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Synthesis, X-ray molecular structure, biological evaluation and molecular docking studies of some N^4-benzyl substituted 5-nitroisatin-3-thiosemicarbazones

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Abstract

A series of fifteen N^4-benzyl substituted 5-nitroisatin-3-thiosemicarbazones **5a-o** was synthesized and evaluated for urease inhibitory, phytotoxic and cytotoxic influences. All the compounds proved to be highly potent inhibitors of the enzyme, showing inhibitory activity (IC_{50} = 0.87 ± 0.25 – 8.09 ± 0.23 μM) much better than the reference inhibitor, thiourea (IC_{50} = 22.3 ± 1.12 μM) and may thus act as persuasive leads for further studies. In phytotoxicity assay, twelve out of fifteen thiosemicarbazones tested i.e. **5a-e**, **5g**, **5i** and **5k-o** appeared to be active, exhibiting weak or non-significant (5-35%) growth inhibition at the highest tested concentrations (1000 or 500 μg/mL). In contrast, only one compound i.e. **5i** was active in the brine shrimp (*Artemia salina*) lethality bioassay, demonstrating cytotoxic activity with LD_{50} value 2.55×10^{-5} M. Molecular docking studies of compounds **5a-o** were also performed to identify their probable binding modes in the active site of the enzyme.

Keywords: Cytotoxicity, 5-Nitroisatin, Phytotoxicity, Thiosemicarbazones, Urease inhibition, Urease inhibitors

1. Introduction

Isatin and its derivatives are reported to exhibit a broad spectrum of chemotherapeutic properties.[1-25] Among isatin derivatives, isatin-thiosemicarbazones have been found to demonstrate diverse biological activities such as analgesic and antiinflammatory, anticancer, antimicrobial, antitubercular, antiulcer, antiviral and enzymatic inhibition.[1-5,7,11,12,17,21-25] In view of these facts and as a part of our synthetic work on potential biologically active isatin derivatives, we have recently synthesized a number of N^4-aryl substituted isatin-3-thiosemicabazones as antibacterial, antifungal, antileishmanial, cytotoxic, phytotoxic and more importantly antiurease compounds.[26-35] Investigation of the structure-activity relationships (SAR) revealed that in some cases, the nature and position of the substituents about the phenyl ring attached to N^4 of the thiosemicarbazone moiety and/or the presence of inductively electron-withdrawing/lipophilic groups (nitro, trifluoromethoxy, fluoro, chloro) at position-5 of the isatin scaffold played an important role in the inducement and/or increment of certain activities. Furthermore, during a preliminary screening of some isatin-thiosemicarbazones prepared in our laboratory, oneviz.N-benzyl-2-(5-nitro-2-oxo-2,3-dihydro-1*H*-indol-3-ylidene)-1-hydrazinecarbothioamidedrew our attention, as it showed about four fold more urease inhibitory activity than the reference inhibitor, thiourea, used in the assay. In view of this, it was envisaged that the attachment of variously substituted benzyl groups (having one or two inductively electron-attracting /-donating substituents about the phenyl ring) to N^4 of the thiosemicarbazone moiety would cause an increase in the urease inhibitory potential of the molecules. Thus, the present work to synthesize a series of fifteen title thiosemicarbazones and screen them mainly for their urease inhibitory as well as phytotoxicity and cytotoxicity potential was carried out.

This paper is one of the first to report on N^4-benzyl substituted isatin-thiosemicarbazones with urease inhibitory potential. In it, we confine our discussion to those compounds, which are having attached concurrently strong inductively electron-attracting/lipophilic nitro group at position-5 of the isatin scaffold and the benzyl substituents (modified by substituting one inductively electron-donating and

one or two electron-attracting substituents about the phenyl ring) to N^4 of the thiosemicarbazone moiety.

2. Experimental

2.1. General

Melting points (uncorrected) were determined on a Fisher-Johns melting point apparatus. Elemental analyses were performed by using a Leco CHNS-9320 (USA) elemental analyzer. FT-IR spectra (KBr disks) were run on Shimadzu Prestige-21 FT-IR spectrophotometer. ^{1}H-NMR spectra were recorded in DMSO-$_{d6}$ on Bruker Avance 300 and Bruker Spectrospin 400 spectrometers, operating at 300 and 400 MHz, respectively, and using TMS as an internal standard. The chemical shifts are reported in δ/ ppm and coupling constants in Hz. ^{13}C-NMR spectra were recorded in DMSO-d_6 on Bruker Avance 300, operating at 75 MHz with the same internal standard. Electron impact (EI) mass spectra were obtained on MAT-312 and JEOL MSRoute mass spectrometers. X-ray crystallographic data were taken on Bruker Kappa APEXII CCD. All the chemicals and solvents used in the present study were procured from Merck-Schuchatdt, Fluka and Sigma-Aldrich.

2.2. Synthesis of *N*-substituted thiosemicarbazides 3

The *N*-substituted thiosemicarbazides used in this study were synthesized according to the reported synthetic route.[36]

2.3. Synthesis of isatin-thiosemicarbazones 5a-o

An appropriate thiosemicarbazide **3** (5 mmol) dissolved in ethanol (10 mL) was added to a hot solution of 5-nitroisatin **4** (5 mmol) in 50% aqueous ethanol (15 mL) under stirring and the reaction mixture was then heated to reflux for 2 h. The crystalline solid formed during heating in each case was collected by suction filtration and washed with hot aqueous ethanol to afford the desired compounds **5a-o**.

2.3.1. *N*-Benzyl-2-(5-nitro-2-oxo-2,3-dihydro-1*H*-indol-3-ylidene)-1-hydrazinecarbothioamide (5a)

Yellow crystals (82%): mp 262-264 °C; IR (KBr, cm^{-1}): 3344, 3118 (NH), 1693 (C=O), 1604 (C=N), 1157 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 4.91 (d, J = 6.0 Hz, 2H, benzyl CH_2), 7.12 (d, J = 8.8 Hz, 1H, indole C_7-H), 7.25-7.39 (m, 5H, benzyl C_2-H, C_3-H, C_4-H, C_5-H, C_6-H), 8.26 (dd, J = 8.8, 2.4 Hz, 1H, indole C_6-H), 8.55 (d, J = 2.0 Hz, 1H, indole C_4-H), 10.13 (t, J = 7.5 Hz, 1H, CSNH), 11.83 (s, 1H, indole NH), 12.44 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 47.24 (CH_2), 111.19 (CH), 116.20 (CH), 120.94, 126.89 (CH), 127.03 (CH), 127.19 (CH), 128.29 (CH), 130.10, 138.14, 142.67, 147.39, 162.95, 177.70; EIMS (70eV) *m/z* (%): 355(M^+, 2), 327 (2), 296 (1), 279 (3), 220 (4), 192 (20), 164 (9), 131 (20), 106 (44), 91 (100), 77 (17), 65 (27), 51 (15). Anal. Calcd for $C_{16}H_{13}N_5O_3S$ (355): C, 54.08; H, 3.66; N, 19.72. Found: C, 53.89; H, 3.62; N, 19.66.

