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Synthesis and antioxidant, antiurease and anti-xanthine oxidase activities of some new benzimidazoles bearing triazole, oxadiazole, thiadiazole and imin function

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In this study, a series of new 2-(4-nitrobenzyl)-1*H*-benzimidazole derivatives bearing thiosemicarbazide, triazole, oxadiazole and thiadiazole moieties at the 1st position of benzimidazole ring have been synthesized and screened for their antioxidant, antiurease and anti-xanthine oxidase activities. Antioxidant activities of the synthesized compounds have been determined with CUPric Reducing Antioxidant Capacity (CUPRAC), ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/persulfate and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays. Most of the compounds show significant antioxidant activity. Also, among the tested compounds, **9c** displays the best inhibitory effect against XO with an IC_{50} value of 12.30±0.33 µg/mL and compound **7a**shows the best inhibitory effect against urease with an IC_{50} value of 13.04±0.89 µg/mL.

Keywords: Benzimidazole, antioxidant, antiurease, anti-xanthine oxidase

Benzimidazoles and its derivatives are a significant class of bioactive compounds in the field of drugs and pharmaceuticals[1,2]. Benzimidazole nucleus is found in biologically important natural compounds. The structure of vitamin B_{12} contains benzimidazole core and purine has relationship with benzimidazole. Benzimidazoles play an role important in the medicinal chemistry with their biological actions like antitumor, antiviral, antimicrobial, antioxidant, anti-xanthine oxidase, antiurease, anticonvulsant, antilipase, and anti-inflammatory activities[3-10]. In addition to these, some benzimidazole derivatives are in use as drugs such as Mebendazole, Flubendazole (antihelmintic), Astemizole (antihistaminic), Omeprazole, Lansoprazole (antiulcerative drug)[11-13], *etc*.

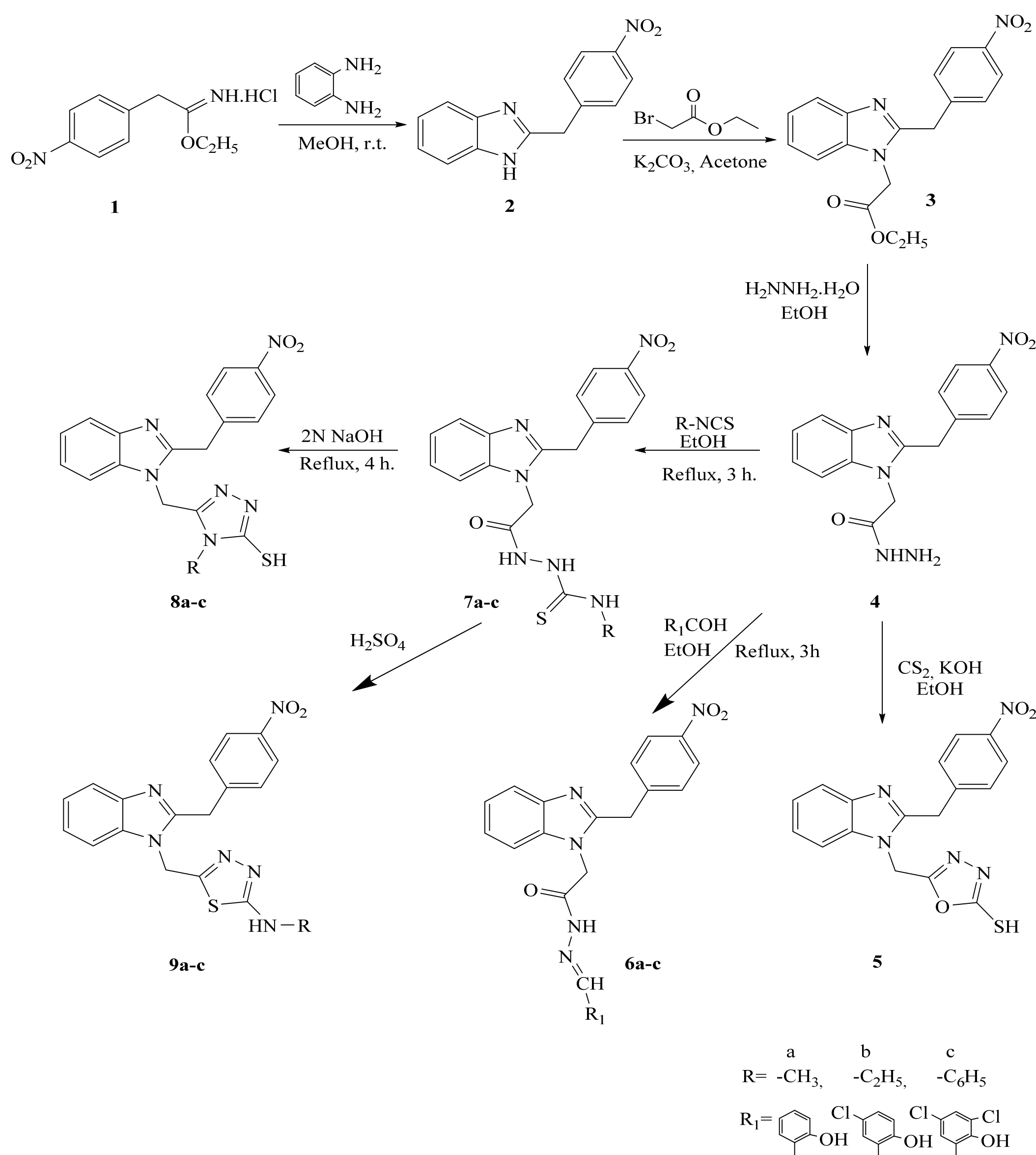
Various drugs contain triazole, thiadiazole, oxadiazole ring like Fluconazole, Itraconazole, Acetazolamide, Methazolamide, Raltegravir[14-17], *etc*. Different heterocyclic rings linked to benzimidazoles show diverse biological activities[5,6,18,19]. Benzimidazoles bearing thiosemicarbazide, triazole, thiadiazole and oxadiazole moiety in the 1st position of imidazole nucleus have demonstrated considerable antioxidant activity[5,6,20]. Besides, it has been found that *p*-fluoro, *p*-chloro or *p*-nitro phenyl groups lead to increas of biological activity[5,21,22].

In view of the above observations, we report herein the synthesis of some new benzimidazoles containing thiosemicarbazide, thiadiazole, triazole, and oxadiazole moieties and the biological evaluation of their antiurease, anti-xanthine oxidase and antioxidant properties.

