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Xanthine oxidase inhibitory activity of nicotino/isonicotinohydrazides: A systematic approach from *in vitro*, *in silico* to *in vivo* studies

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ABSTRACT

Change in life style and eating habits has led to an increased prevalence of hyperuricemia worldwide. The role of hyperuricemia is no more restricted to gout, but it has a central role in progression of CVD, hypertension, metabolic syndrome, and arthritis. Among the different factors involved in regulation of serum uric acid, xanthine oxidase (XO) is the best pharmacological target to control the levels of serum uric acid as it catalyzes the final steps in uric acid production. In the current study, a systemic search for the inhibitors of xanthine oxidase, starting from synthesis to *in vitro* screening and leading to *in vivo* studies is presented. Benzylidene nicotino/isonicotinohydrazides (**1**–**54**) were synthesized by treating nicotinic/isonicotinic hydrazides with substituted aromatic aldehyde, and characterized by EI-MS and ^{1}H NMR. Elemental analysis was also performed. All synthetic compounds were screened for xanthine oxidase inhibitory activity initially using an *in vitro* spectroscopic XO inhibition assay. Among them twenty-two derivatives were found to be active with IC_{50} values between 0.96 and 330.4 μM, as compared to standard drug allopurinol $IC_{50} = 2.00 \pm 0.01$ μM. Kinetic studies of five most active compounds (**8**, **35**, **36**, **39**, and **45**) with low IC_{50} values between 0.96 and 54.8 μM showed a competitive mode of inhibition. Further *in silico* molecular docking was carried out to study the interactions of these inhibitors with catalytically important amino acid residues in XO. Three compounds **8**, **35**, and **36** with IC_{50} values of 10, 12.4, and 0.96 μM, respectively, were also found to be non-cytotoxic, and thus selected for *in vivo* studies. A simple and physiologically relevant animal model was used to analyze the *in vivo* XO inhibitory activity of these compounds. Among these, two compounds **35**, and **36** showed a significant inhibition in male Wistar rats, and identified as potential lead molecules for anti-hyperuricemic drug development.

1. Introduction

Uric acid, a heterocyclic organic compound, is the end product of purine metabolism in humans. The weak acidic pKa of uric acid (5.8) limits its solubility at physiological pH, and it exist in salt form *i.e.* urate ion. Therefore an elevated level of serum uric acid increases the risk for monosodium urate crystal formation in connective tissues, kidneys, and joints. This increase in serum uric acid from the normal concentration (6.8 mg/dL) is referred as hyperuricemia.[1] Hyperuricemia is involved in the pathophysiology of many diseases, including gout, hypertension, and renal dysfunction. The role of increased serum uric acid is still debatable in cardiovascular diseases (CVD).[2] However, various researchers have established a link between increased xanthine oxidase (XO) activity, and increased reactive oxygen species (ROS) production in the progression of CVD due to serum uric acid.[2,3]

The regulation of uric acid production and excretion are complex processes involving various factors. Among these factors, XO is responsible for the formation of uric acid in purine catabolism, and is therefore involved in the regulation of uric acid levels in serum. Consequently XO catalyzed reactions are the most relevant pathway responsible for uric acid overproduction. Hence XO inhibitors are excellent pharmacological agents for the treatment of

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0968-0896/

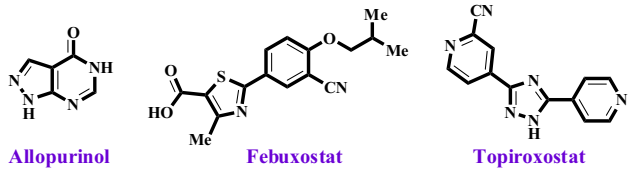


Fig. 1. Clinical drugs for XO inhibition.

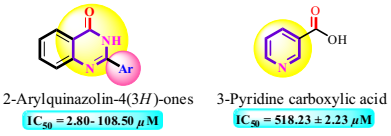


Fig. 2. General representation of our previous study.

hyperuricemia, rather than uricosuric agents that increases excretion of uric acid with severe adverse effects.[4,5]

Allopurinol, febuxostat, and recently developed topiroxostat are the clinical drugs for XO inhibition. However, these drugs have several adverse effects, such as hypersensitivity drug reactions. Adverse effects related to allopurinol includes interstitial nephritis, peripheral neuritis, renal toxicity, and congenital malformations when used during pregnancy. So, there is still a need to develop new drugs with better pharmacological profile and lesser side effects.[6,7]

Several classes of synthetic and natural compounds have been reported as XO inhibitors. The synthetic inhibitors of XO are broadly classified into purine and non-purine based inhibitors. Non-purine based inhibitors have advantage over purine based inhibitors as the former is not being metabolized by the enzymes of purine metabolism. Although there are several studies reported on XO inhibitors using *in vitro* assays, still very few of them reached to the clinical trials. One reason behind this limited success could be a poor correlation between *in vitro* and *in vivo* studies[8–10] (See Fig. 1).

Our group previously reported 3-pyridine carboxylic acid as an inhibitor of XO.[11] The compound was isolated from *Pyrenacantha staudtii* Engl. and its IC_{50} value was found to be 518.23 ± 2.23 μM. We have also reported nitrogen containing heterocycles as potential XO inhibitors,[12,13] such as 2-arylquinazolin-4(3*H*)-ones (Fig. 2).[12]

It is worth noting that these synthetic derivatives, and the recently developed drug topiroxostat have pyridine ring in the structures. Therefore, in the light of our previous study and the structural resemblance of the synthetic benzylidene nicotino/isonicotinohydrazides (**1**–**54**) (Fig. 3) with the drug topiroxostat, we were encouraged to evaluate this library of compounds for their XO inhibitory activity. We, herein, report the synthesis and XO inhibitory activity *in vitro*, as well as *in vivo*.

The results of *in vitro* spectrophotometric inhibition assay encouraged us to carry out *in vivo* studies. Significant results were obtained in *in vivo* studies. The mode of inhibition was determined by kinetic studies and to predict the interaction of these compounds with specific amino acids residues in the active site of XO, *in silico* molecular docking studies were performed.

The most common *in vivo* model for XO inhibitors is based on the use of potassium oxonate (uricase inhibitor) that increases the amount of uric acid in experimental animals. The inhibitors identified through this model still lack the clear indication that the decrease in serum uric acid is due to inhibition of XO (*i.e.*, decreased production) or test compounds have increased the excretion of uric acid.[11]

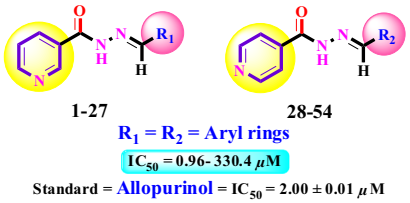
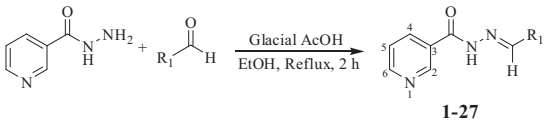
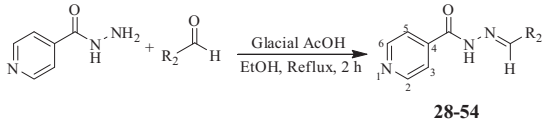


Fig. 3. General representation of the synthetic library and their bioactivity.



Scheme 1. Synthesis of benzylidene nicotinohydrazides **1**–**27**.



Scheme 2. Synthesis of benzylidene isonicotinohydrazides **28**–**54**.

In current study, we used a simple and reliable *in vivo* assay that measures the XO inhibition in animals through drug-drug interaction.[14] 6-Mercaptopurine (6-MP) is a pro-drug used in the treatment of leukemia, and it is metabolized by three pathways.[15] One of these pathways is catalyzed by XO that convert 6-MP into 6-thiouric acid which is also inactive product. The basic mechanism of action of allopurinol is that it lowers the formation of 6-thiouric acid by inhibiting the enzyme XO, and thus increase the concentration of 6-MP.[16,17]

2. Results and discussion

2.1. Chemistry

Benzylidene nicotino/isonicotinohydrazides (**1**–**54**) were synthesized from nicotinic and isonicotinic hydrazide by condensing with different aromatic aldehydes in ethanol under reflux (Schemes 1 and 2). Glacial acetic acid was used as a catalyst. The structures of compounds **1**–**54** were elucidated by different spectroscopic techniques, such as ^{1}H NMR and EI-MS. Elemental analysis was also performed and found satisfactory. Except compounds **7** and **16**, all other derivatives were previously reported.[18]

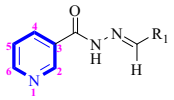
2.2. In vitro XO inhibitory activity

All the compounds **1**–**54** were evaluated for their XO inhibitory activity. Compounds **1**, **2**, **5**, **7**, **8**, **11**, **12**, **18**–**22**, **24**, **29**, **35**, **36**, **38**, **39**, **45**, **48**, and **49** showed their inhibitory potential in the range of IC_{50} = 0.96–330.4 μM, as compared to standard drug allopurinol IC_{50} = 2.00 ± 0.01 μM (Table 1). The limited structure-activity relationship was rationalized by observing the effect of different substituted rings 'R_1/R_2' on inhibitory activity.

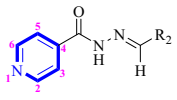
2.3. Structure-activity relationship of benzylidene nicotinohydrazides

The parent compound **1** has a phenyl ring with no substitution, and showed an IC_{50} value of 210.7 ± 1.2 μM. The better activity of

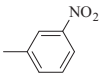
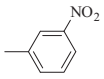
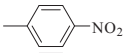
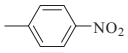
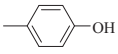
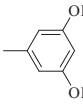
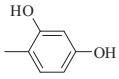
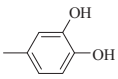
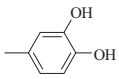
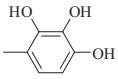
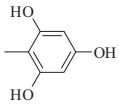
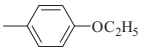
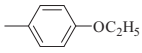
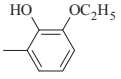
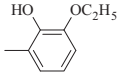
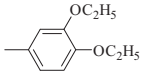
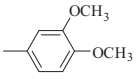
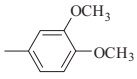
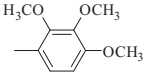
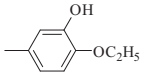
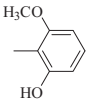
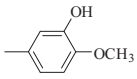
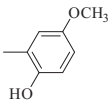
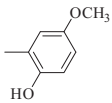
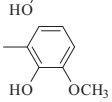
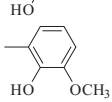
Table 1
XO inhibitory activity of benzylidene nicotino (**1**–**27**), and isonicotinohydrazides (**28**–**54**).



1-27

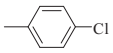
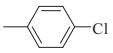
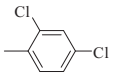
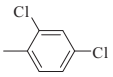
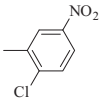
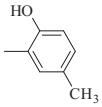
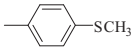
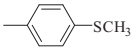
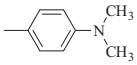
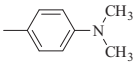


28-54

Comp.	R_1	IC_{50} ± SEM[a] (μM)	Comp.	R_2	IC_{50} ± SEM[a] (μM)
1		210.7 ± 1.2	**28**		NA[b]
2		183.7 ± 0.8	**29**		173.56 ± 2.1
3		NA[b]	**30**		NA[b]
4		NA[b]	**31**		NA[b]
5		183.7 ± 0.4	**32**		NA[b]
6		NA[b]	**33**		NA[b]
7		161 ± 4.5	**34**		NA[b]
8		10 ± 0.2	**35**		12.4 ± 0.1
9		NA[b]	**36**		0.9 ± 0.01
10		NA[b]	**37**		NA[b]
11		242 ± 6.3	**38**		330.4 ± 2.1
12		264 ± 4.5	**39**		54.8 ± 1.2
13		NA[b]	**40**		NA[b]
14		NA[b]	**41**		NA[b]
15		NA[b]	**42**		NA[b]
16		NA[b]	**43**		NA[b]
17		NA[b]	**44**		NA[b]
18		175.5 ± 2.7	**45**		21.1 ± 1.3
19		197.6 ± 1.2	**46**		NA[b]

(*continued on next page*)

Table 1 (*continued*)

Comp.	R_1	IC_{50} ± SEM[a] (μM)	Comp.	R_2	IC_{50} ± SEM[a] (μM)
20		173.8 ± 1.7	**47**		NA[b]
21		205.1 ± 0.1	**48**		206 ± 1.0
22		195 ± 0.8	**49**		179.3 ± 0.3
23		NA[b]	**50**		NA[b]
24		180.3 ± 3.1	**51**		NA[b]
25		NA[b]	**52**		NA[b]
26		NA[b]	**53**		NA[b]
27		NA[b]	**54**		NA[b]
Standard[c] = Allopurinol				2.00 ± 0.01	

SEM[a] (Standard error of the mean); NA[b] (Not active); Standard[c] = Allopurinol (Inhibitor for Xanthine Oxidase activity).

compound **1**, in comparison to 3-pyridine carboxylic acid (IC_{50} = 518.23 ± 2.23 μM), might be due to its increased lipophilicity. This is in consistent with the previous results where Biagi et al. reported that the addition of lipophilic substituents (phenyl ring in this case) on the unsubstituted heterocyclic ring system increases the activity.[19]

Among the nitrobenzene containing derivatives, only *meta* substituted compound **2** showed a weak inhibition with an IC_{50} value of 183.7 ± 0.8 μM, as compared to standard allopurinol (IC_{50} = 2.00 ± 0.01 μM). Whereas, *ortho* and *para* substituted derivatives **3** and **4** were found to be inactive. It shows that nitro group plays an important role in the activity, when it is present at *meta* position. Might be other positional isomers **3** and **4** attained the conformation which is not fitted well into the active site of the enzyme.

Among the phenol containing compounds, hydroxyl group was only favorable at *para* position, as in compound **5** (IC_{50} value = 183.7 ± 0.4 μM). The substitution of two OH groups *meta* to each other, increased the activity of compound **7** (IC_{50} = 161.0 ± 4.5 μM). Interestingly, compound **8** with two hydroxyl groups *ortho* to each other showed several folds increased activity with IC_{50} of 10.0 ± 0.2 μM which is comparable to the standard inhibitor, allopurinol (IC_{50} = 2.0 ± 0.01 μM). Tri-hydroxy substituted compound **9** was found to be inactive, indicating the importance of number as well as position of OH groups on benzene ring.

Amongst the ethylphenyl ether derivatives, compound **11** (IC_{50} = 242 ± 6.3 μM) with OC_2H_5 group at *para* position showed a weak inhibitory activity. While compound **12** with OH and OC_2H_5 groups showed further decrease in the activity (IC_{50} = 264.03 ± 4.5 μM).

Compounds **13**, **14**, and **15** with mono, di, and tri-OCH_3 groups were found to be inactive. Among the OCH_3 *cum* OH group derivatives, only compound **18** with OH and OCH_3 groups *ortho* to each other, was weakly active (IC_{50} = 175.5 ± 2.7 μM).

Among the halobenzene containing derivatives, compound **19** with a Br group at *ortho* position showed an IC_{50} of 197.6 ± 2.1 μM, while the presence of F at *ortho* position in compound **20** increases the activity (IC_{50} = 173.8 ± 1.7 μM), as F is more electronegative than Br and can better interact with the active site *via* hydrogen bonding interactions or polar interactions. Among the chlorobenzene derivatives, the presence of Cl at *meta* position (compound **21**) showed IC_{50} value of 205.1 ± 0.1 μM, while the presence of Cl group at *para* position, as in compound **22**, showed an IC_{50} value of 195 ± 0.8 μM. Interestingly, the dichloro substitution at *ortho* and *para* positions, as in compound **23**, made compound inactive.

The presence of CH_3 group at *ortho* position in compound **24** showed a weak activity with an IC_{50} value of 180.3 ± 3.1 μM. The presence of OH at *ortho* and CH_3 at *meta* position in compound **25** was found to be inactive. Compounds **26**, and **27** with dimethylamine and thiomethyl at *para* positions, respectively, were also found to be inactive.

2.4. Structure-activity relationship of benzylidene isonicotinohydrazides

Benzylidene isonicotinohydrazides were also evaluated for XO inhibitory activity. The parent compound **28** with no substitution on phenyl ring was found to be inactive. Might be the compound attained a conformation which doesn't fit well in the active site. Among the nitrobenzene containing compounds, compound **29** with a nitro group at *meta* position, showed a weak inhibition of XO. Activity results of compounds **2** and **29** indicated that NO_2 group is only favorable for XO inhibitory activity when it is at *meta* position.