2.3.2. *N*-(2-Methylbenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5b)

Yellow fluffy crystals (82%): mp 240-242 °C; IR (KBr, cm^{-1}): 3319, 3246 (NH), 1701 (C=O), 1601 (C=N), 1163 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 2.35 (s, 3H, CH_3), 4.87 (d, J = 6.0 Hz, 2H, benzyl CH_2), 7.12 (d, J = 9.0 Hz, 1H, indole C_7-H), 7.19 (s, 4H, benzyl C_3-H, C_4-H, C_5-H, C_6-H), 8.26 (dd, J = 9.0, 3.0 Hz, 1H, indole C_6-H), 8.56 (d, J = 3.0 Hz, 1H, indole C_4-H), 10.05 (t, J = 7.5 Hz, 1H, CSNH), 11.83 (s, 1H, indole NH), 12.47 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 55.27 (CH_2), 112.95 (CH), 113.59 (CH), 114.13 (CH), 122.24, 123.65 (CH), 127.20 (CH), 130.62, 131.06, 133.23 (CH), 141.38, 144.07 (CH), 157.43, 162.26, 176.45; EIMS (70eV) *m/z* (%): 369 (M^+, 2), 341(2), 293(1), 220 (3), 192 (23), 178 (17), 163 (12), 145 (25), 120 (38), 105 (100), 91 (38), 79 (52), 77 (65), 65 (20). Anal.Calcd for $C_{17}H_{15}N_5O_3S$ (369): C, 55.28; H, 4.07; N, 18.97. Found: C, 55.12; H, 4.04; N, 18.93.

2.3.3. *N*-(3-Methylbenzyl))-2-[5-nitro-2-oxo-2,3-dihydro-1*H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5c)

Yellow crystals (72%): mp 210-212 °C; IR (KBr, cm^{-1}): 3323, 3069 (NH), 1699 (C=O), 1610 (C=N), 1159 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 2.56 (DMSO, CH_3), 3.35 (DMSO, benzyl CH_2), 6.96 (d, J = 7.1 Hz, 1H, indole C_7-H), 7.35-7.33 (m, 1H, benzyl C_4-H), 7.39-7.50 (m, 4H,

benzyl C_2-H, C_5-H, C_6-H, indole C_6-H), 7.80 (d, *J* = 2.0 Hz, 1H, indole C_4-H), 10.82 (s, 1H, CSNH), 11.36 (s, 1H, indole NH), 12.71 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 112.64 (CH), 116.96 (CH), 120.83 (CH), 121.67, 126.55, 130.80 (CH), 131.75, 141.33, 162.36, 177.89; EIMS (70eV) *m/z* (%): 369 (M^+, 3), 341 (2), 293 (2), 248 (2), 220 (8), 206 (6), 192 (46), 178 (20), 163 (17), 145 (46), 144 (11), 121 (65), 105 (100), 90 (18), 91 (55), 79 (19), 77 (73), 65 (34). Anal.Calcd for $C_{17}H_{15}N_5O_3S$ (369): C, 55.28; H, 4.07; N, 18.97. Found: C, 54.99; H, 4.02; N, 18.94.

2.3.4. *N*-(4-Methylbenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5d)

Yellow crystals (89%): mp 208-210 °C; IR (KBr, cm^{-1}): 3316, 3163 (NH), 1678 (C=O), 1600 (C=N), 1159 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 2.29 (s, 3H, CH_3), 4.86 (d, *J* = 5.6 Hz, 2H, benzyl CH_2), 6.84 (d, *J* = 9.0 Hz, 1H, indole C_7-H), 7.07-7.34 (m, 4H, benzyl C_2-H, C_3-H, C_5-H, C_6-H), 8.27 (d, *J* = 9.0 Hz, 1H, indole C_6-H), 8.55 (s, 1H, indole C_4-H), 10.08 (t, *J* = 7.5 Hz, 1H, CSNH), 11.83 (s, 1H, indole NH), 12.43 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 20.67 (CH_3), 47.02 (CH_2), 111.19 (CH), 113.44 (CH), 116.20 (CH), 120.95, 126.88 (CH), 127.21 (CH), 128.51 (CH), 128.83 (CH), 130.11, 135.08, 142.66, 147.37, 162.96, 177.54; EIMS (70eV) *m/z* (%): 369 (M^+, 19), 341 (3), 293 (7), 220 (13), 206 (76), 192 (44), 178 (47), 163 (34), 145 (44), 136 (27), 121 (96), 105 (100), 91 (22), 79 (17), 77 (28), 65 (9). Anal.Calcd for $C_{17}H_{15}N_5O_3S$ (369): C, 55.28; H, 4.07; N, 18.97. Found: C, 54.91; H, 4.05; N, 18.91.

2.3.5. *N*-(2-Methoxybenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5e)

Yellow crystals (89%): mp 290-292 °C; IR (KBr, cm^{-1}): 3356, 3093 (NH), 1737 (C=O), 1606 (C=N), 1137 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 3.85 (s, 3H, CH_3), 4.41 (d, *J* = 5.0 Hz, 2H, benzyl CH_2), 6.91-6.99 (m, 2H, benzyl C_3-H, C_5-H), 7.11 (d, *J* = 9.0 Hz, 1H, indole C_7-H), 7.21-7.29 (m, 2H, benzyl C_4-H, C_6-H), 8.24 (d, *J* = 9.0 Hz, 1H, indole C_6-H), 8.42-8.49 (m, 2H, indole C_4-H, NH), 11.73 (s, 2H, CSNH, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 37.75 (CH_3), 55.34 (CH_2), 110.30 (CH), 110.97 (CH), 115.43, 115.58 (CH), 120.17 (CH), 120.69 (CH), 126.87 (CH), 127.75 (CH),

142.59, 146.60, 148.38, 153.75, 156.37, 162.92, 165.04; EIMS (70eV) *m/z* (%): 369 (3), 339 (3), 206 (100), 178 (80), 163 (93), 149 (20), 136 (51), 121 (97), 104 (26), 91 (91), 77 (26), 65 (17). Anal. Calcd for $C_{17}H_{15}N_5O_4S$ (385): C, 52.99; H, 3.90; N, 18.18. Found: C, 52.74; H, 3.85; N, 18.12.

2.3.6. *N*-(3-Methoxybenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5f)

Yellow crystals (81%): mp 260-262 °C; IR (KBr,cm^{-1}): 3302, 3221 (NH), 1714 (C=O), 1597 (C=N), 1147 (C=S); ^{1}H-NMR (DMSO-d_6, 300 MHz, δ, ppm): 3.74 (s, 3H, CH_3), 4.87 (d, *J* = 6.3 Hz, 2H, benzyl CH_2), 6.84 (dd, *J* = 8.4, 2.4 Hz, 1H, benzyl C_4-H), 6.92-6.95 (m, 2H, benzyl C_2-H, C_6-H), 7.11 (d, *J* = 8.7 Hz, 1H, indole C_7-H), 7.27 (t, *J* = 8.1Hz, 1H, benzyl C_5-H), 8.26 (dd, *J* = 8.7, 2.4 Hz, 1H, indole C_6-H), 8.54 (d, *J* = 2.4 Hz, 1H, indole C_4-H), 10.11 (t, *J* = 6.3 Hz, 1H, CSNH), 11.83 (s, 1H, indole NH), 12.44 (s,1H, NNH); ^{13}C- NMR (DMSO-d_6, δ, ppm): 113.03 (CH), 114.17, 120.74, 122.05, 123.75 (CH), 124.44 (CH), 127.86 (CH), 128.79 (CH), 130.24 (CH), 131.27, 133.50 (CH), 139.82, 141.58, 162.27, 176.19; EIMS (70eV) *m/z* (%): 385 (M^+, 5), 309 (2), 220 (12), 193 (23), 161 (29), 136 (49), 121 (100), 109 (31), 104 (19), 91 (92), 77 (84), 65 (65). Anal.Calcd for $C_{17}H_{15}N_5O_4S$ (385): C, 52.99; H, 3.90; N, 18.18. Found: C, 52.85; H, 3.88; N, 18.10.