Results and Discussion

Chemistry

Synthesis of all compounds has been carried out as depicted in Scheme I. Firstly, compound **1** was prepared according to the Pinner method[23]. Then, the treatment of compound **1** with *o*-phenylenediamine resulted in the formation of 2-(4-nitrobenzyl)-1*H*-benzimidazole **2**. The reaction of compound **2** with ethylbromoacetate in the presence of potassium carbonate produced ethyl[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetate (**3**), then treated with hydrazine monohydrate to obtain 2-[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide (**4**). The reaction of compound **4** with carbon disulfide in the presence of potassium hydroxide resulted in the formation of 5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-1,3,4-oxadiazole-2-thiol (**5**). In the next step, Schiff base compounds **6a-c** were obtained by reacting corresponding salicylaldehydes with compound **4**. Also, the nucleophilic addition of compound **4** to methyl-, ethyl- and phenylisothiocyanate afforded the corresponding hydrazinecarbothioamide derivatives (**7a-c**). In the final step, the intramolecular cyclization



Scheme I — Synthetic pathway for the preparation of compounds**2-5**, **6a-c**, **7a-c**, **8a-c** and **9a-c**

of the compounds **7a-c**led to the formation of 1,2,4-triazole compounds (**8a-c**) in the presence of 2N NaOH and 1,3,4-thiadiazole derivatives (**9a-c**) in the presence of conc. H_2SO_4.

The structures of newly synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, mass spectroscopy and elemental analyses. ^{1}H and ^{13}C NMR spectra of all compounds exhibited the suitable signals with the proposed structures. N-H, NH_2 and O-H signals were established by D_2O exchange. The compounds containing aryldien-hydrazide structure may exist as *E*/*Z* geometrical isomers around the C=N double bond and as *cis*/*trans* amide conformers[24,25]. In the ^{1}H NMRspectra of the compounds **6a-c**, $-CH_2$, N-CH_2, N=CH, -OH and -NH were observed in two sets of signals because of *cis*/*trans* conformers. The ^{1}H NMR spectrum of compound **6a** is given in Figure 1.

In addition, all compounds have suitable molecular ion peaks and reasonable elemental analysis data.

Antioxidant Activity

In this study, antioxidant activities of the synthesized compounds were clarified using various *in vitro* antioxidant assays including Cupric Reducing Antioxidant Capacity (CUPRAC), ABTS

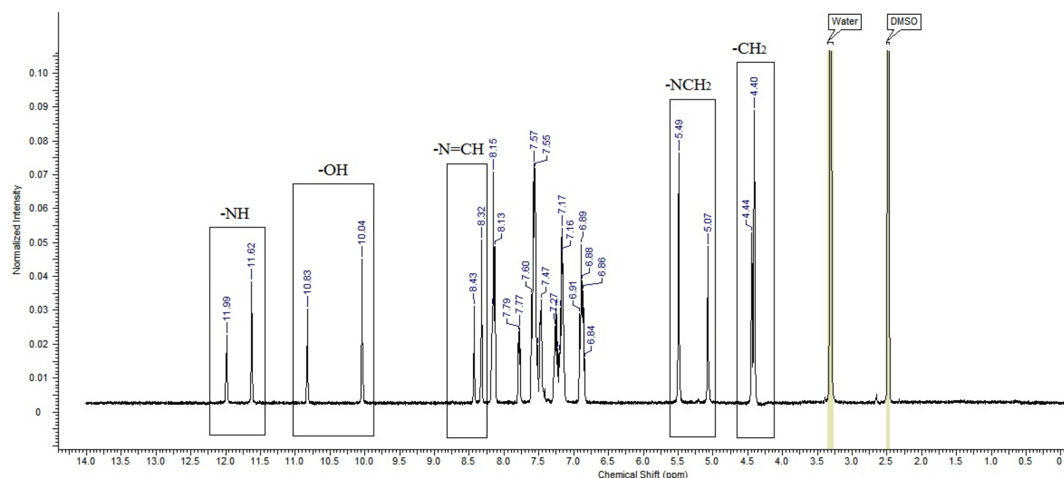


Figure 1 — ^{1}H NMR spectrum of compound **6a**

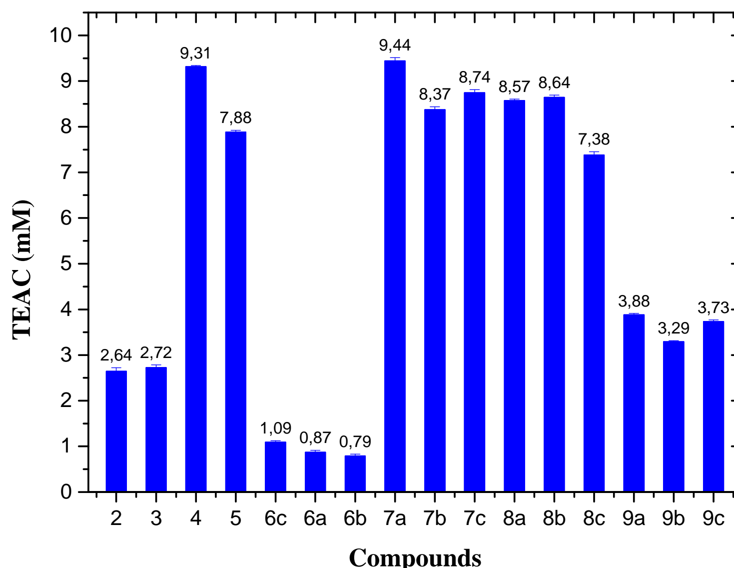


Figure 2 — CUPRAC test results of all the synthesized compounds as mM TEAC (Trolox equivalent antioxidant capacity) values obtained from [Trolox]- absorbance calibration graph (r^2=0.998). CUPRAC values of compounds are expressed as the mean ±SD in triplicate.

(2,2-azinobis(3-ethylbenzothiazoline-6-sulfonicacid)/ Persulfate and DPPH (1,1-diphenyl-2-picrylhydrazyl) assays. Catechin, BHT (butylated hydroxytoluene) and Ascorbic acid (AA) were used as positive antioxidant and radical scavenger molecules.

CUPRAC Antioxidant Activity Assay

The CUPRAC method is based on the absorbance measurement of the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine)copper(II) cation [Cu(II)-Nc], where absorbance is recorded at the maximal light absorption wavelength of 450 nm. The orange–yellow colour is due to the Cu(I)-Nc charge-transfer complex formed. Increased absorbance of the reaction mixture indicates increased reduction capability[26]. The antioxidant effects were classified by two groups; compounds **4**, **5**, **7a**, **7b**, **7c**, **8a**, **8b** and **8c** the most effective, was the first. The others, evaluated by the range of moderately, were the second (Figure 2).

DPPH Scavenging Assay

The total radical scavenging capacity of the compounds were determined and compared to that of BHT, ascorbic acid and catechin by using the $DPPH^•$ and $ABTS^{•+}$ radical scavenging methods. The DPPH method is based on the fact that the free radical is

purple in color, and that the purple color of DPPH decays in the presence of an antioxidant. The color changed from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. The results were expressed as SC_{50} (μg/mL), which was calculated from the curves by plotting absorbance values, the SC_{50} values representing the concentration of the compound (μg/mL) required to scavenge 50% of the radicals. Because of having the lowest SC_{50} value, the compound **4** was the best compared to the other synthesized compounds. The compounds **7a**, **7b** and **7c** showed fairly well DPPH radical scavenging activity. Also, it has been determined that SC_{50} values of these compounds were lower than BHT as a standard (Figure 3). On the other hand, the compounds **5**, **8a** and **8c** showed good activity.

