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ORIGINAL ARTICLE

Synthesis of 3,4,5-trihydroxybenzohydrazone and evaluation of their urease inhibition potential

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KEYWORDS

3,4,5-Trihydroxybenzohydrazones;
Urease inhibition;
Kinetic study

Abstract In the continuation of our work to synthesize enzyme inhibitors, we synthesized 3,4,5-trihydroxybenzohydrazones (**1–19**) from 3,4,5-trihydroxybenzohydrazide, which were obtained from methyl 3,4,5-trihydroxybenzoate by refluxing with hydrazine hydrate. All the synthesized compounds were characterized by different spectroscopic methods. The synthesized compounds were evaluated for urease inhibition and showed excellent results, close to the standards thiourea. The kinetic studies on the five most active compounds **6**, **10**, **14**, **16** and **18** were carried out to determine their mode of inhibition and dissociation constant K_i. The compounds **6** and **16** were found to be competitive inhibitors with K_i values 19.1 and 10.53 μM, respectively, while the compounds **10**, **14** and **18** were found to be mixed-type of inhibitors with K_i values in the range of 18.4–21.7 μM.

1. Introduction

Applications of benzohydrazones are reported in medicinal and analytical chemistry (Cimerman et al., 2000; Taha et al., 2014; Tarafder et al., 2002). Benzohydrazones having heterocyclic rings were reported to have antiglycation, anticonvulsant, Phosphodiesterase-1 inhibitors, antiproliferative, antifungal and anti-HIV activities (Kabak et al., 1999; Küçükgüzel et al., 2004; Khan et al., 2014a; Jamil et al., 2015; Pandeya et al., 1999). Several benzohydrazones reported interesting bioactivities, such as antifungal, anticonvulsant, anti-inflammatory,

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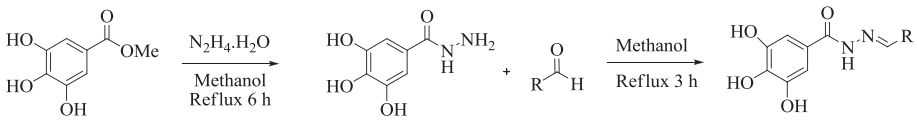


Scheme 1 Synthesis of 3,4,5-Trihydroxybenzohydrazones (**1–19**).

antibacterial, antimalarial, antiplatelets, analgesic, antituberculosis, antioxidant (Loncle et al., 2004; Küçükgüzel et al., 2003; Todeschini et al., 1998; Melnyk et al., 2006; Lima et al., 2000; Cunha et al., 2003; Kaymakçıoğlu et al., 2006; Aziz et al., 2014), antileishmanial, insecticidal, immunoconjugates, antimycobacterial, adriamycin proteinase inhibition and activity against protozoan parasite (Sawada et al., 2003; Taha et al., 2013; Küçükgüzel and Rollas, 2002; Greenfield et al., 1990; Caffery et al., 2002). Other benzohydrazone derivatives have reported *β*-glucuronidase (Jamil et al., 2014) and *α*-glucosidase inhibition activity (Taha et al., 2015a). On the other hand, substituted acylhydrazide Schiff bases have shown a broad range of bioactivities, including antiurease (Taha et al., 2015b), and antibacterial activities (Imran et al., 2014). Hydrazine derivatives also have several commercial applications (Ragnarsson, 2001).

Urease (E.C 3.5.1.5) plays an important role in the virulence of some bacterial pathogens as well as determinant in pathogenesis of many diseases in human. It is involved in the production of infectious stones; add to the pathogenesis of urolithiasis, pyelonephritis, and hepatic encephalopathy (Weatherburn, 1967). The urease results in pathologies by

Table 1 *In vitro* urease inhibition **1–19.**

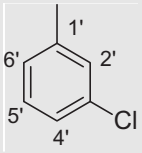
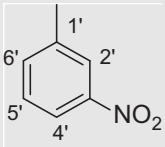
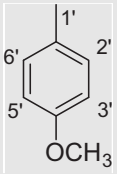
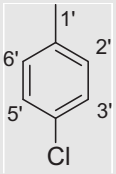
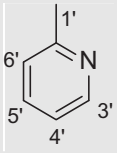
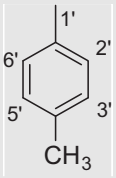
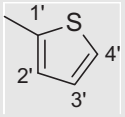
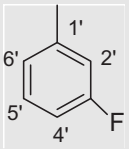
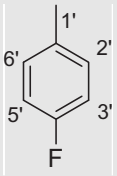
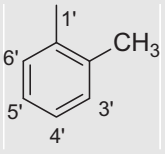
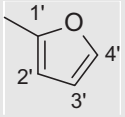
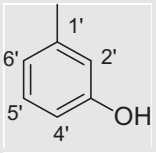
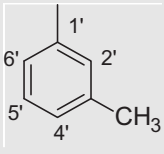
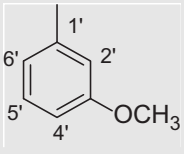
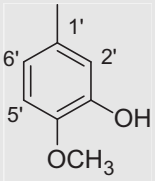
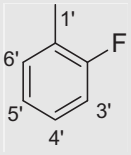
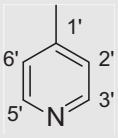
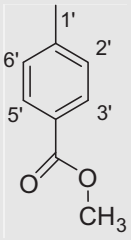
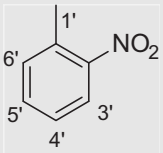
Compound	R	IC_{50} (μM ± SEM[a])	Compound	R	IC_{50} (μM ± SEM[a])
1		37.30 ± 1.4	11		40.20 ± 1.6
2		57.40 ± 2.2	12		53.90 ± 1.8
3		47.40 ± 1.8	13		55.40 ± 1.9
4		38.40 ± 1.4	14		30.20 ± 1.3
5		39.70 ± 1.4	15		54.00 ± 1.8
6		28.90 ± 1.2	16		27.20 ± 1.2
7		38.30 ± 1.5	17		83.20 ± 2.2

Table 1 (*continued*)

Compound	R	IC_{50} (μM ± SEM[a])	Compound	R	IC_{50} (μM ± SEM[a])
8		50.80 ± 1.9	18		30.10 ± 1.2
9		42.80 ± 1.5	19		48.80 ± 1.6
10		30.80 ± 1.2	Standard Thiourea[b]		21.20 ± 1.30

[a] Standard deviation.
[b] Standard drug.