2.3.7. *N*-(4-Methoxybenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5g)

Orange crystals (87%): mp 274-276 °C; IR (KBr, cm^{-1}): 3369, 3267 (NH), 1687 (C=O), 1620 (C=N), 1164 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 3.30 (DMSO, CH_3, benzylCH_2), 6.90 (d, *J* = 8.3 Hz, indole C_7-H), 7.11 (t, *J* = 9.0 Hz, benzyl C_5-H), 7.49-7.50 (m, 4H, benzyl C_2-H, C_3-H, C_6-H, indole C_6-H), 7.99 (d, *J* = 2.4 Hz, 1H, indole C_4-H), 10.92 (s, 1H, CSNH), 11.37 (s, 1H, indole NH), 12.67 (s, 1H, NNH); ^{13}C- NMR (DMSO-d_6, δ, ppm): 112.15 (CH), 112.48 (CH), 112.65 (CH), 113.03 (CH), 114.17, 121.34 (CH), 122.06, 123.78 (CH), 129.87 (CH), 129.99 (CH), 131.23, 133.49 (CH), 139.99, 141.57, 159.96, 162.28, 163.18, 176.14; EIMS (70eV) *m/z* (%): 385 (M^+, 2), 309 (2), 248 (2), 206 (13), 194 (22), 161(57), 136 (34), 121 (100), 109 (22), 104 (11), 91 (38), 77 (74), 65

(28). Anal.Calcd for $C_{17}H_{15}N_5O_4S$ (385): C, 52.99; H, 3.90; N, 18.18. Found: C, 52.88; H, 3.87; N, 18.14.

2.3.8. *N*-(2-Fluorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5h)

Yellow crystals (82%): mp 264-266 °C; IR (KBr, cm^{-1}): 3352, 3052 (NH), 1701 (C=O), 1607 (C=N), 1172 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 3.85 (s, 2H, benzyl CH_2), 6.90 (d, J = 8.3 Hz, 1H, indole C_7-H), 7.01 (t, J = 7.5 Hz, 1H, benzyl C_4-H), 7.14 (d, J = 8.0 Hz, 1H, benzyl C_6-H), 7.29 (td, J = 8.0, 1.2 Hz, 1H, benzyl C_5-H), 7.51-7.54 (m, 2H, benzyl C_3-H, indole C_6-H), 7.93 (d, J = 2.4 Hz, 1H, indole C_4-H), 10.56 (s, 1H, CSNH), 11.34 (s, 1H, indole NH), 12.59 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 55.65 (CH_2), 111.89 (CH), 112.98 (CH), 114.13, 120.06 (CH), 122.21, 123.52 (CH), 126.88, 127.97 (CH), 128.10 (CH), 130.73, 133.28 (CH), 141.46, 153.85, 162.24, 176.90; EIMS (70eV) *m/z* (%): 373 (M^+, 4), 345 (2), 297 (2), 220 (4), 192 (26), 149 (47), 124 (53), 109 (100), 90 (12), 83 (55), 77 (21). Anal.Calcd for$C_{16}H_{12}FN_5O_3S$ (373): C, 51.47; H, 3.22; N, 18.77. Found: C, 51.39; H, 3.19; N, 18.69.

2.3.9. *N*-(3-Fluorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5i)

Yellow crystals (86%): mp 256-258 °C; IR (KBr, cm^{-1}): 3359, 3201 (NH), 1697 (C=O), 1606 (C=N), 1161 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 4.92 (d, J = 5.0 Hz, 2H, benzyl CH_2), 7.07-7.23 (m, 4H, benzyl C_2-H, C_5-H, C_6-H, indole C_7- H), 7.37-7.46 (m, 1H, benzyl C_4-H), 8.27 (d, J = 10 Hz, 1H, indole C_6-H), 8.54 (d, J = 2.5 Hz, 1H, indole C_4-H), 10.13 (t, J = 7.5Hz, 1H, CSNH), 11.84 (s, 1H, indole NH), 12.47 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 46.71 (CH_2), 111.21 (CH), 113.68 (CH), 116.20 (CH), 120.90, 123.16 (CH), 126.96 (CH), 130.24, 130.29, 130.37 (CH), 141.07, 142.67, 147.42, 162.95, 177.89; EIMS (70eV) *m/z* (%): 373 (M^+, 4), 345 (2), 297 (2), 191 (24), 149 (20), 125 (52), 111 (74), 97 (90), 83 (73), 71 (85), 57 (100). Anal.Calcd for$C_{16}H_{12}FN_5O_3S$ (373): C, 51.47; H, 3.22; N, 18.77. Found: C, 51.38; H, 3.20; N, 18.75.

2.3.10. *N*-(4-Fluorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5j)

Yellow fluffy crystals (89%): mp 276-278 °C; IR (KBr, cm^{-1}): 3342, 3130 (NH), 1722 (C=O), 1605 (C=N), 1161 (C=S)); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 3.30 (DMSO, benzyl CH_2), 6.91 (d, J = 8.0 Hz, 1H, indole C_7-H), 7.39-7.72 (m, 5H, benzyl C_2-H, C_3-H, C_5-H, C_6-H, indole C_6-H), 7.83 (d, J = 2.5 Hz, 1H, indole C_4-H), 10.90 (s, 1H, CSNH), 11.35 (s, 1H, indole NH), 12.64 (s, 1H, NNH); ^{13}C- NMR (DMSO-d_6, δ, ppm): 113.04 (CH), 114.15, 122.16, 123.65 (CH), 127.60 (CH), 129.02 (CH), 129.61 (CH), 130.61, 131.19, 131.57, 133.41 (CH), 135.94, 141.41, 162.22, 177.68; EIMS (70eV) *m/z* (%): 373 (M^+, 2), 345 (2), 297 (2), 192 (24), 182 (9), 149 (25), 124 (40), 109 (100), 83 (70), 57 (100). Anal.Calcd for$C_{16}H_{12}FN_5O_3S$ (373): C, 51.47; H, 3.22; N, 18.77. Found: C, 51.39; H. 3.19; N, 18.68.

2.3.11. *N*-(2-Chlorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5k)

Yellow crystals (91%): mp 268-270 °C; IR (KBr, cm^{-1}): 3348, 3157 (NH), 1692 (C=O), 1616 (C=N), 1188 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 4.96 (d, J = 5.0 Hz, 2H, benzyl CH_2), 7.13 (d, J = 9.0 Hz, 1H, indole C_7-H), 7.27-7.37 (m, 3H, benzyl C_4-H, C_5-H, C_6-H), 7.49 (d, J = 7.5 Hz, 1H, benzyl C_3-H), 8.28 (d, J = 9.0 Hz, 1H, indole C_6-H), 8.55 (s, 1H, indole C_4-H), 10.12 (t, J = 6.0 Hz, 1H, CSNH), 11.85 (s, 1H, indole NH), 12.50 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 45.05 (CH_2), 111.24 (CH), 116.24 (CH), 120.89, 126.99 (CH), 127.01 (CH), 127.79, 128.65, 129.00 (CH), 130.00, 131.00, 135.01, 142.68, 147.45, 162.95, 178.19; EIMS (70eV) *m/z* (%): 354 (71), 325 (5), 268 (7), 206 (62), 192 (18), 183 (18), 178 (12), 163 (13), 140 (25), 125 (100), 106 (17), 89 (14), 77 (10), 63 (7). Anal.Calcd for $C_{16}H_{12}ClN_5O_3S$ (389.5): C, 49.29; H, 3.08, N, 17.97. Found: C, 49.38; H, 3.07; N, 17.95.

