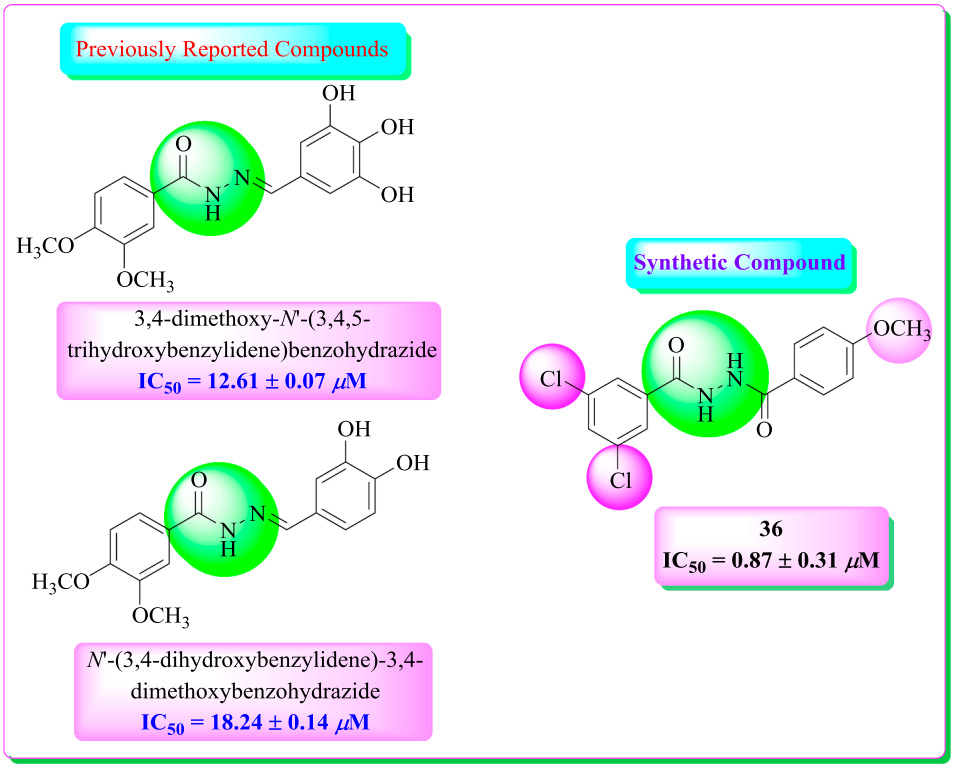


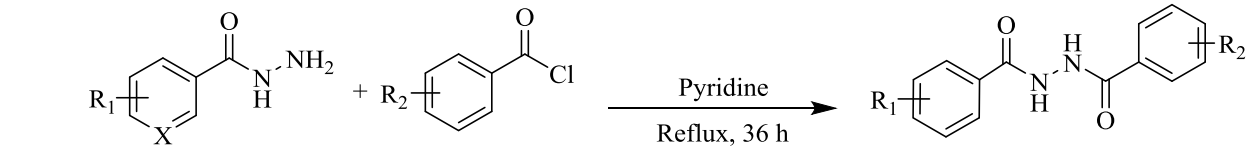
Fig. 1. Rationale of current study.

synthesized libraries of compounds having different functionalities and core structures in order to have a molecule which may serve as lead compounds for urease inhibition [11–15]. In the recent past we had synthesized benzohydrazide derivatives and identified them as urease inhibitors [16], therefore, in search of potentially active compounds we had synthesized benzohydrazide derivatives **1–43** and evaluated their *in vitro* urease inhibitory activity and found good results. Further modifications and studies on these compounds might be helpful in bringing a number of lead compounds which may serve as potential drug candidates (Fig. 1).

2. Results and discussion

2.1. Chemistry

Substituted *N*-benzoyl/nicotinoyl and aryl benzohydrazides were synthesized by treating different hydrazide with various benzoyl chlorides in pyridine. The reaction mixture was refluxed for 36 h. The products thus obtained were purified *via* crystallization [17]. The synthetic molecules were then characterized with various spectral studies such as EI-MS, H^1-NMR (Scheme 1).



Scheme 1. Synthesis of *N'*-benzoyl/nictinyl arcylhydrazide derivatives **(1–42)**.

2.2. In vitro urease inhibitory activity

All synthetic benzohydrazide derivatives **1–43** were screened for their *in vitro* urease inhibitory activity. It is noteworthy that except seven compounds **17**, **18**, **23**, **24**, **29**, **30**, and **41**, rest of the derivatives were found to be significantly urease inhibitors with IC_{50} values in the range of 0.87 ± 0.31 to 19.0 ± 0.25 μM as compared to the standard thiourea (IC_{50} = 21.25 ± 0.15 μM). The variable activity of synthetic derivatives was might be due to the different substituents on rings A and B. To understand the better structure-activity relationship we have divided the synthetic compounds into different categories on the basis of their substitution on ring A (Table 1) (Fig. 2).

2.3. Structure-activity relationship (SAR)

2.3.1. Dichloro substituted ring A

Compound **36** (IC_{50} = 0.87 ± 0.31 μM) having two chloro groups at *meta* positions of ring A and methoxy group at *para* position of ring B was the most active compound of library and exhibited potential inhibitory activity as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 μM). The activity of this compound might be due

Table 1
Synthetic derivatives of benzohydrazide/nicotinoydrazide (**1–43**).

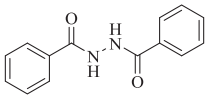
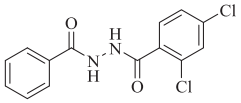
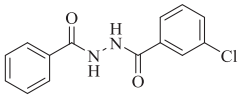
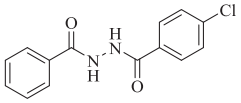
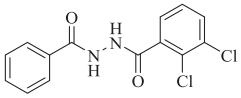
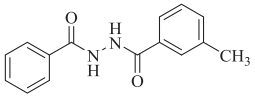
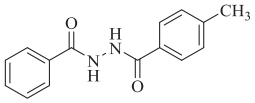
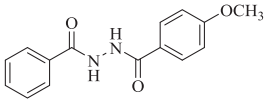
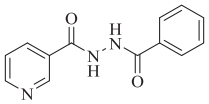
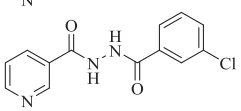
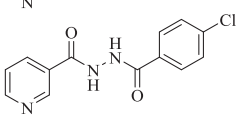
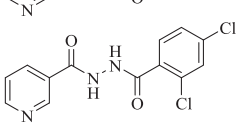
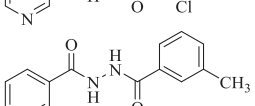
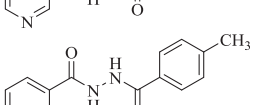
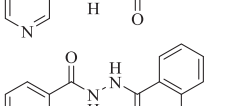
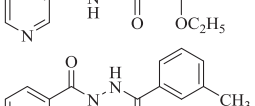
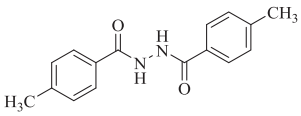
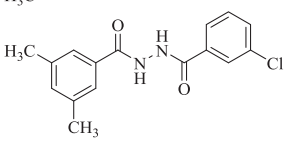
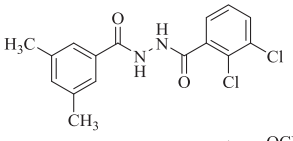
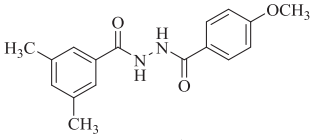
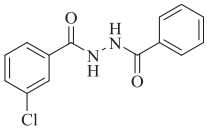
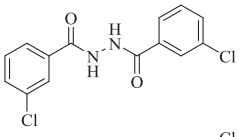
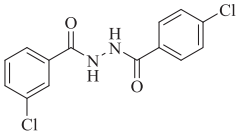
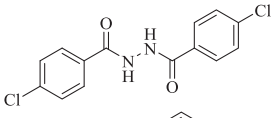
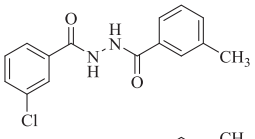
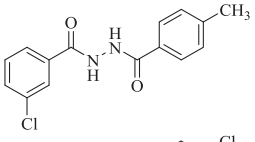
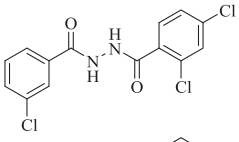
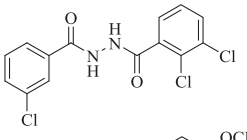
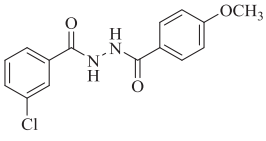
S. No.	Structures	IC_{50} ± SEM[a] (μM)
1		6.78 ± 0.60
2		5.30 ± 0.42
3		7.57 ± 0.28
4		9.32 ± 0.36
5		9.48 ± 0.23
6		12.7 ± 0.57
7		13.6 ± 0.66
8		9.63 ± 0.41
9		9.64 ± 0.19
10		14.7 ± 0.19
11		3.83 ± 0.33
12		12.3 ± 0.19
13		10.5 ± 0.83
14		9.87 ± 0.24
15		13.3 ± 0.72
16		9.07 ± 0.39
17		–[b]
18		–[b]
19		2.72 ± 0.11
20		1.64 ± 0.48
21		6.23 ± 0.28
22		13.1 ± 0.68
23		–[b]
24		–[b]
25		8.90 ± 0.73
26		4.62 ± 0.62
27		19.0 ± 0.25
28		7.35 ± 0.28
29		–[b]

Table 1 (*continued*)

(*continued on next page*)

Table 1 (*continued*)

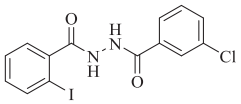
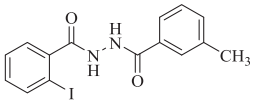
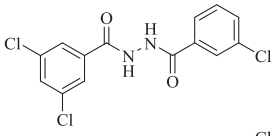
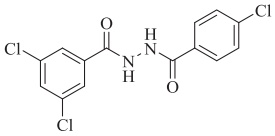
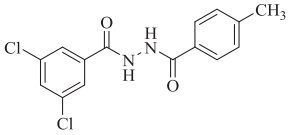
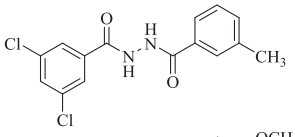
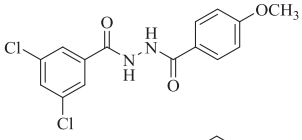
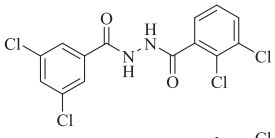
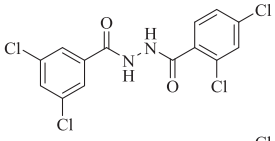
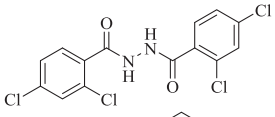
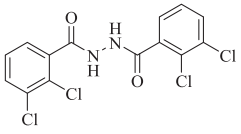
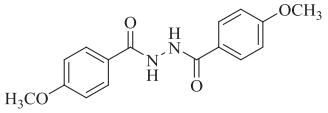
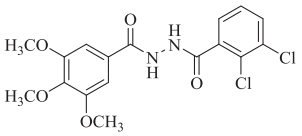
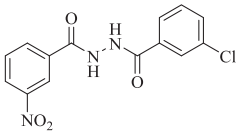
S. No.	Structures	IC_{50} ± SEM[a] (μM)
30		–[b]
31		13.7 ± 0.65
32		16.7 ± 0.36
33		1.82 ± 0.61
34		2.13 ± 0.55
35		9.71 ± 0.48
36		0.87 ± 0.31
37		2.66 ± 0.09
38		4.65 ± 0.45
39		5.01 ± 0.18
40		1.97 ± 0.90
41		–[b]
42		9.12 ± 0.14
43		9.15 ± 0.24

Table 1 (*continued*)

S. No.	Structures	IC_{50} ± SEM[a] (μM)
Thiourea[(std)]		21.25 ± 0.15

SEM[a] (Standard error of mean); -[b](Not active);Urea (Standard inhibitor for urease enzyme).

to the presence of methoxy group at ring B which may have better binding interactions within the enzyme pocket. Compound **34** (IC_{50} = 2.13 ± 0.55 μM) bearing methyl group at *para* position of ring B also exhibited good inhibitory activity, nonetheless, it was less active as compared to methoxy substituted analogue **36**, this shows that the oxygen of methoxy is binding well with the enzyme active site through hydrogen bond interactions. The activity was decreased up to 4 folds when the position of methyl was shifted from *para* to *meta* as in compound **35** (IC_{50} = 9.71 ± 0.48 μM) which showed that substitution at *para* position is actively participating in the activity.