All the phenol containing derivatives (**32**, **33** and **52**) were found to be inactive. Compound **34** with OH groups at *ortho* and *para* positions was also inactive. Interestingly, the presence of two OH groups at *ortho* and one at *para* in compound **36** resulted

in a potent XO inhibitory activity (IC_{50} = 0.96 ± 0.01 μM). Its activity was compared with its positional isomer compound **9** with three OH at *ortho*, *meta*, and *para* positions which was found to be completely inactive. Might be compound **9** attained a conformation which is not fitted properly into the active site of enzyme. Compound **35** with two OH groups at *meta* and *para* positions also showed activity (IC_{50} = 12.4 ± 0.1 μM) which is comparable to compound **8** and almost similar in structure but only has a nicotine ring, instead of isonicotine (Table 2).

The XO inhibitory activity of compounds **35**, and **36** indicated that positions as well as number of OH substituent play an important role in the activity of these compounds. This was in accordance with earlier reports by Leigh et al., and by Pauf and Hille that the OH group is involved in XO inhibitory activity of different classes, such as hydrazide, thiosemicarbazides, curcumins, *etc.*[20,21]

Among the ethylphenyl ether derivatives, **37** with ethoxy at *ortho* position was found to be inactive, as was observed in case of compound **10**. Inactivity of the compounds **10** and **37** might be due to the steric hindrance created by the ethoxy group in binding with the active site of enzyme. Compound **38** with OC_2H_5 at *para* position was weakly active (IC_{50} = 330.4 ± 2.1 μM). Activity results of compounds **11**, and **38** showed that ethoxy group is only favorable when it is present at the *para* position. Compound **39** with OH and OC_2H_5 (*ortho* and *meta*) was a moderate inhibitor with an IC_{50} value of 54.8 ± 1.2 μM, and showed almost five times better inhibition as compared to structurally similar compound **12** which has a nicotine ring instead of isonicotine.

Compounds **41**, **43**, and **44** with OCH_3 and OH *cum* OCH_3 group substitutions were found to be inactive. Only compound **45** with OH and OCH_3 at *ortho* (1′) and *meta* (2′) positions, respectively,

Table 2
Results of kinetics and molecular docking studies on selected inhibitors.

Compounds	IC_{50} ± SEM (μM)	*Ki* ± SEM (μM)	Type of Inhibition	Docking Score	ΔG_{bind} (kcal/mol)
8	10 ± 0.26	7.27 ± 0.021	Competitive	−7.4	−42
35	0.96 ± 0.01	0.82 ± 0.0789	Competitive	−6.8	−40
36	12.4 ± 0.1	15.46 ± 0.0052	Competitive	−5.9	−32
39	54.8 ± 1.2	51.34 ± 0.032	Competitive	−5.7	−16
45	21.1 ± 1.3	15.02 ± 0.008	Competitive	−5.3	−22

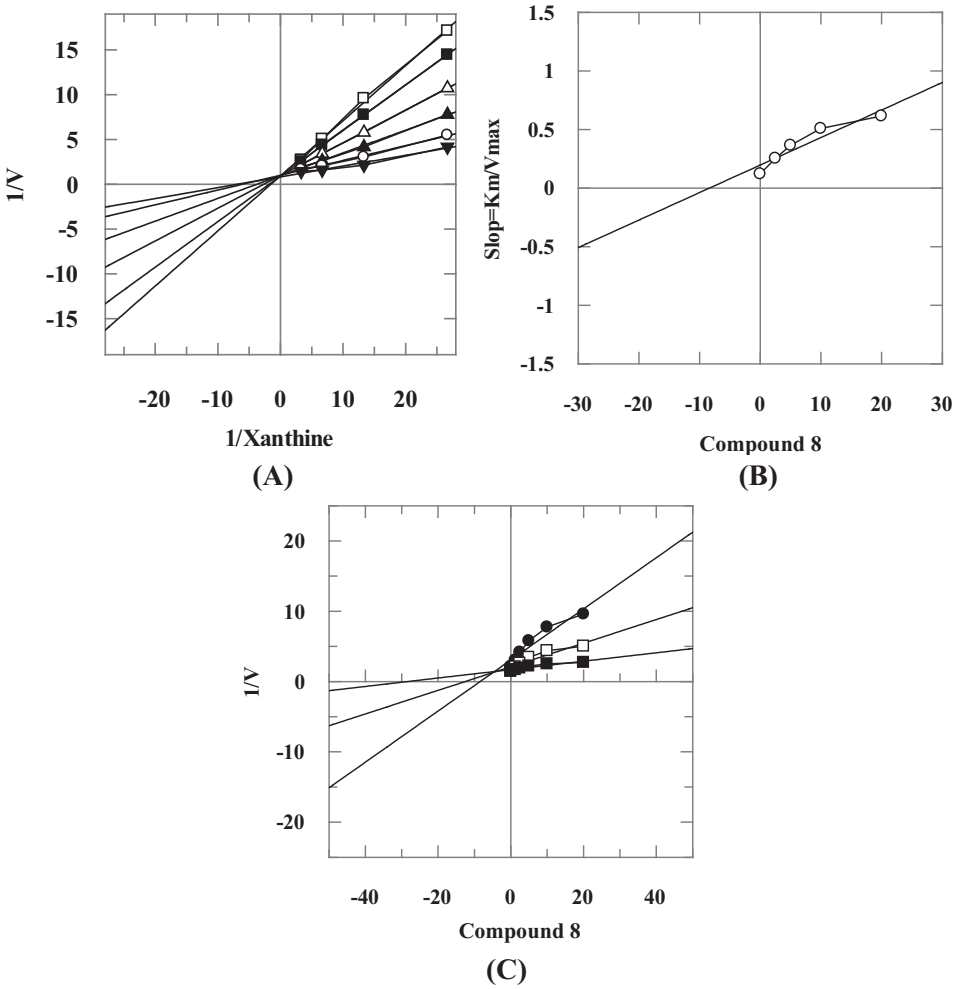


Fig. 4. The inhibition of XO by compound **8**, (A) Lineweaver-Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (xanthine) in the absence (-), and in presence of 20 μM (■), 10 μM (□), 5 μM (Δ), 2.5 μM (▲), and 1.25 μM (○) of compound **8**. (B) Secondary replot of Lineweaver-Burk plot between the slopes of each line on Lineweaver-Burk plot *vs* different concentrations of compound **8**. (C) Dixon plot of reciprocal of rate of reaction (velocities) *vs* different concentrations of compound **8**.

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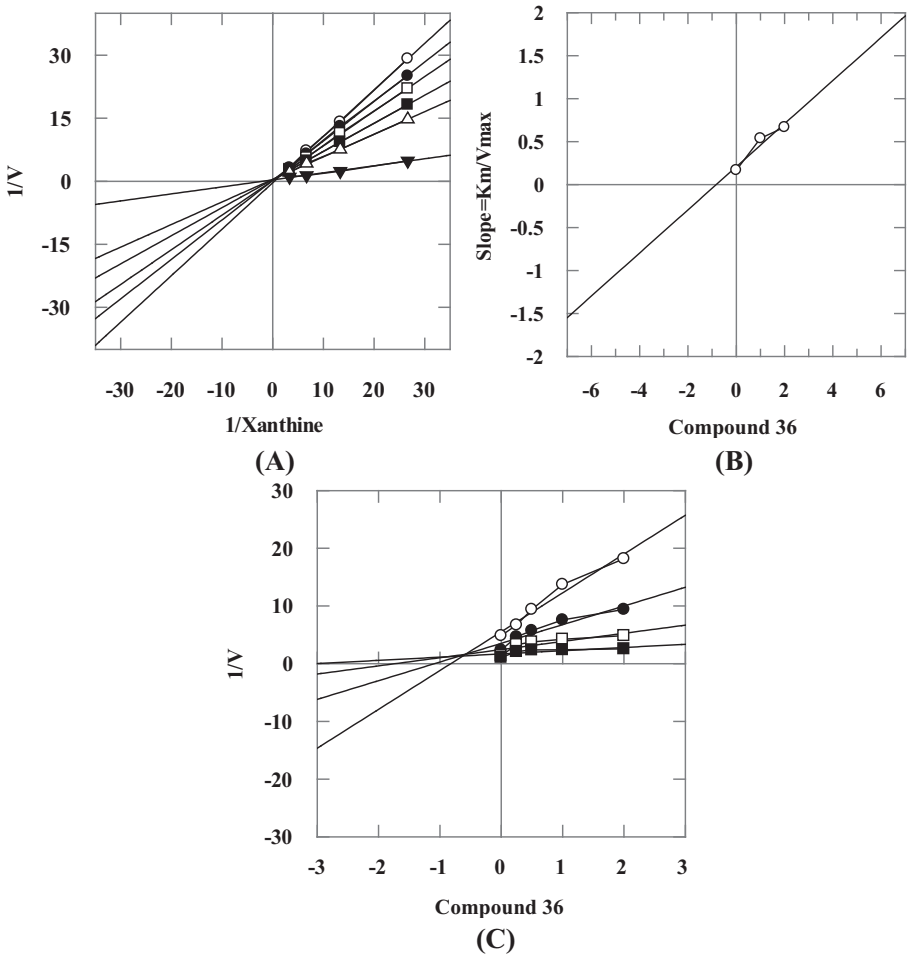

Fig. 5. The inhibition of XO by compound **36**, (A) Lineweaver-Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (xanthine) in the absence (-), and in presence of 16 μM (○), 8 μM (●), 4 μM (□), 2 μM (■), and 1 μM (Δ) of compound **36**. (B) Secondary replot of Lineweaver-Burk plot between the slopes of each line on Lineweaver-Burk plot *vs* different concentrations of compound **36**. (C) Dixon plot of reciprocal of rate of reaction (velocities) *vs* different concentrations of compound **36**.

showed a significant activity (IC_{50} = 21.1 ± 1.3 μM). Activity results of compounds **18**, and **45** indicated that the OH at *ortho* (1′) and OCH_3 at *meta* (2′) are contributing towards the activity of this series of compounds.

Halogen substitutions in benzylidene isonicotinohydrazides were seemingly unable to contribute significantly in inhibiting XO as compounds **46**, **47**, and **50** with Cl, and F, were found to be inactive. The presence of Cl group at *meta* position in compound **48** showed a weak activity with an IC_{50} value of 206 ± 1.0 μM. The presence of Cl group at *para* position in compound **49** lead to a slightly better activity (IC_{50} = 179.3 ± 0.3 μM), in comparison to compound **48**. The same activity pattern was observed in case of compounds **21**, and **22**.

Similarly, compounds **53**, and **54** with dimethylamine and thiomethyl at *para* positions, respectively, were found to be inactive, as was observed in case of benzylidene nicotinohydrazides.

2.5. Kinetic studies

Five compounds **8**, **35**, **36**, **39**, and **45** were selected for kinetic studies, based on their lower IC_{50} values (0.96 to 54.8 μM). The mode of inhibition was analyzed from Lineweaver-Burk plot, and was found to be competitive as *K*m values were increased in the presence of each inhibitor without affecting the *V*max values (Figs. 4–6). *K*i values were calculated from secondary replots of Lineweaver-Burk plot, and also from Dixon plot (Table 2).

2.6. In silico studies

To predict the interaction of these compounds with specific amino acids residues in the active site of XO, *in silico* molecular docking studies were performed. The *in silico* studies were performed using bovine milk XDH with bound competitive inhibitor oxypurinol (PDB ID: 3BDJ).

Oxypurinol is the hydroxylated product of allopurinol that is formed by XO. This oxypurinol then interacts with the Mo atom and catalytically important amino acids thereby inhibit the activity of XO. The important amino acid residues that are involved in the XO catalyzed reactions are Arg880, Phe914 and 1009, Glu1261, Glu802, and Thr1010. Oxypurinol forms hydrogen bonds with Arg880, Thr1010, and π-π interactions with the two Phe residues 914 and 1009 (Fig. 7).[22,23]

The phenyl ring in these inhibitors occupied the same position as that of pyrimidine ring in oxypurinol structure (Fig. 8). There were some common interactions observed in all the five inhibitors. These involved π-π stacking of the benzene ring with two Phe residues (914 and 1009). While the change in substitutions on the benzene ring, resulted in change in the hydrogen bonding pattern.

For instance, compounds **8** and **35** have similar structures with only difference in the position of nitrogen in the pyridine ring. Compound **8** has 3-pyridine, while **35** has a 4-pyridine ring which resulted in some differences in interactions. Both OH groups (*meta* and *para*) in compound **8** were involved in hydrogen bonding with

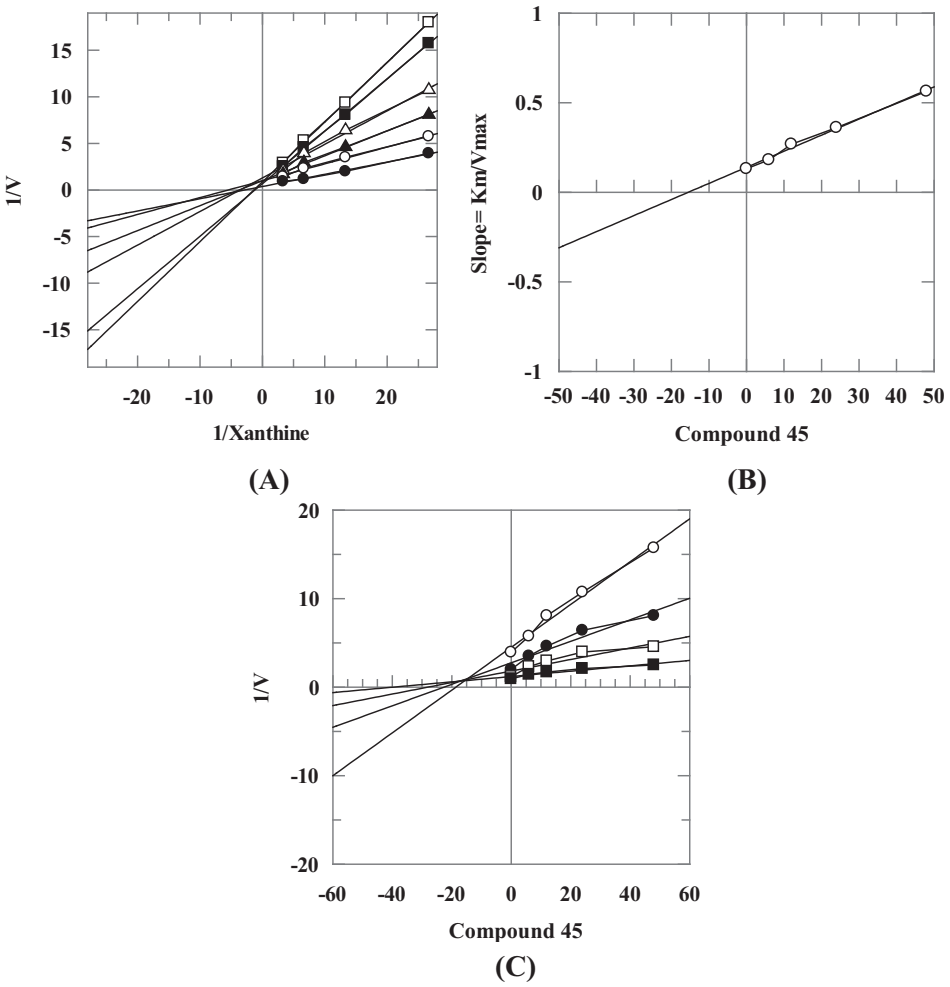

Fig. 6. The inhibition of XO by compound **45**, (A) Lineweaver-Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (xanthine) in the absence (-), and in presence of 96 μM (□), 48 μM (■), 24 μM (Δ), 12 μM (▲), and 6 μM (○) of compound **45**. (B) Secondary replot of Lineweaver-Burk plot between the slopes of each line on Lineweaver-Burk plot *vs* different concentrations of compound **45**. (C) Dixon plot of reciprocal of rate of reaction (velocities) *vs* different concentrations of compound **45**.

the Arg880 (Fig. 9). However, the OH group at *meta* position in compound **35** was able to interact with Thr1010 through hydrogen bond. An additional π-π stacking interaction was also observed through the pyridine ring with Phe1013 (Fig. 10).