All of the compounds active in the CUPRAC antioxidant assay exhibit enhanced radical scavenging activities in the DPPH method. According to the antioxidant test results of compounds, compounds having high CUPRAC value such as **4**, **5**, **7a**, **7b**, **7c**, **8a**, **8b**and **8c**exhibited very good DPPH radical scavenging activity. Consequently, it has been determined that compounds having higher CUPRAC value have lower SC_{50} values. It was observed to have a strong negative correlation between the cuprac values with the SC_{50} values of these compounds (Figure 4). This result could be seen as a normal. There is an inverse ratio between two different methods[27].

$ABTS^{\bullet+}$ Scavenging Assay

The pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity[28]. The compounds **4**, **5**, **7a**, **7b**, **7c**, **8a**, **8b**and**8c** showed more scavenging activity than BHT standard at the 1.5 and 0.38 μg/mL final concentration. Beside this observation, it has been

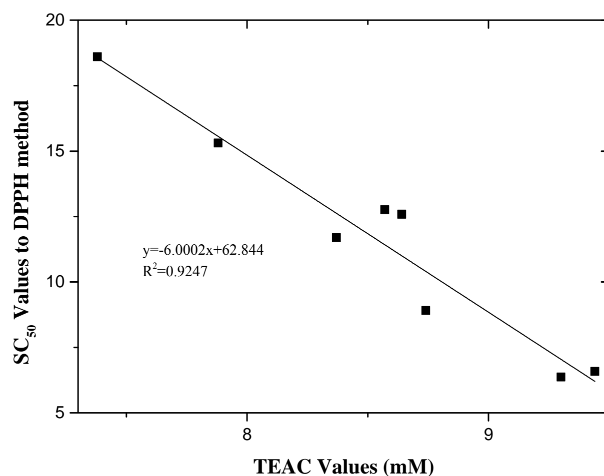


Figure 4 — Basic correlation analysis graph between the results of CUPRAC antioxidant activities test (TEAC values) DPPH radical scavenging activities of the synthesized compounds

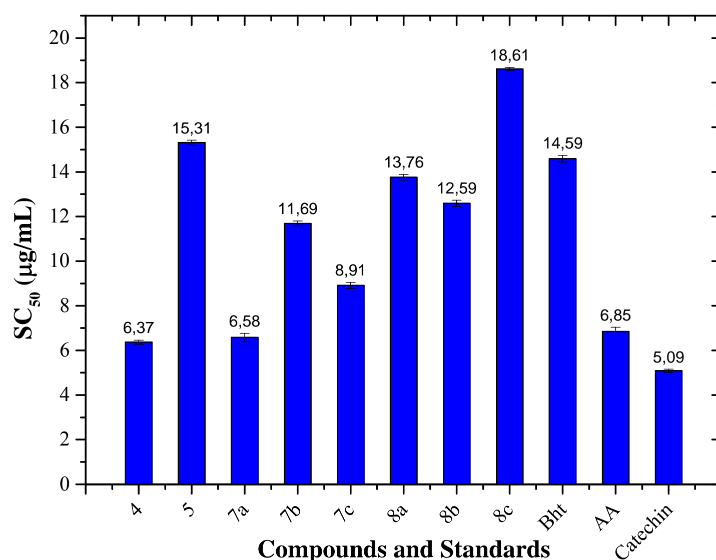


Figure 3 — SC_{50} values to DPPH method of synthesized compounds and standards. SC_{50} valuesof compounds are expressed as the mean ±SD in triplicate.

found that all compounds have greater scavenging activity than BHT and AA at 0.38 µg/mL final concentration (Figure 5).

Basic correlation analysis was performed to investigate the relationship between the $ABTS^{•+}$ radical scavenging and cupric reducing activities of the compounds, as shown in Figure 6. It was expected that the antioxidant activities of the compounds in both assays would show similar trends and that the values of both could, therefore, be correlated. The results of the correlation of the two antioxidant tests are plotted at 6.0, 1.5 and 0.38 µg/mL final concentration (Figure 6), and a relatively good correlation was observed (R^2 = 0.9407-0.9758). All of the compounds active in the CUPRAC antioxidant assay exhibit high radical scavenging activities in the $ABTS^{•+}$ method. Some differences seen between the results of the two antioxidant method results are probably due to the differences between the reaction mechanisms and dependence on the reaction conditions and steric issues in the case of $ABTS^{•+}$ test[29].

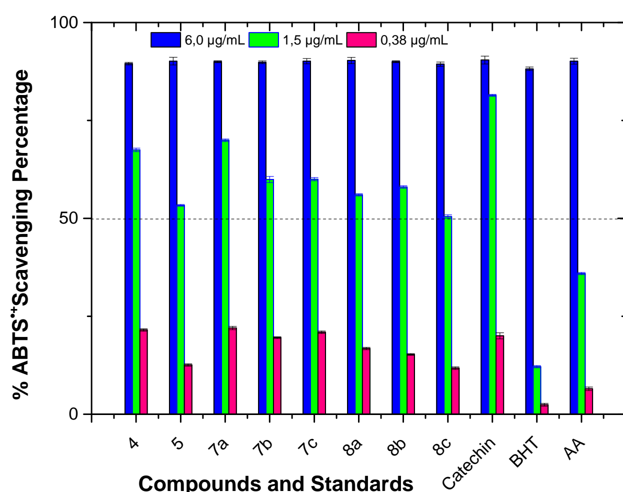


Figure 5 — $ABTS^{•+}$ radical scavenging activity values of the synthesized compounds at 6.0, 1.5 and 0.38 µg/mL final concentration.% $ABTS^{•+}$ scavenging percentage of compounds are expressed as the mean ±SD in triplicate.

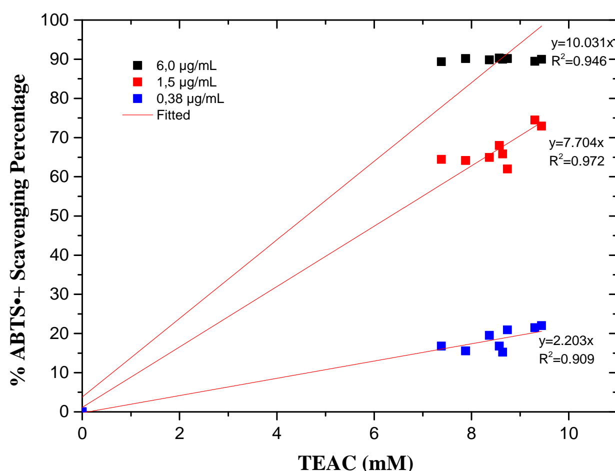


Figure 6 — Basic correlation analysis graph between the results of CUPRAC antioxidant activities test (TEAC values) and $ABTS^{•+}$ radical scavenging activities of the synthesized compounds at 0,38; 1,50 and 6,0 µg/mL final concentration (percentage scavenging of $ABTS^{•+}$ at 5 min incubation time).% $ABTS^{•+}$ scavenging percentage of compounds are expressed as the mean ±SD in triplicate.