Among chloro substituted compounds on ring B, compound **33** (IC_{50} = 1.82 ± 0.61 μM) having one chloro group at *para* position was found to be most active might be due to mesomeric effect of chloro group. Shifting the position of chloro group from *para* to *meta* resulted in decreased activity as in compound **32** (IC_{50} = 16.7 ± 0.36 μM). It indicated that the substituents at *para* position are mainly contributing in the activity and binds well within the active site of enzyme (Fig. 3).

The di-chloro substituted compound **38** (IC_{50} = 4.65 ± 0.45 μM) having two chloro groups at *ortho* and *para* positions also showed good inhibitory activity. Interestingly, compound **39** (IC_{50} = 5.01 ± 0.90 μM) having two chloro groups at *ortho* and *para* positions of both rings was slightly less active as compared to compound **38**, nevertheless, these compounds showed potent activity in comparison with standard thiourea (IC_{50} = 22.3 ± 1.06 μM) (Fig. 3).

2.3.2. Mono chloro substituted ring A

Compound **21** (IC_{50} = 6.23 ± 0.28 μM) bearing unsubstituted aromatic ring B was found to have good inhibitory activity might be the unsubstituted ring fits well within the enzyme pocket. Addition of chloro group at *meta* position of ring B resulted in decreased activity as in compound **22** (IC_{50} = 13.1 ± 0.68 μM). Compound **27** (IC_{50} = 19.6 ± 0.25 μM) bearing two chloro groups at *ortho* and *para* positions of ring B was found to have good inhibitory activity as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 μM). However, changing the position of one of the chloro group from *para* to *meta* of ring B resulted in increased activity as in compound **28** (IC_{50} = 7.35 ± 0.28 μM). Compound **26** (IC_{50} = 4.62 ± 0.62 μM) having methyl group at *para* position of ring B showed good inhibitory activity as compared to chloro substituted analogs. The activity was decreased up to 2 folds when the position of methyl was shifted from *para* to *meta* as in compound **25** (IC_{50} = 8.90 ± 0.73 μM), it shows that the chloro group on ring B is deactivating the ring thus resulting in the decreased activity as compared to its methyl analogs (Fig. 4).

2.3.3. Unsubstituted ring A

Compounds **1–8** have unsubstituted phenyl part as ring A also showed good inhibitory potential as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 μM). Compound **1** (IC_{50} = 6.78 ± 0.60 μM) having unsubstituted phenyl rings both as ring A and B showed superior inhibitory activity as compared to the standard. The most active compound among them was compound **2** (IC_{50} = 5.30 ± 0.42 μM) having two chloro groups at *ortho* and *para* positions of ring B, respectively. Compound **5** (IC_{50} = 9.48 ± 0.23 μM) bearing chloro groups at *ortho* and *meta* positions of ring B exhibited less activity as compared to compound **2** which showed that the particular position of the substituents are helping in the good binding interactions of compounds with enzyme.

Compound **3** (IC_{50} = 7.57 ± 0.28 μM) bearing one chloro group at

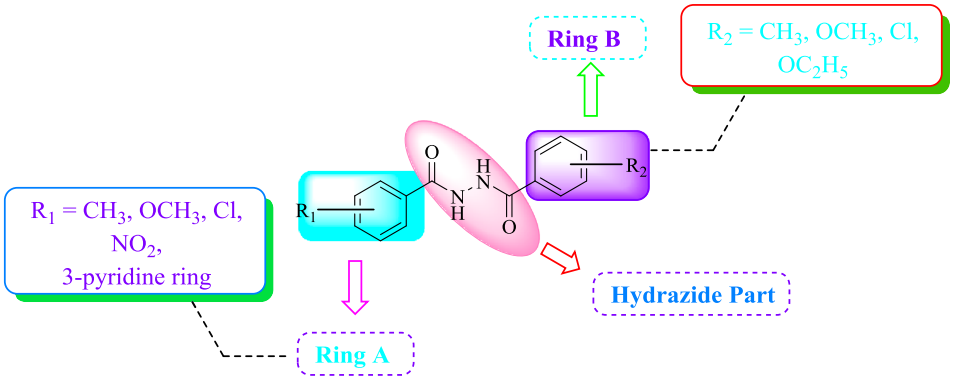


Fig. 2. General structureof synthetic compound.

meta position also showed good inhibition against urease enzyme, however, the activity was decreased when the chloro group was placed at *para* position in compound **4** (IC_{50} = 9.32 ± 0.36 µM). Compound **6** (IC_{50} = 12.7 ± 0.57 µM) having methyl group at *meta* position was found to be less active as compared to the chloro substituted compounds of this category. Shifting the position of methyl group from *meta* to *para* resulted in the decreased activity in compound **7** (IC_{50} = 13.6 ± 0.66 µM), nevertheless, both of these compounds were more active as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 µM). Compound **8** (IC_{50} = 9.63 ± 0.41 µM) having methoxy group at *para* position also showed good inhibitory potential.

Concisely, the variation in activity was observed on the basis of substituents and their particular positions, methyl and chloro substituted compounds were more active among them and *meta* and *para* positions were also actively participating in the activity (Fig. 5).

2.3.4. Nicitinohydrazide as ring A

Amongst them, compound **11** (IC_{50} = 3.83 ± 0.33 µM) having chloro group at *para* position of ring B was most active which again showed that the presence of chloro group is participating in the activity. Compound **10** (IC_{50} = 14.7 ± 0.19 µM) bearing chloro group at *meta* position of ring B showed good inhibitory activity as compared to standard thiourea (IC_{50} = 22.3 ± 1.06 µM), nonetheless, it was less active as compared to compound **9**. Addition of another chloro group at *ortho* position as in compound **12** (IC_{50} = 12.3 ± 0.19 µM) resulted in 3 folds decreased activity which again showed that in these compounds substitution of chloro group at *para* position is favorable and have more binding interactions within enzyme's active site, changing the position of chloro group or addition of another chloro substituent a decreased activity was observed. Compound **9** (IC_{50} = 9.64 ± 0.19 µM) having unsubstituted phenyl ring also showed potential activity as compared to the standard. Compound **13** (IC_{50} = 10.5 ± 0.83 µM) having methyl group at *meta* position exhibited good inhibitory activity, shifting the position of methyl from *meta* to *para* resulted in increased activity as in compound **14** (IC_{50} = 9.87 ± 0.24 µM). Compound **15** (IC_{50} = 13.3 ± 0.72 µM) having ethoxy group at *ortho* position was also found to have good inhibition against urease enzyme as compared to the standard thiourea. It is worth noting that among the nicotinohydrazide derivatives chloro substituted compounds were more active