Compound **36** with three OH groups (*ortho* and *para*) showed some additional interactions. Beside the hydrogen bond with Thr1010, the OH group at *para* position also interacted with Glu1261 through hydrogen bond. The *ortho* OH group was involved in hydrogen bonding with the water molecule (Fig. 11).

Compound **45** with OH *cum* OCH_3 groups at *ortho* and *meta* positions, respectively, showed hydrogen bond between OCH_3 group and Ala1079, and also among NH and Ser876 (Fig. 12). However, the replacement of OCH_3 group by OC_2H_5 in compound **39** resulted in a loss of these hydrogen bonds (Fig. 13).

2.7. In vivo studies of selected inhibitors

The significant XO inhibitory activities of these compounds in competitive manner motivated us to subject them to *in vivo* studies. The compounds were analyzed for their cytotoxic activity using colorimetric MTT assay. This assay measures the MTT dye reduction (yellow color) to formazan (Purple color). The increase in the production of formazan indicated the viability of cells and hence the compound was referred as non-cytotoxic. All the five compounds **8**, **35**, **36**, **39**, and **45** were found to be non-cytotoxic against 3T3- cell lines. Three significant inhibitors **8**, **35**, and **36** were selected among these five for *in vivo* studies.

6-MP, allopurinol, and test compounds **8**, **35**, and **36** were given to test animals at a dose of 50 mg/kg through I.P. route. Rats were sacrificed after 6 h of administration of drug and blood was collected through heart puncture. Plasma samples were prepared (materials and methods) for the analysis of 6-MP. In order to quantify the plasma samples, calibration curve was plotted between various amounts of 6-MP and related peak area. The standard curve demonstrated a linear relationship between the peak areas and concentrations for the pooled rat plasma, spiked with 6-MP standards (Fig. 14).

The blank group that was untreated showed no peak in the total run time of 15 min that could otherwise overlap with the peak of 6-MP. The negative control group in which only 6-MP was injected showed a non-quantifiable peak of 6-MP indicating that XO is active, and has metabolized the drug (Fig. 15A-B).

The positive control group that was injected with 6-MP as well as allopurinol showed a quantifiable peak of 6-MP at the same retention time, indicating the inhibition of XO by allopurinol. The

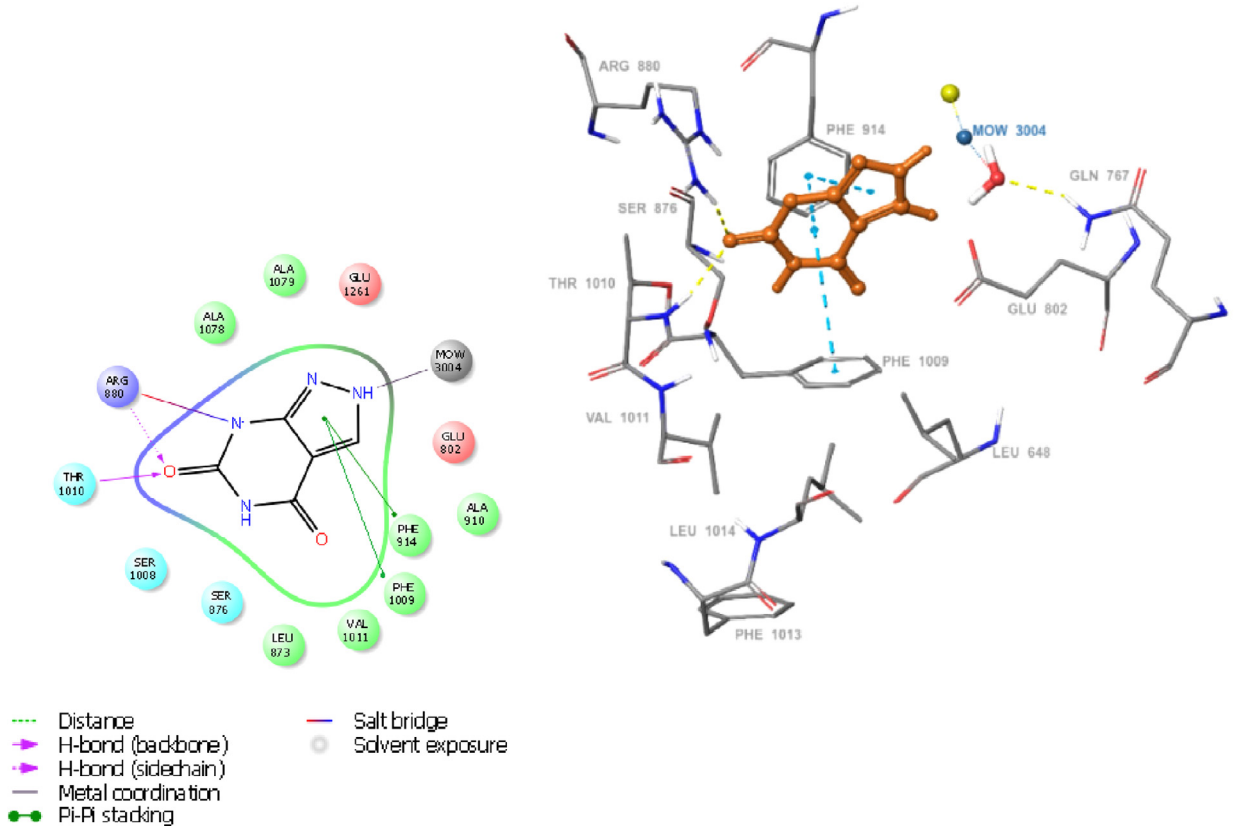

Fig. 7. Ligplot and 3D view of docked pose of oxypurinol (ball and stick) interacting with active site amino acid residues *via* π-π stacking interaction stacking interactions (blue dotted lines) with Phe914 and 1009, and form hydrogen bonds (yellow dotted lines) with Arg880, Thr1010, and also shows metal interaction with molybdenum cofactor.

test group I that was injected with 6-MP as well as compound **8**, showed a non-quantifiable peak of 6-MP indicating that this compound was not able to inhibit XO in *in vivo* conditions (Fig. 15C-D).

Test groups II and III that were injected with compounds **35**, and **36**, respectively, in addition to 6-MP, showed a quantifiable peak indicating the inhibition of XO. The peak areas of 6-MP in the chromatogram of compounds **35** and **36** were found to be 47.4 and 73.5 mAU, respectively (Fig. 15E-F). Compounds **35** and **36** were thus able to inhibit the activity of XO up to 28%, and 44% in comparison to the 100% inhibition of the enzyme activity by allopurinol. Although the percent inhibition was lower than the standard inhibitor allopurinol, but it is still significant (Fig. 16).

3. Conclusion

We screened two series of synthetic benzylidene nicotine/isonicotinohydrazides (**1**–**54**) using *in vitro* spectroscopic XO inhibition assay. Five compounds with lower IC_{50} values (0.96 to 54.8 μM) were selected from the two series and were subjected to mechanism based studies by evaluation of their kinetic parameters. All compounds showed a competitive pattern of inhibition and were further explored by *in silico* molecular docking studies. These inhibitors showed interactions with the catalytically important amino acid residues, such as Phe (914, 1009), Arg880, Thr1010, and Glu1261. These compounds were also found to be non-cytotoxic against 3T3-cell line (Mouse fibroblast cells), and were evaluated for *in vivo* XO inhibition activity *in vivo* using male Wistar rats. Three compounds (**8**, **35**, and **36**) were subjected to the *in vivo* studies and among them two compounds (**35** and **36**) were able to inhibit XO significantly. These two compounds can serve as potential leads for XO inhibition, but more detailed *in vivo* studies are required.

4. Material and methods

XO (bovine milk, EC 1.17.3.2.), 6-MP, Dulbecco's modified eagle medium (DMEM), and cycloheximide were purchased from Sigma Aldrich, USA. Xanthine was acquired from TCI, Japan. Allopurinol and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from MP Biomedicals, LLC, France. All reagents were of analytical grade, and used without any purification. Dimethylsulfoxide (DMSO) was purchased from Fisher Scientific UK. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, and sodium nitroprusside were purchased from Merck, Germany. Mouse fibroblast cell line (3T3) was purchased from American Type Culture Collection (ATCC), USA. 0.25%. Fetal bovine serum (FBS) was purchased from A&E Scientific (PAA), USA. 0.4% Trypan Blue solution was purchased from Amersco, USA. Sterile heparinized capillary tubes were purchased from BD, USA.

*4.1. General procedure for the synthesis of benzylidene nicotino/isonicotinohydrazides (**1**–**54**)*

Nicotinic/isonicotinic hydrazides (1 mmol), substituted aromatic aldehyde (1 mmol) and glacial acetic acid (few drops) were taken in ethanol (10 mL) and refluxed for 3 h. Progress of the reaction was monitored by TLC. After the completion of the reaction, mixture was left for evaporation of solvent, and then crude product

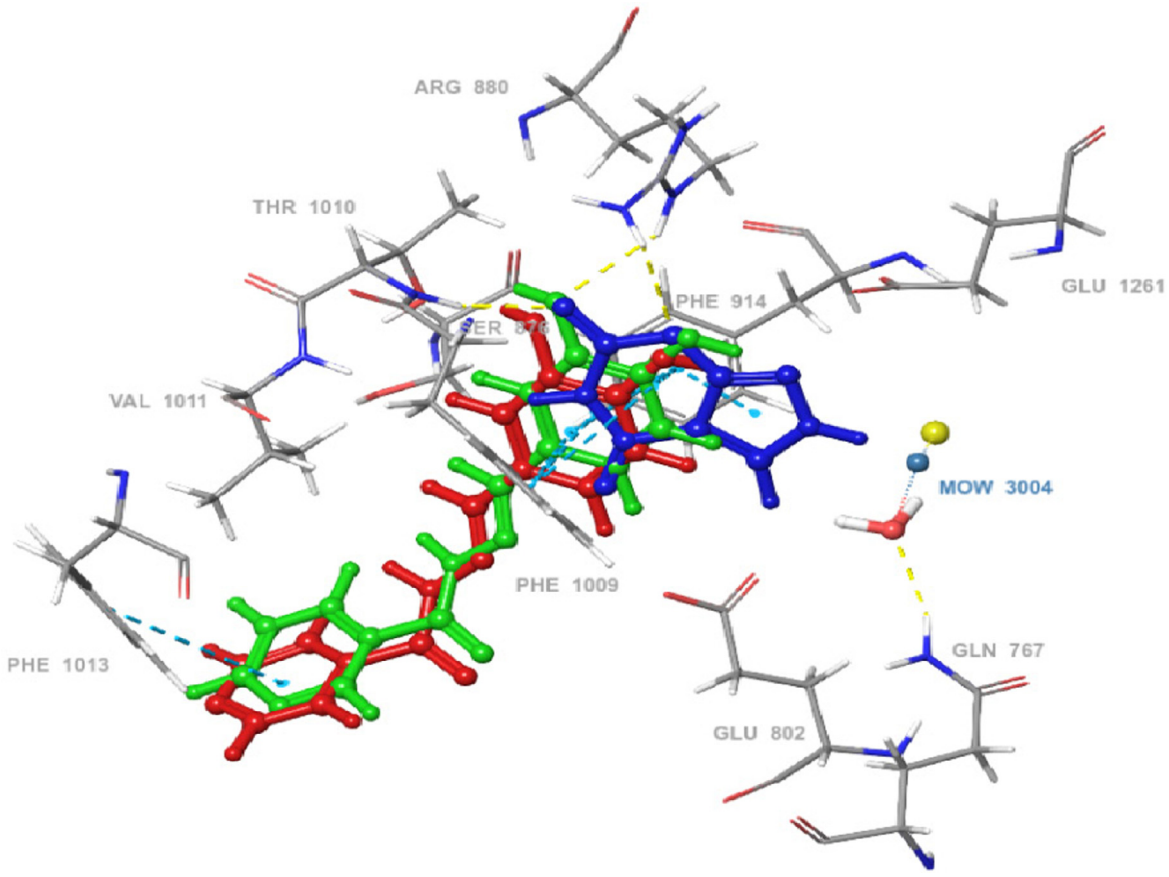

Fig. 8. 3D view of docked pose of allopurinol (blue), compound **8** (green), and **35** (red) and interacting with active site amino acid residues through π-π stacking interactions (blue dotted lines) and hydrogen bonds (yellow dotted lines).

washed with dichloromethane and hexane, and dried to afford compounds **1**–**54**.

4.2. Assay for XO inhibitory activity

The XO inhibitory activity was evaluated in 96-well plate ELISA reader.[24,25] Phosphate buffer (pH 7.4, 50 mM) was used to dissolve XO (0.003 units/well). Test samples were dissolved in DMSO (90%). 10 μL of test compounds, 20 μL of XO and 150 μL of buffer were added in 96-well plate and incubated for 10 min at 30 °C in the spectrophotometer. After incubation, 20 μL of xanthine (0.15 mM) was added, and change in absorbance was monitored for 15 min at 295 nm. Allopurinol drug was used as a positive control, and all experiments were performed in triplicate.

4.3. Protocol for kinetic studies

Kinetic studies were performed in the same way as the inhibition studies were performed, by varying the inhibitor and substrate concentrations. Four concentrations of xanthine were used (0.0375, 0.075, 0.15, and 0.300 mM).

4.4. Protocol for docking studies

Computational studies were performed using Glide 6.9 module of Schrodinger suite of programs. Bovine milk xanthine dehydrogenase (PDB ID: 3BDJ) with bound oxypurinol was used to dock the ligands. Protein was prepared, optimized and minimized using protein preparation wizard and the selected force field was OPLS_2005. Maestro Build and LIGPREP module were used to prepare the ligands. The centroid of the co-crystallized ligand (oxypurinol) was used to define a grid box ($15 \times 15 \times 15$ Å). Ligands were docked using Glide extra precision (XP) module, and the best docked poses were used for the interpretation of final results.[26–28]

4.5. In vitro cytotoxicity measurement assay

Mouse fibroblast (3T3) cell line was grown in DMEM, provided with 5% of FBS, 100 IU/mL of penicillin and 100 μg/mL of streptomycin, and kept at 37 °C in 5% CO_2 incubator. Cell culture (5×10^4 cells/mL) was prepared, and poured into 96-well plates and incubated overnight. After completion of incubation, fresh medium was added (after removing the old medium) with test compound of different concentrations (ranging from 0.1 M to 0.00016 M). After 48 h, 200 μL of MTT (0.5 mg/mL) was added to plate and further incubated for 4 h. Afterward, DMSO (100 μL) was added to each well. Reduction of MTT to formazan was measured spectrophotometrically at 540 nm, using a micro plate reader (Spectra Max plus, Molecular Devices, CA, USA). The experiments were run in triplicate (n = 3). Cycloheximide was used as positive control (IC_{50} = 0.2 ± 0.10 μM).[29,30]

4.6. In vivo measurement of XO inhibitory activity

6-Mercaptopurine (6-MP) is the structural analogue of hypoxanthine and is being metabolized by XO. In this assay, 6-MP was used as a substrate in order to analyze the activity of XO. 6-MP, allopurinol, and our test samples were all administered to the experimental animals by intra-peritoneal injections. 6-MP (50 mg/kg) was injected in male Wistar rats *via* intra-peritoneal