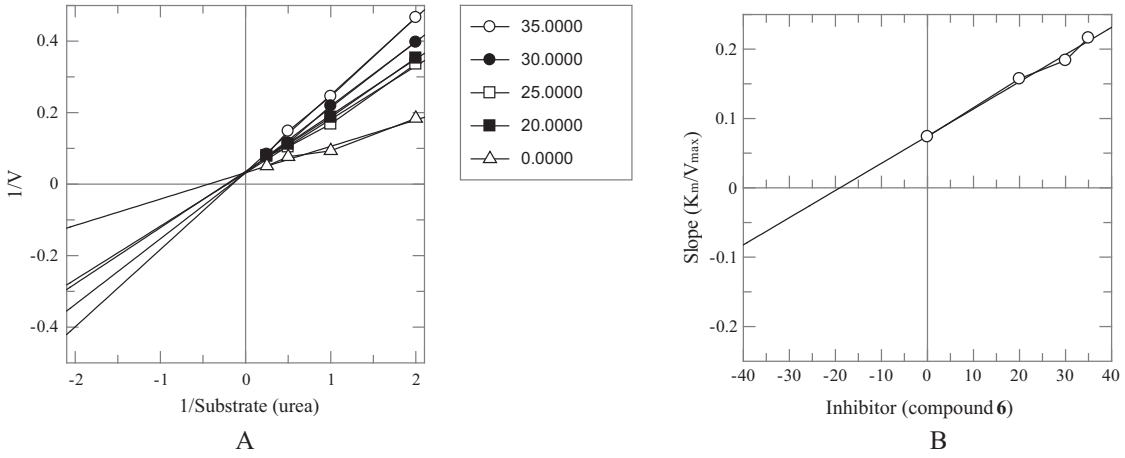


Figure 1 The inhibition of urease by compound **6**. (A) Lineweaver–Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (urea) in the absence (△), and in presence of 20 μM (■), 25 μM (□), 30 μM (●), and 35 μM (○) of compound **6**. (B) Secondary replot of Lineweaver–Burk plot between the slopes of each line on Lineweaver–Burk plot *vs* different concentration of compound **6**.

Helicobacter pylori (HP), by helping the bacteria to endure at low pH of the stomach during colonization. Thus, it plays a vital role in the pathogenesis of the gastric as well as peptic ulcers which may cause cancer (Devesa et al., 1998). Additionally, urease causes kidney stones formation but also engages in the growth of urolithiasis, pyelonephritis, and hepatic encephalopathy (Martelli et al., 1981). In agriculture, during urea fertilization, high urease activity results in significant environmental and economic losses by discharge of abnormally huge amounts of ammonia in atmosphere. This also leads to plant damage by depriving them from essential nutrients, secondary ammonia toxicity and increase in pH of the soil (Mobley and Hausinger, 1989). Urease inhibition, therefore, has been identified as first line of treatment of diseases caused by ureolytic bacteria (Saify et al., 2014). Recently reported urease inhibitors are hydroxamate complex (Cheng et al., 2014), homoserine lactone derivative (Czerwonka et al., 2014), thiophosphoric triamides (Ludden et al., 2000), oxadiazoles derivatives (Akhtar et al., 2014), thioureas (Khan et al., 2014b), ethyl 4-(3-benzothioureido) benzoates derivatives (Saeed et al., 2014), Oxindole derivatives (Taha et al., 2015c) and thiobarbituric acid derivatives (Khan et al., 2014c). However, currently available inhibitors are not efficient and the full potential of urease inhibition is yet to be discovered (Font et al., 2008).

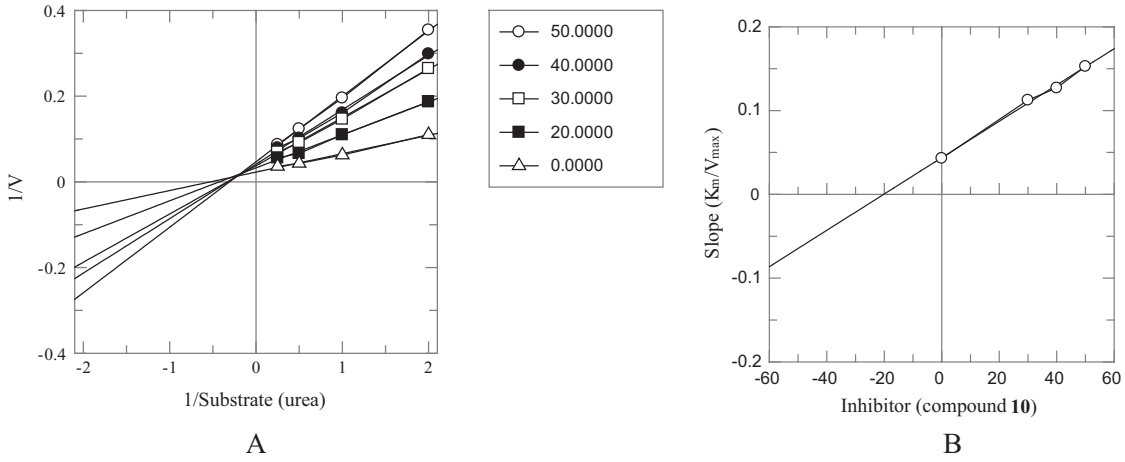


Figure 2 The inhibition of urease by compound **10**. (A) Lineweaver–Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (urea) in the absence (△), and in presence of 20 μM (■), 30 μM (□), 40 μM (●), and 50 μM (○) of compound **10**. (B) Secondary replot of Lineweaver–Burk plot between the slopes of each line on Lineweaver–Burk plot *vs* different concentration of compound **10**.