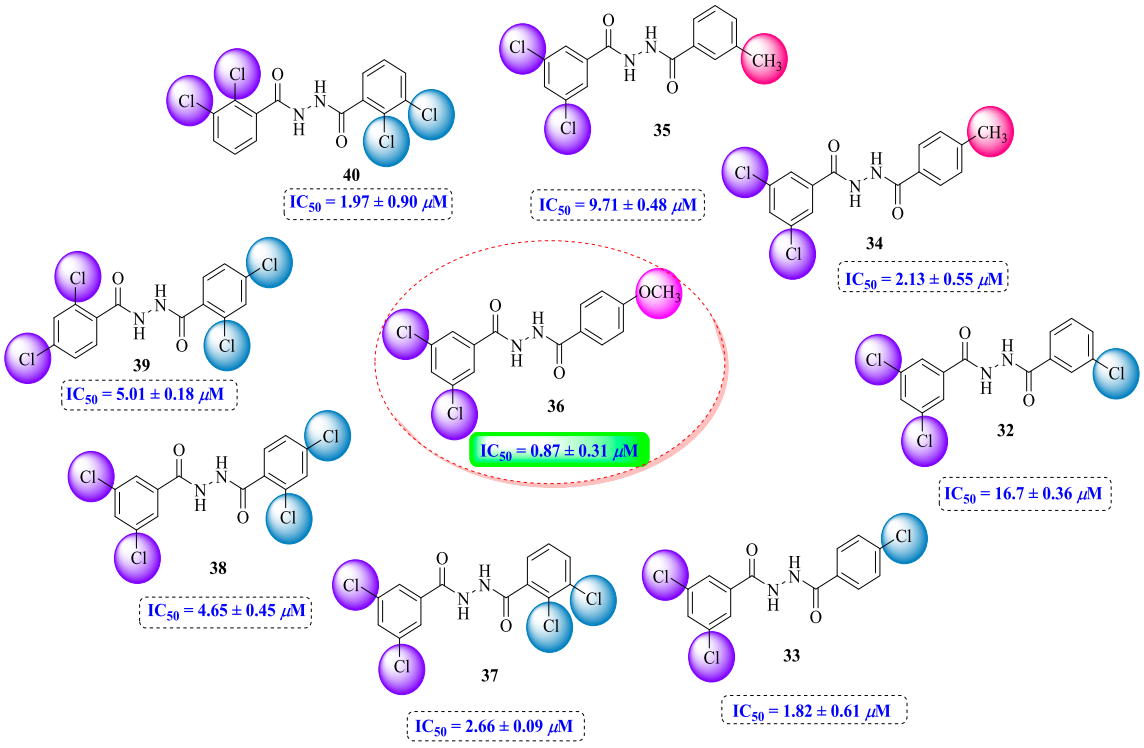


Fig. 3. Structure-activity relationship of compounds **32–40.**

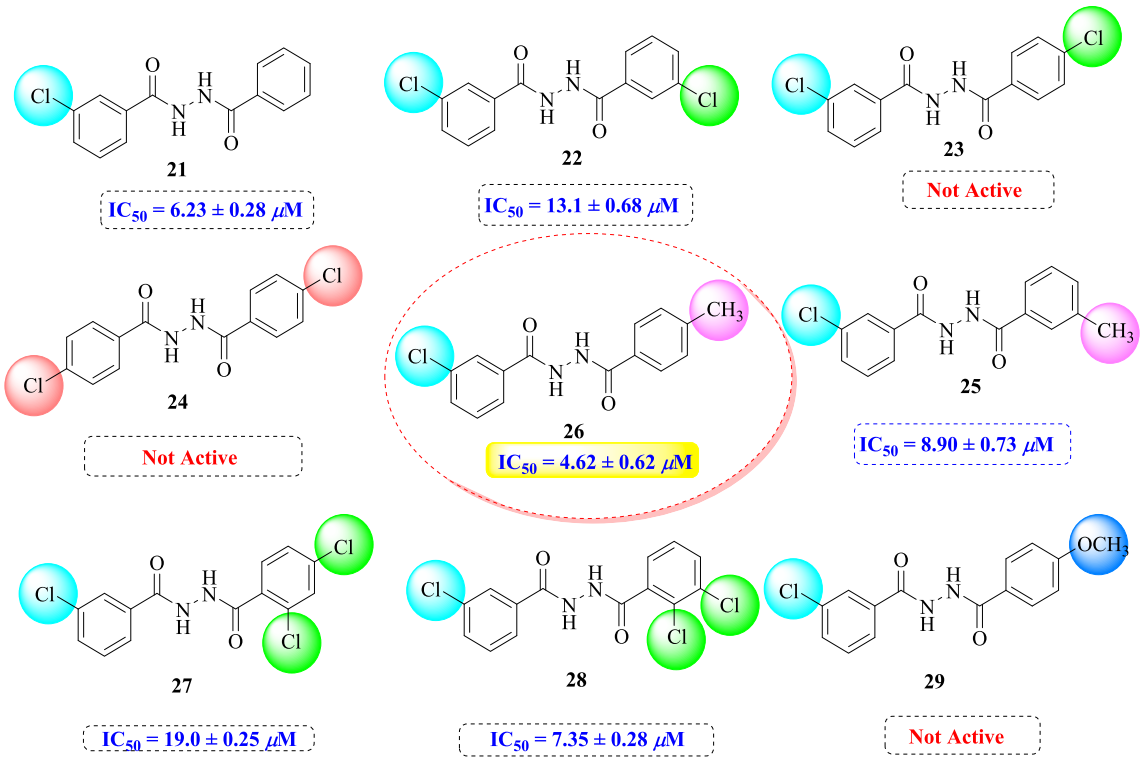


Fig. 4. Structure-activity relationship of compounds **21–29.**

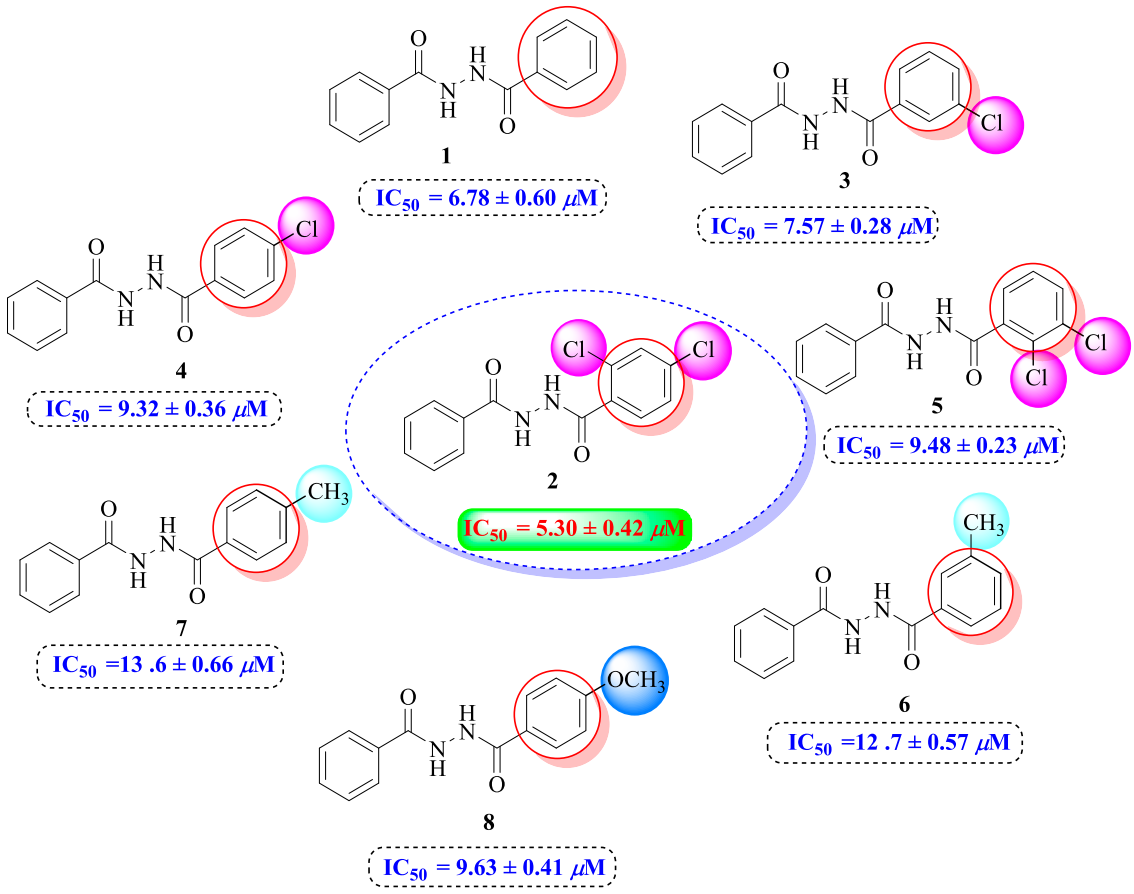


Fig. 5. Structure-activity relationship of compounds **1–18.**

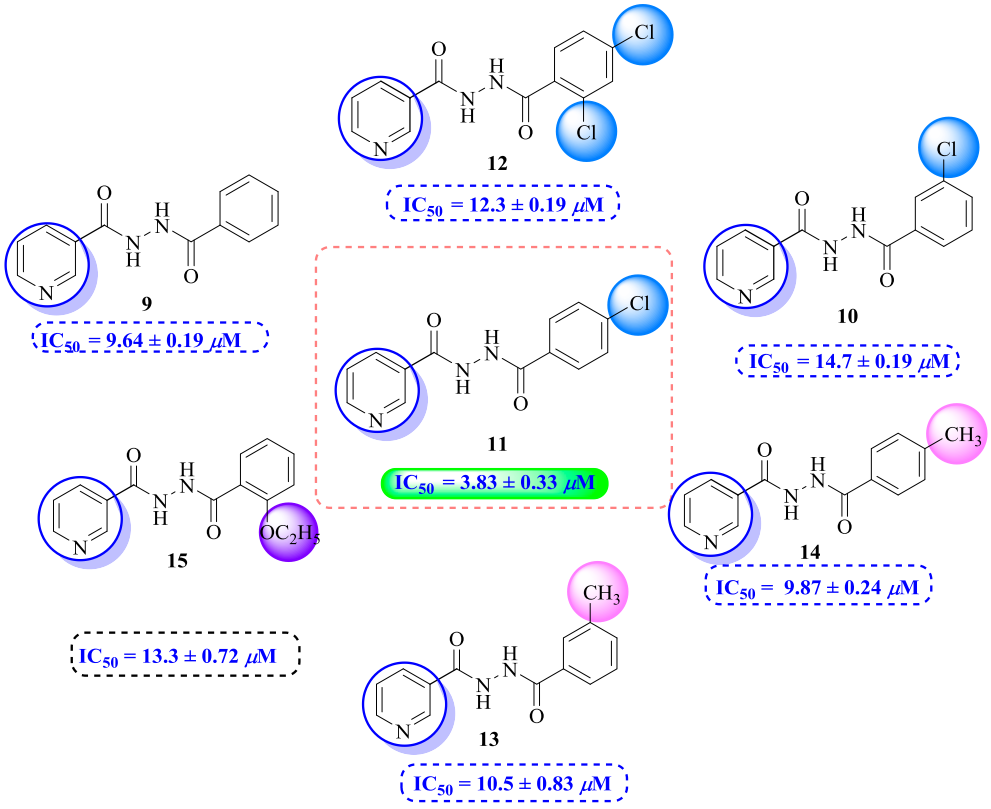


Figure-6: Structure-activity relationship of compounds **9-15**

Fig. 6. Structure-activity relationship of compounds **9–15.**

as compared to other analogs of this category. It was also observed that *para* position of the substituents also resulted in the enhanced activity as in compounds **11** and **14** (Fig. 6).

2.3.5. Methyl substituted ring A

Compound **16** (IC_{50} = 9.07 ± 0.39 μM) having methyl groups at *meta* position of both rings A and B also showed good inhibitory activity as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 μM). However, compound **17** having methyl group at *para* position of both rings was found to be inactive which showed that changing the position of methyl group resulted in the loss of activity (Fig. 7).

Compounds **19**, and **20** with dimethyl groups at *ortho* and *meta* positions of ring A were also found to have good inhibitory activity as compared to the standard. Among them compound **20** (IC_{50} = 1.64 ± 0.48 μM) bearing methoxy group at *para* position was most active and was the second most active compound of the series. Interestingly, compound **19** (IC_{50} = 2.72 ± 0.11 μM) having two

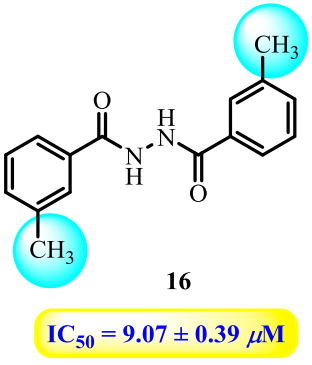


Fig. 7. Structure-activity relationship of compound **16.**

chloro groups at *ortho* and *meta* positions was less active as compared to the methoxy containing compound **20**, might be the electronegative oxygen in methoxy is helping in the hydrogen binding interactions (Fig. 8).

2.3.6. Iodo substituted ring A

Compound **31** (IC_{50} = 13.7 ± 0.65 μM) having iodo group at *ortho* position of ring A and methyl group at *meta* position of ring B showed good inhibitory activity as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 μM). The activity of this compound is less as compared to other halogenated compounds might be due to the presence of methyl group which may help in the formation of hydrogen bonds within the enzyme pocket (Fig. 9).

2.3.7. Methoxy and nitro substituted ring A

Compound **42** (IC_{50} = 9.12 ± 0.14 μM) having tri-methoxy groups at *meta* and *para* positions of ring A and di-chloro groups at *ortho* and *meta* positions of ring B showed good inhibitory potential as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 μM). 3-Nitrobenzohydrazide derivative, compound **43** (IC_{50} = 9.15 ± 0.24 μM) exhibited comparable activity as compound **42** which showed that chloro group at ring B and the oxygen atoms of nitro and methoxy group are easily binding within the active site of enzyme (Fig. 10).

