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A new series of asymmetric bis-isatin derivatives containing urea/thiourea moiety: Preparation, spectroscopic elucidation, antioxidant properties and theoretical calculations

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ABSTRACT

In the present study, design, synthesis, characterization, and investigation of antioxidant properties of novel asymmetric bis-isatin derivatives (**1-8**) containing urea/thiourea moiety are reported for the first time. FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopic methods and elemental analysis were used to elucidate the structures of the synthesized compounds. Their CUPRAC and ABTS cation radical scavenging abilities were investigated for antioxidant activity. The bis-isatins containing urea moiety (compounds **1-4**) did not show ABTS activity, while those containing the thiourea moiety (compounds **5-8**) showed moderate ABTS activity. Besides, all bis-isatins were observed to exhibit CUPRAC activity at a low micromolar level The 1-(5-chloro-2-oxoindolin-3-ylidene)-5-(2-oxoindolin-3-ylidene)thiocarbonohydrazide (compound **5**) showed the highest ABTS activity with IC_{50} value of 18.44 μM; on the other hand, the 1-(5-chloro-2-oxoindolin-3-ylidene)-5-(5-methoxy-2-oxoindolin-3-ylidene)carbonohydrazide (compound **2**) had the strongest CUPRAC activity with $A_{0.50}$ value of 0.600 μM. Both spectroscopic and antioxidant properties of the compounds were examined computationally, and the structure-activity relationship was investigated theoretically by comparing with experimental data. The ground state geometries and chemical reactivity parameters of the compounds were calculated using the B3LYP hybrid functional with 6-311++g(2d,2p) and 6-31g(d) basis sets. After determining the local electron affinity of the compounds, the Laplacian bond order and intrinsic bond strength indexes (independent gradient model-δg^{IGMH} and $IBSI^{IGMH}$ descriptors based on Hirshfeld approach) of hydrogen bonds at possible reactive sites were calculated and associated with the antioxidant properties of the compounds.

1. Introduction

Isatin (1H-indole-2,3-dione), one of the indole-based derivatives, constitutes an important class of aromatic heterocyclic compounds found in plant and human tissue. Emerging as a promising nucleus, these relationships have attracted considerable attention in medicinal chemistry and drug discovery over the past two decades. Isatin nucleus can be considered an important scaffold for the design of biologically active compounds. Various biological activities can be performed by modifying the isatin scaffold, such as anti-cancer [1,2], anti-oxidant [3,4], HIV reverse transcriptase inhibition [5], anti-fungal [6], and anti-bacterial [7]. It has also been shown that the isatin ring can be used as an important part of anti-cancer hybrid molecules [8,9]. Also, the design of many carbonic anhydrase inhibitors based on isatin has been reported [10–12]. Thio/carbohydrazones are another important class of organic chemistry with similar biological effects, and have been reported to have numerous medicinal and biological activities such as antioxidant [13,14], anti-bacterial [15], anti-microbial [16], anti-tumor [17], anti-tuberculosis [18], anti-fungi [19], and antiviral activity [20].

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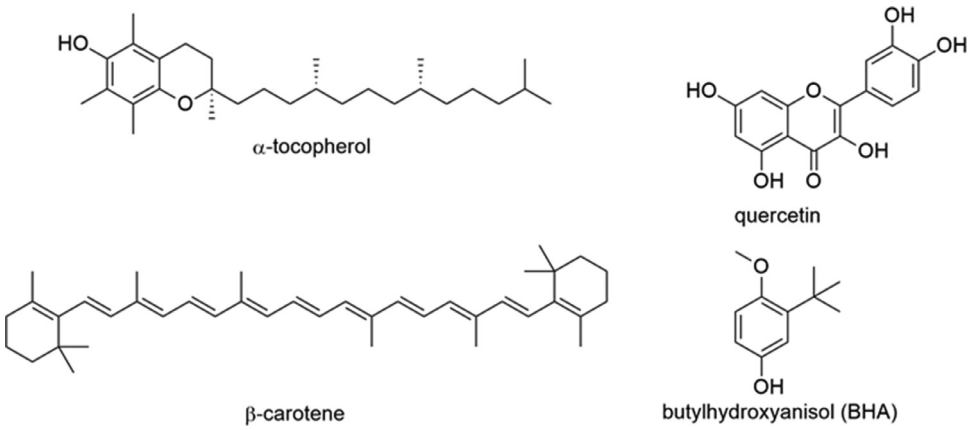


Scheme 1. Chemical structures of some antioxidant compounds.