Table 2 The mode of inhibition of compounds **6**, **10**, **14**, **16**, and **18**.

Compounds	K_i (μM)	Type of inhibition
6	19.10 ± 0.007	Competitive inhibition
10	19.90 ± 0.003	Mixed-type inhibition
14	21.71 ± 0.007	Mixed-type inhibition
16	10.53 ± 0.02	Competitive inhibition
18	18.41 ± 0.003	Mixed-type inhibition
Standard (thiourea)	20.01 ± 0.02	Competitive inhibition

2. Experimental

2.1. General experimental

Melting points were determined on a Büchi 434 melting point apparatus and are uncorrected. NMR was performed on Bruker AV 300, 400, and 500 MHz instruments, respectively. CHN analyses were determined on a Carlo Erba Strumentazion-Mod-1106, Italy instrument. Infrared (IR) spectra were recorded on a JASCO IR-A-302 spectrometer. Electron impact mass spectra (EI MS) were recorded on a Finnigan MAT-311A, Germany spectrometer. Thin layer chromatography (TLC) was performed on precoated silica gel glass plates (Kieselgel 60, 254, E. Merck, Germany).

2.2. Experimental protocol

2.2.1. General procedure for the synthesis of 3,4,5-trihydroxybenzohydrazide

The methyl 3,4,5-trihydroxybenzoate was refluxed with the mixture of hydrazine hydrated (5 mL) and methanol (15 mL) for 6 h. The excess hydrazine and methanol were evaporated to obtain crude product which was recrystallized by methanol and yielded 92% pure 3,4,5-trihydroxybenzohydrazide.

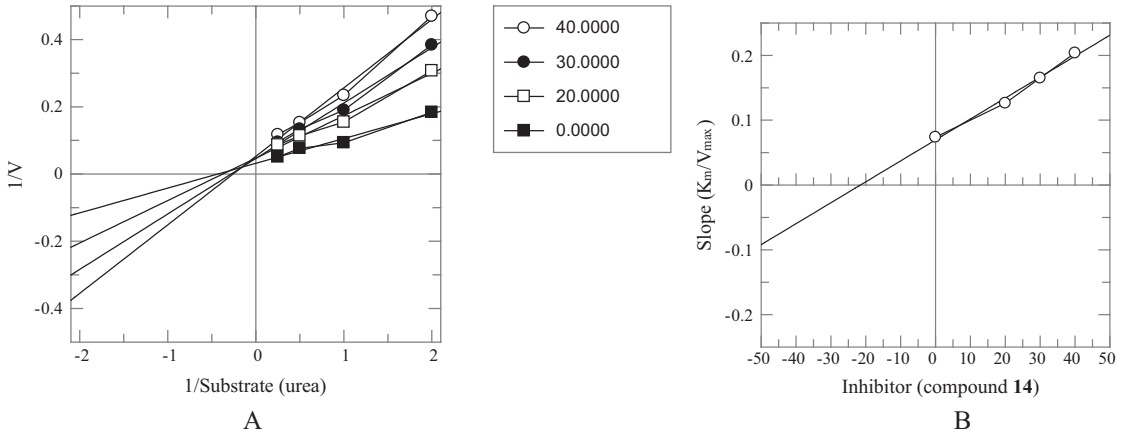


Figure 3 The inhibition of urease by compound **14**. (A) Lineweaver–Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (urea) in the absence (■), and in presence of 20 μM (□), 30 μM (●), and 40 μM (○) of compound **14**. (B) Secondary replot of Lineweaver–Burk plot between the slopes of each line on Lineweaver–Burk plot *vs* different concentration of compound **14**.

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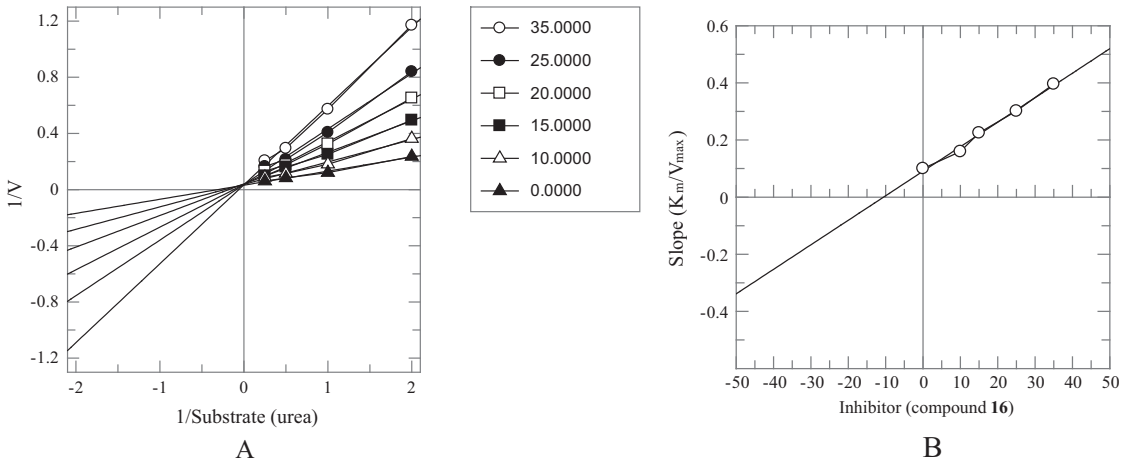

Figure 4 The inhibition of urease by compound **16**. (A) Lineweaver–Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (urea) in the absence (▲), and in presence of 10 μM (△), 15 μM (■), 20 μM (□), 25 μM (●), and 30 μM (○) of compound **16**. (B) Secondary replot of Lineweaver–Burk plot between the slopes of each line on Lineweaver–Burk plot *vs* different concentration of compound **16**.