2.3.8. Kinetic studies

In order to explore the mechanism of inhibition, the detailed kinetics studies were performed on active compound **40**, **20**, **36**, and **33**. The Lineweaver-Burk plot for compound **36** revealed a noncompetitive mode of inhibition, whereas, compounds **40**, **20**, and **33** showed competitive mode of inhibition by illustrating the same y-intercept for uninhibited and inhibited enzymes (Fig. 11). Moreover, Michaelis-Menten kinetic parameters; K_m and V_{max} of urease inhibition were also determined for inhibitors **40**, **20**, **36**, and **33**. In case of compound **36**,

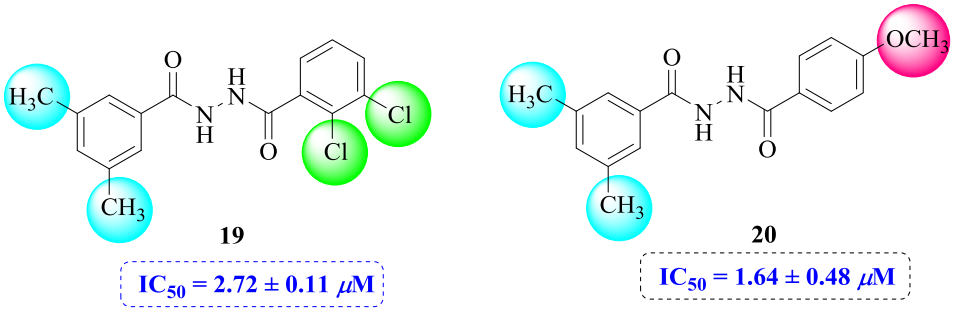


Fig. 8. Structure-activity relationship of compound **19–20.**

the maximal velocity (V_{max}) and Michaelis constant (K_m) were found to decrease with the increasing concentration of inhibitors (Fig. 11c). On the other hand in case of compounds **40**, **20**, and **33**, the maximal velocity (V_{max}) was found almost constant even by using different concentrations of respective inhibitors, however, the Michaelis constant (K_m) was increasing with increase in concentration of inhibitors as presented in Fig. 11a, b and d.

2.3.9. Molecular docking studies

To carry out the further investigation of potent inhibitors revealed by *in vitro* analysis, the docking studies were carried out. The docking studies were carried out by LeadIT, however, visualization was done by discovery studio. The protein structure PDB 3LA4 was selected and docking analysis for the synthetic compounds was performed in the active pocket of jack bean urease. The rational of docking studies was to justify the binding exhibited by enzyme-ligand complex in active site and correlate with *in vitro* assay. The interactions showed by the potent compounds were similar and all the compounds were found to bind in same orientation in the entrance of active pocket of enzyme. The overlay of the potent and selective compounds from the series are presented in Fig. 12.

When individual analysis were made for each compound, the similar results were noticed. The compounds showed same binding interactions in the active site of urease. The active site of urease possess the amino acid residues Ala436, His593, Arg639 and Arg609 in addition to Asp494 and nickel ions. The nickel ions are important for the catalytic activity of the enzyme and compounds were found to bind in near vicinity to the nickel ions in active site of urease (Fig. 12). The diacyl hydrazide group (−NH−N=CH−) in the compounds serves as stabilizing agent in the active site and prevent the binding of substrate. In addition to stabilize the inhibitors, the −NH group of diacyl hydrazide was also involved making strong hydrogen bonds with amino acid Arg439 and Ala636. The interactions were shown in 3D interaction diagrams of inhibitors inside the binding site of urease (Fig. 13a–g).

When detailed investigation was done for the docked poses of inhibitors, it was found that all the potent and selective compounds exhibited di-chloro or tetra-chloro substitution at benzohydrazide. The most potent compound **36**, possess di-chloro at *meta* positions of ring A, in addition to methoxy group at ring B. The replacement of methoxy by chloro or methyl group cause the decrease in inhibitory activity to some extent. Another compound, **20** (*N*′-(4′-methoxybenzoyl)-3,5-dimethylbenzohydrazide) having no chloro group exhibited significant inhibition as well.

For the exploration of binding site interactions of the active compounds inside enzyme's pocket, the comprehensive analysis was done. The compounds having dichloro group at *meta* position on ring A represent the hydrogen bond formation, which result in increased inhibition potential. Moreover, the methyl group substitution at *meta* position on ring A showed significant inhibitory profile. The reason for increased inhibition might be that the mobile flap of the active site in jack bean urease was covered and essential structural modification in the conformation of enzyme occur [18]. These conformational changes are responsible for the regulation of active site. In Fig. 13, it was clearly noticed that the ring A and its substituents are completely fitted in the entrance of active site and thus responsible for this behavior of compounds. In this way substrate was not available to the enzyme and therefore, no product formation occur, and thus the inhibition took place. Fig. 13(a–g) depicted that the hydrogen bonds were noticed between electronegative elements like chloro/oxygen groups of compounds (**40**, **34**, **19**, **20**, **37**, **36** and **33**) with the hydrogen atoms of amino acid residues present inside the active pocket of Jack bean urease. However, the π-π interactions were observed between phenyl ring A and B and the active pocket amino acids. All the selected compounds exhibited the interactions with most important residues of active site like, Ala440, Arg439, Ala436 and Ala636 in addition to Met637. The compounds **33**, **37**, **19**, **40**, and **34** having di-chloro substitution along with methoxy or methyl groups prevent the binding of substrate by occupying the entrance cavity and forming stable hydrogen bonds in addition to π-π interactions. The hydrogen bonds and π-π interactions are playing significant role in stabilizing the enzyme-inhibitor complex. Moreover, the inhibitors were found to be in contact with nickel ions which is another contributing factor for the stability of enzyme-inhibitor complex. Therefore, the substrate entry was prevented and hence no enzyme-substrate complex was formed.

3. HYDE assessment of selective compounds against all the targets

The HYDE affinity evaluation was carried out for the first 30 top

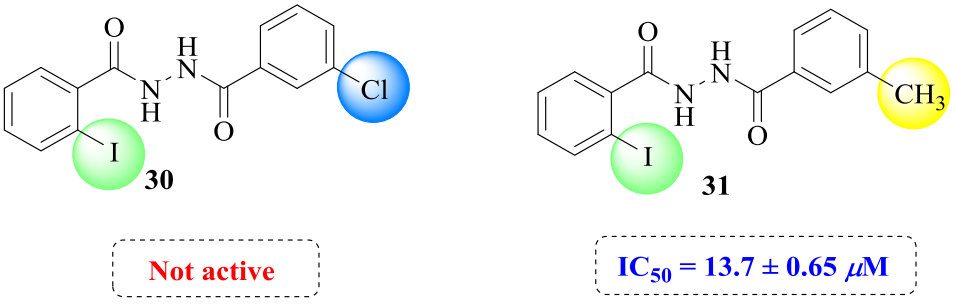


Fig. 9. Structure-activity relationship of compound **30–31.**

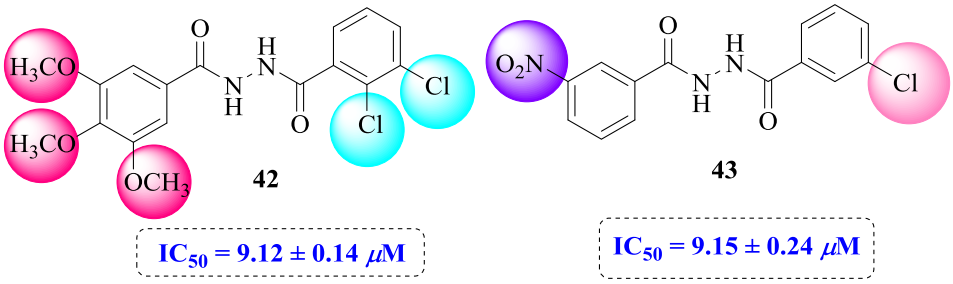


Fig. 10. Structure-activity relationship of compound **42–43.**

ranking docked conformations inside the active pocket of Jack bean urease. The analysis helped in the selection of better binding mode as well as the selective and potent compound. The binding free energy ΔG and FlexX docking score for the active compounds were given in Table 2. The FlexX docking score presented that the potent compound has lower energy scores as compared to non-selective inhibitors. Moreover, the binding free energies ΔG showed that the potent inhibitors exhibited higher affinity towards enzyme. The potent and selective compounds exhibit better binding affinity and give favorable contributions.

4. ADME profile of synthesized derivatives (1–43)

In order to determine the ADME (Absorption, Distribution, Metabolism, and Excretion) properties of the selective and potent compounds (**1–43**), *in silico* evaluation has been carried out with some physiochemical parameters. The results are shown in Table 3. Polar surface area (PSA) is sum of all the polar atoms in compound. It gives us the estimated ability of synthetic derivatives to cross the blood brain barrier (BBB). The value of PSA less than 140 is ideal and all compounds showing the values less than 100 [19–21]. Another parameter, cLogP shows the octanol water partition coefficient (ratio of compound

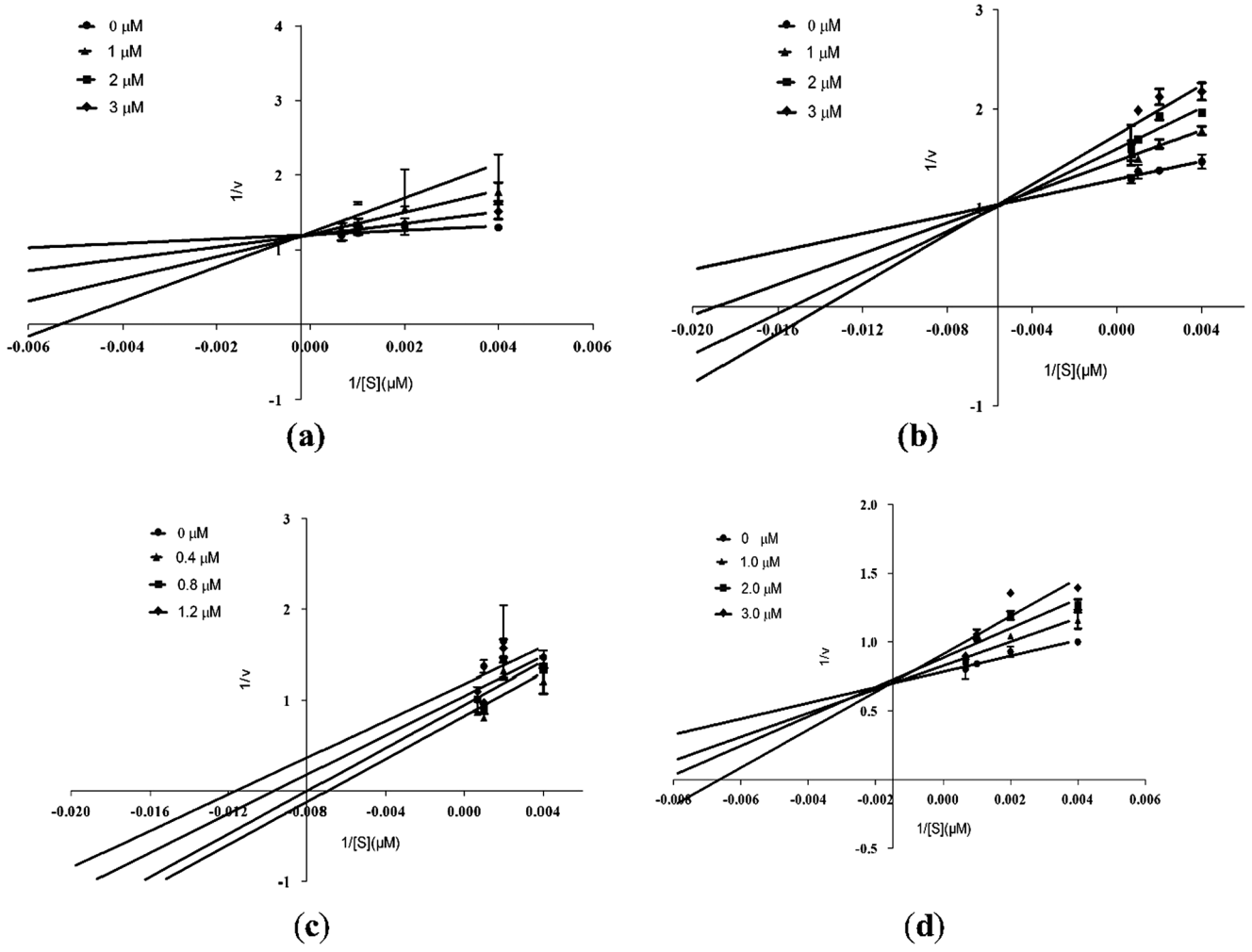


Fig. 11. Lineweaver-Burk plot for urease inhibitory activity by compounds **40** (a); **20** (b); **36** (c); and **33** (d) using different concentrations of substrate (0, 250, 500, 1000 and 15000 μM).