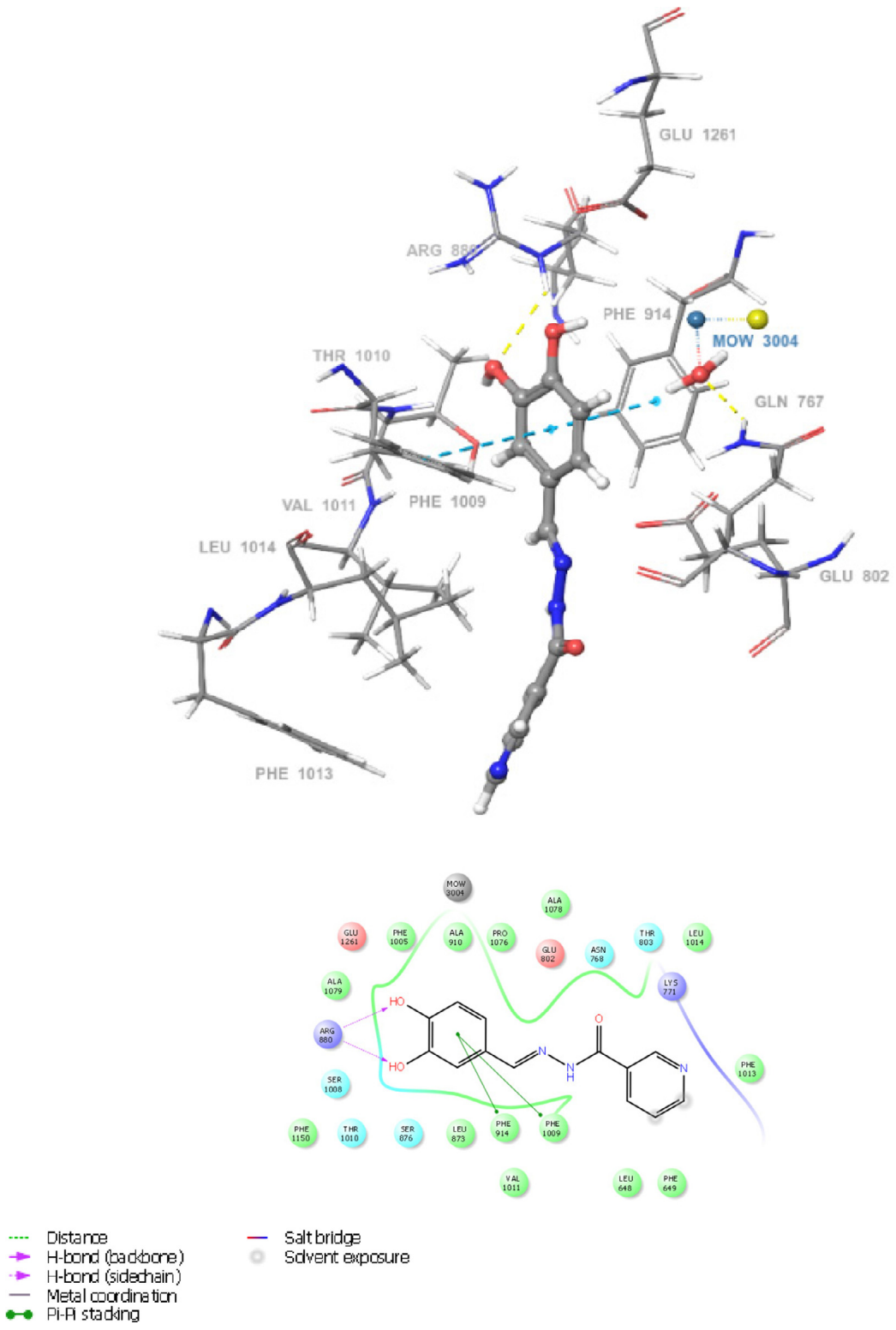

Fig. 9. Ligplot and 3D view of docked pose of compound **8** (ball and stick) interacting with active site amino acid residues *via* π-π stacking interaction with Phe914 and 1009, and form hydrogen bond (yellow dotted lines) with Arg880.

route. Animals were sacrificed 6 h after the administration of the allopurinol. Blood was collected by heart puncture, and plasma samples were prepared. The amount of uncatalyzed 6-MP was analyzed in the plasma samples by ultra-pressure liquid chromatography (UPLC) technique.

4.7. Animals welfare

Male Wistar rats (180–200 g) were acquired from the animal house of Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological

Sciences, University of Karachi, Pakistan. Before performing the experiment, the ethical clearance (00010-2014) was obtained from the Animal Use and Care Committee of International Center of Chemical and Biological Sciences, University of Karachi. Animals were then housed in standard conditions: 12 h light-dark cycle, provision with food and water *ad libitum*.

4.8. Grouping of animals

Animals were dividing into six groups with 6 rats in each group.

Group I (Blank group): was untreated

Group II (Negative control group): Injected with 6-MP at a dose of 50 mg/kg

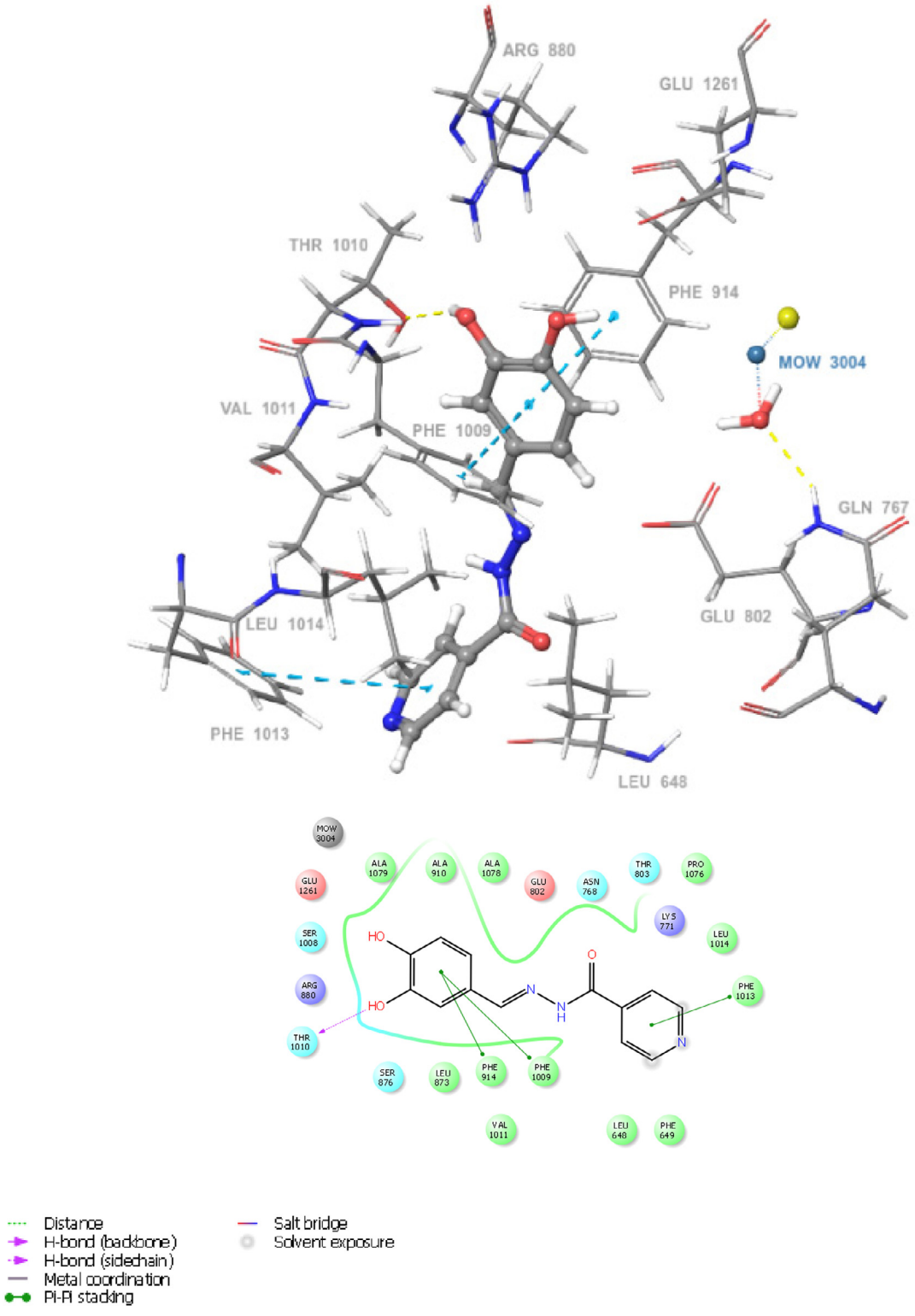

Fig. 10. Ligplot and 3D view of docked pose of compound **35** (ball and stick) interacting with active site amino acid residues *via* π-π stacking interaction with Phe914, 1009, 1013, and form hydrogen bond (yellow dotted lines) with Thr1010.

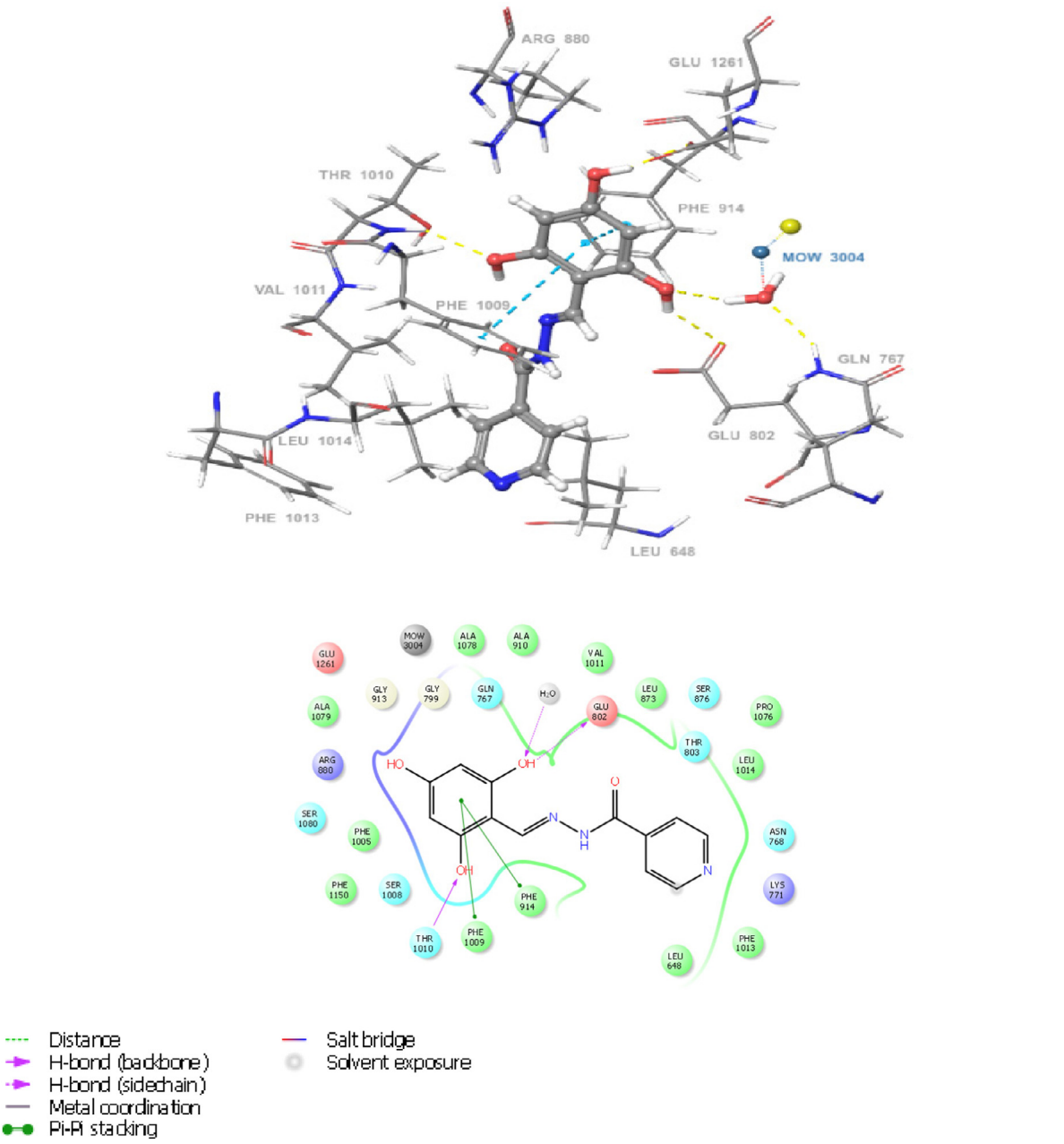

Fig. 11. Ligplot and 3D view of docked pose of compound **36** (ball and stick) interacting with active site amino acid residues *via* π-π stacking interactions (blue dotted lines) with Phe914 and 1009, and forms hydrogen bonds (yellow dotted lines) with Thr1010, and Glu802.

Group III (Positive control group)**:** Injected with 6-MP (50 mg/kg), and allopurinol (50 mg/kg).

Group IV (Test group I): Injected with 6-MP (50 mg/kg), and compound **8** (50 mg/kg).

Group V (Test group II): Injected with 6-MP (50 mg/kg), and compound **35** (50 mg/kg).

Group VI (Test group III): Injected with 6-MP (50 mg/kg), and compound **36** (50 mg/kg).

After 6 h of 6-MP administration, rats were sacrificed and blood was collected by heart puncture in sterile heparinized capillary tubes. Plasma was stored at −20 °C until further analysis (Table 3).

4.9. Preparation of plasma samples

Plasma samples were prepared by adding of dithiothreitol (DTT)-methanol solvent (10 mg/dL) in 1:1 ratio. The samples were then vortexed for few sec, and then centrifuged at 14,000 rpm for 25 min, in order to make the solution clear. Clear supernatant was transferred into the glass tubes for injection into UPLC chromatographic system. In order to quantify the plasma samples, calibration curve was plotted using various concentrations of 6-MP in the range of 10 mg/kg to 50 mg/kg with 50 mg/kg of allopurinol.

4.10. Determination of 6-MP in rat plasma by chromatographic techniques

The quantification of 6-MP was carried out by slight modifications in the previously reported method of Mohammad et al., with slight modifications.[16] The method was then performed on UPLC for better efficiency and quantification.

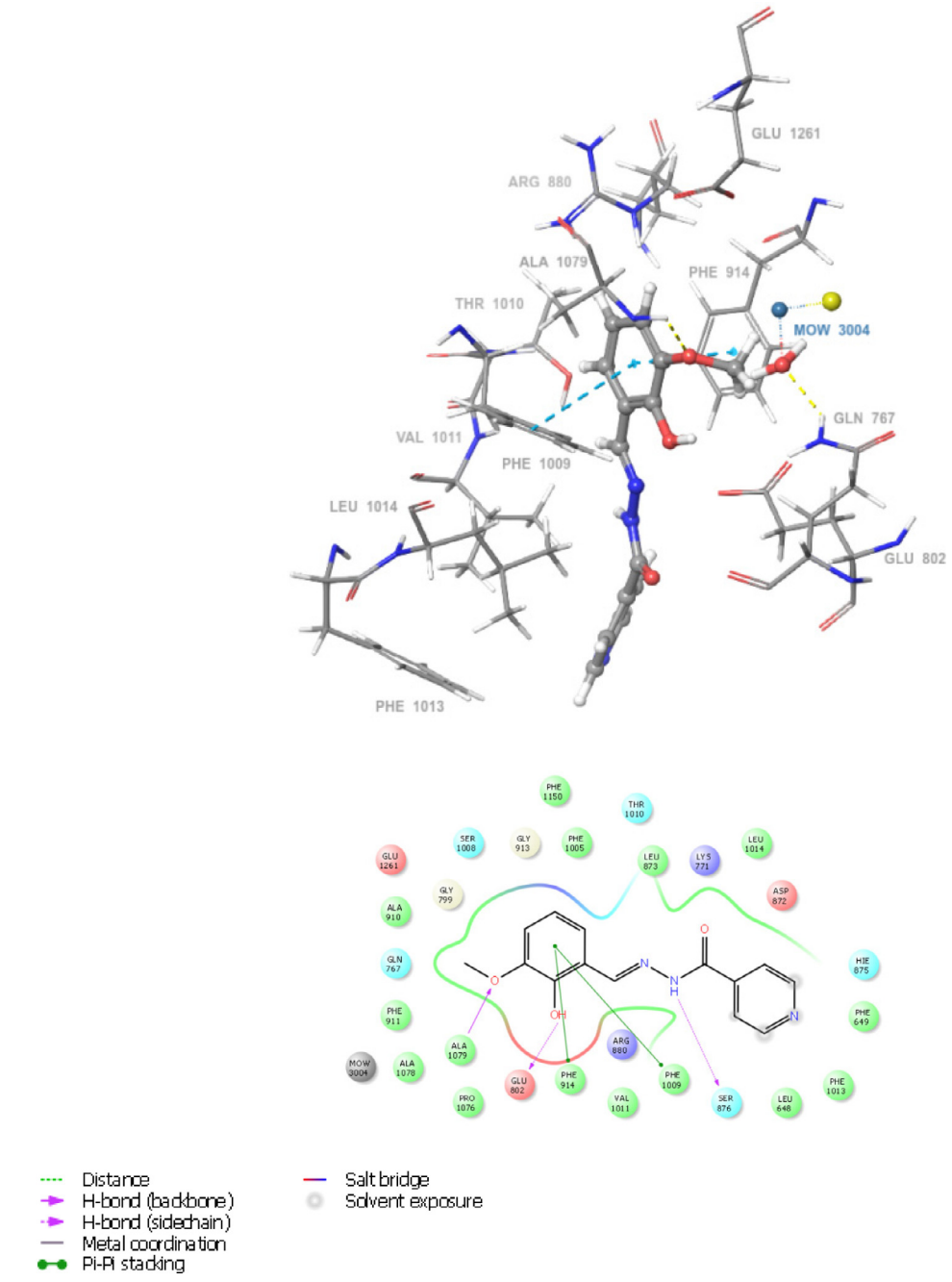

Fig. 12. Ligplot (left) and3D view (right) of docked pose of compound **45** (ball and stick) interacting (blue dotted lines) through π-π stacking interaction with active site amino acid residues Phe914 and 1009, and form hydrogen bonds (yellow dotted lines) with Glu802, Ala1079, and Ser876.