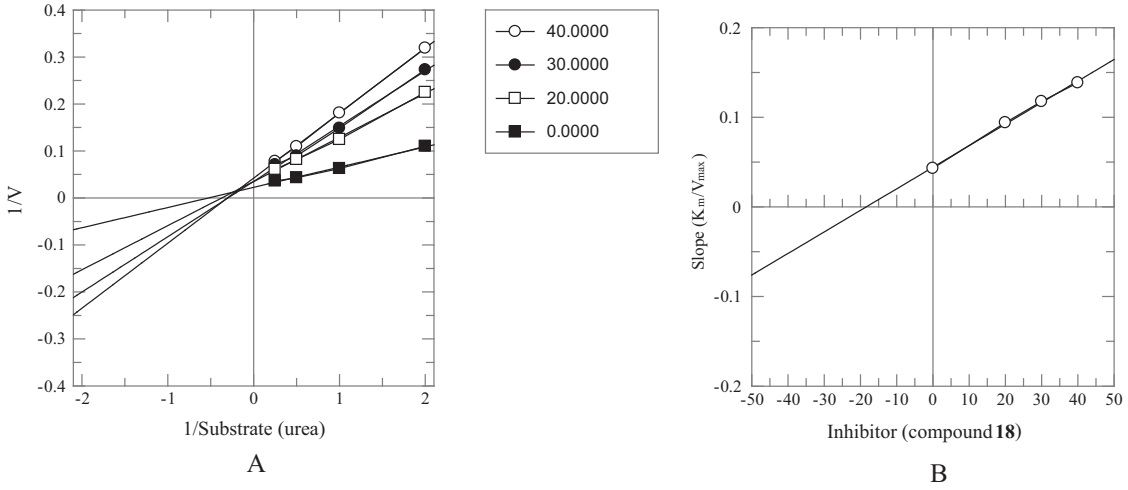

Figure 5 The inhibition of urease by compound **18**. (A) Lineweaver–Burk plot of reciprocal of rate of reaction (velocities) *vs* reciprocal of substrate (urea) in the absence (■), and in presence of 20 μM (□), 30 μM (●), and 40 μM (○) of compound **18**. (B) Secondary replot of Lineweaver–Burk plot between the slopes of each line on Lineweaver–Burk plot *vs* different concentration of compound **18**.

2.2.2. General procedure for the synthesis of 3,4,5-trihydroxybenzohydrazone derivatives

The 3,4,5-trihydroxybenzohydrazone derivatives were synthesized by refluxing in methanol a mixture of 2 mmol each of 3,4,5-trihydroxybenzohydrazide with different aldehydes and catalytic amount of acetic acid for 3 h. After the completion of the reaction, the solvent was evaporated by vacuum to afford crude products which were further recrystallized in methanol and got needle like pure product in most of the cases in good to excellent yields.

2.2.2.1. (E)-N′-(3-chlorobenzylidene)-3,4,5-trihydroxybenzohydrazide (1). Yield: 82%. m.p. 172–173 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.64 (s, 1H, NH), 9.64 (br. s, 3H, OH), 8.34 (s, 1H, N═CH─Ar), 7.77 (s, 1H), 7.66 (d, 1H, J = 5.5 Hz), 7.06 (dd, 1H, J = 5.5, 2.0 Hz), (d, 1H, J = 2.0 Hz), 6.91(s, 2H); Anal. Calcd for Anal. Calcd for $C_{14}H_{11}ClN_2O_4$, C = 54.83, H = 3.62, N = 9.13, Found C = 54.84, H = 3.63, N = 9.15; EI MS m/z (% rel. abund.): 308 (M + 2, 6), 306 (M^+, 20).

2.2.2.2. (E)-3,4,5-trihydroxy-N′-(4-methoxybenzylidene)benzohydrazide (2). Yield: 78%. m.p. 174–175 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.70 (s, 1H, NH), 11.40 (s, 1H, OH) 9.88 (br. s, 3H, OH), 831 (s, 1H, N═CH─Ar), 7.92 (d, 2H, J = 8.5 Hz), 7.06 (d, 2H, J = 8.5 Hz), 6.90 (s, 2H), 3.81 (s, 3H, OCH_3); Anal. Calcd for Anal. Calcd for $C_{15}H_{14}N_2O_5$, C = 59.60, H = 4.67, N = 9.27, Found C = 59.61, H = 4.68, N = 9.26; EI MS m/z (% rel. abund.): 302.

2.2.2.3. (E)-3,4,5-trihydroxy-N′-(pyridin-2-ylmethylene)benzohydrazide (3). Yield: 84%. m.p. 175–176 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.48 (s, 1H, NH), 10.54 (s, 1H,

Table 3 Variation in the IR and H NMR of all functional groups present in compound (**1–19**).