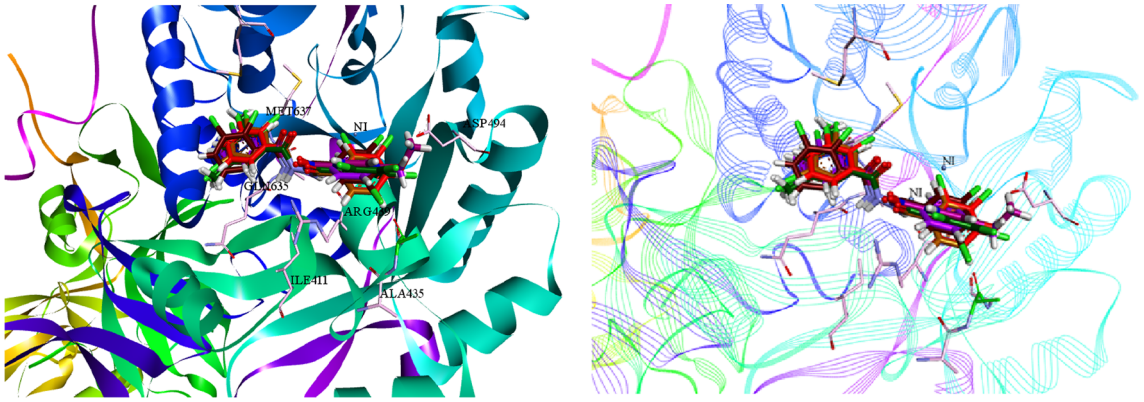


Fig. 12. The overlap of all the docked inhibitors in the active site of urease, compound **40** (red), **34** (purple), **19** (green), **20** (crimson), **37** (chocolate), **36** (blue), **33** (light brown).

concentration in octanol to water concentration). The parameter cLogP is responsible to calculate the compound ability to cross the membrane. Similarly, cLogS is the calculation of water solubility at 25 °C. The number of hydrogen bond donors (HD) and hydrogen bond acceptors (HA) must be less than 5 and 10, respectively. All the compounds presented favorable ADME properties and no violation of Lipinski's rule was observed [22–24].

5. Conclusion

Benzohydrazide derivatives **1–43** were synthesized and screened for *in vitro* urease inhibitory activity. It is worth-mentioning that all synthetic compounds were found to have potent inhibitory activity in the range of (IC_{50} = 0.87 ± 0.31 – 16.7 ± 0.36 μM) as compared to the standard thiourea (IC_{50} = 22.3 ± 1.06 μM), except compounds **17**, **18**, **23**, **24**, **29**, **30**, and **41** which were found to be inactive. Structure-activity relationship (SAR) was established on the basis of varying structural features of the molecules. It showed that almost all structural features are taking part in the inhibition potential but some groups Cl, CH_3, and OMe are contributing an important role. Kinetic studies have been done through various parameters and showed non-competitive or competitive mode of inhibition. However, *in silico* study was performed to confirm the binding interactions of the molecules (ligand) with the active site of enzyme. The current study had identified a range of chemical spaces which may act as lead molecules for future research to explore powerful urease inhibitors for the treatment of ulcer.

6. Experimental

6.1. Materials and methods

Reagents were purchased from Sigma-Aldrich, USA. Thin layer chromatography was carried out on pre-coated silica gel, GF-254 (Merck, Germany). Spots were visualized under ultraviolet light at 254, 366 nm or iodine vapor. Mass spectra were recorded under on MAT 312 and MAT 113D mass spectrometers. The ^{1}H NMR were recorded on a Bruker AM spectrometers, operating at 300 and 400 MHz. The chemical shift values are presented in ppm (δ), relative to tetramethylsilane (TMS) as an internal standard and the coupling constant (J) are in Hz. Melting points of the compounds were determined on a Stuart SMP10 melting point apparatus and are uncorrected.

7. General procedure for the synthesis of benzohydrazide (1–43)

N-benzoyl/nicotinoyl aryl benzohydrazides were obtained by treating different hydrazides with various derivatives of benzoyl chlorides in pyridine and the reaction mixture was refluxed for 36 h. The progress of reaction was monitored by TLC, after completion, the reaction mixture was poured onto 19% HCl, precipitates formed were filtered further washed with hot water and hexane. The solid product was crystallized from ethanol. The compounds thus obtained were characterized *via* EI-MS and H^1-NMR. Among all synthetic derivatives, compounds **19**, **37**, and **40** are new while rest of the compounds are reported in the literature [25–37].

7.1. Spectral data of synthetic compounds *1–43*

7.1.1. N′-Benzoylbenzohydrazide (1) [CAS # 787-84-8]

Yield: 87%; M.p.: 241–242 °C; ^{1}H NMR (300 MHz, DMSO-d_6);δ 10.49 (s, 2H, 1″-NH, 2″-NH), 7.93 (d, $J_{2,6/3,5,2',6'/3',5'}$ = 7.2 Hz, 4H, H-2, H-6, H-2′, H-6′), 7.61 (overlapping multiplete, 6H, H-3, H-4, H-5, H-3′, H-4′, H-5′); EI-MS *m/z* (% rel. abund.): 240 (M^+, 89), 105 (100), 77 (68), 51 (22).

7.1.2. N′-Benzoyl-2′,4′-Dichlorobenzohydrazide (2) [CAS # 195066-99-0]

Yield: 77%; M.p.: 196–198 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.64 (s, 1H, NH), 10.49 (s, 1H, NH), 7.93 (d, $J_{2,6/3,5}$ = 7.2 Hz, 2H, H-2, H-6), 7.75 (s, 1H, H-3′), 7.59 (overlapping multiplete, 5H, H-3, H-4, H-5, H-5′, H-6′); EI-MS *m/z* (% rel. abund.): 308 (M^+, 7), 310 (M + 2, 5), 173 (49), 105 (100), 77 (20).

7.1.3. N′-Benzoyl-3′-chlorobenzohydrazide (3) [CAS # 316142-01-5]

Yield: 87%; M.p.: 215–217 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.63 (s, 1H, NH), 10.55 (s, 1H, NH), 7.94 (overlapping multiplete, 3H, H-2′, H-5′, H-6′), 7.69 (d, $J_{4',5'}$ = 8.4 Hz, 1H, H-4′), 7.62 (d, $J_{2,6/3,5}$ = 8.1 Hz, 2H, H-2, H-6), 7.57 (overlapping multiplete, 3H, H-3, H-4, H-5); EI-MS *m/z* (% rel. abund.): 274 (M^+,41), 276 (M + 2, 14), 139 (55), 111 (23), 105 (1 0 0), 77 (50).

7.1.4. N′-Benzoyl-4′-chlorobenzohydrazide (4) [CAS # 6828-55-3]

Yield: 69%; M.p.: 222–223 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.61 (overlapping multiplete, 2H, NH, NH), 7.94 (m, 4H, H-2, H-6, H-2′, H-6′), 7.61 (d, $J_{3',5'/2',6'}$ = 8.4 Hz, 2H, H-3′, H-5′), 7.57 (overlapping multiplete, 3H, H-3, H-4, H-5); EI-MS *m/z* (% rel. abund.): 274 (M^+, 17), 276 (M + 2, 6), 139 (100), 111 (51), 105 (97), 77 (39).

7.1.5. N′-Benzoyl-2,3-dichlorobenzohydrazide (5) [CAS # 1182292-59-6]

Yield: 67%; M.p.: 190–192 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.64 (s, 1H, NH), 10.49 (s, 1H, NH), 8.73 (s, 1H, H-2), 7.93 (d, $J_{4',5'}$ = 7.6 Hz, 1H, H-4′), 7.78 (m, 1H, H-5′), 7.59 (t, 1H, H-4), 7.53 (overlapping multiplete, 3H, H-5, H-6′), 7.46 (overlapping multiplete, 1H, H-3); EI-MS *m/z* (% rel. abund.): 308 (M^+, 24), 310 (M + 2, 14), 312 (M + 4, 2), 173 (84), 145 (26), 105 (100), 77 (76), 51 (15).

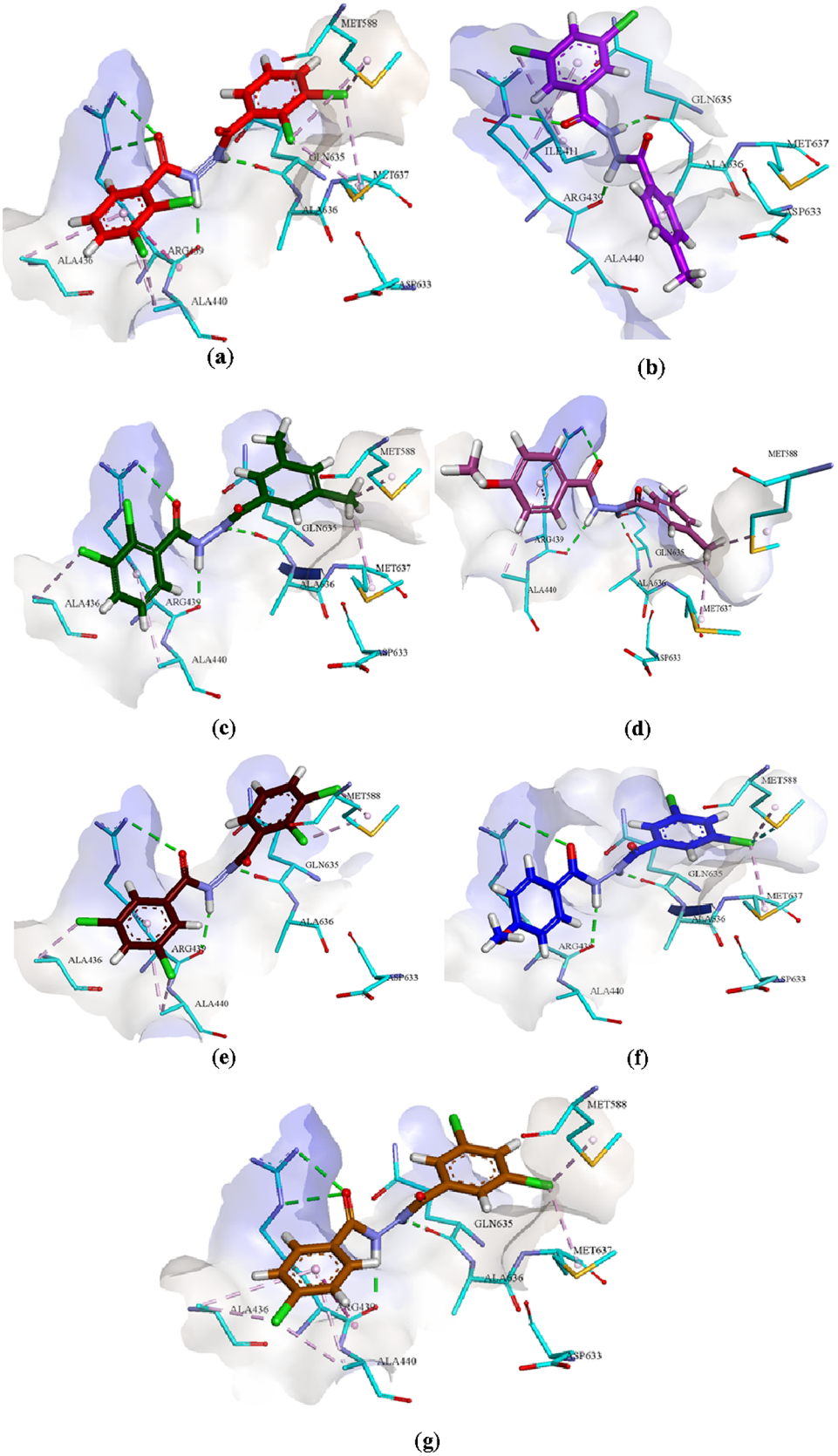


Fig. 13. The putative binding modes for potent compounds in the series against urease; (a) **40**: (b) **34**: (c) **19**: (d) **20**: (e) **37**: (f) **36**: (g) **33**.