4.11. UPLC system and experimental conditions

UPLC (1260 series, Agilent technologies, USA) was used to calculate the concentration of 6-MP in each plasma sample, ZORBAX Eclipse XDB-C18 (Rapid Resolution HT, 3.0 × 50 mm, 1.8-µm, 600 Bar) column was used. Phosphoric acid: water: acetonitrile: DTT in a ratio of 2.5 mL: 500 mL: 15 mL: 50 mg, was used as mobile phase, and was filtered and degassed under vacuum, before its use. Samples were run for a total time of 15 min at a flow rate of 0.08 mL/min, and wavelength of 322 nm.

4.12. Statistical analysis

The results for *in vitro* XO inhibition studies were recorded using microtitre plate reader (SpectraMax plus 384, Molecular Devices, CA, USA). SoftMax Pro 4.8 software (Molecular Devices, CA, USA) and Microsoft excel were used to process the results. Percent inhibition was calculated by using the following formula:

$$\text{Percent inhibition} = 100 - (\text{OD}_{\text{test compound}}/\text{OD}_{\text{control}}) \times 100$$

Where OD is the optical density.

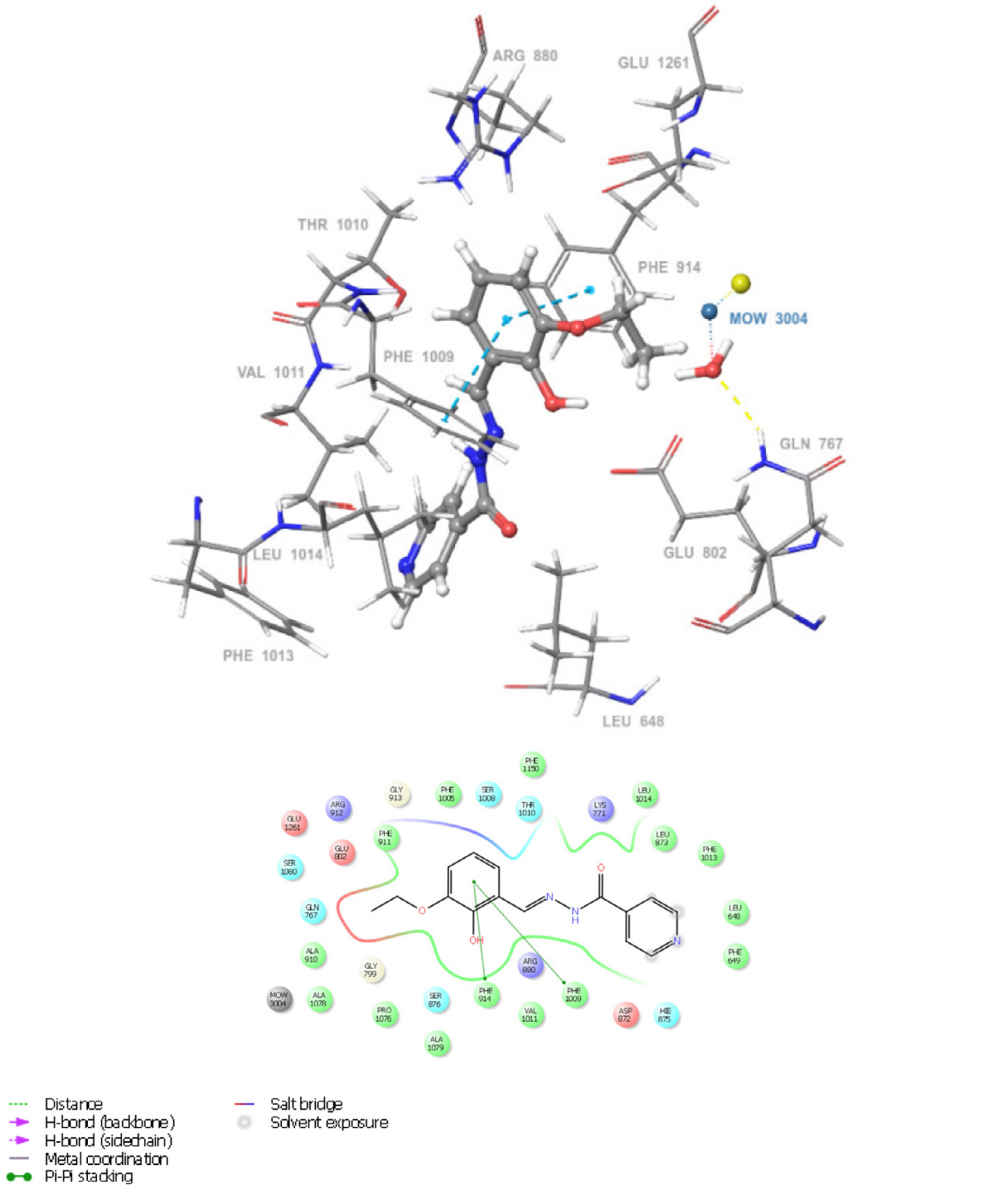

Fig. 13. Ligplot (left) and3D view (right) of docked pose of compound **39** (ball and stick) interacting (blue dotted lines) through π-π stacking with active site amino acid residues Phe914 and 1009.

EZ-FIT, Enzyme kinetics software (Perrella Scientific, Inc., USA), was used to measure the IC_{50} values and kinetic parameters, respectively. One-way ANOVA and Statistica® (Version 5.0) software packages were used for statistical analysis of *in vivo* studies.

4.13. Spectroscopic data of compounds **1**–**54**

4.13.1. (E)-N'-Benzylidenenicotinohydrazide (**1**)[18a]

Yield: 76%; m.p. 146–148 °C (lit. 148–149 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.00 (s, 1H, NH), 9.09 (s, 1H, H-2), 8.76 (dd, 1H, $J_{6,5}$ = 4.8 Hz, $J_{6,2}$ = 1.2 Hz, H-6), 8.44 (s, 1H, =CH), 8.25 (br.d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.75 (m, 2H, H-2′, H-6′), 7.56 (m, 1H, H-5), 7.45 (m, 3H, H-3′, H-4′, H-5′); EI-MS: *m*/*z* (rel. abund.%), 225 (M^+, 6), 122 (92), 106 (100), 78 (70); Anal. Calcd for $C_{13}H_{11}N_3O$ (225.25): C, 69.32; H, 4.92; N, 18.66; Found: C, 69.30; H, 4.91; N, 18.68.

4.13.2. (E)-N'-(3'-Nitrobenzylidene) nicotinohydrazide (**2**)[18b]

Yield: 89%; m.p. 196–198 °C (lit. 195–197 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.27 (s, 1H, NH), 9.27 (s, 1H, H-2′), 8.76

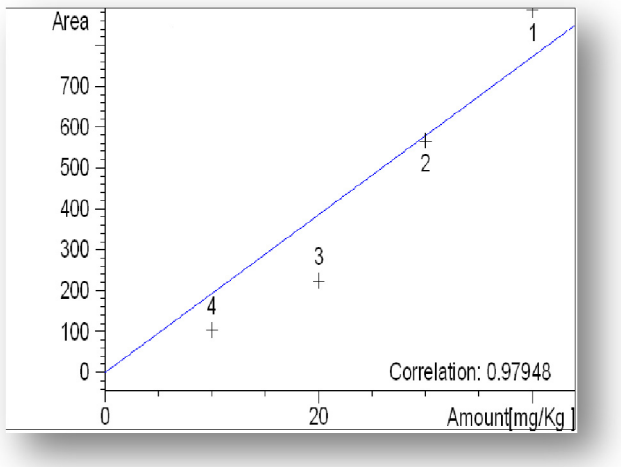

Fig. 14. Calibration curve between the amount of 6-MP and peak area.

(s, 1H, H-2), 8.55 (m, 2H, =CH, H-6), 8.26 (m, 3H, H-4, H-5, H-6′), 7.76 (m, 1H, H-5′), 7.56 (m, 1H, H-4′); EI-MS: *m/z* (rel. abund.%), 270 (M^+, 5), 122 (35), 106 (100), 78 (79); Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.78; H, 3.73; N, 20.73; Found: C, 57.76; H, 3.74; N, 20.74.

*4.13.3. (E)-N′-(2′-Nitrobenzylidene) nicotinohydrazide (**3**)*[18c]

Yield: 77%; m.p. 191–192 °C (lit. 190–192 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.33 (s, 1H, NH), 9.08 (s, 1H, H-2), 8.86 (s, 1H, =CH), 8.77 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.27 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 8.13 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 8.09 (d, 1H, $J_{3',4'}$ = 8.5 Hz, H-3′), 7.83 (t, 1H, $J_{4',3'/4',5'}$ = 8.0 Hz, H-4′), 7.69 (t, 1H, $J_{5',4'/5',6'}$ = 8.0 Hz, H-5′), 7.57 (m, 1H, H-5); EI-MS: *m/z* (rel. abund. %), 270 (M^+, 5), 122 (24), 106 (100), 78 (50); Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.78; H, 3.73; N, 20.73; Found: C, 57.79; H, 3.74; N, 20.70.

*4.13.4. (E)-N′-(4′-Nitrobenzylidene) nicotinohydrazide (**4**)*[18c]

Yield: 81%; m.p. 257–259 °C (lit. 258–260 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.29 (s, 1H, NH), 9.07 (s, 1H, H-2), 8.77 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.54 (s, 1H, =CH), 8.29 (m, 3H, H-4, H-2′, H-6′), 8.02 (d, 2H, $J_{3',2'/5',6'}$ = 8.0 Hz, H-3′, H-5′), 7.57 (m, 1H, H-5); EI-MS: *m/z* (rel. abund.%), 270 (M^+, 3), 122 (66), 106 (100), 78 (81); Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.78; H, 3.73; N, 20.73; Found: C, 57.77; H, 3.71; N, 20.75.

*4.13.5. (E)-N′-(4′-Hydroxybenzylidene) nicotinohydrazide (**5**)*[18b]

Yield: 70%; m.p. 230–232 °C (lit. 231–232 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.78 (s, 1H, NH), 9.03 (s, 1H, H-2), 8.74 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.42 (s, 1H, =CH), 8.27 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.56 (m, 3H, H-5, H-2′, H-6′), 6.83 (d, 2H, $J_{3',2'/5',6'}$ = 8.0 Hz, H-3′, H-5′); EI-MS: *m/z* (rel. abund.%), 241 (M^+, 25), 123 (86), 106 (100), 78 (55); Anal. Calcd for $C_{13}H_{11}N_3O_2$ (241.25): C, 64.72; H, 4.60; N, 17.42; Found: C, 64.71; H, 4.62; N, 17.41.

*4.13.6. (E)-N′-(3′-Hydroxybenzylidene) nicotinohydrazide (**6**)*[18b]

Yield: 74%; m.p. 203–205 °C (lit. 202–204 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.94 (s, 1H, NH), 9.62 (s, 1H, H-2′), 9.05 (s, 1H, H-2), 8.77 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.42 (s, 1H, =CH), 8.27 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.70 (m, 1H, H-5), 7.55 (m, 1H, H-5′), 7.12 (d, 1H, $J_{6',5'}$ = 7.5 Hz, H-6′), 6.83 (d, 1H, $J_{4',5'}$ = 7.5 Hz, H-4′); EI-MS: *m/z* (rel. abund.%), 241 (M^+, 10), 123 (63), 106 (100), 78 (26); Anal. Calcd for $C_{11}H_{13}N_3O_2$ (241.25): C, 64.72; H, 4.60; N, 17.42; Found: C, 64.73; H, 4.61; N, 17.41.

*4.13.7. (E)-N′-(3′,5′-Dihydroxybenzylidene) nicotinohydrazide (**7**)*

Yield: 77%; m.p. 245–247 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.10 (s, 1H, NH), 10.23 (s, 1H, OH), 9.06 (s, 1H, OH), 8.96 (s, 1H, H-2), 8.76 (d, 1H, $J_{6,5}$ = 4.4 Hz, H-6), 8.57 (s, 1H, =CH), 8.25 (d, 1H, $J_{4,5}$ = 4.4 Hz, H-4), 7.56 (m, 1H, H-5), 7.00 (d, 1H, $J_{4',6'/4',2'}$ = 2.0 Hz, H-4′), 6.74 (m, 2H, H-2′, H-6′); EI-MS: *m/z* (rel. abund.%), 258 (M^+, 18), 122 (78), 106 (100), 78 (61); HREI-MS calcd for $C_{13}H_{11}N_3O_3$: *m/z* = 257.0800, found 257.0803; Anal. Calcd for $C_{13}H_{11}N_3O_3$ (257.24): C, 60.70; H, 4.31; N, 16.33; Found: C, 60.71; H, 4.33; N, 16.32.

*4.13.8. (E)-N′-(3′,4′-Dihydroxybenzylidene) nicotinohydrazide (**8**)*[18d]

Yield: 70%; m.p. 198–200 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.07 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.73 (d, 1H, $J_{4,5}$ = 3.6 Hz, H-4), 8.23 (s, 1H, =CH), 8.11 (d, 1H, $J_{6,5}$ = 8.5 Hz, H-6), 7.54 (m, 1H, H-5), 7.24 (s, 1H, H-2′), 6.92 (dd, 1H, $J_{6',5'}$ = 8.0 Hz, $J_{6',2'}$ = 2.0 Hz, H-6′), 6.77 (d, 1H, $J_{5',6'}$ = 8.0 Hz, H-5′); EI-MS: *m/z* (rel. abund.%), 257 (M^+, 80), 135 (77), 123 (79), 106 (100); Anal. Calcd for $C_{13}H_{11}N_3O_3$ (257.24): C, 60.70; H, 4.31; N, 16.33; Found: C, 60.71; H, 4.32; N, 16.3O.

*4.13.9. (E)-N′-(2′,3′,4′-Trihydroxybenzylidene) nicotinohydrazide (**9**)*[18e]

Yield: 80%; m.p. 205–207 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.06 (s, 1H, OH), 8.74 (s, 1H, H-2), 8.74 (d, 1H, $J_{6,5}$ = 4.8 Hz, H-6), 8.37 (s, 1H, =CH), 8.35 (d, 1H, $J_{4,5}$ = 4.8 Hz, H-4), 7.61 (m, 1H, H-5), 6.78 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 6.44 (d, 1H, $J_{5',6'}$ = 8.5 Hz, H-5′); EI-MS: *m/z* (rel. abund.%), 273 (M^+, 50), 123 (31), 106 (100), 78 (51); Anal. Calcd for $C_{13}H_{11}N_3O_4$ (273.24): C, 57.14; H, 4.06; N, 15.38; Found: C, 57.12; H, 4.07; N, 15.39.