Reactive oxygen species (ROS) are chemicals produced by cellular metabolism, and have types such as superoxide anion, oxygen, hydroxyl radical, singlet oxygen and hydrogen peroxide. Low levels of ROS play an essential role in many critical biological processes including growth, development, and death, but high ROS levels become fatal. At this level, it destroys the structure of biological macromolecules such as DNA and proteins by inducing oxidative stress [21,22]. The ROS cause peroxidation damage in the organism and this can cause many diseases such as diabetes, atherosclerosis, especially malignancies [23]. Every organism in the human body has set up its own antioxidant system to protect against oxidative stress. However, in some cases, supplementing with antioxidants can eliminate the damage caused by excess free radicals and delay disease formation. Therefore, the development of effective antioxidants to scavenge free radicals is an important issue (Scheme 1) [24,25].

In this study, novel asymmetric bis-isatin derivatives containing urea/thiourea moiety were synthesized and their antioxidant activity by CUPRAC and ABTS methods were evaluated. The antioxidant capacity of a compound is related to the number of quenched radicals or electrons transferred per antioxidant molecule, and the limits of this relationship are directly determined by reaction thermodynamics. In general, the thermodynamic parameters are affected by many variables such as electrostatic force, the concentration of reductants, solvent media, the acidity of solutions, ionic medium, temperature conditions, etc. The antioxidant property of a molecule can be said to have a multivariate sensitivity, but the reactions are controlled by the dominant variables, so that the experimental results repeat themselves consistently within an acceptable range of errors. From this point on, in determining the antioxidant activities of the synthesized compounds by the CUPRAC method, the distinguishing data of the electronic and structural properties of the compounds were analyzed and interpreted. The effects of substituted groups on the electronic properties of the compounds were investigated by Natural Bond Orbital (NBO), Quantum Theory Atom in Molecule (QTAIM), and Non-covalent Interaction (NCI) calculations. The effectiveness of electronic properties such as Highest Occupied Molecular Orbital (HOMO)-Lowest Unoccupied Molecular Orbital (LUMO) energies, electron affinity (EA), electronegativity (χ), ionization potential (IP), and chemical hardness (η) in the reaction of compounds with Cu(II)-neocuproine (Nc) was investigated. Besides, to analyze the hydrogen bond reactive in the reduction reaction of Cu(II)-Nc, in addition to local electron affinity (LEA, or EA_L) and Laplacian bond order (LBO) calculations, independent gradient model (IGM) based on Hirshfeld partition of molecular density (IGMH) [26] and intrinsic bond strength index (IBSI) [27] calculations were also performed, and associated with the antioxidant properties of the compounds determined by the CUPRAC method.

2. Materials and Methods

2.1. Instruments and chemicals

All chemical materials were purchased from Sigma, Aldrich, Acros Organics, or Merck Chemical Company, and were used without further purification. The solvents were of spectroscopic grade. A Stuart SMP 30 melting point apparatus was utilized for determining melting points °C. The elemental analysis was performed on a Eurovector EA3000-Single. A Bruker Alpha Fourier transform IR (FT-IR) spectrometer was used to record for infrared spectra. ^{1}H and ^{13}C-NMR spectra were taken on a Bruker Avance DPX-400 spectrophotometer (400 and 101 MHz) in DMSO-d_6. Antioxidant spectrophotometric measurements were performed with BioTek Synergy H1 Hybrid Multi-Mode Reader.