7.1.6. N′-Benzoyl-3′-methylbenzohydrazide (6) [CAS # 108013-58-7]

Yield: 78%; M.p.: 208-210 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.48 (overlapping multiplete, 2H, NH, NH), 7.93 (d, $J_{2,6/3,5}$ = 7.2 Hz, 2H, H-2, H-6), 7.73 (overlapping multiplete, 2H, H-4′, H-6′), 7.59 (m, 3H, H-3, H-4, H-5), 7.40 (d, $J_{5',4'/6',5'}$ = 4.8 Hz, 2H, H-5′, H-4′), 2.37 (s, 3H, CH_3); EI-MS *m/z* (% rel. abound.): 254 (M^+, 15), 119 (100), 105

Table 2
Docking and Hyde scores and their corresponding ranks by Hyde affinity estimation.

Code	FlexX score of the top ranking pose	Binding free energy ΔG (KJ mol^{-1})
19	−22.62	−10
20	−20.98	−22
33	−22.55	−16
34	−22.80	−21
36	−23.46	−29
37	−23.02	−19
40	−23.71	−20

Table 3
In silico calculation of some ADME parameters of the selective and potent compounds **19**, **20**, **33**, **34**, **36**, **37**, and **40**.

Codes	Mol. Wt	PSA	cLogP	cLogS	HD	HA
19	337.20	58.20	4.405	−5.9998	2	2
20	298.34	67.43	3.181	−4.5816	2	3
33	343.59	58.20	4.405	−5.7863	2	2
34	323.17	58.20	4.111	−5.5259	2	2
36	339.17	67.43	3.769	−5.1024	2	3
37	378.04	58.20	4.993	−6.5206	2	2
40	378.04	58.20	4.878	−6.5206	2	2

(50), 91 (33), 77 (20).

7.1.7. N′-Benzoyl-4′-methylbenzohydrazide (7) [CAS # 19338-21-7]

Yield: 81%; M.p.: 220–221 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.45 (s, 1H, NH), 10.41 (s, 1H, NH), 7.92 (d, $J_{2',6'/3',5'}$ = 7.2 Hz, 2H, H-2′, H-6′), 7.83 (d, $J_{3',5'/2',6'}$ = 8.1 Hz, 2H, H-3′, H-5′), 7.61 (m, 3H, H-3, H-4, H-5), 7.33 (d, $J_{2,6/3,5}$ = 8.1 Hz, 2H, H-2, H-6), 2.37 (s, 3H, CH_3); EI-MS *m/z* (% rel. abound.): 254 (M^+, 30), 119 (100), 105 (75), 91 (61), 77 (35), 65 (13).

7.1.8. N′-Benzoyl-4′-methoxybenzohydrazide (8) [CAS # 6781-59-5]

Yield: 89%; M.p.: 186–187 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.42 (s, 1H, NH), 10.34 (s, 1H, NH), 7.91 (overlapping multiplete, 4H, H-2, H-6, H-2′, H-6′), 7.58 (overlapping multiplete, 1H, H-4), 7.53 (t, $J_{3,2/5,6/3,4/5,4}$ = 7.5 Hz, 2H, H-3, H-5), 7.06 (d, $J_{3',5/2',6'}$ = 8.7 Hz, 2H, H-3′, H-5′), 3.82 (s, 3H, OCH_3); EI-MS *m/z* (% rel. abund.): 270 (M^+, 48), 135 (100), 105 (64), 92 (16), 77 (47).

7.1.9. N′-Benzoylnicotinohydrazide (9) [CAS # 56352-76-2]

Yield: 79%; M.p.: 258–259 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.70 (s, 1H, NH), 10.57 (s, 1H, NH), 9.07 (d, $J_{6,3}$ = 1.6 Hz, 1H, H-2), 8.77 (dd, $J_{4,2}$ = 1.2, $J_{4,5}$ = 4.8 Hz, 1H, H-4), 8.26 (d, $J_{6,5}$ = 7.6 Hz, 1H, H-6), 7.92 (d, $J_{2',6'/3',5'}$ = 7.6 Hz, 2H, H-2′, H-6′), 7.60 (overlapping multiplete, 4H, H-5, H-3′, H-4′, H-5′); EI-MS *m/z* (% rel. abund.): 241 (M^+, 12), 105 (100), 77 (45), 51 (15).

7.1.10. N′-(3′-Chlorobenzoyl)nicotinohydrazide (10) [CAS # 258264-84-5]

Yield: 71%; M.p.: 230–232 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.78 (overlapping multiplete, 2H, NH, NH), 10.72 (s, 1H, NH), 9.06 (s, 1H, H-2), 8.78 (d, $J_{6,5}$ = 3.9 Hz, 1H, H-6), 8.27 (d, $J_{6,5}$ = 1.8 Hz, 1H, H-5), 7.89 (s, 1H, H-2′) 7.87 (d, $J_{4,5}$ = 7.5 Hz, 1H, H-4), 7.67 ($J_{6',5'}$ = 8.1 Hz, 1H, H-6′), 7.60 (t, 2H, H-5, H-5′); EI-MS *m/z* (% rel. abund.): 275 (M^+, 41), 277 (M + 2, 14), 139 (100), 113 (13), 111 (42), 106 (73), 78 (32), 75 (11), 51 (10).

7.1.11. N′-(4′-Chlorobenzoyl)nicotinohydrazide (11) [CAS # 56352-79-5]

Yield: 69%; M.p.: 192–194 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.73 (overlapping multiplete, 2H, NH, NH), 9.06 (s, 1H, H-2), 8.77 (d, $J_{6,5}$ = 3.6 Hz, 1H, H-6), 8.25 (d, $J_{4,5}$ = 7.6 Hz, 1H, H-4), 7.94 (d, $J_{2',6'/3',5'}$ = 8.0 Hz, 2H, H-2′, H-6′), 7.61 (overlapping multiplete, 3H, H-5, H-3′, H-5′); EI-MS *m/z* (% rel. abund.): 275 (M^+, 7), 277 (M + 2, 3), 139 (100), 111 (19), 78 (12).

7.1.12. N′-(2′,4′-Dichlorobenzoyl)nicotinohydrazide (12) [CAS # 392314-26-0]

Yield: 65%; M.p.: 254–256 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.87 (s, 1H, NH), 10.58 (s, 1H, NH), 9.06 (s, 1H, H-2), 8.77 (d, $J_{6,5}$ = 4.4 Hz, 1H, H-6), 8.27 (d, $J_{4,5}$ = 2.4 Hz, 1H, H-4), 7.76 (m, 1H, H-5), 7.55 (overlapping multiplate, 3H, H-3′, H-5′ H-6′); EI-MS *m/z* (% rel. abund.): 309 (7), 311 (M + 2, 4), 173 (100), 145 (13), 106 (37), 78 (24).

7.1.13. N′-(3′-Methylbenzoyl) nicotinohydrazide (13) [CAS # 821018-69-3]

Yield: 69%; M.p.: 216–218 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.70 (s, 1H, NH), 10.52 (s, 1H, NH), 9.06 (s, 1H, H-2), 8.77 (d, $J_{4/5}$ = 2.7 Hz, 1H, H-4), 8.26 (d, $J_{6/5}$ = 7.5 Hz, 1H, H-6), 7.73 (d, $J_{6'/5'}$ = 8.7 Hz, 2H, H-6′, H-5), 7.57 (s, 1H, H-2′), 7.40 (s, 2H, H-5′,H-4′); EI-MS *m/z* (% rel. abund.): 255 (M^+, 34), 119 (100), 106 (23), 91 (84), 78 (22), 65 (16), 51 (11).

7.1.14. N′-(4′-Methylbenzoyl) nicotinohydrazide (14) [CAS # 56352-79-5]

Yield: 68%; M.p.: 238–240 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.68 (s, 1H, NH), 10.50 (s, 1H, NH), 9.06 (s, 1H, H-2), 8.77 (d, $J_{4,5}$ = 4.2 Hz, 1H, H-4), 8.26 (d, $J_{6,5}$ = 7.8 Hz, 1H, H-6), 7.83 (d, $J_{6',2'/5',3'}$ = 8.1 Hz, 2H, H-2′, H-6′), 7.58 (m, 1H, H-5), 7.33 (d, $J_{3',5'/2',6'}$ = 7.8 Hz, 2H, H-3′, H-5′), 2.37 (s, 3H, CH_3); EI-MS *m/z* (% rel. abund.): 255 (M^+, 10), 119 (100), 106 (10), 91 (57), 78 (14), 78 (14), 65 (13).

7.1.15. N′-(2′-Ethoxybenzoyl) nicotinohydrazide (15) [CAS # 713490-22-3]

Yield: 70%; M.p.: 202–202 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.03 (s, 1H, NH), 10.17 (s, 1H, NH), 9.06 (s, 1H, H-2), 8.76 (d, $J_{4,5}$ = 4.2 Hz, 1H, H-4), 8.27 ($J_{6,5}$ = 6.6 Hz, 1H, H-6), 7.77 (dd, $J_{4,2/4,5}$ = 1.5 Hz, 1H, H-4), 7.57 (overlapping multiplete, 2H, H-6′, H-4′), 7.19 (d, $J_{3',4'}$ = 8.1 Hz, 1H, H-3′), 7.09 (t, 1H, H-5′), 4.24 (q, $J_{OCH2,CH3}$ = 6.9 Hz, 2H, OCH_2), 1.41 (t, $J_{CH3,CH2}$ = 7.2 Hz, 3H, CH_3). EI-MS *m/z* (% rel. abund.): 285 (M^+, 9), 149 (100), 121 (74), 93 (10).