Compound	R	IR (cm^{-1})	^1H NMR
1	3-Cl	715	Due to chlorine electronegativity adjacent proton shifted to downfield
2	4-OCH3	1210	The presence of OCH_3 at δ 3.81
3	2-C═N─C	1640	Due to nitrogen electronegativity adjacent proton shifted to downfield
4	Thiophene C─S─C	540	Due to small ring its proton coupling constants are 4–5 Hz only
5	4-F	1340	Its adjacent proton gave multiple splitting due to fluorine
6	Furan C─O─C	1230	Due to small ring its proton coupling constants are 4–5 Hz only
7	3-C─CH_3	2950 1440	The presence of CH_3 at δ 2.36
8	3-OH 4-OCH_3	3320 1190	The presence of OH δ 9.20 and at 3.86 for OCH_3
9	4-C═N─C	1635	Due to nitrogen electronegativity adjacent proton shift to downfield
10	2-NO_2	1460	Due to nitrogen electronegativity adjacent proton shift to downfield
11	3-NO_2	1430	Due to nitrogen electronegativity adjacent proton shift to downfield
12	4-Cl	740	Due to chlorine electronegativity adjacent proton shift to downfield
13	4-CH_3	2930 1460	The presence of CH_3 at δ 2.33
14	3-F	1342	Its adjacent proton gave multiple splitting due to fluorine
15	2-CH_3	2937 1452	The presence of CH_3 at δ 2.35
16	3-OH	3340	The presence of OH δ 10.21
17	4-OCH_3	1190	The presence of OCH_3 at δ 3.80
18	2-F	1310	Its adjacent proton gave multiple splitting due to fluorine
19	4-COOMe	1710 1220	The presence of OCH_3 at δ 3.84

OH), 9.70 (br. s, 2H, OH), 9.06 (d, 1H, J = 2.0 Hz), 8.70 (dd, 1H, J = 7.0, 2.0 Hz), 8.41 (dd, 2H, J = 2.0, 6.5 Hz), 831 (s, 1H, N═CH─Ar), 7.82 (dd, 1H, J = 7.0, 6.5 Hz), 6.94 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{13}H_{11}N_3O_4$, C = 57.14, H = 4.06, N = 15.38, Found C = 57.15, H = 4.08, N = 15.37; EI MS m/z (% rel. abund.): 273.

2.2.2.4. (E)-3,4,5-trihydroxy-N′-(thiophen-2-ylmethylene)benzohydrazide (4). Yield: 85%. m.p. 179–180 °C; ^1H NMR (500 MHz, DMSO-d_6): δ 11.48 (s, 1H, NH), 9.60 (br. s, 3H, OH), 8.61 (s, 1H, N═CH─Ar), 7.49 (d, 1H, J = 5.0 Hz), 7.41 (d, 1H, J = 3.0 Hz), 7.22 (dd, 1H, J = 3.0, 5.0 Hz), 6.89 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{12}H_{10}N_2O_4S$, C = 51.79, H = 3.62, N = 10.07, Found C = 59.78, H = 3.63, N = 10.09; EI MS m/z (% rel. abund.): 278.

2.2.2.5. (E)-N′-(4-fluorobenzylidene)-3,4,5-trihydroxybenzohydrazide (5). Yield: 86%. m.p. 171–172 °C; ^1H NMR (500 MHz, DMSO-d_6): δ 11.60 (s, 1H, NH), 9.80 (br. s, 3H, OH), 8.37 (s, 1H, N═CH─Ar), 7.78 (t, 2 H, J = 5.5 Hz), 7.33 (t, 1H, J = 6.0 Hz), 6.91 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{14}H_{11}FN_2O_4$, C = 57.93, H = 3.82, N = 9.65, Found C = 57.94, H = 3.83, N = 9.67; EI MS m/z (% rel. abund.): 290.

2.2.2.6. (E)-N'-(furan-2-ylmethylene)-3,4,5-trihydroxybenzohydrazide (6). Yield: 81%. m.p. 178–179 °C; ^1H NMR (500 MHz, DMSO-d_6): δ 11.42 (s, 1H, NH), 9.70 (br. s, 3H, OH), 8.42 (s, 1H, N═CH─Ar), 7.60 (d, 1H, J = 6.0 Hz), 7.35 (d, 1H, J = 4.0 Hz), 7.41 (d, 1H, J = 3.0 Hz), 7.10 (dd, 1H, J = 4.0, 6.0 Hz), 6.92 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{12}H_{10}N_2O_5$, C = 54.97, H = 3.84, N = 10.68, Found C = 54.96, H = 3.84, N = 10.69; EI MS m/z (% rel. abund.): 262.

2.2.2.7. (E)-3,4,5-trihydroxy-N′-(3-methylbenzylidene)benzohydrazide (7). Yield: 83%. m.p. 175–176 °C; ^1H NMR (500 MHz, DMSO-d_6): δ 11.51 (s, 1H, NH), 9.42 (br. s, 3H, OH), 8.42 (s, 1H, N═CH─Ar), 7.52 (s, 1H), 7.48 (d, 1H, J = 7.5 Hz), 7.35 (t, 1H, J = 7.5 Hz), 7.10 (d, 1H, J = 8.0 Hz), 6.93 (s, 2H), 2.36 (3H, CH_3); Anal. Calcd for Anal. Calcd for $C_{15}H_{14}N_2O_4$, C = 62.93, H = 4.93, N = 9.79, Found C = 62.94, H = 3.94, N = 9.81; EI MS m/z (% rel. abund.): 286.

2.2.2.8. (E)-3,4,5-trihydroxy-N′-(3-hydroxy-4-methoxybenzylidene)benzohydrazide (8). Yield: 88%. m.p. 172–173 °C; ^1H NMR (500 MHz, DMSO-d_6): δ 11.34 (s, 1H, NH), 9.51 (br. s, 3H, OH), 9.21 (s, 1H, OH), 8.25 (s, 1H, N═CH─Ar), 7.23 (s, 1H), 7.03 (d, 1H, J = 8.5 Hz), 6.97 (t, 1H, J = 8.5 Hz), 6.90 (s, 2H), 3.86 (3H, OCH_3); Anal. Calcd for Anal. Calcd for $C_{15}H_{14}N_2O_6$, C = 56.60, H = 4.43, N = 8.80, Found C = 56.62, H = 4.44, N = 8.82; EI MS m/z (% rel. abund.): 318.