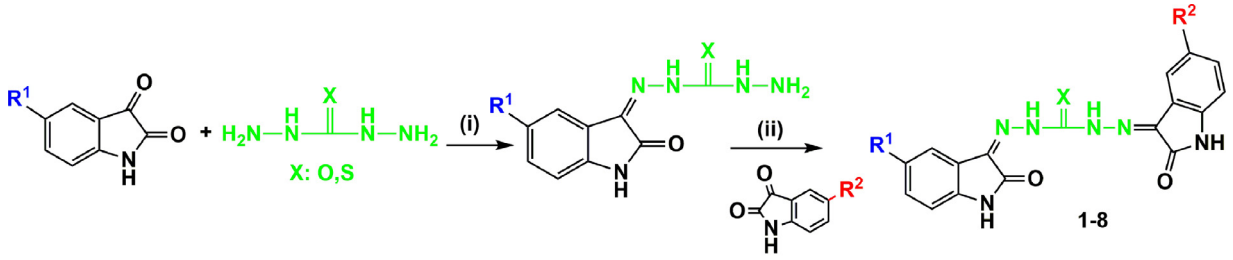
2.2. Synthesis of the compounds 1-8

Isatin thiocarbohydrazides/carbohydrazides were prepared with minor modifications according to a reported procedure [28]. Asymmetric bis-isatin thiocarbohydrazone/carbohydrazones were prepared from isatin-thiocarbohydrazide/carbohydrazides (0.01 mol) and 5-substituted isatins (0.01 mol) in the presence of concentrated HCl (three drops) in 20 mL of absolute ethanol. The mixture was refluxed for 4 h. The formed solid products were filtered and washed with hot ethanol and dried (Scheme 2). The compounds were successfully obtained in high yields (60–81%).

2.3. Antioxidant Activity Assay

2.3.1. $ABTS^{\cdot+}$ Cation Radical Decolorization Assay

$ABTS^{\cdot+}$ scavenging activities of the compounds were determined according to the literature [29]. 7 mM of $ABTS^{\cdot+}$ radical solution was prepared from 2,2-azino-bis(3- ethylbenzothiazoline- 6-sulphonic acid and $K_2S_2O_3$. The solution was kept in dark for 24 h at room temperature, and the absorbance of the solution was fixed to ~0.70 at 734 nm by dilution. The solutions of the samples were prepared in 4 different concentrations in DMSO, and added to make a final concentration of 50, 25, 12.5, 6.25 µM, respectively, in a flat-bottomed 96-well plate. The $ABTS^{\cdot+}$ solution was then added and incubated for 15 min at room temperature in the dark, and the absorbance was measured at 734 nm (Each absorbance was taken to be the mean of triplicate measurements). DMSO and BHA were used as the control solvent and standard, respectively. The decrease



Comp.	R^1	R^2	X
1	H	Cl	O
2	OCH_3	Cl	O
3	H	F	O
4	H	OCH_3	O
5	H	Cl	S
6	OCH_3	Cl	S
7	Cl	F	S
8	OCH_3	F	S

Scheme 2. Synthetic route for the compounds **1-8**. Reagents and conditions: (i) conc.HCl / EtOH/reflux 3 h; (ii) conc.HCl/EtOH/reflux 4 h.

Table 1
The physical data for the synthesized compounds.

Comp.	Compounds Name's	M.P. (°C)	Colour	Yield (%)
1	(*Z*)-1-(5-chloro-2-oxoindolin-3-ylidene)-5-(2-oxoindolin-3-ylidene)carbonohydrazide	317-318	Yellow	62
2	(*Z*)-1-(5-chloro-2-oxoindolin-3-ylidene)-5-(5-methoxy-2-oxoindolin-3-ylidene)carbonohydrazide	306-307	Orange	81
3	(*Z*)-1-(5-fluoro-2-oxoindolin-3-ylidene)-5-(2-oxoindolin-3-ylidene)carbonohydrazide	320-321	Yellow	74
4	(*Z*)-1-(5-methoxy-2-oxoindolin-3-ylidene)-5-(2-oxoindolin-3-ylidene)carbonohydrazide	294-295	Orange	60
5	(*Z*)-1-(5-chloro-2-oxoindolin-3-ylidene)-5-(2-oxoindolin-3-ylidene)thiocarbonohydrazide	280-281	Orange	70
6	(*Z*)-1-(5-chloro-2-oxoindolin-3-ylidene)-5-(5-methoxy-2-oxoindolin-3-ylidene)thiocarbonohydrazide	278-279	Brown	67
7	(*Z*)-1-(5-fluoro-2-oxoindolin-3-ylidene)-5-(5-chloro-2-oxoindolin-3-ylidene)thiocarbonohydrazide	291-292	Red	69
8	(*Z*)-1-(5-fluoro-2-oxoindolin-3-ylidene)-5-(5-methoxy-2-oxoindolin-3-ylidene)thiocarbonohydrazide	277-278	Brown	65

in the absorption was used to calculate the activities. The results were calculated as IC_{50} with Graphpad Prism 5.00 (San Diego, CA, USA).