*4.13.10. (E)-N′-(2′-Ethoxybenzylidene) nicotinohydrazide (**10**) CAS # 292180-67-7*

Yield: 78%; m.p. 186–188 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.00 (s, 1H, NH), 9.06 (s, 1H, H-2), 8.79 (s, 1H, =CH), 8.75 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.25 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.87 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.56 (m, 1H, H-5), 7.40 (t, 1H, $J_{5',4'/5',6'}$ = 8.0 Hz, H-5′), 7.10 (d, 1H, $J_{3',4'}$ = 8.5 Hz, H-3′), 7.01 (t, 1H, $J_{4',3'/4',5'}$ = 8.0 Hz, H-4′), 4.12 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 1.38 (t, 3H, $J_{2'',1''}$ = 7.2 Hz, CH_3-2″); EI-MS: *m/z* (rel. abund.%), 269 (M^+, 7), 147 (82), 119 (100), 78 (81); Anal. Calcd for $C_{15}H_{15}N_3O_2$ (269.30): C, 66.90; H, 5.61; N, 15.60; Found: C, 66.89; H, 5.61; N, 15.62.

*4.13.11. (E)-N′-(4′-Ethoxybenzylidene)nicotinohydrazide (**11**) CAS # 324531-29-5*

Yield: 81%; m.p. 155–157 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.85 (s, 1H, NH), 9.04 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.75 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.37 (s, 1H, =CH), 8.23 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.667(d, 2H, $J_{2',3'/6',5'}$ = 8.0 Hz, H-2′, H-6′), 7.57 (m, 1H, H-5), 7.01 (d, 2H, $J_{3',2'/5',6'}$ = 8.0 Hz, H-3′, H-5′), 4.10 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 1.35 (t, 3H, $J_{2'',1''}$ = 6.8 Hz, CH_3-2″); EI-MS: *m/z* (rel. abund.%), 269 (M^+, 37), 147 (100), 119 (74), 106 (53); Anal. Calcd for $C_{15}H_{15}N_3O_2$ (269.30): C, 66.90; H, 5.61; N, 15.60; Found: C, 66.89; H, 5.60; N, 15.61.

*4.13.12. (E)-N′-(3′-Ethoxy-2′-hydroxybenzylidene) nicotinohydrazide (**12**) CAS # 292180-68-8*

Yield: 80%; m.p. 247–249 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.20 (s, 1H, NH), 10.78 (s, 1H, OH), 9.09 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.76 (dd, 1H, $J_{6,5}$ = 4.8 Hz, $J_{6,2}$ = 1.2 Hz, H-6), 8.64 (s, 1H, =CH), 8.27 (m, 1H, H-4), 7.57 (m, 1H, H-5), 7.17 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.04 (d, 1H, $J_{4',5'}$ = 8.5 Hz, H-4′), 7.86 (t, 1H, $J_{5',4'/5',6'}$ = 8.5 Hz, H-5′), 4.06 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 1.34 (t, 3H, $J_{2'',1''}$ = 7.2 Hz, CH_3-2″); EI-MS: *m/z* (rel. abund.%), 285 (M^+, 55), 163 (20), 106 (100), 78 (67); Anal. Calcd for $C_{15}H_{15}N_3O_3$ (285.30): C, 63.15; H, 5.30; N, 14.73; Found: C, 63.16; H, 5.29; N, 14.74.

*4.13.13. (E)-N′-(2′-Methoxybenzylidene) nicotinohydrazide (**13**)*[18f]

Yield: 94%; m.p. 157–159 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.99 (s, 1H, NH), 9.07 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.79 (s, 1H, =CH), 8.75 (dd, 1H, $J_{6,5}$ = 6.5 Hz, $J_{6,2}$ = 1.6 Hz, H-6), 8.26 (dt, 1H, $J_{6',5'}$ = 8.0, $J_{6',4'}$ = 1.6 Hz, H-6), 7.88 (dd, 1H, $J_{3',4'}$ = 6.5 Hz, $J_{3',5'}$ = 1.6 Hz, H-3′), 7.55 (m, 1H, H-4), 7.18 (td, 1H, $J_{5',4'/5',6'}$ = 8.0 Hz, $J_{5',3'}$ = 1.6 Hz, H-5′), 7.12 (t, 1H, $J_{4',3'/4',5'}$ = 8.0 Hz, H-4′), 7.03 (t, 1H, $J_{5,4/5,6}$ = 8.0 Hz, H-5), 3.86 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund. %), 255 (M^+, 32), 133 (100), 106 (100), 78 (89); Anal. Calcd for $C_{14}H_{13}N_3O_2$ (255.27): C, 65.87; H, 5.13; N, 16.46; Found: C, 65.86; H, 5.11; N, 16.48.

*4.13.14. (E)-N′-(3′,4′-Dimethoxybenzylidene) nicotinohydrazide (**14**)*[18g]

Yield: 84%; m.p. 166–168 °C (lit. 166–167 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.88 (s, 1H, NH), 9.04 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.75 (dd, 1H, $J_{6,2}$ = 1.2 Hz, $J_{6,5}$ = 4.8 Hz, H-6), 8.35 (s, 1H, =CH), 8.23 (td, 1H, $J_{4,5}$ = 8.0 Hz, $J_{4(2,6)}$ = 2.0 Hz, H-4),

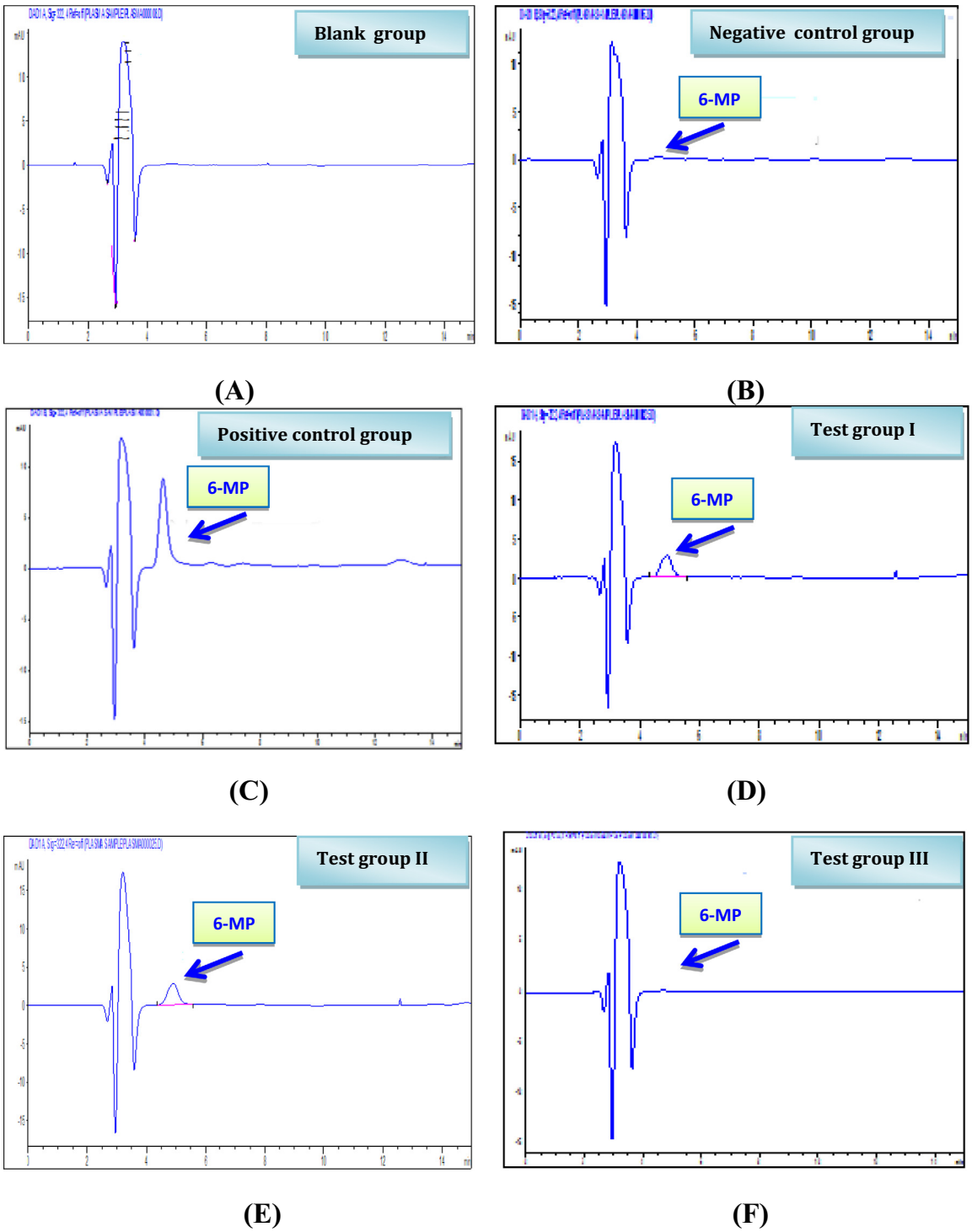

Fig. 15. UPLC chromatograms of (A) Blank group, (B) Negative control group, (C) Positive control group, (D) Test group I, (compound **8**; 50 mg/kg), (E) Test group II, (compound **35**; 50 mg/kg), and (F) Test group III (compound **36**; 50 mg/kg), showing the corresponding peaks of 6-MP.

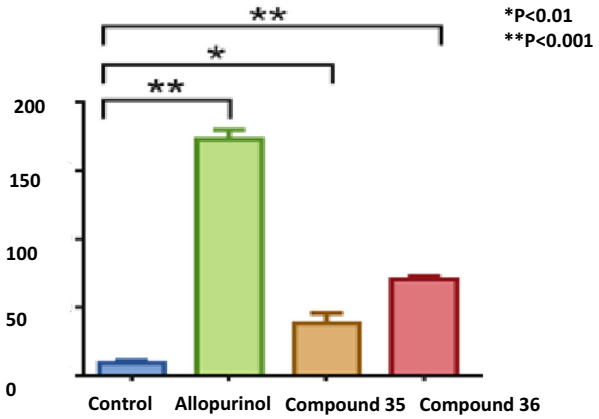

Fig. 16. Comparison of standard inhibitor allopurinol, compounds **35**, and **36** with the control group showing significant peak areas of 6-mercaptopurine (6-MP) with P values <0.01, and 0.001, respectively.

Table 3
Cytotoxicity and *in vivo* XO inhibitory activity of selected inhibitors.

S. No.	Group	MTT assay	Retention Time	Peak Area (mAU)	Amount (6-MP) (mg/mL)
1	Control group	–	4.912	7.3	–
2	Allopurinol (50 mg/kg)	–	4.929	166.3	1.67 (100%)
3	Compound **8** (50 mg/kg)	Non-cytotoxic	4.901	0.001	–
4	Compound **35** (50 mg/kg)	Non-cytotoxic	4.946	47.4	0.475 (28%)
5	Compound **36** (50 mg/kg)	Non-cytotoxic	4.918	73.5	0.76 (44%)

7.57 (m, 1H, H-5), 7.34 (d, 1H, $J_{2',6'}$ = 2.0 Hz, H-2′), 7.74 (dd, 1H, $J_{6',5'}$ = 8.0 Hz, $J_{6',2'}$ = 2.0 Hz, H-6′), 7.03 (d, 1H, $J_{5',6'}$ = 8.0 Hz, H-5′), 3.81 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund.%), 285 (M^+, 73), 163 (100), 148 (40), 106 (44); Anal. Calcd For $C_{15}H_{15}N_3O_3$ (285.30): C, 63.15; H, 5.30; N, 14.73; Found: C, 63.17; H, 5.32; N, 14.70.

*4.13.15. (E)-N′-(2′,3′,4′-Trimethoxybenzylidene) nicotinohydrazide (**15**) CAS # 556786-23-3*

Yield: 76%; m.p. 182–184 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.90 (s, 1H, NH), 9.05 (s, 1H, H-2), 8.75 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.24 (s, 1H, =CH), 7.65 (d, 1H, $J_{6',5'}$ = 8.0 Hz, H-6′), 7.70 (m, 1H, H-5), 6.94 (d, 1H, $J_{5',6'}$ = 8.0 Hz, H-5′); EI-MS: *m/z* (rel. abund.%), 315 (M^+, 19), 193 (100), 178 (48), 78 (26); Anal. Calcd for $C_{16}H_{17}N_3O_4$ (315.32): C, 60.94; H, 5.43; N, 13.33; Found: C, 60.95; H, 5.41; N, 13.34.

*4.13.16. (E)-N′-(2′-Hydroxy-6′-methoxybenzylidene) nicotinohydrazide (**16**)*

Yield: 77%; m.p. 278–280 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.21 (s, 1H, NH), 10.55 (s, 1H, OH), 9.00 (d, 1H, $J_{2,6}$ = 1.4 Hz, H-2), 8.72 (dd, 1H, $J_{6,5}$ = 5.0 Hz, $J_{6,4}$ = 1.4 Hz, H-6), 8.60 (s, 1H, =CH), 8.11 (m, 1H, H-4), 7.48 (m, 1H, H-5), 7.14 (d, 1H, $J_{3',4'}$ = 8.0 Hz, H-3′), 7.05 (m, 2H, H-4′, H-5′), 3.81 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 98), 149 (74), 106 (100), 78 (84); HREI-MS calcd for $C_{14}H_{13}N_3O_3$: *m/z* = 271.0957, found 271.0961; Anal. Calcd for $C_{14}H_{13}N_3O_3$ (271.27): C, 61.99; H, 4.83; N, 15.49; Found: C, 61.97; H, 4.84; N, 15.50.

*4.13.17. (E)-N′-(2′-Hydroxy-5′-methoxybenzylidene) nicotinohydrazide (**17**)*[18h]

Yield: 92%; m.p. 242–244 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.20 (s, 1H, NH), 10.78 (s, 1H, OH), 9.07 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.77 (d, 1H, $J_{6',4'}$ = 3.6 Hz, H-6′), 8.65 (s, 1H, =CH), 8.26 (m, 1H, H-6), 7.57 (m, 1H, H-4), 7.18 (d, 1H, $J_{3',4'}$ = 8.0 Hz, H-3′), 7.04 (d, 1H, $J_{4',3'}$ = 8.0 Hz, H-4′), 6.86 (t, 1H, $J_{5,4/5,6}$ = 8.0 Hz, H-5), 3.81 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 89), 123 (81), 106 (100), 51 (39); Anal. Calcd for $C_{14}H_{13}N_3O_3$ (271.27): C, 61.99; H, 4.83; N, 15.49; Found: C, 61.98; H, 4.81; N, 15.51.

*4.13.18. (E)-N′-(2′-Hydroxy-3′-methoxybenzylidene) nicotinohydrazide (**18**)*[18b]

Yield: 73%; m.p. 197–199 °C (lit. 198–199 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.20 (s, 1H, NH), 10.76 (s, 1H, OH), 9.07 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.77 (dd, 1H, $J_{6,5}$ = 4.8 Hz, $J_{6,4}$ = 1.2 Hz, H-6), 8.56 (s, 1H, =CH), 8.27 (td, 1H, $J_{4(2,6)}$ = 2.0 Hz, $J_{4,5}$ = 8.0 Hz, H-4), 7.57 (m, 1H, H-5), 7.17 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.05 (d, 1H, $J_{4',5'}$ = 8.5 Hz, H-4′), 7.86 (t, 1H, $J_{5'(4',5')}$ = 8.5 Hz, H-5′), 3.81 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 50), 149 (86), 106 (100), 83 (68); Anal. Calcd for $C_{14}H_{13}N_3O_3$ (271.27): C, 61.99; H, 4.83; N, 15.49; Found: C, 61.97; H, 4.84; N, 15.50.

*4.13.19. (E)-N′-(2′-Bromobenzylidene) nicotinohydrazide (**19**)*[18i]

Yield: 69%; m.p. 203–205 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.23 (s, 1H, NH), 9.02 (s, 1H, H-2), 8.81 (s, 1H, =CH), 8.76 (d, 1H, $J_{6,5}$ = 4.8 Hz, H-6), 8.27 (d, 1H, $J_{4,5}$ = 5.0 Hz, H-4), 8.01 (d, 1H, $J_{6',5'}$ = 7.5 Hz, H-6′), 7.70 (d, 1H, $J_{3',4'}$ = 7.5 Hz, H-6′), 7.57 (m, 1H, H-5), 7.48 (t, 1H, $J_{4'(3',5')}$ = 8.0 Hz, H-4′), 7.48 (t, 1H, $J_{5'(4',6')}$ = 8.0 Hz, H-6′); EI-MS: *m/z* (rel. abund.%), 303 (M^+, 3), 122 (70), 106 (100), 78 (68); Anal. Calcd for $C_{13}H_{10}BrN_3O$ (304.14): C, 156.34; H, 3.31; N, 13.82; Found: C, 51.32; H, 3.32; N, 13.81.