2.2.2.9. (E)-3,4,5-trihydroxy-N′-(pyridin-4-ylmethylene)benzohydrazide (9). Yield: 90%. m.p. 174–175 °C; ^1H NMR (500 MHz, DMSO-d_6): δ 11.48 (s, 1H, NH), 10.54 (s, 1H, OH), 9.70 (br. s, 2H, OH), 8.79 (d, 2H, J = 6.0 Hz), 8.35 (s, 1H, N═CH─Ar), 7.66 (d, 2H, J = 6.0, Hz), 6.93 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{13}H_{11}N_3O_4$, C = 57.14, H = 4.06, N = 15.38, Found C = 57.16, H = 4.09, N = 15.36; EI MS m/z (% rel. abund.): 273.

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2.2.2.10. (E)-3,4,5-trihydroxy-N′-(2-nitrobenzylidene)benzohydrazide (10). Yield: 87%. m.p. 170–171 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.92 (s, 1H, NH), 9.84 (br. s, 3H, OH), 8.82 (s, 1H, N═CH─Ar), 8.13 (d, 1H, J = 7.5 Hz), 8.08 (dd, 1H, J = 2.0, 7.0 Hz), 7.89 (t, 1H, J = 7.5 Hz), 7.68 (ddd, 1H, J = 7.5, 2.0, 2.0 Hz), 6.92 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{14}H_{11}N_3O_6$, C = 53.00, H = 3.49, N = 13.24, Found C = 53.02, H = 3.50, N = 13.26; EI MS m/z (% rel. abund.): 317.

2.2.2.11. (E)-3,4,5-trihydroxy-N′-(3-nitrobenzylidene)benzohydrazide (11). Yield: 92%. m.p. 171–172 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.79 (s, 1H, NH), 9.74 (br. s, 3H, OH), 8.52 (s, 1H), 8.46 (s, 1H, N═CH─Ar), 8.26 (dd, 1H, J = 6.5, 2.0 Hz), 8.13 (d, 1H, J = 7.5 Hz), 7.77 (t, 1H, J = 8.0 Hz), 6.95 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{14}H_{11}N_3O_6$, C = 53.00, H = 3.49, N = 13.24, Found C = 53.01, H = 3.51, N = 13.25; EI MS m/z (% rel. abund.): 317.

2.2.2.12. (E)-N′-(4-chlorobenzylidene)-3,4,5-trihydroxybenzohydrazide (12). Yield: 90%. m.p. 180–181 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.60 (s, 1H, NH), 9.65 (br. s, 3H, OH), 8.35 (s, 1H, N═CH─Ar), 7.74 (d, 2H, J = 8.0 Hz), 7.50 (d, 2H, J = 8.0, Hz), 6.90 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{14}H_{11}ClN_2O_4$, C = 54.83, H = 3.62, N = 9.13, Found C = 54.84, H = 3.61, N = 9.14; EI MS m/z (% rel. abund.): 308 (M + 2, 20), 306 (M^+, 60).

2.2.2.13. (E)-3,4,5-trihydroxy-N′-(4-methylbenzylidene)benzohydrazide (13). Yield: 87%. m.p. 177–178 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.44 (s, 1H, NH), 9.80 (br. s, 3H, OH), 8.33 (s, 1H, N═CH─Ar), 7.60 (d, 2H, J = 8.0 Hz), 7.27 (d, 2H, J = 8.0, Hz), 6.90 (s, 2H), 2.33 (3H, CH_3); Anal. Calcd for Anal. Calcd for $C_{15}H_{14}N_2O_4$, C = 62.93, H = 4.93, N = 9.79, Found C = 62.94, H = 4.92, N = 9.80; EI MS m/z (% rel. abund.): 286.

2.2.2.14. (E)-N′-(3-fluorobenzylidene)-3,4,5-trihydroxybenzohydrazide (14). Yield: 80%. m.p. 175–176 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.63 (s, 1H, NH), 9.70 (br. s, 3H, OH), 8.37 (s, 1H, N═CH─Ar), 7.54–7.46 (m, 3 H), 7.33 (td, 1H, J = 6.5, 2.0 Hz), 6.91 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{14}H_{11}FN_2O_4$, C = 57.93, H = 3.82, N = 9.65, Found C = 57.95, H = 3.81, N = 9.66; EI MS m/z (% rel. abund.): 290.

2.2.2.15. (E)-3,4,5-trihydroxy-N′-(2-methylbenzylidene)benzohydrazide (15). Yield: 82%. m.p. 169–170 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.40 (s, 1H, NH), 9.60 (br. s, 3H, OH), 8.69 (s, 1H, N═CH─Ar), 7.85 (d, 1H, J = 7.5 Hz), 7.31–7.23 (m, 3H), 6.92 (s, 2H), 2.35 (3H, CH_3); Anal. Calcd for Anal. Calcd for $C_{15}H_{14}N_2O_4$, C = 62.93, H = 4.93, N = 9.79, Found C = 62.94, H = 4.94, N = 9.81; EI MS m/z (% rel. abund.): 286.