2.3.12. *N*-(3-Chlorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5l)

Orange crystals (72%): mp 258-260 °C; IR (KBr,cm^{-1}): 3348, 3064 (NH), 1701 (C=O), 1605 (C=N), 1163 (C=S); ^{1}H-NMR (DMSO-d_6, 300 MHz, δ, ppm): 4.90 (d, J = 6.0 Hz, 2H, benzyl CH_2), 7.12 (d, J = 9.0 Hz, 1H, indole C_7-H), 7.32-7.43 (m, 4H, benzyl C_2-H, C_4-H, C_5-H, C_6-H), 8.28 (dd, J = 8.7,

2.4 Hz, 1H, indole C_6-H), 8.54 (d, *J* = 2.4 Hz, 1H, indole C_4-H), 10.14 (t, *J* = 6.0 Hz, 1H, CSNH), 11.84 (s, 1H, indole NH), 12.47 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 47.14 (CH_2), 111.74 (CH), 116.70 (CH), 121.41, 126.43 (CH), 127.43 (CH), 127.50 (CH), 130.76 (CH), 130.91, 133.44, 141.25, 143.16, 147.97, 163.48, 178.34; EIMS (70eV) *m/z* (%): 389 (M^+, 3), 313 (2), 220 (6), 192 (50), 178 (4), 165 (16), 140 (49), 125 (100), 89 (80), 77 (58), 64 (25). Anal.Calcd for $C_{16}H_{12}ClN_5O_3S$ (389.5): C, 49.29; H, 3.08, N, 17.97. Found: C, 49.46; H, 3.09; N, 17.99.

2.3.13. *N*-(4-Chlorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5m)

Orange crystals (89%): mp 238-240 °C; IR (KBr,cm^{-1}): 3336, 3188 (NH), 1697 (C=O), 1604 (C=N), 1166 (C=S); ^{1}H-NMR (DMSO-d_6, 400 MHz, δ, ppm): 4.89 (d, *J* = 9.0 Hz, 2H, benzyl CH_2), 7.13 (d, *J* = 7.5 Hz, 1H, indole, C_7-H), 7.37-7.48 (m, 4H, benzyl C_2-H, C_3-H, C_5-H, C_6-H), 8.27 (d, *J* = 8.0 Hz, 1H, indole C_6-H), 8.54 (s, 1H, indole C_4-H), 10.14 (t, *J* = 9.0 Hz, 1H, CSNH), 11.84 (s, 1H, indole NH), 12.45 (s, 1H, NNH); ^{13}C-NMR ((DMSO-d_6, δ, ppm): 46.56 (CH_2), 111.22 (CH), 116.07 (CH), 120.83, 126.77 (CH), 127.92 (CH), 129.09 (CH), 129.95, 131.52, 137.02, 142.30, 147.48, 162.35, 177.75; EIMS (70eV) *m/z* (%): 391/389 (M^+, 2/7), 361/359 (3/2), 220 (4), 206 (46), 192 (25), 178 (7), 163 (7), 140 (25), 125 (100), 89 (13), 77 (8), 65 (16). Anal.Calcd for $C_{16}H_{12}ClN_5O_3S$ (389.5): C, 49.29; H, 3.08, N, 17.97. Found: C, 49.36; H, 3.05; N, 17.93.

2.3.14. *N*-(2,4-Dichlorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5n)

Orange fluffy crystals (80%): mp 260-262 °C; IR (KBr,cm^{-1}): 3336, 3130 (NH), 1681 (C=O), 1606 (C=N), 1159 (C=S); ^{1}H-NMR (DMSO-d_6, 300 MHz, δ, ppm): 4.91 (d, *J* = 6.0 Hz, 2H, benzyl CH_2), 7.13 (d, *J* = 8.4 Hz, 1H, indole C_7-H), 7.28 (d, *J* = 9.0 Hz, 1H, benzyl C_6-H), 7.46 (dd, *J* = 8.1, 2.1 Hz, 1H, benzyl C_5-H), 7.66 (d, *J* = 2.1 Hz, 1H, benzyl C_3-H), 8.28 (dd, *J* = 8.7, 2.4 Hz, 1H, indole C_6-H), 8.55 (d, *J* = 2.4 Hz, 1H, indole C_4-H), 10.13 (t, *J* = 6.0 Hz, 1H, CSNH), 11.86 (s, 1H, indole NH), 12.51 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm): 45.18 (CH_2), 111.77 (CH), 116.73 (CH), 121.34 (CH), 127.55 (CH), 127.85 (CH), 129.15 (CH), 129.74, 130.96, 132.76, 132.95, 134.79,

143.17, 147.99, 163.45, 178.70;EIMS (70eV) *m/z* (%): 390/388 (M^+-HCl, 10/13), 208/206 (8/2), 192 (35), 176 (22), 159 (100), 140 (20), 123 (28), 103 (22), 89 (40), 75 (37), 63 (48), 51 (22). Anal.Calcd for $C_{16}H_{11}Cl_2N_5O_3S$ (424): C, 45.28; H, 2.59; N, 16.51. Found: C, 45.22; H, 2.58; N, 16.49.

2.3.15. *N*-(3,4-Dichlorobenzyl)-2-[5-nitro-2-oxo-2,3-dihydro-*1H*-indol-3-ylidene]-1-hydrazinecarbothioamide (5o)

Orange crystals (79%): mp 270-272 °C; IR (KBr,cm^{-1}): 3336, 3228 (NH), 1693 (C=O), 1612 (C=N), 1166 (C=S); ^{1}H-NMR (DMSO-d_6, 300 MHz, δ, ppm): 4.87 (d, J = 6.0 Hz, 2H, benzyl CH_2), 7.12 (d, J = 8.7 Hz, 1H, indole C_7-H), 7.35 (dd, J = 8.4, 2.1 Hz, 1H, benzyl C_6-H), 7.60-7.64 (m, 2H, benzyl C_2-H, C_5-H), 8.26 (dd, J = 8.7, 2.4 Hz, 1H, indole C_6-H), 8.52 (d, J = 2.1 Hz, 1H, indole C_4-H), 10.12 (t, J = 6.0 Hz, 1H, CSNH), 11.84 (s, 1H, indole NH), 12.47 (s, 1H, NNH); ^{13}C-NMR (DMSO-d_6, δ, ppm):): 46.64 (CH_2), 111.69 (CH), 116.64 (CH), 121.30 (CH), 127.46 (CH), 128.10 (CH), 129.65 (CH), 130.06, 130.86, 131.01, 131.36, 139.89, 143.13, 147.91, 163.41, 178.75;EIMS (70eV) *m/z* (%): 425/423 (M^+, 2/2), 395 (4), 347 (3), 234/232 (4/5), 219/217 (17/21), 206 (84), 192 (29), 176/174 (27/36), 161/159 (94/100). 149/147 (91/61), 140 (39), 125/123 (15/7), 105/103 (15/7), 91/89 (10/10). Anal.Calcd for $C_{16}H_{11}Cl_2N_5O_3S$ (424): C, 45.28; H, 2.59; N, 16.51. Found: C, 45.25; H, 2.54; N, 16.47.