Anti-xanthine oxidase activity

All the synthesized compounds were evaluated with regard for bovine milk xanthine oxidase activity. The results had shown that the compound **9c** had promising activity to inhibit XO up to 96.57% at concentration of 60 mg/mL (Table I). Among these compounds, **9c** displayed the best inhibitory effect against XO with an IC_{50} value of 12.30±0.33 µg/mL. All other compounds except compound **7a** showed moderate XO enzyme inhibition activity.

Anti-urease activity

The synthesized compounds were assayed for their *in vitro* inhibitory activity against Jack Bean urease. Thiourea and acetohydroxamic acid were used as standards. Initially, all the synthesized compounds were screened at the final concentration of 100 µg/mL. Among these compounds, **7a** exhibited the best inhibitory effect against urease (Table II).

Until now, only one compound, acetohydroxamic acid, has been clinically used for the treatment of

Table I — Results of % residual XO activity and IC_{50} values of the synthesized chemical compounds

Compd	Residual XO activity (%)	
	(60 µg/mL)	IC_{50} (µg/mL)
Control[a]	100.00	–
2	11.67±0.89	19.53±0.21
3	18.00±0.77	22.62±0.11
4	18.39±1.13	21.77±0.23
7a	31.06±0.58	36.85±0.17
7b	19.13±1.21	25.11±0.73
7c	13.94±1.27	20.50±0.39
9a	19.44±1.98	26.00±0.57
9b	15.42±2.01	18.53±0.89
9c	3.43±2.01	12.30±0.33
Allopurinol (7.81 µg/mL)	2.19 ±0.15	0.62±0.03

[a] Control, bovine milk xanthine oxidase without inhibitor; Allopurinol, positive control.

Table II — Inhibitory activities and IC_{50} values of the synthesized compounds against Jack Bean urease

Compd	% Inhibition	
	(100 µg/mL)	$IC_{50}{}^{a}$ (µg/mL)
2	85.28±1.09	26.12±0.73
3	71.56±0.89	48.53±0.65
4	88.33±0.91	24.67±0.29
5	99.00±1.21	22.52±0.96
6a	77.89±0.69	48.32±0.52
6b	87.11±1.11	26.27±0.29
6c	79.17±0.87	47.09±0.72
7a	98.67±0.39	13.04±0.89
7b	92.43±0.77	20.42±0.19
7c	94.00±0.88	17.35±0.21
9a	80.31±0.39	43.55±0.37
9b	80.89±1.23	43.00±0.43
9c	84.22±1.71	21.65±0.81
Standard 1	98.00±2.11	15.07±0.18
Standard 2	88.17±0.95	23.53±0.31

[a] IC_{50} values of compounds are expressed as the mean ±SD in triplicate. Standard 1(Thiourea) and Standard 2 (Acetohydroxamic acid) were used as standard inhibitors.

urinary tract infections by urease inhibition. Unfortunately, it exhibits severe side effects. Thus, it seems that the full potential of urease inhibition has not yet been fully explored. Compared to other compounds, **5**, **7a**, **7b**, **7c**and**9c** had a high inhibitory effect on urease and exhibited lower IC_{50} inhibition values and they have been found to be more effective inhibitors than acetohydroxamic acid (Table II, Figure 7). After wide spectrum research, these compounds can be considered as potential antibiotics to treat *Helicobacter pylori* infections. The other compounds have moderate or little inhibitory activity. Dose-dependent inhibitory effect of compounds **7a-c**, thiourea and acetohydroxamic acid are depicted in Figure 8.

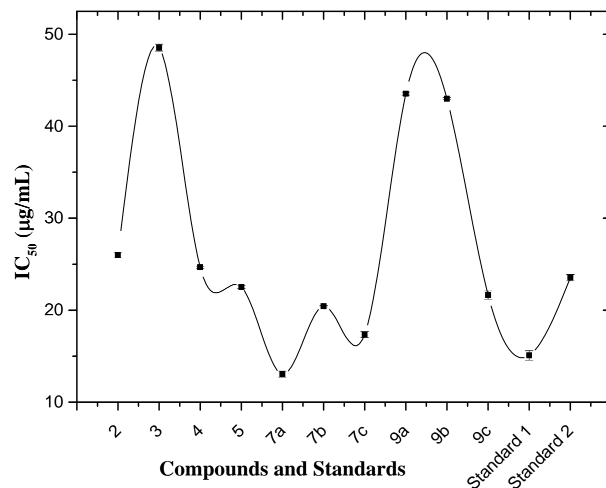


Figure 7 — IC_{50} values of anti-urease activity of compounds and standards

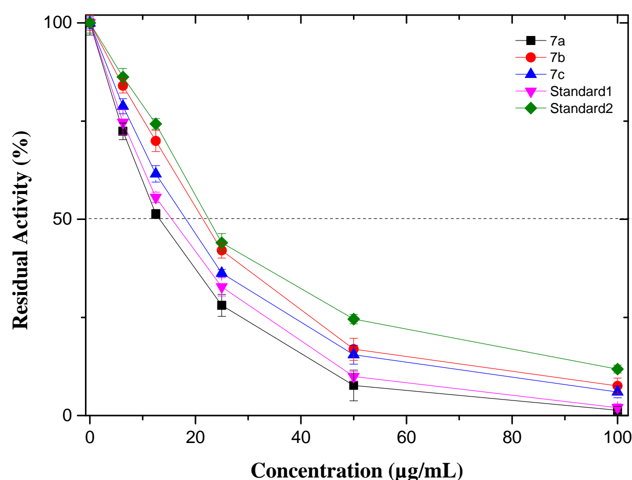


Figure 8 — Dose-dependent inhibitory effect of compounds **7a-c**, Standard 1(Thiourea) and Standard 2 (Acetohydroxamic acid) were used as standard inhibitors. Inhibitory effect of compounds and standards were measured in the range of 100 to 6.25 µg/mL concentrations. Residual activities of compounds are expressed as the mean ±SD in triplicate.