2.2.2.16. (E)-3,4,5-trihydroxy-N′-(3-hydroxybenzylidene)benzohydrazide (16). Yield: 90%. m.p. 172–173 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.47 (s, 1H, NH), 10.21 (s, 1H, OH), 9.74 (br. s, 3H, OH), 8.32 (s, 1H, N═CH─Ar), 7.26 (t, 1H, J = 8.0 Hz), 7.16 (s, 1H), 7.07 (d, 1H, J = 8.0 Hz), 6.92 (s, 2H), 7.16 (dd, 1H, J = 8.0, 2.0 Hz); Anal. Calcd for Anal. Calcd for $C_{14}H_{12}N_2O_5$, C = 58.33, H = 4.20, N = 9.72, Found C = 58.34, H = 4.21, N = 9.71; EI MS m/z (% rel. abund.): 288.

2.2.2.17. (E)-3,4,5-trihydroxy-N′-(3-methoxybenzylidene)benzohydrazide (17). Yield: 82%. m.p. 170–171 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.53 (s, 1H, NH), 9.74 (br. s, 3H, OH), 8.33 (s, 1H, N═CH─Ar), 7.26 (t, 1H, J = 7.5 Hz), 7.30 (s, 1H), 7.26 (d, 1H, J = 8.0 Hz), 6.99 (dd, 1H, J = 8.0, 2.0 Hz) 6.91 (s, 2H), (s, 3H, OCH_3); Anal. Calcd for Anal. Calcd for $C_{15}H_{14}N_2O_5$, C = 59.60, H = 4.67, N = 9.27, Found C = 59.61, H = 4.66, N = 9.28; EI MS m/z (% rel. abund.): 302.

2.2.2.18. (E)-N′-(2-fluorobenzylidene)-3,4,5-trihydroxybenzohydrazide (18). Yield: 90%. m.p. 171–172 °C; ^{1}H-NMR (500 MHz, DMSO-d_6): δ 11.68 (s, 1H, NH), 9.50 (br. s, 3H, OH), 8.66 (s, 1H, N═CH─Ar), 7.92 (t, 3 H, J = 6.5 Hz), 7.48–7.46 (m, 1H), 7.31–7.26 (m, 2H), 6.94 (s, 2H); Anal. Calcd for Anal. Calcd for $C_{14}H_{11}FN_2O_4$, C = 57.93, H = 3.82, N = 9.65, Found C = 57.94, H = 3.83, N = 9.64; EI MS m/z (% rel. abund.): 290.

2.2.2.19. (E)-Methyl 4-((2-(3,4,5-trihydroxybenzo)hydrazono)methyl)benzoate (19). Yield: 92%. m.p. 180–181 °C; ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.60 (s, 1H, NH), 9.40 (br. s, 3H, OH), 8.36 (s, 1H, N═CH─Ar), 8.20 (d, 2H, J = 8.0 Hz), 7.84 (d, 2H, J = 8.0, Hz), 6.91 (s, 2H), 3.80 (3H, OCH_3); Anal. Calcd for Anal. Calcd for $C_{16}H_{14}N_2O_6$, C = 58.18, H = 4.27, N = 8.48, Found C = 58.19, H = 4.28, N = 8.49; EI MS m/z (% rel. abund.): 314.

2.2.3. Urease assay and inhibition

The reaction mixtures, comprising 25 μL of enzyme (jack bean urease) solution and 55 μL of buffers containing 100 mM urea, were incubated with 5 μL of the test compounds (0.5 mM concentration) at 30 °C for 15 min in 96-well plates. For the kinetics assessment the urea concentrations were changed from 2 to 24 mM. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn (1967). Briefly, 45 μL of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and, 70 μL of alkali reagent (0.5% w/v NaOH and 0.1% active chloride NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Device, USA). All reactions were performed in triplicate in a final volume of 200 μL. The results (change in absorbance per min) were processed by using SoftMaxPro software (molecular Device, USA). The entire assays were performed at pH 6.8. Percentage inhibition was calculated from the formula $100\text{-}(OD_{test\ well}/OD_{control}) \times 100$. Thiourea was used as the standard inhibitor for urease (Khan et al., 2014d).

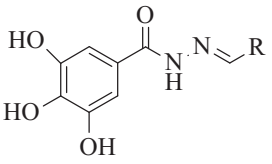
3. Results and discussion

3.1. Chemistry

In the continuation of our research on enzyme inhibition (Rahim et al., 2015a,b; Taha et al., 2015d; Abdullah et al., 2015; Khan et al., 2014e) 3,4,5-trihydroxybenzohydrazones

(**1–19**) were synthesized from 3,4,5-trihydroxybenzohydrazide which were obtained from methyl 3,4,5-trihydroxybenzoate by refluxing with hydrazine hydrate for 4 h. The 3,4,5-trihydroxybenzohydrazide obtained was recrystallized from methanol. 3,4,5-Trihydroxybenzohydrazones were prepared by refluxing 3,4,5-trihydroxybenzohydrazide with differently substituted aldehydes **1–19** in methanol for 3–4 h (Scheme 1). The crude products obtained were recrystallized in methanol and mostly needle like crystals were obtained in 74–87% yield. The structures of 3,4,5-trihydroxybenzohydrazones were deduced by using various spectroscopic techniques and CHN analyses.

Table 1 Derivatives of 3,4,5-Trihydroxybenzohydrazone **1–19**.



3.2. Urease inhibition

In the continuation of our work, on enzyme inhibition we synthesized **1–19** 3,4,5-trihydroxybenzohydrzones. They were evaluated for urease inhibition. The compounds **16**, **6**, **18**, **14** and **10** showed good activities. The compounds **1**, **4**, **7**, **5**, **11** and **9**, **3**, **19** showed moderate activities while, compounds **8**, **12**, **15**, **13** and **17** showed weak activity (Table 1).