2.4. X-ray structure determination

2.4.1. Crystallographic data collection and structural refinement

Specimen of **5g** of good quality and size was mounted on a thin glass fiber at room temperature and the reflection data were collected on a Bruker Kappa APE XII CCD diffractometer equipped with graphite mono-chromated Mo Kα radiation (λ = 0.71073 Å). The data were corrected to Lorentz and polarization effect. The structure was solved using SHELXS-97.[37] Final refinement on F^2 was carried out by full-matrix least-squares techniques using SHELXL-97[37]

2.5. Biological assays

In vitro urease inhibitory, phytotoxicity and cytotoxicity potential was determined in accordance with the literature protocols given in our published research articles.[38,34,31]

2.6. Molecular docking studies

Molecular docking studies as well as HYDE assessment were carried out according to the literature method provided in our very recently published research paper.[39] In order to perform docking studies, the crystallographic structure of Jack bean urease (PDB ID: 3LA4) was obtained from the RCSB PDB database.[40]

3. Results and discussion

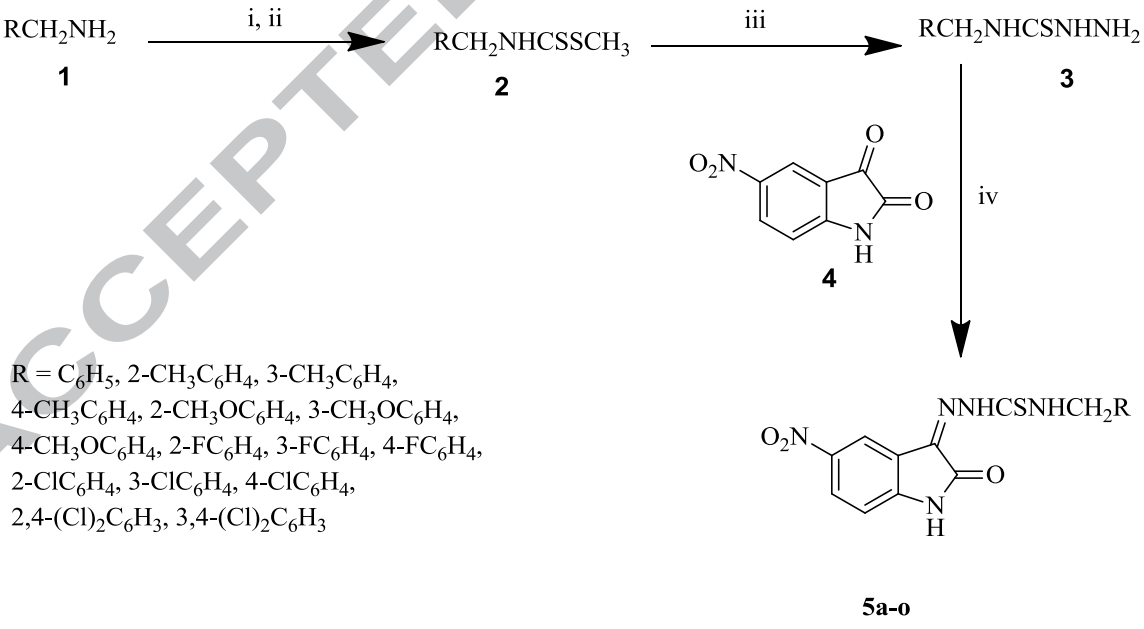
This study describes the synthesis and *in vitro* evaluation of antiurease, phytotoxic and cytotoxic potential of fourteen presently and one previously reported[41] N^4-benzyl substituted 5-nitroisatin-thiosemicarbazones **5b-o** and **5a**, respectively.

3.1. Chemistry of compounds 5a-o

Appropriate *N*-substituted thiosemicarbazides **3** were treated with 5-nitroisatin **4** in 50% ethanol (aq.) to afford the respective isatin-3-thiosemicarbazones **5a-o** (Scheme 1) in good to excellent yields (72-91%).

The structures of the synthesized isatin-thiosemicarbazones **5a-o** were deduced by analytical and spectral (IR, ^{1}H-NMR, ^{13}C-NMR, EIMS) as well as single crystal X-ray diffraction analyses. Satisfactory elemental (CHN) data were obtained in all the cases. Also, the spectral data were in tune with the respective literature.[22,24,42,43] The IR spectra of **5a-o** showed NH stretchings of indole and thioamide functions in the 3359-3302 and 3250-3052 cm^{-1} regions. The lactam C = O, azomethine C = N and thioamide C = S stretchings were observed in the 1722-1678, 1616-1597 and 1188-1147 cm^{-1} regions, respectively.[22,42,43] The ^{1}H-NMR spectra exhibited two separate singlets at δ 11.34-11.86 and 12.43-12.71 for indole NH and thiosemicarbazone N^2-H, respectively, in all the cases except **5e**, wherein the indole NH resonance was observed as a multiplet at δ 8.42-8.49 due to overlapping of indole C_4-Hsignal, while the thiosemicarbazone N^2-H resonated as a singlet at δ 11.73 together with thiosemicarbazone N^4-H. Similarly, the thiosemicarbazone N^4-H appeared as a triplet at δ 10.05-10.14 in all the cases[22,24] except **5c**, **5g**, **5h** and **5j**, wherein it resonated as a singlet at δ 10.82, 10.92,

10.56 and 10.90, respectively. Furthermore, the benzyl CH_2 protons were observed as a doublet at δ 4.41-4.96 in all the cases[22,24] except **5c**, **5g**, **5h** and **5j;** in the case of **5h,** they appeared as a singlet at δ 3.85, whereas in **5c**, **5g** and **5j**, they resonated together with the CH_3 group protons and/or the residual protons of DMSO. The ^{13}C-NMR spectra of compounds **5a-o** supported the IR and ^{1}H-NMR findings. Also, the electron impact (EI) mass spectra of all the compounds except **5e**, **5k** and **5n** demonstrated molecular ions of varied intensity, which validated their molecular weights. The structures of **5e**, **5k** and **5n** were, however, made certain by the existence of the fragments related to thiosemicarbazone moieties, formed by the breakage of N-N and NH-CS bonds. The major fragmentation pathway involved the breakage of endocyclic NH-CO and exocyclic N-N, NH-CS bonds.[43] The proposed fragmentation pattern of **5c** is depicted in Figure S1 (see the SI). X-ray structure of a representative compound **5g** was determined to substantiate the assigned structures of the synthesized thiosemicarbazones **5a-o**.



Scheme 1. Reagents and conditions: (i) Et_3N, MeOH, CS_2, stir, 30 ^{o}C (internal), 75 min., r.t., 1 h (ii) CH_3I, MeOH, stir, -10 ^{o}C, 20 min., r.t., 2 h (iii) $NH_2NH_2.H_2O$, EtOH, reflux, 2 h (iv) 50% EtOH (aq.), reflux, 2 h.

3.1.1. X-ray analysis of compound 5g

For the X-ray structure determinations, X-ray quality single crystals of **5g** (Table 1) were obtained from its DMSO solution by slow evaporation at room temperature. The X-ray analysis demonstrates that it crystallizes in triclinic crystal lattice with the $P\bar{1}$ space group. The molecular structure (ORTEP diagram) of **5g** along with crystallographic numbering scheme is depicted in Figure 1.The central *N*-iminothiourea (thiosemicarbazone) moiety is almost planar adopting preferably the S-*cis*/S-*trans* conformation, most probably due to the formation of intramolecular hydrogen bond [N(5)-H(4)···N(3) 2.259 Å].[44-46] The dihedral angles between S(1)-C(9)-N(4)-H(3), S(1)-C(9)-N(5)-H(4) and C(9)-N(4)-N(3)-C(7) are -1.61°, 176.07° and 173.08°, respectively. This planarity around the central *N*-iminothiourea moiety can be ascribed to extensive delocalization of N lone pairs onto the thiocarbonyl (C=S) function, which is clearly shown by the shorter N-C bond lengths [N(4)-C(9) 1.376(3) Å and N(5)-C(9) 1.325(3) Å], suggestive of the partial double bond character of N-C bonds. The slightly longer bond length in the case of N-C bond, directly connected to the imino (-C=N-) function of the *N*-iminothiourea moiety, indicates less delocalization of the N lone pair onto the thiocarbonyl (C=S) group, presumably due to the presence of inductively electron-attracting sp^2-hybridized N atom in its vicinity. Notably, it is the planarity and the mentioned intramolecular hydrogen bonding due to which the central *N*-iminothiourea moiety in such molecules adopts S-*cis*/S-*trans* conformation, thus exposing the thioamide function to form centrosymmetric supramolecular dimers in their solid state packing.[44-46] However, compound **5g** has an additional amide moiety, offering a competing environment for the appearance of otherwise two reliable centrosymmetric dimer synthons [$R_2^2(8)\{\cdots H–N–C=S\}_2$ and $R_2^2(8)\{\cdots H–N–C=O\}_2$] in crystal engineering.[44-47]