7.1.16. N′-(3′-Methylbenzoyl)-3-methylbenzohydrazide (16) [CAS # 59646-36-5]

Yield: 26%; M.p.: 220–222 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.40 (s, 2H, NH, NH), 7.73 (overlapping multiplete, 4H, H-2, H-6, H-2′, H-6′), 7.40 (d, $J_{5,4/4,5/5',4'/4',5'}$ = 4.5 Hz, 4H, H-5, H-4, H-5′, H-4′), 2.37 (s, 6H, CH_3); EI-MS *m/z* (% rel. abund.): 268 (M^+, 18), 119 (100), 91 (62), 65 (16).

7.1.17. N′-(4′-Methylbenzoyl)-4-methylbenzohydrazide (17) [CAS # 1530-73-0]

Yield: 70%; M.p.: 253–254 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.36 (s, 2H, NH, NH), 7.82 (d, $J_{2,6/3,5/2',6'/3',5'}$ = 8.1 Hz, 4H, H-2, H-6, H-2′, H-6′), 7.32 (d, $J_{3,5/2,6/3',5'/2',6'}$ = 7.8 Hz, 4H, H-3, H-5, H-3′, H-5′), 2.36 (s, 6H, CH_3); EI-MS *m/z* (% rel. abund.): 268 (M^+, 22), 119 (100), 91 (69), 65 (19).

7.1.18. N′-(3′-Chlorobenzoyl)-3,5-dimethylbenzohydrazide (18) [CAS # 1002706-72-0]

Yield: 63%; M.p.: 232–234 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.59 (s, 1H, NH), 10.41 (s, 1H, NH), 7.94 (s, 1H, H-2′), 7.88 (d, $J_{6',5'}$ = 7.6 Hz ,1H, H-6′), 7.68 (d, $J_{4',5'}$ = 6.8 Hz, 1H, H-4′), 7.58 (overlapping multiplete, 1H, H-5′), 7.52 (s, 2H, H-2, H-6), 7.22 (s, 1H, H-4), 2.32 (s, 6H, CH_3, CH_3); EI-MS *m/z* (% rel. abund.): 302 (M^+, 19), 304 (M + 2, 8), 139 (80), 133 (100), 105 (59), 77 (19).

*7.1.19. 2′,3′-Dichloro-N′-(3,5-dimethylbenzoyl)benzohydrazide **(19)***

Yield: 67%; M.p.: 208–210 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.84 (m, 2H, NH, NH), 7.94 (overlapping multiplete, 2H, H-4′, H-6′), 7.69 (s, 1H, H-4), 7.65 (overlapping multiplete, 3H, H-2, H-6, H-5′), 2.65 (s, 6H, 3-CH_3, 5-CH_3); EI-MS *m/z* (% rel. abund.): 336 (M^+, 8), 338 (M + 2, 5), 173 (76), 145 (12), 133 (100), 105 (21).

*7.1.20. N′-(4′-Methoxybenzoyl)-3,5-dimethylbenzohydrazide **(20)** [CAS # 892004-94-3]*

Yield: 66%; M.p.: 195 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.29 (overlapping multiplete, 2H, NH, NH), 7.90 (d, $J_{2′,4′/3′,5′}$ = 8.4 Hz, 2H, H-2′,H-6′), 7.51 (s, 2H, H-2, H-6), 7.21 (s, 1H, H-4), 7.04 (d, $J_{3′,5′/2′,6′}$ = 8.4 Hz, 2H, H-3′, H-5′), 3.82 (s, 3H, OCH_3), 2.34 (s, 6H, CH_3, CH_3); EI-MS *m/z* (% rel. abund.): 298 (M^+, 41), 135 (100), 105 (26), 92 (13), 77 (22).

*7.1.21. N′-Benzoyl-3′-chlorobenzohydrazide **(21)** [CAS # 316142-01-5]*

Yield: 62%; M.P.: 215–217 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.62 (s, 1H, NH), 10.55 (s, 1H, NH), 7.94 (overlapping multiplete, 4H, H-2, H-6, H-2′, H-6′), 7.69 (d, $J_{4,5}$ = 8.0 Hz, 1H, H-4), 7.59 (overlapping multiplete, 4H, H-5, H-3′, H-4′, H-5′); EI-MS *m/z* (% rel. abund.): 274 (M^+, 22), 276 (M + 2, 8), 139 (94), 111 (40), 105 (100), 77 (73), 51 (20).

*7.1.22. 3-Chloro-N′-(3′-chlorobenzoyl) benzohydrazide **(22)** [CAS # 38192-14-2]*

Yield: 68%; M.p.: 234–236 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.68 (s, 2H, 2NH), 7.94 (d, $J_{2,4/2′,4′}$ = 1.6 Hz, 2H, H-2, H-2′), 7.88 (d, $J_{6,5/6′,5′}$ = 7.6 Hz, 2H, H-6, H-6′), 7.69 (d, $J_{4,5/4′,5′}$ = 8.0 Hz, 2H, H-4, H-4′), 7.58 (t, $J_{5,4/5,6/5′,4′/5′,6′}$ = 8.0 Hz, 2H, H-5, H-5′); EI-MS *m/z* (% rel. abund.): 308 (M^+, 42), 310 (M + 2, 30), 312 (M + 4, 5), 139 (100), 111 (67), 75 (20)

*7.1.23. 3-Chloro-N′-(4′-chlorobenzoyl)benzohydrazide **(23)** [CAS # 316146-11-9]*

Yield: 71%; M.p.: 206–208 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.64 (s, 2H, NH), 7.93 (overlapping multiplete, 4H, H-2, H-6, H-2′ H-6′), 7.69 (d, $J_{4,5}$ = 8.0 Hz, 1H, H-4), 7.61 (overlapping multiplete, 3H, H-5, H-5′, H-3′); EI-MS *m/z* (% rel. abund.): 308 (M^+, 10), 310 (M + 2, 5), 312 (M + 4, 1), 139 (100), 111 (44), 75 (13).

*7.1.24. 4-Chloro-N′-(4′-chlorobenzoyl) benzohydrazide **(24)** [CAS # 895-84-1]*

Yield: 75%; M.p.: 220–222 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.61 (s, 2H, NH, NH), 7.93 (d, $J_{2,6/3,5/2′,6′/3′,5′}$ = 8.4 Hz, 4H, H-2, H-6, H-2′, H-6′), 7.61 (d, $J_{3,5/2,6/3′,5′/2′,6′}$ = 8.4 Hz, 4H, H-3, H-5, H-3′, H-5′); EI-MS *m/z* (% rel. abund.): 308 (M^+, 6), 310 (M + 2, 5), 139 (100), 111 (31), 75 (13).

*7.1.25. 3-Chloro-N′-(3′-methylbenzoyl) benzohydrazide **(25)** [CAS # 304456-89-1]*

Yield: 66%; M.p.: 202–204 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.60 (s, 1H, NH), 10.48 (s, 1H, NH), 7.94 (s, 1H, H-2), 7.88 (d, $J_{6,5}$ = 7.6 Hz, 1H, H-6), 7.73 (overlapping multiplete, 3H, H-4, H-2′, H-6′), 7.58 (d, $J_{5,4}$ = 7.6 Hz, 1H, H-5), 7.40 (overlapping multiplete, 2H, H-4′, H-5′), 2.37 (s, 3H, CH_3); EI-MS *m/z* (% rel. abund.): 288 (M^+, 44), 290 (M + 2, 13), 139 (75) 119 (100), 111 (28), 91 (90), 65 (14).

*7.1.26. 3-Chloro-N′-(4′-methylbenzoyl) benzohydrazide **(26)** [CAS # 316142-02-6]*

Yield: 68%; M.p.: 202–202 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.58 (s, 1H, NH), 10.45 (s, 1H, NH), 7.93 (s, 1H, H-2), 7.68 (d, $J_{4,5}$ = 8.0 Hz, 1H, H-4), 7.82 (d, $J_{2′,6′/3′,5′}$ = 8.0 Hz, 2H, H-2′, H-6′), 7.58 (t, $J_{5,4/5,6}$ = 7.6 Hz, 1H, H-5). 7.32 (d, $J_{3′,5′/2′,6′}$ = 8.0 Hz, 2H, H-3′, H-5′), 2.37 (s, 3H, CH_3), EI-MS *m/z* (% rel. abund.): 288 (M^+, 21), 290 (M + 2, 7), 139 (57), 119 (100), 111 (26), 91 (70), 75 (10), 65 (13).

*7.1.27. 2′,4′-Dichloro-N′-(3-chlorobenzoyl)benzohydrazide **(27)** [CAS # 316143-31-4]*

Yield: 75%; M.p.: 212–214 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.77 (s, 1H, NH), 10.51 (s, 1H, NH), 7.95 (s, 1H, H-3′), 7.89 (d, $J_{6′,5′}$ = 7.6 Hz, 1H, H-6′), 7.75 (s, 1H, H-2), 7.68 (d, $J_{5′,6′}$ = 7.6 Hz, 1H, H-5′), 7.58 (overlapping multiplete, 3H, H-4, H-5, H-6); EI-MS *m/z* (% rel. abund.): 344 (M^+, 5), 346 (M + 2, 1), 173 (100), 139 (58), 111 (25), 83 (15).

*7.1.28. 2′,3′-Dichloro-N′-(3-chlorobenzoyl)benzohydrazide **(28)** [CAS # 1182660-05-4]*

Yield: 74%; M.p.: 186–188 °C; ^{1}H NMR (400 MHz, DMSO-d_6):δ 10.79 (s, 1H, NH), 10.57 (s, 1H, NH), 7.96 (s, 1H, H-2), 7.89 (d, $J_{4′,5′}$ = 7.6 Hz, 1H, H-4′), 7.78 (m, 1H, H-5′), 7.68 (d, $J_{6′,5′}$ = 8.0 Hz, 1H, H-6′), 7.58 (t, $J_{5,4/5,6}$ = 8.0 Hz, 1H, H-5) 7.50 (overlapping multiplete, 2H, H-4, H-6); EI-MS *m/z* (% rel. abund.): 342 (M^+, 9), 344 (M + 2, 8), 346 (M + 4, 3), 173. (83), 139 (100), 111 (27), 75 (12).

*7.1.29. 3-Chloro-N′-(4′-methoxybenzoyl)benzohydrazide **(29)** [CAS # 316151-65-2]*

Yield: 70%; M.p.: 206–208 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.55 (s, 1H, NH), 10.39 (s, 1H, NH), 7.93 (overlapping multiplete, 4H, H-2H-6, H-4′, H-2′), 7.68 (d, $J_{6′,5′}$ = 8.0 Hz, 1H, H-6′), 7.58 (t, $J_{5′,6′/5′,4′}$ = 7.6 Hz, 1H, H-5′), 7.05 (d, $J_{3,5/2,6}$ = 8.4 Hz, 2H, H-3, H-5), 3.82 (s, 3H, OCH_3); EI-MS *m/z* (% rel. abund.): 304 (M^+, 11), 306 (M + 2, 4), 135 (100), 111 (32), 92 (19), 77 (25).

*7.1.30. N′-(3′-Chlorobenzoyl)-2-iodobenzohydrazide **(30)** [CAS # 812686-07-0]*

Yield: 68%; M.p.: 202–204 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.70 (s, 2H, NH, NH), 7.94 (overlapping multiplete, 2H, H-2′, H-6′), 7.86 (d, $J_{6,5/5,6}$ = 7.8 Hz, 2H, H-5, H-6), 7.70 (d, $J_{4,3/5,4}$ = 8.7 Hz, 2H, H-3, H-4), 7.59 (overlapping multiplete, 2H, H-4′, H-5′); EI-MS *m/z* (% rel. abund.): 308 (32), 310 (M + 2, 20), 231 (19), 139 (100), 111 (56), 75 (17).