*4.13.20. (E)-N′-(2′-Fluorobenzylidene) nicotinohydrazide (**20**)*[18j]

Yield: 90%; m.p. 149–151 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.12 (s, 1H, NH), 9.07 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.79 (d, 1H, $J_{6,5}$ = 3.6 Hz, H-6), 8.68 (s, 1H, =CH), 8.27 (td, 1H, $J_{4,5}$ = 8.0 Hz, $J_{4(2,6)}$ = 2.0 Hz, H-4), 7.95 (t, 1H, $J_{3'(4',F)}$ = 8.0 Hz, H-3′), 7.53 (m, 2H, H-5, H-6′), 7.31 (m, 2H, H-4′, H-5′); EI-MS: *m/z* (rel. abund.%), 243 (M^+, 15), 122 (97), 106 (100), 78 (85); Anal. Calcd for $C_{13}H_{10}FN_3O$ (243.24):C, 64.19; H, 4.14; N, 17.28; Found: C, 64.2O; H, 4.13; N, 17.27.

*4.13.21. (E)-N′-(3′-Chlorobenzylidene) nicotinohydrazide (**21**)*[18k]

Yield: 88%; m.p. 131–133 °C (lit. 130–132 °C); ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.14 (s, 1H, NH), 9.05 (s, 1H, H-2), 8.77 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.42 (s, 1H, =CH), 8.27 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.79 (s, 1H, H-2′), 7.70 (m, 1H, H-5), 7.55 (m, 3H, H-4′, H-5′, H-6′); EI-MS: *m/z* (rel. abund.%), 259 (M^+, 3), 261 (M+2, 1.2), 122 (58), 106 (100), 78 (52); Anal. Calcd for $C_{13}H_{10}ClN_3O$ (259.69): C, 60.12; H, 3.88; N, 16.18; Found: C, 60.13; H, 3.87; N, 16.19.

*4.13.22. (E)-N′-(4′-Chlorobenzylidene) nicotinohydrazide (**22**)*[18b]

Yield: 85%; m.p. 226–228 °C (lit. 227–229 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.09 (s, 1H, NH), 9.05 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.76 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.42 (s, 1H, =CH), 8.25 (br.d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.77 (d, 2H, $J_{2',3'/6',5'}$ = 8.0 Hz, H-2′, H-6′), 7.58 (m, 1H, H-5), 7.54 (d, 2H, $J_{3',2'/5',6'}$ = 8.0 Hz, H-3′, H-5′); EI-MS: *m/z* (rel. abund.%), 259 (M^+, 7), 261 (M + 2, 3), 122 (69), 106 (100), 78 (63); Anal. Calcd for $C_{13}H_{10}ClN_3O$ (259.69): C, 60.12; H, 3.88; N, 16.18; Found: C, 60.10; H, 3.89; N, 16.17.

*4.13.23. (E)-N'-(2',4'-Dichlorobenzylidene) nicotinohydrazide (**23**)*[18c]

Yield: 81%; m.p. 139–141 °C (lit. 138–140 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.28 (s, 1H, NH), 9.07 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.79 (s, 1H, =CH), 8.78 (d, 1H, $J_{4,5}$ = 3.6 Hz, H-4), 8.27 (d, 1H, $J_{6,5}$ = 8.5 Hz, H-6), 8.03 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.75 (d, 1H, $J_{5',6'}$ = 8.0 Hz, H-3′), 7.59 (m, 2H, H-5, H-3′); EI-MS: *m/z* (rel. abund.%), 293 (M^+, 8), 295 (M + 2, 5), 258 (30), 122 (88), 106 (100); Anal. Calcd for $C_{13}H_9Cl_2N_3O$ (294.14): C, 53.08; H, 3.08; N, 14.29; Found: C, 53.07; H, 3.07; N, 14.31.

*4.13.24. (E)-N'-(2'-Methylbenzylidene) nicotinohydrazide (**24**)*[18l]

Yield: 83%; m.p. 152–154 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.97 (s, 1H, NH), 9.07 (d, 1H, $J_{2,6}$ = 1.5 Hz, H-2), 8.76 (dd, 1H, $J_{6,5}$ = 4.8 Hz, $J_{6,2}$ = 1.5 Hz, H-6), 8.73 (s, 1H, =CH), 8.26 (td, 1H, $J_{4,5}$ = 5.0 Hz, $J_{4(2,6)}$ = 1.5 Hz, H-4), 7.58 (m, 1H, H-5), 7.29 (m, 4H, H-3′, H-4′, H-5′, H-6′), 2.17 (s, 3H, CH_3); EI-MS: *m/z* (rel. abund. %), 239 (M^+, 23), 123 (90), 106 (100), 78 (87); Anal. Calcd for $C_{14}H_{13}N_3O$ (239.27): C, 70.28; H, 5.48; N, 17.56; Found: C 70.27; H, 5.47; N, 17.58.

*4.13.25. (E)-N'-(2'-Hydroxy-5'-methylbenzylidene) nicotinohydrazide (**25**)*[18h]

Yield: 78%; m.p. 200–202 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.20 (s, 1H, NH), 10.87 (s, 1H, OH), 9.07 (s, 1H, H-2), 8.76 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.59 (s, 1H, =CH), 8.26 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.38 (s, 1H, H-6′), 7.11 (d, 1H, $J_{4',3'}$ = 8.0 Hz, H-4′), 6.82 (d, 1H, $J_{3',4'}$ = 8.0 Hz, H-3′), 2.24 (s, 3H, CH_3); EI-MS: *m/z* (rel. abund.%), 255 (M^+, 100), 122 (80), 106 (100), 78 (100); Anal. Calcd for $C_{14}H_{13}N_3O_2$ (255.27): C, 65.87; H, 5.13; N, 16.46; Found: C, 65.85; H, 5.14; N, 16.45.

*4.13.26. (E)-N'-(4'-(Methylthio)benzylidene) nicotinohydrazide (**26**)*[18m]

Yield: 81%; m.p. 149–151 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.97 (s, 1H, NH), 9.04 (s, 1H, H-2), 8.76 (d, 1H, $J_{6,5}$ = 4.2 Hz, H-6), 8.38 (s, 1H, =CH), 8.24 (d, 1H, $J_{4,5}$ = 8.0 Hz, H-4), 7.67 (d, 2H, $J_{3',2'/5',6'}$ = 8.0 Hz, H-3′, H-5′) 7.57 (m, 1H, H-5), 7.33 (d, 2H, $J_{2',3'/6',5'}$ = 8.0 Hz, H-2′, H-6′), 2.51 (s, 3H, SCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 67), 149 (100), 106 (65), 78 (38); Anal. Calcd for $C_{14}H_{13}N_3OS$ (271.34); C, 61.97; H, 4.83; N, 15.49; Found: C, 61.98; H, 4.81; N, 15.51.

*4.13.27. (E)-N'-(4'-(Dimethylamino)benzylidene) nicotinohydrazide (**27**)*[18b]

Yield: 78%; m.p. 144–146 °C (lit. 145–147 °C); ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.67 (s, 1H, NH), 9.03 (d, 1H, $J_{2,6}$ = 1.2 Hz, H-2), 8.72 (dd, 1H, $J_{6,5}$ = 4.8 Hz, $J_{6,2}$ = 1.2 Hz, H-6), 8.29 (s, 1H, =CH), 8.21 (d, 1H, $J_{4,6}$ = 1.2 Hz, H-2), 7.54 (m, 3H, H-5, H-2′, H-6′), 6.75 (d, 2H, $J_{3',2'/5',6'}$ = 8.0 Hz, H-3′, H-5′), 2.97 (s, N $(CH_3)_2$); EI-MS: *m/z* (rel. abund.%), 268 (M^+, 24), 146 (32), 83 (23), 43 (15); Anal. Calcd for $C_{15}H_{16}N_4O$ (268.31): C, 67.15; H, 6.01; N, 20.88; Found: C, 67.14; H, 6.02; N, 20.87.

*4.13.28. (E)-N'-Benzylideneisonicotinohydrazide (**28**)*[18n]

Yield: 71%; m.p. 200–202 °C (lit. 201–202 °C); Yield: 0.20 (68%); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.04 (s, 1H, NH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.46 (s, 1H, =CH), 7.82 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.75 (m, 2H, H-2′, H-6′), 7.47 (m, 3H, H-2′, H-3′, H-4′); EI-MS: *m/z* (rel. abund.%), 225 (M^+, 15), 122 (94), 106 (100), 78 (64); Anal. Calcd for $C_{13}H_{11}N_3O$ (225.25): C, 69.32; H, 4.92; N, 18.66; Found: C, 69.31; H, 4.93; N, 18.65.

*4.13.29. (E)-N'-(3'-Nitrobenzylidene)isonicotinohydrazide (**29**)*[18n]

Yield: 92%; m.p. 231–233 °C (lit. 231–232 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.31 (s, 1H, NH), 8.80 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.57 (s, 1H, =CH), 8.56 (s, 1H, H-2′), 8.28 (dd, 1H, $J_{4',5'}$ = 8.5 Hz, $J_{4',2'}$ = 2.5 Hz, H-4′), 8.18 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.83 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.76 (t, 1H, $J_{5'(4',6')}$ = 8.5 Hz, H-5′); EI-MS: *m/z* (rel. abund.%), 270 (M^+, 04), 122 (95), 106 (100), 78 (80); Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.78; H, 3.73; N, 20.73; Found: C, 57.75; H, 3.75; N, 20.74.

*4.13.30. (E)-N'-(2'-Nitrobenzylidene)isonicotinohydrazide (**30**)*[18n]

Yield: 92%; m.p. 234–236 °C (lit. 235–236 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.39 (s, 1H, NH), 8.88 (s, 1H, =CH), 8.79 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.13 (m, 2H, H-3′, H-4′), 7.84 (m, 3H, H-6′, H-2, H-6), 7.72 (t, 1H, $J_{5'(4',6')}$ = 8.5 Hz, H-5′); EI-MS: *m/z* (rel. abund.%), 270 (M^+, 2), 122 (60), 106 (100), 78 (75); Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.78; H, 3.73; N, 20.73; Found: C, 57.77; H, 3.71; N, 20.75.

*4.13.31. (E)-N'-(4'-Nitrobenzylidene)isonicotinohydrazide (**31**)*[18n]

Yield: 77%; m.p. 280–282 °C (lit. 281–282 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.20 (s, 1H, NH), 8.80 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.56 (s, 1H, =CH), 8.32 (d, 2H, $J_{3',2'/5',6'}$ = 8.5 Hz, H-3′, H-5′), 8.01 (d, 2H, $J_{2',3'/6',5'}$ = 8.5 Hz, H-2′, H-6′), 7.83 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6); EI-MS: *m/z* (rel. abund. %), 270 (M^+, 8), 122 (98), 106 (100), 78 (88); Anal. Calcd for $C_{13}H_{10}N_4O_3$ (270.24): C, 57.78; H, 3.73; N, 20.73; Found: C, 57.77; H, 3.75; N, 20.72.

*4.13.32. (E)-N'-(4'-Hydroxybenzylidene)isonicotinohydrazide (**32**)*[18n]

Yield: 74%; m.p. 294–296 °C (lit. 293–294 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.84 (s, 1H, NH), 9.96 (s, 1H, OH), 8.76 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.34 (s, 1H, =CH), 7.80 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.58 (d, 2H, $J_{2',3'/6',5'}$ = 8.5 Hz, H-2′, H-6′), 6.84 (d, 2H, $J_{3',2'/5',6'}$ = 8.5 Hz, H-3′, H-5′); EI-MS: *m/z* (rel. abund. %), 241 (M^+, 23), 165 (17), 106 (100), 78 (71); Anal. Calcd for $C_{13}H_{11}N_3O_2$ (241.25): C, 64.72; H, 4.60; N, 17.42; Found: C, 64.70; H, 4.59; N, 17.44.

*4.13.33. (E)-N'-(3'-Hydroxybenzylidene)isonicotinohydrazide (**33**)*[18n]

Yield: 78%; m.p. 269–271 °C (lit. 268–269 °C); ^{1}H NMR: (300 MHz, DMSO-d_6): δ 11.99 (s, 1H, NH), 9.65 (s, 1H, OH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.36 (s, 1H, =CH), 7.81 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.26 (t, 1H, $J_{5'(4',6')}$ = 8.5, H-5′), 7.22 (s, 1H, H-2′), 7.11 (d, 1H, $J_{4',5'}$ = 8.5, H-4′), 6.84 (d, 1H, $J_{6',5'}$ = 8.5, H-6′); EI-MS: *m/z* (rel. abund.%), 239 (M^+, 58), 122 (92), 91 (34), 44 (22); Anal. Calcd for $C_{13}H_{11}N_3O_2$ (241.25): C, 64.72; H, 4.60; N, 17.42; Found: C, 64.71; H, 4.61; N, 17.40.

*4.13.34. (E)-N'-(2',4'-Dihydroxybenzylidene)isonicotinohydrazide (**34**)*[18n]

Yield: 70%; m.p. 296–298 °C (lit. 294–295 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.09 (s, 1H, OH), 11.25 (s, 1H, NH), 9.99 (s, 1H, OH), 8.77 (s, 1H, H-3′), 8.52 (s, 1H, =CH), 7.81 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 7.35 (d, 1H, $J_{6',5'}$ = 7.5 Hz, H-6′), 6.33 (m, 3H, H-2, H-6, H-5′); EI-MS: *m/z* (rel. abund.%), 257 (M^+, 51), 135 (100), 106 (63), 78 (42); Anal. Calcd for $C_{13}H_{11}N_3O_3$ (257.24): C, 60.70; H, 4.31; N, 16.33; Found: C, 60.71; H, 4.30; N, 16.32.

*4.13.35. (E)-N'-(3',4'-Dihydroxybenzylidene)isonicotinohydrazide (**35**)*[18o]

Yield: 73%; m.p. 221–223 °C (lit. 224–225 °C); ^{1}H NMR: (300 MHz, DMSO-d_6): δ 11.92 (s, 1H, NH), 8.73 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.38 (s, 1H, =CH), 7.80 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.35 (d, 1H, $J_{2',6'}$ = 2.5 Hz, H-2′), 7.22 (dd, 1H, $J_{6',5'}$ = 8.5 Hz, $J_{6',2'}$ = 2.5 Hz, H-6′), 7.03 (d, 1H, $J_{5',6'}$ = 8.5 Hz, H-5′); EI-MS: *m/z* (rel. abund.%), 257 (M^+, 23), 163 (100), 106 (25), 78 (28); Anal. Calcd for $C_{15}H_{15}N_3O_3$ (285.30): C, 63.15; H, 5.30; N, 14.73; Found: C, 63.17; H, 5.29; N, 14.71.

*4.13.36. (E)-N′-(2′,4′,6′-Trihydroxybenzylidene)isonicotinohydrazide (**36**)*[18p]

Yield: 77%; m.p 239–241 °C (lit. 238–240 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.11 (s, 1H, OH), 11.05 (s, 1H, OH), 9.86 (s, 1H, OH), 8.81 (s, 1H, =CH), 8.77 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 7.83 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 5.85 (s, 2H, H-3′, H-5′); EI-MS: *m/z* (rel. abund.%), 273 (M^+, 100), 151 (79), 106 (89), 78 (55); Anal. Calcd for $C_{13}H_{11}N_3O_4$ (273.24): C, 57.14; H, 4.06; N, 15.38; Found: C, 57.12; H, 4.08; N, 15.37.

*4.13.37. (E)-N′-(2′-Ethoxybenzylidene)isonicotinohydrazide (**37**)*[18n]

Yield: 81%; m.p. 201–203 °C (lit. 204–205 °C); ^{1}H NMR: (300 MHz, DMSO-d_6): δ 12.05 (s, 1H, NH), 8.81 (s, 1H, =CH), 8.77 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 7.88 (dd, 1H, $J_{6',4'}$ = 2.0 Hz, $J_{6',5'}$ = 8.0 Hz, H-6′), 7.83 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.40 (t, 1H, $J_{5'(4',6')}$ = 8.5 Hz, H-5′), 7.10 (d, 1H, $J_{3',4'}$ = 8.5 Hz, H-3′), 7.01 (t, 1H, $J_{4'(3',5')}$ = 8.5 Hz, H-4′), 4.15 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 1.40 (t, 3H, $J_{2'',1''}$ = 6.8 Hz, CH_3-2″); EI-MS: *m/z* (rel. abund.%), 269 (M^+, 07), 147 (94), 119 (100), 106 (74), 78 (59); Anal. Calcd for $C_{15}H_{15}N_3O_2$ (269.30): C, 66.90; H, 5.61; N, 15.60; Found: C, 66.91; H, 5.63; N, 15.58.