Table 1. Crystallographic data of **5g**

Crystal data	**5g**
CCDC	1005939
Chemical formula	$C_{19}H_{21}N_5O_5S_2$

M_r	463.53
Crystal system, space group	Triclinic, $P\bar{1}$
Temperature (K)	296
a, b, c (Å)	8.6754 (4), 11.9082 (6), 12.2545 (10)
α, β, γ (°)	112.750 (3), 98.066 (3), 106.130 (2)
V (Å^3)	1076.79 (12)
Z	2
Radiation type	Mo $K\alpha$
μ (mm^{-1})	0.29
Crystal size (mm)	0.38 × 0.23 × 0.20
Data collection	
Diffractometer	Bruker Kappa APEXII CCD
Absorption correction	Multi-scan (*SADABS*; Bruker, 2005)
T_{min}, T_{max}	0.898, 0.944
No. of measured, independent and observed [$I > 2\sigma(I)$] reflections	17062, 5051, 3780
R_{int}	0.022
$(\sin \theta/\lambda)_{max}$ (Å^{-1})	0.658
Refinement	
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.045, 0.150, 1.07
No. of reflections	5051
No. of parameters	283
H-atom treatment	H-atom parameters constrained
$\Delta\rangle_{max}$, $\Delta\rangle_{min}$ (e Å^{-3})	0.85, -0.41

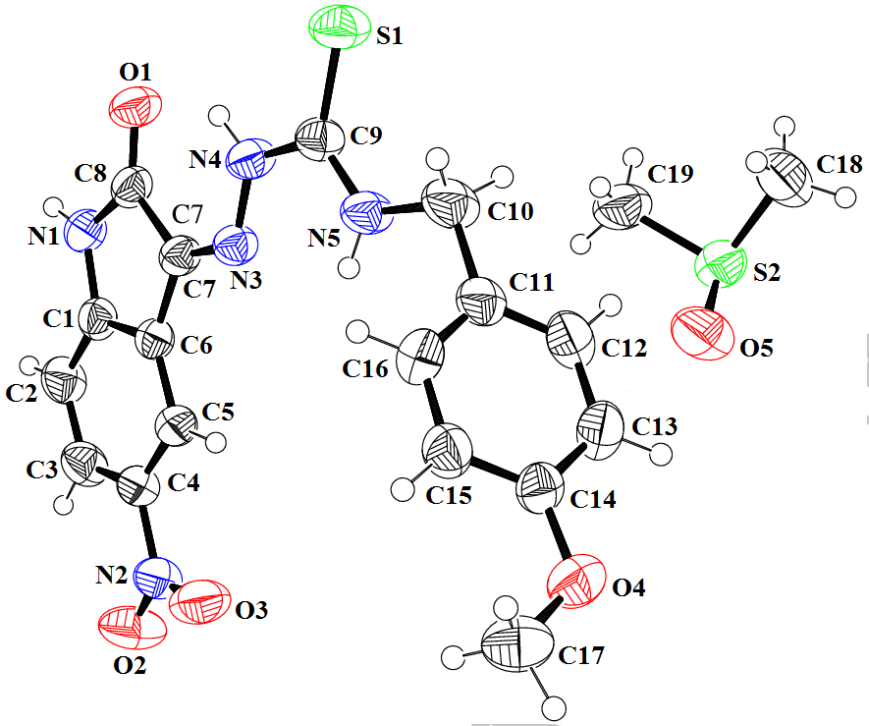


Figure 1. The molecular structure (ORTEP diagram) of **5g**.

3.2. Biology of compounds 5a-o

3.2.1. Urease inhibition (*in vitro*)

The synthesized N^4-benzyl substituted 5-nitroisatin-thiosemicarbazones **5a-o** were tested for their urease inhibitory potential against Jack bean urease. Thiourea served as a reference inhibitor in this assay. All the compounds proved to be highly potent inhibitors of the enzyme, demonstrating inhibitory activity (IC_{50} = 0.87 ± 0.25 – 8.09 ± 0.23 µM) much better than the reference inhibitor, thiourea (IC_{50} = 22.3 ± 1.12 µM) (Table 2) and may thus act as convincing leads for further studies.

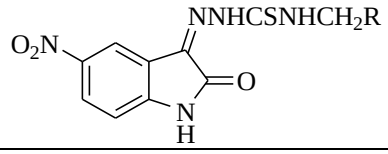
The structure-activity relationship (SAR) studies in the isatin-thiosemicarbazones **5a-o** revealed that in comparison to compound **5a** having no substituent about the phenyl ring of the benzyl group attached to N^4 of the thiosemicarbazone moiety, all the other compounds except **5k** and **5m**, irrespective of the type, number and place of the functional groups present on the phenyl ring, showed increased enzymatic activity (IC_{50} values 0.87 ± 0.25 – 2.54 ± 0.18 µM vs. 5.92 ± 0.25 µM). Compound **5g** possessing methoxy substituent at position-4 of the phenyl ring was found to be the

most potent urease inhibitor of the series in the present assay, exhibiting about seven and twenty six fold more activity than compound **5a** and the reference inhibitor, thiourea (IC_{50} = 0.87 ± 0.25 μM vs. 5.92 ± 0.25 and 22.3 ± 1.12 μM, respectively). Next most potent urease inhibitor was compound **5c** with methyl substituent at position-3 of the phenyl ring. This compound displayed somewhat less inhibitory activity than the most potent inhibitor **5g** but much more than the reference inhibitor, thiourea (IC_{50} value 1.27 ± 0.19 μM vs.0.87 ± 0.25 and 22.3 ± 1.12 μM, respectively). The third most potent urease inhibitor was compound **5j** having a fluoro substituent at position-4 of the phenyl ring. This compound, regardless of the nature and position of the substituents about the phenyl ring, exhibited almost the same activity as that of compound **5c** (IC_{50} value 1.29 ± 0.27 vs.1.27 ± 0.19 μM). However, like compound **5c**, it was found to be somewhat less active than the most potent urease inhibitor of the series i.e. compound **5g** (IC_{50} value 1.29 ± 0.27 vs. 0.87 ± 0.25 μM) but about five and seventeen times more active than compound **5a** and the reference inhibitor, thiourea (IC_{50} value 1.29 ± 0.27 vs. 5.92 ± 0.25 and 22.3 ± 1.12 μM, respectively). The remaining relatively more potent urease inhibitors were the mono- and dichloro-substituted derivatives **5l** and **5n**. Among these, **5l** having the substituent at position-3 of the phenyl ring was found to be a little bit less active than the corresponding methyl-substituted derivative **5c** but about four and fourteen times more active than compound **5a** and the reference inhibitor, thiourea (IC_{50} value 1.63 ± 0.18 μM vs. 1.27 ± 0.19, 5.92 ± 0.25 and 22.3 ± 1.106 μM, respectively). In contrast, the dichloro-substituted derivative **5n** with the substituents at positions-2,4 (*ortho, para*) of the phenyl ring was four times more active than the monochloro-substituted derivative **5k** bearing the substituent at position-2 of the phenyl ring (IC_{50} value 1.94 ± 0.13 vs. 8.09 ± 0.23 μM). This indicated that compound **5n** with an additional inductively electron-withdrawing chloro function at position-4 of the phenyl ring meddled with the enzyme in a different manner, giving rise to an extensive increase in its inhibitory activity.

The above results showed that the attachment of variously substituted benzyl groups (having one or two inductively electron-donating or –withdrawing functionalities about the phenyl ring) to N^4 of the

thiosemicarbazone moieties caused the molecules to interfere with the enzyme differently and in some instances much more competently.