*7.1.31. N′-(3′-methylbenzoyl)-2-iodobenzohydrazide **(31)** [CAS # 304480-86-2]*

Yield: 65%; M.p.: 194 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.53 (s, 1H, NH), 10.30 (s, 1H, NH), 7.94 (d, $J_{6,5}$ = 7.8 Hz, 1H, H-6), 7.70 (overlapping multiplete, 2H, H-3, H-6′), 7.40 (overlapping multiplete, 2H, H-5, H-4), 7.25 (m, 1H, H-5′), 7.23 (overlapping multiplete, 3H, H-2′, H-3′, H-4′), 2.37 (s, 3H, CH_3); EI-MS *m/z* (% rel. abund.): 380 (M^+, 32), 231 (78), 202 (12), 119 (100), 91 (28).

*7.1.32. 3,5-Dichloro-N′-(3′-chlorobenzoyl)benzohydrazide **(32)** [CAS # 1182308-11-17]*

Yield: 78%; M.p.: 280–282 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.80 (overlapping multiplete, 2H, NH, NH), 7.93 (overlapping multiplete, 5H, H-2, H-4, H-6, H-2′, H-6′), 7.69 (d, $J_{4′,5′}$ = 7.6 Hz, 1H, H-4′), 7.58 (t, $J_{5′,6′/5′,4′}$ = 7.6 Hz, 1H, H-5′); EI-MS *m/z* (% rel.abund.): 344 (M^+, 21), 346 (M + 2, 7), 173 (80), 140 (100), 111 (80), 75 (24).

*7.1.33. 3,5-Dichloro-N′-(4′-chlorobenzoyl)benzohydrazide **(33)** [CAS # 909221-15-4]*

Yield: 65%; M.p.: 180 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.76 (s, 1H, NH), 10.71 (s, 1H, NH), 7.93 (overlapping multiplete, 5H, H-2, H-4, H-6, H-2′, H-6′), 7.61 (d, $J_{3′,5′/2′,6′}$ = 8.4 Hz, 2H, H-3′, H-5′); EI-MS *m/z* (% rel. abund.): 342 (M^+, 10), 344 (M + 2, 9), 346 (M + 4, 3), 173 (39), 139 (100), 111 (50), 75 (22).

*7.1.34. 3,5-Dichloro-N′-(4′-methylbenzoyl)benzohydrazide **(34)** [CAS # 909221-07-4]*

Yield: 70%; M.p.: 214–216 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ

10.69 (s,1H, NH), 10.52 (s, 1H, NH), 10.35 (s, 1H, OH), 7.92 (overlapping multiplete, 3H, H-2, H-4, H-6), 7.82 (d, $J_{2',6'/3',5'}$ = 8.0 Hz, 2H, H-2′, H-6′), 7.32 (d, $J_{3',5'/2',6'}$ = 7.6 Hz, 2H, H-3′ H-5′), 2.37 (s, 3H, CH_3); EI-MS *m/z* (% rel. abund.): 322 (M^+, 3), 324 (M + 2, 2), 173(10), 119 (100), 91 (26).

7.1.35. 3,5-Dichloro-N'-(3′-methylbenzoyl)benzohydrazide ***(35)*** *[CAS # 909220-95-7]*

Yield: 59%; M.p.: 244–246 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.71 (s, 1H, NH), 10.54 (s, 1H, NH), 7.92 (overlapping multiplete, 3H, H-2, H-4, H-6), 7.73 (overlapping multiplete, 2H, H-6′, H-2′), 7.40 (overlapping multiplete, 2H, H-5′, H-4′), 2.37 (s, 3H, CH_3); EI-MS *m/z* (% rel. abund.): 322 (M^+, 16), 324 (M + 2, 8), 326 (M + 4, 2), 173 (34), 145 (13), 119 (100), 91 (74), 65 (13).

7.1.36. 3,5-Dichloro-N'-(4′-methoxybenzoyl)benzohydrazide ***(36)*** *[CAS # 909221-09-6]*

Yield: 68%; M.p.: 192 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.66 (overlapping multiplete, 2H, NH, NH), 7.91 (overlapping multiplete, 4H, H-2, H-6, H-2′, H-6′), 7.05 (overlapping multiplete, 3H, H-4, H-3′, H-5′), 3.82 (s, 3H, OCH_3); EI-MS *m/z* (% rel. abund.): 338 (M^+, 9), 340 (M + 2, 5), 300 (31), 173 (44), 135 (100), 107 (15), 92 (20), 77 (24).

7.1.37. 2′,3′-Dichloro-N'-(3,5-dichlorobenzoyl)benzohydrazide ***(37)***

Yield: 69%; M.p.: 240–242 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.91 (s, 1H, NH), 10.65 (s, 1H, NH), 7.94 (overlapping multiplete, 2H, H-4, H-4′), 7.79 (overlapping multiplete, 2H, H-3′, H-6′), 7.50 (d, $J_{2,4/6,4}$ = 4.8 Hz, 2H, H-2, H-6); EI-MS *m/z* (% rel. abund.): 378 (M^+, 7), 380 (M + 2, 3), 173 (100), 145 (19), 109 (6).

7.1.38. 2′,4′-Dichloro-N'-(3,5-dichlorobenzoyl)benzohydrazide ***(38)*** *[CAS # 909221-11-0]*

Yield: 68%; M.p.: 270–271 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.88 (s, 1H, NH), 10.62 (s, 1H, NH), 7.93 (overlapping multiplete, 4H, H-2, H-6, H-5′, H-6′), 7.75 (s,1H, H-3′), 7.56 (s, 1H, H-4); EI-MS *m/z* (% rel. abund.): 378 (M^+, 8), 380 (M + 2, 4), 173 (100), 145 (26), 109 (10).

7.1.39. 2,4-Dichloro-N'-(2′,4′-dichlorobenzoyl)benzohydrazide ***(39)*** *[CAS # 76114-78-8]*

Yield: 74%; M.p.: 264–265 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.64 (s, 2H, NH, NH), 7.74 (s, 2H, H-3, H-3′), 7.55 (s, 4H, H-6, H-5, H-6′, H-5′); EI-MS *m/z* (% rel. abund.): 378 (M^+, 7), 380 (M + 2, 3), 173 (100), 145 (25), 109 (12).

7.1.40. 2,3-Dichloro-N'-(2′,3′-dichlorobenzoyl)benzohydrazide ***(40)***

Yield: 77%; M.p.: 270–272 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.68 (s, 2H, NH, NH), 7.78 (m, 2H, H-4, H-4′), 7.49 (overlapping multiplete, 4H, H-5, H-6, H-5′, H-6′); EI-MS *m/z* (% rel. abund.): 378 (M^+, 8), 380 (M + 2, 2), 173 (100), 139 (28), 111 (14), 75 (15).

7.1.41. 4-Methoxy-N'-(4′-methoxybenzoyl)benzohydrazide ***(41)*** *[CAS # 849-82-1]*

Yield: 78%; M.p.: 224–225 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.26 (s, 2H, NH, NH), 7.90 (d, $J_{3,5/2,6/3',5'/2',6'}$ = 8.8 Hz, 4H, H-3, H-5, H-3′, H-5′), 7.05 (d, $J_{2,6/3,5/2',6'/3',5'}$ = 8.8 Hz, 4H, H-2, H-6, H-2′, H-6′), 3.82 (s, 6H, OCH_3); EI-MS *m/z* (% rel. abund.): 300 (M^+, 57), 135 (100), 107 (30), 92 (31), 77 (42), 64 (12).

7.1.42. N'-(2′,3′-Dichlorobenzoyl)-3,4,5-trimethoxybenzohydrazide ***(42)*** *[CAS # 1182681-57-7]*

Yield: 68%; M.p.: 208–210 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.58 (s, 1H, NH), 10.49 (s, 1H, NH), 7.79 (m, 1H, H-5′), 7.50 (overlapping multiplete, 2H, H-4′, H-6′), 7.27 (s, 2H, H-2, H-6), 3.72 (s, 3H, 4′–OCH_3), 3.84 (s, 6H, 3′–OCH_3, 5′–OCH_3); EI-MS *m/z* (% rel. abund.): 398 (M^+, 24), 400 (M + 2, 21), 402 (M + 4, 4), 195 (100), 173 (21), 152 (10).

7.1.43. 3′-Chloro-N'-(3-nitrobenzoyl)benzohydrazide ***(43)*** *[CAS # 3161-49-08-3]*

Yield: 80%; M.p.: 217 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.97 (s, 1H, NH), 10.77 (s, 1H, NH), 8.73 (s, 1H, H-2), 8.46 (d, $J_{4,5}$ = 8.0 Hz ,1H, H-4), 8.35 (d, $J_{6,5}$ = 7.2 Hz, 1H, H-6), 7.95 (s, 1H, H-2′), 7.67 (m, 2H, H-5, H-6′), 7.59 (overlapping multiplete, 2H, H-4′, H-5′); EI-MS *m/z* (% rel. abund.): 319 (M^+, 11), 321 (M + 2, 4), 150 (28), 139 (100), 120 (13), 111 (31).

7.2. Urease inhibition assay

In vitro urease inhibition assay of the synthetic compounds was determined according to the previously reported literature protocols [38,39].

7.3. Kinetic studies

In order to characterize the interaction of the potent inhibitors of urease, the type of inhibition was determined using Michaelis-Menten kinetics. For this purpose, the initial rates of the enzyme inhibition were measured at different substrate concentrations in the absence and presence of different concentrations of the selected representative inhibitors against respective enzymes. The results were depicted as double reciprocal Lineweaver-Burk plots using PRISM 5.0 (GraphPad, San Diego, California, USA).

8. Molecular docking studies

8.1. Selection of protein structure and preparation of ligands

Ligand-bound crystallographic structures of Jack bean urease (PDB ID: 3LA4) was downloaded from Protein Data Bank (PDB) [18]. The MOE builder tool was used to generate the 3D structures of the compounds [40]. The minimization of protein was done by MOE (MOE 2014.09) by keeping the four histidine residues surrounding two nickel ions in the active site pocket of enzyme [40]. Protein structure was protonated using AMBER99 force field and minimized with a RMSD gradient of 0.05 kcal/mol, prior to docking studies [41]. Afterwards, addition of hydrogen atoms was done to all the compounds and charges were assigned to respective atom. At the end the energy minimization of generated 3D structure was carried out by applying force field MMFF94x at the RMSD gradient of 0.01 kcal/mol Å [41].

8.2. Docking experiment

The docking analysis was done by LeadIT (BioSolveIT GmbH, Germany) [42], after the compound and proteins preparation. The docking analysis and studies were carried out for the selected inhibitors and reference standards without modifying the default parameters. Top 50 ranking poses were visualized and selected for each ligand and further analysis was done using HYDE assessment to investigate the favorable and unfavorable interactions [43]. Poses with lowest binding energy and favorable affinity were chosen and further analyzed by Discovery Studio Visualizer [44].

Acknowledgement

This work was financially supported by the Higher Education Commission (HEC) Pakistan (Project No. 20-1910), under the National Research Program for Universities. J. Iqbal is grateful to the Organization for the Prohibition of Chemical Weapons (OPCW), The Hague, The Netherlands and Higher Education Commission of Pakistan for the financial support through Project No. D/14/520.

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