Experimental Section

All the chemicals were obtained from commercial suppliers and used without further purification. Melting points are uncorrected and determined in open capillaries on a Büchi oil-heated melting point apparatus. Reactions were monitored by thin-layer chromatography (TLC) using precoated aluminum sheets (silica gel 60 F 2.54 0.2 mm thickness). The mobile phase was ethyl acetate and hexane (2:1 or 3:1) and detection was made using UV light. ^{1}H and ^{13}C NMR spectra were recorded on a Varian-Mercury 400 (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) spectrometer using DMSO-d_6 as solvent and TMS as internal standard. All chemical shifts are reported in ppm. The mass spectra were obtained for the synthesized compounds on Thermo Scientific Quantum Access max LC-MS spectrometer. Elemental analyses were performed on a Carlo Erba 1106 CHN analyser (Heraeus, Hanau, Germany); the experimental values were in agreement (± 0.4%) with calculated ones.

Procedure for the preparation of 2-(4-nitrobenzyl)-1*H*-benzimidazole, 2

A mixture of 1,2-phenylenediamine (0.010 mol) and ethylimido-*p*-nitrophenyl acetate hydrochloride

(**1**) (0.012 mol) in dry methanol (25 mL) was stirred at RT for 6 h (monitored by TLC, ethyl acetate/hexane, 2:1). The resulting reaction mixture was poured onto water. The precipitate formed was filtered off, dried and purified by recrystallization from ethanol/water (1:2) to give the desired product **2** in 85% yield (2.15g). m.p.211-12°C (CAS Registry Number: 7291-96-5). ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.46 (s, 2H, NCH_2), 7.26 (d, 2H, ArH, J = 2.8 Hz), 7.60-7.74 (m, 4H, ArH), 8.32 (d, 2H, ArH, J = 8Hz), 12.50 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 34.98, 121.92, 124.04, 130.62, 146.02, 146.77, 152.69; LC-MS: m/z 253.30 $[M+1]^+$. Anal. Calcd for $C_{14}H_{11}N_3O_2$: C, 66.40; H, 4.38; N, 16.59. Found: C, 66.43; H, 4.34; N, 16.63%.

Procedure for the preparation of ethyl [2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetate, 3

To a solution of compound **2** (0.010 mol) in dry acetone, K_2CO_3 (0.025 mol) was added and the mixture was stirred at RT for 1 h. Then, ethyl bromoacetate (0.011 mol) was added to this mixture and stirred at RT for an additional 8 h. After the reaction was completed (monitored by TLC, ethyl acetate/hexane, 2:1), the reaction mixture was poured onto water. The precipitate formed was filtered off and purified by recrystallization from ethanol/water (1:3) to give the desired product **3** in 80% yield (2.71g). m.p.125-26°C (CAS Registry Number 1193045-86-1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.29 (t, 3H, CH_3, J = 6.8 Hz), 4.18 (q, 2H, CH_2, J = 6.8 Hz), 4.61 (s, 2H, CH_2), 5.42 (s, 2H, NCH_2), 7.37 (d, 2H, ArH, J = 6.4 Hz), 7.65 (d, 2H, ArH, J = 4 Hz), 7.76 (d, 2H, ArH, J = 7.6 Hz), 8.36 (d, 2H, ArH, J = 8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.87, 32.38, 44.49, 61.22, 110.17, 118.67, 121.77, 122.20, 123.46, 130.37, 130.39, 135.55, 142.07, 144.70, 146.32, 152.77, 167.84; LC-MS: m/z 340.35 $[M+1]^+$. Anal. Calcd for $C_{18}H_{17}N_3O_4$: C, 63.71; H, 5.05; N, 12.38. Found: C, 3.67; H, 5.09; N, 12.42%.

Procedure for the preparation of 2-[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide, 4

To a solution of compound **3** (0.010 mol) in dry ethanol (10 mL), hydrazine monohydrate (0.035 mol) was added and was stirred at RT for 2 h. The reaction was completed (monitored by TLC, ethyl acetate), and the reaction mixture was filtered, dried and then purified by recrystallization from ethanol/water (1:2) to give the desired product **4** in 68% yield (2.21 g); m.p.149-50°C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.31 (s, 2H, CH_2), 4.42 (s, 2H, NH_2), 4.86 (s, 2H, CH_2), 7.13-7.21 (m, 2H, ArH), 7.42 (d, 1H, ArH, J = 7.6 Hz), 7.53 (d, 1H, ArH, J = 4.6 Hz), 7.56- 7.60 (m, 2H, ArH), 8.17 (d, 2H, ArH, J = 8.8 Hz), 9.52 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.00, 44.95, 110.47, 119.05, 122.04, 122.41, 123.87, 130.94, 135.90,142.60, 145.52, 146.75, 153.61, 166.28; LC-MS: m/z 326.35 $[M+1]^+$. Anal. Calcd for $C_{16}H_{15}N_5O_3$: C, 59.07; H, 4.65; N, 21.53. Found: C, 59.11; H, 4.68; N, 21.49%.

Procedure for the preparation of 5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-1,3,4-oxadiazole-2-thiol, 5

To a solution of compound **4** (0.010 mol) in dry ethanol (20 mL), CS_2 (0.60 mL, 0.010 mol) and a solution of KOH (0.56 g, 0.010 mol) in 50 mL water were added. Then the reaction mixture was refluxed for 3 h. The mixture was cooled at RT and acidified with conc. HCl. Then the solid formed was filtered off, washed with plenty of water and dried. The product was purified by recrystallization from ethanol/water (1:1) to give the desired product **5**. Yield 65% (2.39 g). m.p.210-12°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.58 (s, 2H, CH_2), 5.81 (s, 2H, N-CH_2), 7.26-7.34 (m, 2H, ArH), 7.57-7.69 (m, 4H, ArH), 8.20 (d, 2H, ArH, J = 8 Hz); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.78, 38.57, 110.75, 119.28, 122.66, 123.09, 123.89, 130.64, 135.63, 142.36, 144.76, 146.76, 153.03, 159.36, 178.19; LC-MS: m/z 367.39 $[M]^+$. Anal. Calcd for $C_{17}H_{13}N_5O_3S$: C, 55.58; H, 3.57; N, 19.06. Found: C, 55.62; H, 3.60; N, 19.05%.

Procedure for the preparation of Schiff base derivatives of benzimidazole, 6a-c

To a solution of compound **4** (0.010 mol) with 0.5 mL glacial acetic acid in dry ethanol, corresponding salicylaldehyde (0.010 mol) was added. Then, the mixture was refluxed for 4 h (monitored by TLC, ethyl acetate/hexane, 2:1). The mixture was cooled to RT and a white solid appeared. Then the solid was filtered off, washed with plenty of water and dried. The product was purified by recrystallization from ethanol/water (1:1) to give the desired product **6a-c**.