Table 2. Inhibition of urease by compounds **5a-o**



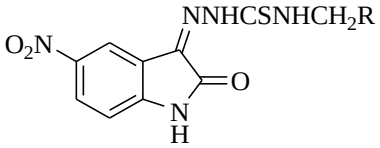
Compound	R	IC_{50} ± SEM* (µM)
5a	C_6H_5	5.92 ± 0.25
5b	2-$CH_3C_6H_4$	1.39 ± 0.13
5c	3-$CH_3C_6H_4$	1.27 ± 0.19
5d	4-$CH_3C_6H_4$	1.52 ± 0.11
5e	2-$CH_3OC_6H_4$	1.33 ± 0.12
5f	3-$CH_3OC_6H_4$	1.34 ± 0.17
5g	4-$CH_3OC_6H_4$	0.87 ± 0.25
5h	2-FC_6H_4	1.58 ± 0.16
5i	3-FC_6H_4	2.54 ± 0.18
5j	4-FC_6H_4	1.29 ± 0.27
5k	2-ClC_6H_4	8.09 ± 0.23
5l	3-ClC_6H_4	1.63 ± 0.18
5m	4-ClC_6H_4	6.34 ± 0.18
5n	2,4-$(Cl)_2C_6H_3$	1.94 ± 0.13
5o	3,4-$(Cl)_2C_6H_3$	2.11 ± 0.07
Thiourea**		22.3 ± 1.06

* Standard error of the mean; ** standard inhibitor for urease.

3.2.2. Phytotoxicity (*in vitro*)

The synthesized 5-nitroisatin-thiosemicarbazones **5a-o** were further tested for their phytotoxicity potential at 1000, 100, 10 or 500, 50, 5 µg / mL concentrations. All the compounds of this series except **5f**, **5h** and **5j** appeared to be active in the present assay, displaying weak or non-significant (5-35%) plant growth inhibition at the highest tested concentrations (1000 or 500 µg/mL) (Table 3).

Table 3. Growth inhibition of *Lemna aequinocitalis* by compounds **5a-o*** at different concentrations



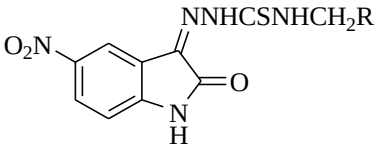
Compound	R	1000 µg/mL (% G.I)	100 µg/mL (% G.I)	10 µg/mL (% G.I)	500 µg/mL (% G.I)	50 µg/mL (% G.I)	5 µg/mL (% G.I)
5a	C_6H_5	–	–	–	10	00	00
5b	2-$CH_3C_6H_4$	15	05	00	–	–	–
5c	3-$CH_3C_6H_4$	20	05	00	–	–	–
5d	4-$CH_3C_6H_4$	05	00	00	–	–	–
5e	2-$CH_3OC_6H_4$	35	15	00	–	–	–
5f	3-$CH_3OC_6H_4$	00	00	00	–	–	–
5g	4-$CH_3OC_6H_4$	10	06	00	–	–	–
5h	2-FC_6H_4	00	00	00	–	–	–
5i	3-FC_6H_4	10	00	00	–	–	–
5j	4-FC_6H_4	00	00	00	–	–	–
5k	2-ClC_6H_4	05	00	00	–	–	–
5l	3-ClC_6H_4	05	00	00	–	–	–
5m	4-ClC_6H_4	05	00	00	–	–	–
5n	2,4-$(Cl)_2C_6H_3$	–	–	–	30	00	00
5o	3,4-$(Cl)_2C_6H_3$	–	–	–	10	00	00

*The standard drug, paraquat, shows 100% growth inhibition at a concentration of 0.015 µg/mL; G.I: growth inhibition.

3.2.3. Cytotoxicity (*in vitro*)

The synthesized thiosemicarbazones **5a-o** were also evaluated for their cytotoxicity potential by a brine shrimp lethality bioassay, using etoposide (a standard anticancer drug) as a reference point. From the data presented in Table 4, it is evident that all the compounds of this series except **5i** gave LD_{50} values >2.36 x 10^{-4} – 2.82 x 10^{-4} M against *Artemia salina* in the present assay and, therefore, can be considered to be almost inactive. Compound **5i** bearing 3-fluorobenzyl function at N^4 of the thiosemicarbazone moiety, however, proved to be active, exhibiting cytotoxic activity with LD_{50} value 2.55 × 10^{-5} M.

Table 4. Brine shrimp (*Artemia salina*) lethality bioassay for compounds **5a-o**



Compound	R	LD_{50} (M)
5a	C_6H_5	$>2.82 \times 10^{-4}$
5b	2-$CH_3C_6H_4$	$>2.71 \times 10^{-4}$
5c	3-$CH_3C_6H_4$	$>2.71 \times 10^{-4}$
5d	4-$CH_3C_6H_4$	$>2.71 \times 10^{-4}$
5e	2-$CH_3OC_6H_4$	$>2.60 \times 10^{-4}$
5f	3-$CH_3OC_6H_4$	$>2.60 \times 10^{-4}$
5g	4-$CH_3OC_6H_4$	$>2.60 \times 10^{-4}$
5h	2-FC_6H_4	$>2.68 \times 10^{-4}$
5i	3-FC_6H_4	2.55×10^{-5}
5j	4-FC_6H_4	$>2.68 \times 10^{-4}$
5k	2-ClC_6H_4	$>2.57 \times 10^{-4}$
5l	3-ClC_6H_4	$>2.57 \times 10^{-4}$
5m	4-ClC_6H_4	$>2.57 \times 10^{-4}$
5n	2,4-$(Cl)_2C_6H_3$	$>2.36 \times 10^{-4}$
5o	3,4-$(Cl)_2C_6H_3$	$>2.36 \times 10^{-4}$

*Tested at 100, 10, 1 µg/mL concentrations.

3.3. Molecular docking

3.3.1. Molecular docking studies of compounds 5a-o

To rationalize the obtained *in vitro* biological results, molecular docking studies of all the inhibitors **5a-o** were carried out against Jack bean urease PDB ID: 3LA4. It was rationalized that most of the compounds were found to bind in the same region of the active site. Before docking the most active inhibitor **5g** in the active site of the urease enzyme, molecular docking studies of the co-crystallized ligand KCX (Lysine NZ-carboxylic acid) was carried out. The ligand was reproduced successfully with an RMSD value of 1.5 Å. Molecular docking studies of all the test compounds **5a-o** were carried out to investigate the

interactions inside the active site. The most potent inhibitor **5g** was successfully oriented inside the active site of the target enzyme. The putative binding mode of **5g** is shown in Figure 2. After reproducing the co-crystallized reference ligand into the active site of the receptor, all the derivatives were docked inside the active site. The docking studies supported the experimental results that most of the inhibitors inhibit the catalytic site of the enzyme. As described previously,[38,39] the most important interactions i.e., hydrogen bonding were seen between the oxygen and hydrogen atoms of the inhibitors and the amino acid residues Arg609, Asp494, Ala440 and His593. Similarly, as reported earlier by us,[48] stable hydrogen bonding interactions within the active pocket were noticed with the amino acid residues His492, His593 and His594. The 4-methoxy benzyl substituent was oriented towards bottom of the active site, while the isatin scaffold located towards opening of the active site and the mid-gorge area. Moreover, the thiosemicarbazone part was found to interact with Arg439 and Ala636 in the middle of the active site. Similarly, weak interactions were shown by all the compounds with Ni842 in the catalytic site of the urease enzyme. Furthermore, the benzyl substituent also showed π-π interaction with the imidazole ring of His594 and π-charge interactions were identified by the thiosemicarbazone moieties of the inhibitors. The putative binding pose of compound **5g** was selected after HYDE assessment and visualization of top 30 poses and is represented in Figure 2.