The compound **16** (IC_{50} = 27.20 ± 1.2 μM) was found to be the most active among the series of nineteen compounds. It was found that this activity is due to the hydroxyl group present at the 3' position and this was confirmed by compound **17** (IC_{50} = 83.20 ± 2.2 μM) in which hydroxyl group is replaced by methoxy substituent which resulted in the decrease of activity by almost 3-fold. Compound **8** (IC_{50} = 50.80 ± 1.9 μM) having 3′-hydroxy-4′-methoxy showed less activity than compound **16**. This might be due to the bulky methoxy group at 4'-position, which causes compound 8 to become structurally non-compatible toward urease enzyme. The compound **2** (IC_{50} = 57.40 ± 2.2 μM) having 4′-methoxy showed only weak activity further proved the significance of hydroxy at position 3'.

The compound **6** (IC_{50} = 28.90 ± 1.2 μM) was found to be the second most active compound of the series and its activity is due to the furan ring which would be having interaction with enzyme.

Compound **4** (IC_{50} = 38.40 ± 1.4 μM), which is a thiophene derivative, showed less activity as compared to the compound **6** (consists of furan ring) and this may be due to large size and low electronegative nature of sulfur as compared to oxygen. Higher electronegativity effect of oxygen allows furan-containing derivative **6** to bind better through hydrogen bonding with urease enzyme as compared to thiophene moiety of compound **4**.

Among the pyridine derivatives, compound **9** (IC_{50} = 42.80 ± 1.5 μM), which is 4′-pyridinyl derivative, showed good activity as compared to its positional isomer 2′-pyridene analogue **3** (IC_{50} = 47.40 ± 1.8 μM).

The compound **18** (IC_{50} = 30.10 ± 1.2 μM) having fluorine atom at 2′-postion and compound **14** (IC_{50} = 30.20 ± 1.3 μM) having fluorine atom at 3′-postion showed similar activity while interestingly its other positional isomer compound **5** (IC_{50} = 39.70 ± 1.4 μM), having fluorine atom at 4′-postion, exhibited slightly lower reactivity. Therefore, the order of reactivity of fluorinated derivative was found to be 2′-F > 3′-F > 4′-F.

Other halogen substituted, compounds **1** (IC_{50} = 37.30 ± 1.4 μM) and **12** (IC_{50} = 53.90 ± 1.8 μM) exhibited the same activity trend, such as, 3′-Cl > 4′-Cl. The compound **10** (IC_{50} = 30.80 ± 1.2 μM) having 2′-nitro substituent showed good activity but its other isomer having 3′-nitro showed weak activity.

The compound **7** having 3′-methyl showed good activity as compared to its other analogues having 2′-methyl, compound **15** (IC_{50} = 54.00 ± 1.8 μM) and 4′-methyl (compound **13** IC_{50} = 55.40 ± 1.9 μM). Their order of reactivity was found to be 3′-methyl > 2′-methyl > 4′-methyl, while the compound **19** (IC_{50} = 48.80 ± 1.6 μM) having ester group showed moderate activity.

To investigate the inhibition mechanism of this series, the kinetic studies on five most active compounds **6**, **10**, **14**, **16** and **18** were performed, with different concentrations of test compounds and substrates. Enzyme reaction is the first order reaction and the enzyme kinetics were used for only the determination of type of inhibition and Ki value. From kinetic studies, it was inferred that compounds **6** and **16** are competitive inhibitors with K_i values 19.1 ± 0.007 and 10.53 ± 0.02 μM, respectively (Figs. 1 and 2). The type of inhibition was determined by Lineweaver–Burk plots. The reciprocal of the rate of the reaction was plotted against the reciprocal of substrate concentration to monitor the effect of inhibitor on both K_m and V_{max}. Figs. 1 and 2 showed that in the presence of compounds **6** and **16**, the V_{max} of jack bean urease enzyme was not affected, while the K_m of enzyme increased, which indicates the competitive inhibition (Table 2).

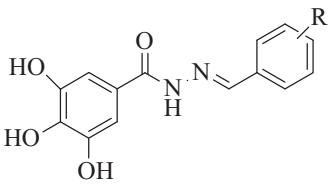
The secondary replots of Lineweaver–Burk plots (Lodhi et al., 2007; Muhammad Et al., 2014) were plotted to determine the K_i value (Fig. 2). The K_i values were calculated by plotting the slope of each line in the Lineweaver–Burk plots against different concentrations of compounds **6** and **16**. The K_i value was confirmed from Dixon plot, by plotting the reciprocal of the rate of reaction against the different concentrations of compounds **6** and **16**.

The kinetic studies of compounds **10**, **14** and **18** indicated that these are mixed-type of inhibitors with K_i values between 18.41 and 21.71 μM (Figs. 3–5). The Lineweaver–Burk plots of compounds **10**, **14** and **18** showed that in the presence of compounds **10**, **14** and **18**, both the V_{max} and K_m of jack bean urease were affected. In the presence of compounds **10**, **14** and **18** the V_{max} of jack bean urease was decreased, while the K_m was increased, which indicated the mixed-type of inhibition. Again for K_i determination of compounds **10**, **14** and **18** the secondary replots of Lineweaver–Burk plots and Dixon plot were used.

4. Difference in IR and NMR data responsible for inhibition activity

The first ring of compounds (**1–19**) is common. The variation in inhibition potential is mainly due to the functional group present on second ring. We can easily distinguish by IR and

NMR by observing the pick of functional group. In case of NMR inactive functional group we can see the variation in Chemical shift of adjacent Proton Table 3.



5. Conclusion

In this study, we synthesized 3,4,5-trihydroxybenzohydrazones (**1–19**) and evaluated them for their urease inhibition activity. The results for urease inhibition showed excellent activity, close to the standard thiourea. We found new class of urease inhibitors. The kinetic studies on the five most active compounds **6**, **10**, **14**, **16** and **18** were carried out. The compounds **6** and **16** were found to be competitive inhibitors and the compounds **10**, **14** and **18** were found to be mixed-type of inhibitors.

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