N′-[(1*E*)-(2-Hydroxyphenyl)methylene]-2-[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetohydrazide, 6a: Yield 88% (3.78 g). m.p.286-87°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.40, 4.44, (s, 2H, CH_2,

trans/*cis* conformers, ration 53/47), 5.07, 5.49 (s, 2H, CH_2, *trans*/*cis* conformers, ration 53/47), 6.84-6.91 (m, 2H, ArH), 7.16-7.23 (m, 3H, ArH), 7.47-7.60 (m, 4H, ArH), 7.78 (d, 1H, ArH, J = 8 Hz), 8.13-8.15 (m, 2H, ArH), 8.32, 8.43 (s, 1H, N=CH, *trans*/*cis* conformers, ration 53/47), 10.04, 10.83 (s, 1H, OH, *trans*/*cis* conformers, ration 86/14), 11.62, 11.99 (s, 1H, NH, *trans*/*cis* conformers, ration 53/47); ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.06, 44.68, 110.51, 116.58, 118.96, 119.79, 120.55, 122.37, 123.81, 126.93, 130.90, 131.71, 136.47, 141.99, 142.64, 145.38, 146.73, 148.04, 153.70, 156.88, 168.16; LC-MS: *m/z* 430.45 $[M+1]^+$. Anal. Calcd for $C_{23}H_{19}N_5O_4$: C, 64.33; H, 4.46; N, 16.31. Found: C, 64.29; H, 4.48; N, 16.29%.

N′-[(1*E*)-(5-Chloro-2-hydroxyphenyl)methylene]-2-[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]aceto-hydrazide, 6b: Yield 79% (3.66 g). m.p.292-93°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.40, 4.44, (s, 2H, CH_2, *trans*/*cis* conformers, ration 63/37), 5.07, 5.54 (s, 2H, CH_2, *trans*/*cis* conformers, ration 62/38), 6.90-6.93 (m, 1H, ArH), 7.15-7.28 (m, 3H, ArH), 7.47-7.60 (m, 4H, ArH), 7.82 (s, 1H, ArH), 8.13-8.15 (m, 2H, ArH), 8.27, 8.39 (s, 1H, N=CH, *trans*/*cis* conformers, ration 63/37), 10.36, 10.92 (s, 1H, OH, *trans*/*cis* conformers, ration 60/40), 11.70, 12.05 (s, 1H, NH, *trans*/*cis* conformers, ration 65/35); ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.05, 44.80, 110.76, 118.37, 118.94, 121.88, 122.33, 122.47, 123.71, 123.80, 125.52, 130.90, 131.14, 136.49, 139.94, 142.64, 145.47, 146.71, 153.71, 155.63, 168.46; LC-MS: *m/z* 464.88 $[M+1]^+$. Anal. Calcd for $C_{23}H_{18}ClN_5O_4$: C, 59.55; H, 3.91; N, 15.10. Found: C, 59.58; H, 3.89; N, 15.11%.

N′-[(1*E*)-(3,5-Dichloro-2-hydroxyphenyl)methylene]-2-[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]aceto-hydrazide, 6c: Yield 85% (4.23 g). m.p.281-82°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.41, 4.44, (s, 2H, CH_2, *trans*/*cis* conformers, ration 71/29), 5.12, 5.57 (s, 2H, CH_2, *trans*/*cis* conformers, ration 71/29), 7.16-7.23 (m, 1H, ArH), 7.50-7.63 (m, 6H, ArH), 7.79 (s, 1H, ArH), 8.13-8.15 (m, 2H, ArH), 8.28, 8.36 (s, 1H, N=CH, *trans*/*cis* conformers, ration 72/28), 10.42, 11.86 (s, 1H, OH, *trans*/*cis* conformers, ration 72/28), 11.94, 12.34 (s, 1H, NH, *trans*/*cis* conformers, ration 77/23); ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.05, 45.29, 110.79, 118.94, 121.09, 121.93, 122.00, 122.34, 123.46, 123.85, 124.39, 125.81, 130.90, 140.82, 142.60, 145.45, 146.70, 147.25, 152.53, 163.91, 168.47; LC-MS: *m/z* 499.34 $[M+1]^+$. Anal. Calcd for $C_{23}H_{17}Cl_2N_5O_4$: C, 55.44; H, 3.44; N, 14.05. Found: C, 55.46; H, 3.41; N, 14.08%.

Procedure for the preparation of N-(*R*)-2-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetyl}hydrazine carbothioamide, 7a-c

To a solution of compound **4** (0.010 mol) in dry ethanol (25 mL), methylisothicyanate for **7a**, ethylisothiocyanate for **7b** or phenylisothiocyanate for **7c** was added. Then, the mixture was refluxed for 3 h. The reaction was complete (monitored by TLC, ethyl acetate/hexane, 2:1). The mixture was cooled at RT and was filtered, dried and then purified by recrystallization from ethanol/water (1:2) to give the desired products **7a-c**.

N-Methyl-2-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetyl}hydrazinecarbothioamide, 7a: Yield 77% (3.07 g). m.p.241-42°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.99 (s, 3H, NCH_3), 4.48 (s, 2H, CH_2), 5.06 (s, 2H, NCH_2), 7.25-7.14 (m, 2H, ArH), 7.54-7.68 (m, 4H, ArH), 8.15-8.29 (m, 2H, ArH), 9.42 (s, 1H, NH), 9.77 (s, 1H, NH), 10.35 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 31.37, 33.03, 44.85, 110.58, 119.07, 122.11, 122.46, 123.93, 130.91, 135.98, 142.55, 145.37, 146.78, 153.57, 166.94, 170.62; LC-MS: *m/z* 398.45 $[M]^+$. Anal. Calcd for $C_{18}H_{18}N_6O_3S$: C, 54.26; H, 4.55; N, 21.09. Found: C, 54.30; H, 4.52; N, 21.04%.

N-Ethyl-2-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]acetyl}hydrazinecarbothioamide, 7b: Yield 78% (3.22 g). m.p.231-33°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.06 (t, 3H, CH_3, J = 7.2 Hz), 3.46 (brs, 2H, CH_2), 4.39 (s, 2H, CH_2), 4.98 (s, 2H, CH_2), 7.14-7.22 (m, 2H, ArH), 7.46-7.59 (m, 4H, ArH), 8.17-8.19 (m, 2H, ArH), 9.25 (s, 1H, NH), 9.65 (s, 1H, NH), 10.25 (s, 1H,NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.87, 33.03, 38.94, 44.86, 110.60, 119.06, 122.10, 122.44, 123.91, 130.82, 135.89, 142.56, 145.39, 146.77, 153.85, 166.84, 170.61; LC-MS: *m/z* 412.48 $[M]^+$. Anal. Calcd for $C_{19}H_{20}N_6O_3S$: C, 55.33; H, 4.89; N, 20.38. Found: C, 55.29; H, 4.93; N, 20.32%.