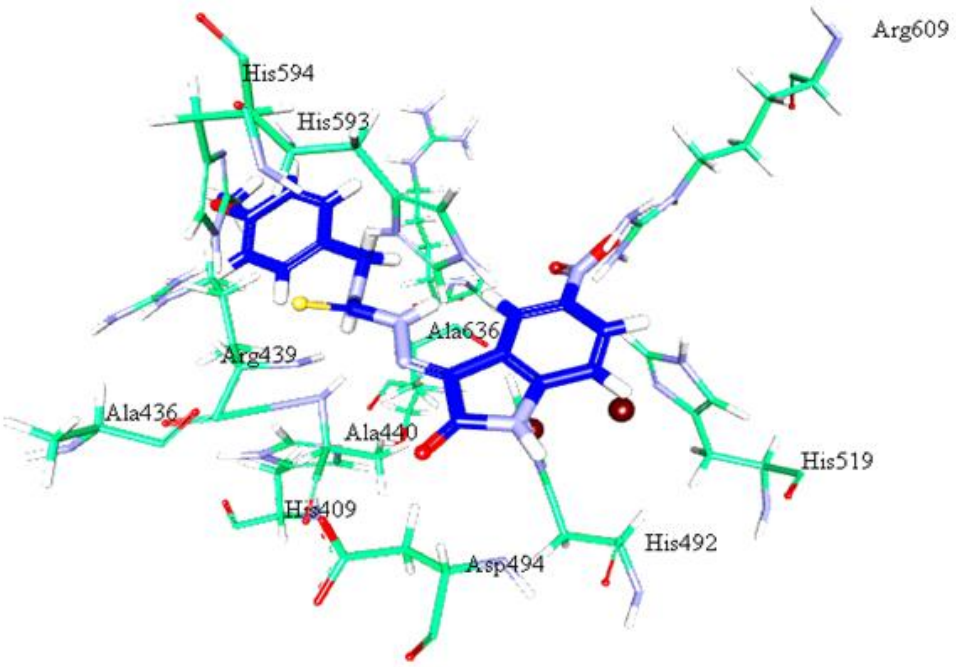


Figure 2. A possible binding mode of compound **5g** is shown. Carbon atoms of **5g** are colored blue, while that of protein light green; oxygen, sulfur and nitrogen atoms are colored red, yellow and blue, respectively. The two nickel ions in the active site are represented as small dark brown spheres.

3.3.2. HYDE assessment of compounds 5a-o

The HYDE affinity assessment was done for the first 20 top ranking docked conformations. The binding free energy ΔG, FlexX docking score and the most favorable poses for all the derivatives **5a-o** are given in Table 5. Most of the compounds bind to the receptor with a very high binding affinity.

Table 5. Docking scores and their corresponding ranks by HYDE affinity assessment

Compound	FlexX score of the top ranking pose	Poser rank	Binding free energy ΔG (kJ mol^{-1})
5a	-20.82	10	-12
5b	-20.78	8	-5
5c	-20.45	17	-5
5d	-20.75	12	-14
5e	-18.60	15	-5

5f	-22.62	2	-2
5g	-19.62	19	-13
5h	-20.88	9	-5
5i	-22.25	8	-2
5j	-20.78	12	-13
5k	-20.30	16	-11
5l	-21.10	8	-4
5m	-20.97	7	-12
5n	-20.33	9	-12
5o	-19.94	15	-7

4. Conclusions

In this study, a series of fifteen N^4-benzyl substituted 5-nitroisatin-thiosemicarbazones **5a-o** have been synthesized and evaluated for *in vitro* urease inhibitory, phytotoxic and cytotoxic activities. All the synthesized thiosemicarbazones proved to be highly potent inhibitors of the enzyme, exhibiting inhibitory activity even better than the reference inhibitor (i.e. thiourea) used in the assay. These compounds, by demonstrating no or weak/non-significant phytotoxicity, attract much attention to their utility as valuable soil ureases inhibitors because they could be mixed with fertilizers in small quantities to enhance the overall effectiveness of nitrogen utilization. Also, being non toxic, they could prove to be credible candidates for orally effective remedial agents used for the treatment of certain clinical conditions induced by microbial ureases.

This study presents the first case of display of urease inhibition by N^4-benzyl substituted 5-nitroisatin-thiosemicarbazones and provides a tangible basis for further studies on such compounds to develop more potent and safe urease inhibitors of agricultural/ medicinal interest.

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Graphical abstract

Synthesis, X-ray molecular structure, biological evaluation and molecular docking studies of some N^4-benzyl substituted 5-nitroisatin-3-thiosemicarbazones

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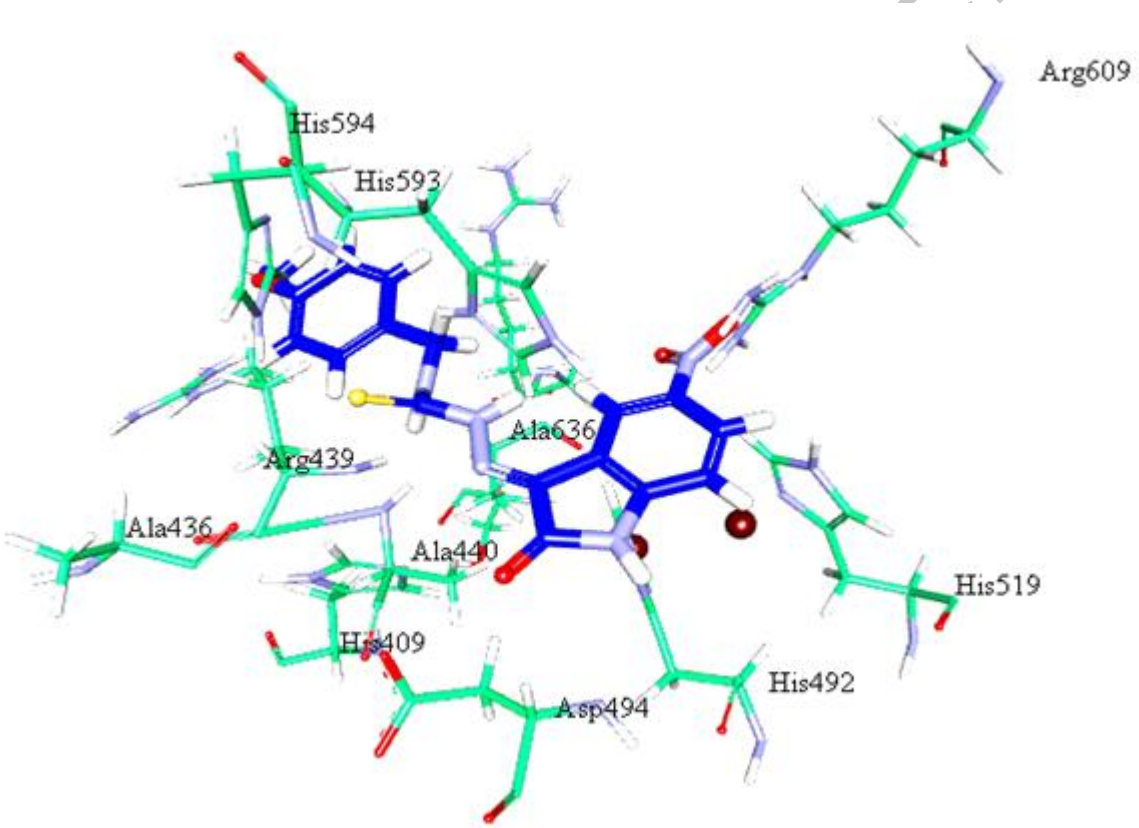

A possible binding mode of compound **5g** bound to active pocket of Jack bean urease