*4.13.38. (E)-N′-(4′-Ethoxybenzylidene)isonicotinohydrazide (**38**)*[18q]

Yield: 74%; m.p. 176–178 °C (lit. 177 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.90 (s, 1H, NH), 8.76 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.39 (s, 1H, =CH), 7.80 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.67 (d, 2H, $J_{2',3'/6',5'}$ = 8.5 Hz, H-2′, H-6′), 7.00 (d, 2H, $J_{3',2'/5',6'}$ = 8.5 Hz, H-3′, H-5′), 4.10 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 1.35 (t, 3H, $J_{2'',1''}$ = 6.8 Hz, CH_3-2″); EI-MS: *m/z* (rel. abund.%), 269 (M^+, 38), 147 (93), 119 (100), 78 (36); Anal. Calcd for $C_{15}H_{15}N_3O_2$ (269.30): C, 66.90; H, 5.61; N, 15.60; Found: C, 66.88; H, 5.62; N, 15.61.

*4.13.39. (E)-N′-(3′-Ethoxy-2′-hydroxybenzylidene) isonicotinohydrazide (**39**)*[18r]

Yield: 87%; m.p. 228–230 °C; ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.25 (s, 1H, NH), 10.66 (s, 1H, -OH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.67 (s, 1H, =CH), 7.84 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.16 (d, 1H, $J_{6',5'}$ = 8.0 Hz, H-6′), 7.04 (d, 1H, $J_{4',5'}$ = 8.0 Hz, H-4′), 6.84 (t, 1H, $J_{5'(4',6')}$ = 8.0 Hz, H-5′), 4.09 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 1.36 (t, 3H, $J_{2'',1''}$ = 6.8 Hz, CH_3-2″); EI-MS: *m/z* (rel. abund.%), 285 (M^+, 45), 163 (63), 135 (97), 106 (100); Anal. Calcd for $C_{15}H_{15}N_3O_3$ (285.30): C, 63.15; H, 5.30; N, 14.73; Found: C, 63.14; H, 5.32; N, 14.72.

*4.13.40. (E)-N′-(3′,4′-Diethoxybenzylidene)isonicotinohydrazide (**40**)*[18n]

Yield: 84%; m.p. 193–195 °C (lit. 193–194 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.92 (s, 1H, NH), 8.77 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.38 (s, 1H, =CH), 7.80 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.34 (d, 1H, $J_{2',6'}$ = 1.5 Hz, H-2′), 7.35 (dd, 1H, $J_{6',5'}$ = 8.5 Hz, $J_{6',2'}$ = 1.5 Hz, H-6′), 7.03 (d, 1H, $J_{5',6'}$ = 8.5 Hz, H-5′), 4.12 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 4.10 (q, 2H, $J_{1''',2'''}$ = 7.2 Hz, OCH_2-1‴), 1.37 (t, 3H, $J_{2'',1''}$ = 6.8 Hz, CH_3-2″), 1.35 (t, 3H, $J_{2''',1'''}$ = 6.8 Hz, CH_3-2‴); EI-MS: *m/z* (rel. abund.%), 313 (M^+, 08), 202 (69), 140 (100),83 (100); Anal. Calcd for $C_{17}H_{19}N_3O_3$ (313.35): C, 65.16; H, 6.11; N, 13.41; Found: C, 65.15; H, 6.13; N, 13.40.

*4.13.41. (E)-N′-(3′,4′-Dimethoxybenzylidene)isonicotinohydrazide (**41**)*[18s]

Yield: 70%; m.p. 190–192 °C (lit. 189–190 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.92 (s, 1H, NH), 8.77 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.38 (s, 1H, =CH), 7.80 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.35 (d, 1H, $J_{2',6'}$ = 2.0 Hz, H-2′), 7.24 (dd, 1H, $J_{6',5'}$ = 8.0 Hz, $J_{6',2'}$ = 2.0 Hz, H-6′), 7.04 (d, 1H, $J_{5',6'}$ = 8.5 Hz, H-5′), 3.81 (s, 3H, OCH_3), 3.80 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund. %), 285 (M^+, 77), 163 (100), 148 (44), 106 (39); Anal. Calcd For $C_{15}H_{15}N_3O_3$ (285.11): C, 63.15; H, 5.30; N, 14.73; Found: C, 63.16; H, 5.33; N, 14.71.

*4.13.42. (E)-N′-(4′-Ethoxy-3′-hydroxybenzylidene) isonicotinohydrazide (**42**) CAS # 680578-78-3*

Yield: 73%; m.p. 225–227 °C; ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.85 (s, 1H, NH), 8.76 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.32 (s, 1H, =CH), 7.79 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.30 (d, 1H, $J_{2',6'}$ = 2.0 Hz, H-2′), 7.10 (dd, 1H, $J_{6',5'}$ = 8.5 Hz, $J_{6',2'}$ = 2.0 Hz, H-6′), 6.85 (d, 1H, $J_{5',6'}$ = 8.5 Hz, H-5′), 4.05 (q, 2H, $J_{1'',2''}$ = 7.2 Hz, OCH_2-1″), 1.34 (t, 3H, $J_{2'',1''}$ = 6.8 Hz, CH_3-2″); EI-MS: *m/z* (rel. abund.%), 285 (M^+, 81), 163 (98), 135 (100) 106 (88), 78 (40); Anal. Calcd for $C_{15}H_{15}N_3O_3$ (285.30): C, 63.15; H, 5.30; N, 14.73; Found: C, 63.16; H, 5.31; N, 14.71.

*4.13.43. (E)-N′-(3′-Hydroxy-4′-methoxybenzylidene) isonicotinohydrazide (**43**)*[18t]

Yield: 73%; m.p. 204–206 °C; ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.86 (s, 1H, NH), 8.74 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.30 (s, 1H, =CH), 7.80 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.27 (d, 1H, $J_{2',6'}$ = 1.5 Hz, H-2′), 7.08 (dd, 1H, $J_{6',5'}$ = 8.5 Hz, $J_{6',2'}$ = 1.5 Hz, H-6′), 6.98 (d, 1H, $J_{5',6'}$ = 8.5 Hz, H-5′), 3.80 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 40), 149 (100), 106 (75), 78 (60); Anal. Calcd for $C_{14}H_{13}N_3O_3$ (271.27): C, 61.99; H, 4.83; N, 15.49; Found: C, 61.97; H, 4.84; N, 15.50.

*4.13.44. (E)-N′-(2′-Hydroxy-5′-methoxybenzylidene) isonicotinohydrazide (**44**)*[18h]

Yield: 85%; m.p. 251–253 °C; ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.24 (s, 1H, NH), 10.48 (s, 1H, OH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.65 (s, 1H, =CH), 7.83 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.16 (d, 1H, $J_{6',4'}$ = 2.0 Hz, H-6′), 6.93 (dd, 1H, $J_{4',3'}$ = 8.0 Hz, $J_{4',6'}$ = 2.0 Hz, H-4′), 6.87 (d, 1H, $J_{3',4'}$ = 8.0 Hz, H-3′), 3.72 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 100), 149 (83), 106 (79), 78 (68); Anal. Calcd for $C_{14}H_{13}N_3O_3$ (271.27): C, 61.99; H, 4.83; N, 15.49; Found: C, 61.97; H, 4.84; N, 15.48.

*4.13.45. (E)-N′-(2′-Hydroxy-3′-methoxybenzylidene) isonicotinohydrazide (**45**)*[18n]

Yield: 81%; m.p. 235–237 °C (lit. 236–237 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.80 (s, 1H, NH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.69 (s, 1H, =CH), 7.84 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.17 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.04 (d, 1H, $J_{4',5'}$ = 8.5 Hz, H-4′), 6.85 (t, 1H, $J_{5'(4',6')}$ = 8.5 Hz, H-4′), 3.81 (s, 3H, OCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 71), 149 (95), 106 (100), 78 (93); Anal. Calcd for $C_{14}H_{13}N_3O_3$ (271.27): C, 61.99; H, 4.83; N, 15.49; Found: C, 61.98; H, 4.85; N, 15.48.

*4.13.46. (E)-N′-(2′-Chlorobenzylidene)isonicotinohydrazide (**46**)*[18n]

Yield: 77%; m.p. 222–224 °C (lit. 221–222 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.26 (s, 1H, NH), 8.87 (s, 1H, =CH), 8.79 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.04 (dd, 1H, $J_{3',4'}$ = 8.5 Hz, $J_{3',5'}$ = 1.5 Hz, H-3′), 7.84 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.55 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.49 (m, 2H,H-4′, H-5′); EI-MS: *m/z* (rel. abund.%), 257 (M^+, 4), 259 (M + 2, 3.5), 122 (80), 106 (100), 78 (80); Anal. Calcd for $C_{13}H_{10}ClN_3O$ (259.69): C, 60.12; H, 3.88; N, 16.18; Found: C, 60.10; H, 3.87; N, 16.19.

*4.13.47. (E)-N′-(2′-Fluorobenzylidene)isonicotinohydrazide (**47**)*[18p]

Yield: 86%; m.p. 190–191 °C (lit. 191–193 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.16 (s, 1H, NH), 8.79 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.70 (s, 1H, =CH), 7.95 (t, 1H, $J_{5'(4',6')}$ = 8.0, H-5′), 7.83 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.52 (m, 1H, H-4′), 7.31 (m, 2H, H-3′, H-6′); EI-MS: *m/z* (rel. abund.%), 243

(M^+, 19), 122 (96), 106 (98), 78 (100); Anal. Calcd for $C_{13}H_{10}FN_3O$ (243.24): C, 64.19; H, 4.14; N, 17.28; Found: C, 64.17; H, 4.15; N, 17.29.

*4.13.48. (E)-N′-(3′-Chlorobenzylidene)isonicotinohydrazide (**48**)*[18u]

Yield: 85%; m.p. 222–224 °C (lit. 224–225 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.24 (s, 1H, NH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.46 (s, 1H, =CH), 7.83 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.79 (s, 1H, H-2′), 7.71 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.50 (m, 2H, H-4′, H-5′); EI-MS: *m/z* (rel. abund.%), 259 (M^+, 12), 122 (91), 106 (100), 78 (52); Anal. Calcd for $C_{13}H_{10}ClN_3O$ (259.69): C, 60.12; H, 3.88; N, 16.18; Found: C, 60.13; H, 3.86; N, 16.19.

*4.13.49. (E)-N′-(4′-Chlorobenzylidene) isonicotinohydrazide (**49**)*[18n]

Yield: 92%; m.p. 216–218 °C (lit. 217–218 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.10 (s, 1H, NH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.45 (s, 1H, =CH), 7.81 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.77 (d, 2H, $J_{2',3'/6',5'}$ = 8.5 Hz, H-2′, H-6′), 7.54 (d, 2H, $J_{3',2'/5',6'}$ = 8.5 Hz, H-3′, H-5′); EI-MS: *m/z* (rel. abund.%), 259 (M^+, 13), 122 (83), 106 (100), 78 (55); Anal. Calcd for $C_{13}H_{10}ClN_3O$ (259.69): C, 60.12; H, 3.88; N, 16.18; Found: C, 60.11; H, 3.86; N, 16.19.

*4.13.50. (E)-N′-(2′,4′-Dichlorobenzylidene)isonicotinohydrazide (**50**)*[18n]

Yield: 78%; m.p. 230–231 °C (lit. 228–229 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.32 (s, 1H, NH), 8.76 (s, 1H, =CH), 8.74 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.04 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′), 7.84 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.70 (d, 1H, $J_{3',5'}$ = 2.5 Hz, H-3′), 7.51 (d, 1H, $J_{5',6'}$ = 8.5 Hz, H-5′), 7.76 (t, 1H, $J_{5'(4',6')}$ = 8.5 Hz, H-5′); EI-MS: *m/z* (rel. abund.%), 293 (M^+, 5), 295 (M + 2, 3), 122 (88), 106 (100), 78 (89); Anal. Calcd for $C_{13}H_9Cl_2N_3O$ (294.14): C, 53.08; H, 3.08; N, 14.29; Found: C, 53.06; H, 3.07; N, 14.31.

*4.13.51. (E)-N′-(2′-Chloro-5′-nitrobenzylidene)isonicotinohydrazide (**51**)*[18v]

Yield: 88%; m.p. 235–237 °C; ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.51 (s, 1H, NH), 8.90 (s, 1H, =CH), 8.81 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.73 (d, 1H, $J_{6',4'}$ = 2.0 Hz, H-6′), 8.26 (dd, 1H, $J_{4',3'}$ = 8.0 Hz, $J_{4',6'}$ = 2.0 Hz, H-4′), 7.86 (m, 3H, H-2, H-6, H-3′); EI-MS: *m/z* (rel. abund.%), 304 (M^+, not observed), 268 (15), 106 (100), 78 (57); Anal. Calcd for $C_{13}H_9ClN_4O_3$ (304.69): C, 51.25; H, 2.98; N, 18.39; Found: C, 51.24; H, 2.96; N, 18.41

*4.13.52. (E)-N′-(2′-Hydroxybenzylidene)isonicotinohydrazide (**52**)*[18n]

Yield: 75%; m.p. 251–253 °C (lit. 252–253 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.26 (s, 1H, OH), 11.13 (s, 1H, NH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.36 (s, 1H, =CH), 7.81 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.26 (t, 1H, $J_{5'(4',6')}$ = 8.5 Hz, H-5′), 7.22 (s, 1H, H-3′), 7.11 (d, 1H, $J_{4',5'}$ = 8.5 Hz, H-4′), 6.84 (d, 1H, $J_{6',5'}$ = 8.5 Hz, H-6′); EI-MS: *m/z* (rel. abund.%), 241 (M^+, 36), 123 (92), 106 (100), 78 (64); Anal. Calcd for $C_{13}H_{11}N_3O_2$ (241.25): C, 64.72; H, 4.60; N, 17.42; Found: C, 64.73; H, 4.61; N, 17.40.

4.13.53. (E)-N′-(4′-(Methylthio)benzylidene)isonicotinohydrazide (53) CAS # 314072-18-9

Yield: 70%; m.p. 197–199 °C; ^{1}H NMR: (400 MHz, DMSO-d_6): δ 12.00 (s, 1H, NH), 8.78 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.41 (s, 1H, =CH), 7.81 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.67 (d, 2H, $J_{2',3'/6',5'}$ = 8.5 Hz, H-2′, H-6′), 7.33 (d, 2H, $J_{3',2'/5',6'}$ = 8.5 Hz, H-3′, H-5′), 2.51 (s, 3H, SCH_3); EI-MS: *m/z* (rel. abund.%), 271 (M^+, 82), 148 (100), 106 (156), 78 (67); Anal. Calcd for $C_{14}H_{13}N_3OS$ (271.34): C, 61.97; H, 4.83; N, 15.49; Found: C, 61.98; H, 4.81; N, 15.47.

*4.13.54. (E)-N′-(4′-(Dimethylamino)benzylidene) isonicotinohydrazide (**54**)*[18n]

Yield: 70%; m.p. 206–208 °C (lit. 206–207 °C); ^{1}H NMR: (400 MHz, DMSO-d_6): δ 11.73 (s, 1H, NH), 8.75 (d, 2H, $J_{3,2/5,6}$ = 6.0 Hz, H-3, H-5), 8.31 (s, 1H, =CH), 7.78 (d, 2H, $J_{2,3/6,5}$ = 6.0 Hz, H-2, H-6), 7.54 (d, 2H, $J_{2',3'/6',5'}$ = 8.5 Hz, H-2′, 6′), 6.75 (d, 2H, $J_{3',2'/5',6'}$ = 8.5 Hz, H-3′, H-5′), 2.92 (s, 6H, $2CH_3$); EI-MS: *m/z* (rel. abund.%), 268 (M^+, 83), 146 (100), 78 (17), 44 (45); Anal. Calcd for $C_{15}H_{16}N_4O$ (268.31): C, 67.15; H, 6.01; N, 20.88; Found: C, 67.14; H, 6.00; N, 20.89.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2017.02.044.

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