2-{[2-(4-Nitrobenzyl)-1*H*-benzimidazol-1-yl]acetyl}-N-phenylhydrazinecarbothioamide, 7c: Yield 72% (3.32 g). m.p.219-20°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.57 (s, 2H, CH_2), 5.19 (s, 2H, CH_2), 7.31-7.74 (m, 6H, ArH), 8.31-8.33 (m, 2H, ArH), 9.84 (s, 1H, NH), 10.48 (s, 1H, NH), 10.67 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 33.04, 44.93, 110.62, 119.05, 122.14, 122.49, 123.91, 128.70, 130.82, 130.91, 135.99, 139.34, 142.53, 145.37, 146.77, 153.59,

166.95, 170.63; LC-MS: *m/z* 460.50 $[M]^+$. Anal. Calcd for $C_{23}H_{20}N_6O_3S$: C, 59.99; H, 4.38; N, 18.25. Found: C, 60.04; H, 4.42; N, 18.23%.

Procedure for the preparation of 4-(*R*)-5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-4*H*-1,2, 4-triazole-3-thiol, 8a-c

A solution of the corresponding carbothioamide derivatives **7a-c** (0.010 mol) in dry ethanol (15 mL) was refluxed in the presence of 2N 15 mL NaOH for 4 h. Then, the mixture was cooled to RT and acidified to *p*H 4-5 with 37% HCl. The separated product was filtered, washed with plenty of water and purified by recrystallization from ethanol–water (1:2) to give the desired products **8a-c**.

4-Methyl-5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-4*H*-1,2,4-triazole-3-thiol, 8a: Yield 65% (2.47 g). m.p.290°C (decomp.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 3.42 (s, 3H, NCH_3), 4.25 (s, 2H, CH_2), 5.35 (s, 2H, NCH_2), 7.09-8.11 (m, 8H, ArH), 13.55 (s, 1H, SH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 30.42, 39.30-40.55 (DMSO-d_6+CH_2), 41.18, 111.92, 112.88, 123.07, 123.79, 130.72, 134.37, 135.67, 142.68, 147.71, 151.70, 153.27, 167.57, 182.36; LC-MS: *m/z* 380.43 $[M]^+$. Anal. Calcd for $C_{18}H_{16}N_6O_2S$: C, 56.83; H, 4.24; N, 22.09. Found: C, 56.79; H, 4.27; N, 22.12%.

4-Ethyl-5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-4*H*-1,2,4-triazole-3-thiol, 8b: Yield 55% (2.17 g). m.p.282°C (decomp.). ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.07 (brs, 3H, CH_3), 3.81 (brs, 2H, CH_2), 4.83 (s, 2H, CH_2), 5.46 (s, 2H, CH_2), 7.17-8.11 (m, 8H, ArH), 13.52 (s, 1H, SH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.63, 38.92, 39.31-40.56 (DMSO-d_6+CH_2), 41.20, 111.86, 112.96, 123.07, 123.82, 130.90, 134.34, 135.49, 141.45, 147.14, 151.12, 167.03, 182.27; LC-MS: *m/z* 394.45 $[M]^+$. Anal. Calcd for $C_{19}H_{18}N_6O_2S$: C, 57.85; H, 4.60; N, 21.31. Found: C, 57.80; H, 4.63; N, 21.34%.

5-{[2-(4-Nitrobenzyl)-1*H*-benzimidazol-1-yl] methyl}-4-phenyl-4*H*-1,2,4-triazole-3-thiol, 8c. Yield 62% (2.74 g). m.p.269-70°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.48 (s, 2H, CH2), 5.63 (s, 2H, CH_2), 6.93-7.02 (m, 2H, ArH), 7.2-7.65 (m, 9H, ArH), 8.04-8.10 (m, 2H, ArH), 13.73 (s, 1H, SH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 39.32-40.57 (DMSO-d_6+CH_2), 41.19, 111.85, 112.76, 121.33, 123.07, 123.57, 123.71, 125.44, 128.33, 129.92, 133.41, 134.40, 136.25, 141.38, 147.21, 149.34, 155.15, 168.59, 182.01; LC-MS: *m/z* 443.50 $[M+1]^+$. Anal. Calcd for $C_{23}H_{18}N_6O_2S$: C, 62.43; H, 4.10; N, 18.99. Found: C, 62.48; H, 4.07; N, 19.02%.

Procedure for the preparation of N-R-5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-1,3,4-thiadiazol-2-amine, 9a-c

A mixture of the corresponding carbothioamide **7a-c** (0.010 mol) and conc. H_2SO_4 (0.06 mol) was stirred at 0-5°C for 30 min. Then, the mixture was stirred for an additioanal 1 h at RT. The resulting mixture was poured slowly into ice-cold water and then the mixture solution made alkaline to *p*H 8 with dilute ammonia. The precipitate was filtreted off, washed with plenty of water and purified by recrystallization from ethanol/water (1:2) to give the desired products **9a-c**.

N-Methyl-5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-1,3,4-thiadiazol-2-amine, 9a: Yield 45% (1.71 g). m.p.184-85°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.74 (s, 3H, NCH_3), 4.51 (s, 2H, CH_2), 5.72 (s, 2H, NCH_2), 7.16-7.24 (m, 2H, ArH), 7.53-7.59 (m, 4H, ArH), 8.14 (d, 2H, ArH, *J* = 8 Hz),9.52 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 31.55, 39.32-40.57 (DMSO-d_6+ CH_2), 42.40, 110.79, 119.30, 122.44, 122.85, 123.93, 130.79, 135.29, 142.67, 145.06, 146.77, 152.79, 153.07, 170.63; LC-MS: *m/z* 380.41 $[M]^+$. Anal. Calcd for $C_{18}H_{16}N_6O_2S$: C, 56.83; H, 4.24; N, 22.09. Found: C, 56.87; H, 4.20; N, 22.12%.

N-Ethyl-5-{[2-(4-nitrobenzyl)-1*H*-benzimidazol-1-yl]methyl}-1,3,4-thiadiazol-2-amine, 9b: Yield 30% (1.18 g). m.p.110-11°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.05 (t, 3H, CH_3, *J* = 8 Hz), 3.14 (q, 2H, CH_2, *J* = 8 Hz), 4.51 (s, 2H, CH_2), 5.72 (s, 2H, CH_2), 7.16-7.24 (m, 2H, ArH), 7.52-7.62 (m, 4H, ArH), 8.14 (d, 2H, ArH, *J* = 8 Hz), 9.54 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 14.51, 33.01, 39.32-40.58 (DMSO-d_6+ CH_2), 42.37, 110.80, 119.30, 122.43, 122.84, 123.92, 130.76, 135.31, 142.67, 145.06, 146.74, 152.78, 152.90, 169.68; LC-MS: *m/z* 394.44 $[M]^+$. Anal. Calcd for $C_{19}H_{18}N_6O_2S$: C, 57.85; H, 4.60; N, 21.31. Found: C, 57.88; H, 4.64; N, 21.28%.

5-{[2-(4-Nitrobenzyl)-1*H*-benzimidazol-1-yl] methyl}-N-phenyl-1,3,4-thiadiazol-2-amine, 9c: Yield 47% (2.08 g). m.p.244-45°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 4.55 (s, 2H, CH_2), 5.86 (s, 2H, CH_2), 6.97-7.22 (m, 5H, ArH), 7.44-7.61 (m, 6H,ArH), 8.15 (d, 2H, ArH, *J* = 8 Hz), 10.33 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 32.96, 42.21, 110.91, 116.74, 119.17, 123.04, 123.95, 126.94, 130.87, 135.24, 140.73,

142.30, 142.44, 144.94, 146.80, 152.93, 155.21, 165.32; LC-MS: *m/z* 442.50 $[M]^+$. Anal. Calcd for $C_{23}H_{18}N_6O_2S$: C, 62.43; H, 4.10; N, 18.99. Found: C, 62.47; H, 4.12; N, 18.96%.

Phamacology

Cupric reducing antioxidant capacity (CUPRAC) assay

The cupric ions (Cu^{2+}) reducing ability of the synthesized compounds was determined according to the literature[30]. First, a test tube 1 mL each of 10 mM Cu(II) chloride (Sigma Chemical Co, USA), 7.5 mM neocuprine (Sigma Chemical Co, USA), and NH_4Ac (Fluka Chemical Co., Switzerland buffer (1 M, *p*H 7.0) solutions were added. About 5 µL of compound solution in DMSO and 1.095 mL of water were added to the initial mixture so as to make the final volume 4.1 mL. The tubes were stoppered, and after 30 min, the absorbance at 450 nm was recorded against a reagent blank containing no compound. Trolox® (Sigma Chemical Co, USA) was also tested under the same conditions as a standard antioxidant compound. The standard curve was linear between 8 mg/mL and 0.03125 mg/mL Trolox® (r^2=0.9989). CUPRAC values were expressed as mM Trolox® equivalent of 1 mg synthesized compound.

DPPH-Free radical scavenging assay

The DPPH radical scavenging activity of the synthesized compounds was measured using the method of Brand-Williams[6,31]. Briefly, 0.1 mM DPPH (2,2-diphenyl-1-picrylhydrazyl, Aldrich-Germany) was prepared in methanol. To 1200 µL of this solution was added 0.3 mL of the sythesized compound solution in DMSO. After 50 min in the dark for incubation at RT, the decrease in absorbance at 517 nm was measured, using a UV-Vis spectrophorometer (1601UV-Shimadzu, Australia). All determinations were repeated three times. Radical scavenging activity was measured by using ascorbic acid (AA), BHT and catechin (Sigma Chemical Co, USA) as standards and all values are expressed as SC_{50} (µg compound per mL), the concentration of the samples that causes 50% scavenging of DPPH radical. The DPPH radical stock solution was prepared fresh daily.

$ABTS^{\bullet+}$ Radical Cation Decolorization Assay

The ability of the synthesized compounds to scavenge $ABTS^{\bullet+}$ radical was determined according to the literature[28-32]. ABTS [2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid)] was dissolved in water to a 7 mM concentration. ABTS (Sigma Chemical Co, USA) radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (Sigma Chemical Co, USA), (final concentration) and allowing the mixture to stand in the dark for 16–18 h at RT. Before using, the ABTS solution was diluted to get an absorbance of 0.700 ± 0.020 at 734 nm with PBS at *p*H 7.4. The compound solution of 200 µL was added to 1.8 mL of the resulting blue-green ABTS radical solution. After incubation for 5 min in the dark at RT, the decrease of absorbance at 734 nm was measured by using a UV-Vis spectrophorometer (1601UV-Shimadzu, Australia). All determinations were repeated three times. ABTS radical scavenging activity was measured by using BHT, ascorbic acid (AA) and catechin (Sigma Chemical Co, USA) as standards and the percentage scavenging was calculated from the formula

% Scavenging = [(ODcontrol-ODtest)/(ODcontrol)×100].

In vitro anti-xanthine oxidase assay

The inhibition of xanthine oxidase was measured by UV-Vis spectroscopy technique at 295 nm which is attributed to released uric acid from xanthine. The inhibitory activity of each compound was determined using a slight modification of the reference methods[33,34]. Briefly, the reaction mixture consisted of 0.5 mL of the test compound, 0.77 mL of phosphate buffer (*p*H 7.8) and 0.07 mL of bovine milk xanthine oxidase (Sigma–Aldrich, St. Louis, USA), which was prepared immediately before use. After preincubation at 25°C for 15 min, the reaction was initiated by the addition 0.66 mL of substrate solution into the mixture. The assay mixture was incubated at 25°C for 15 min. The reaction was stopped by adding 0.2 mL of 0.5N HCl and the absorbance was measured at 295 nm employing UV-Vis spectrophotometer (1601UV-Shimadzu, Australia). A well known XO inhibitor (XOI), allopurinol (Sigma–Aldrich, St. Louis, USA) was used as a positive control for the inhibition test. Residual activity was calculated by comparing the control sample without inhibitor. The assay was carried out in triplicate for calculating standard deviation. The IC_{50} value was determined as the concentration of compound that gives 50% inhibition of maximal activity.

Urease inhibition assay

Urease is an enzyme that catalyzes the hydrolysis of urea into carbon dioxide and ammonia. The production of ammonia was measured by indophenol

method and used to determine the urease inhibitory activity[35]. Reaction mixtures including 200 μL of Jack Bean Urease, 500 μL of buffer (100 mM urea, 0.01 M K_2HPO_4, 1 mM EDTA and 0.01 M LiCl, *p*H 8.2) and 100 μL of the test compounds were incubated at RT for 20 min. The phenol reagent (550 μL, 1% w/v phenol and 0.005% w/v sodium nitroprusside) and alkali reagent (650 μL, 0.5% w/v sodium hydroxide and 0.1% v/v NaOCl) were added to each tube and the increasing absorbance at 625 nm was measured after 50 min, using a UV-Vis spectrophotometer (1601 UV-Shimadzu, Australia). The percentage inhibition was calculated from the formula

% Inhibition = [(ODcontrol-ODtest)/(ODcontrol)×100]

Thiourea and acetohydroxamic acid were used as standard inhibitors. In order to calculate IC_{50} values, different concentrations of synthesized compounds and standard were assayed under the same reaction conditions.

Conclusion

In this study, we have reported the synthesis and characterization of a series of new 2-(4-nitrobenzyl)-1*H*-benzimidazole derivatives containing 1-substituted thiosemicarbazide, triazole, oxadiazole, thiadiazole and imine groups. The synthesized compounds were evaluated *in vitro* for their xanthine oxidase (XO) and urease inhibitory activities. Also, antioxidant activities of the synthesized compounds were clarified using various *in vitro* antioxidant assays. They also showed excellent antioxidant activities in DPPH scavenging and cupric reducing/antioxidant capacity tests.

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