2.3.2. Cupric reducing antioxidant capacity assay (CUPRAC)

Cupric reducing antioxidant capacities of the compounds were determined according to the literature method [30]. The solutions of compounds and standards were prepared in DMSO at four different concentrations. And then added to make a final concentration of 40, 20, 10, 5 µM, respectively, in a flat-bottomed 96-well plate. 61 µL of 10 mM $CuCl_2$, 61 µL 7.5 mM neocuproine and 61 µL of 1.0 mM NH_4CH_3COO buffer (pH 7), respectively. The absorbance was measured at room temperature at 450 nm, after an hour. The results were calculated as $A_{0.50}$. DMSO was used as a solvent to controls and BHA was used as a standard.

2.4. Computational procedure

Gaussian 09 software [31] was used to perform the Kohn-Sham density functional theory (KS-DFT) [32,33] calculations. B3LYP/6-311++g(2d,2p) level of theory was used, without any geometry restriction, for determination of ground-state electronic properties, IR, NMR, QTAIM and NCI calculations. LEA, LBO, and IBSI calculations were performed at the B3LYP/6-31g(d) level using Multiwfn software [34]. There are no imaginary frequencies in the IR data of the optimization calculations, and the analyzes were performed at the real minimum energy level. ^{1}H and ^{13}C-NMR calculations were carried out in the DMSO phase in parallel with the experimental procedure. The conductor-like polarizable continuum model (CPCM) was used to model the solvation effects. Relative chemical shift values were noted by subtracting the TMS shielding, 31.8149 ppm for ^{1}H-NMR and 183.7737 ppm for ^{13}C-NMR. Non-spectral electronic calculations of compounds were performed in gas phase. The charge distribution on individual atoms of the compounds was evaluated using NBO population analysis [35]. Bader's quantum theory of atoms in molecules (QTAIM) [36,37] was used to determine electron density distributions in compounds and analyze intramolecular interactions. The electron density at ring-critical points (RCPs), and the bond-critical points (BCPs) of the reactive N-H bonds were also determined by QTAIM analysis. Bond strength, bond order, and electron affinity are related concepts, and IBSI calculations are generally not dependent on the basis set [27], but using diffuse functions in LEA calculations can cause misleading results. For this reason, LEA, LBO and IBSI calculations were performed on structures optimized at B3LYP/6-31g(d) level in order to CPU time, facilitate the calculations, and compare data.

3. Results and Discussion

3.1. Physical properties

Physical appearances, melting points, yields, and elemental analysis data of the compounds are summarized in Tables 1 and 2.

3.2. Vibrational frequencies

In the FT-IR spectra of the compounds, the asymmetric and symmetric stretching bands of the thio/carbohydrazide amino group (-NH_2) were not observed at 3450–3250 cm^{-1}. Also, the characteristic peak for the C=N stretching vibrations was showed at around 1630–1620 cm^{-1}. These results indicated a successful reaction as expected. For compounds **1–8**, the amino peaks (-NH) of the carbohydrazide region and isatin ring were observed at 3202-3164 and 3296-3248 cm^{-1}, respectively. The carbonyl group signals (C=O) of the isatin ring were observed at 1698-1692 cm^{-1}. The -C–N stretching vibrations were observed at 1283-1189 cm^{-1}. For compounds **5-8**, the thiocarbonyl group signals (C=S) of carbohydrazide region were observed at 1388-1337 cm^{-1}. The other remarkable peaks were observed at the spectrum of compounds

Table 2
The results for elemental analysis and solubility for the synthesized compounds.

Comp	Solubility	Mol. formula	Calculated			Experimental		
			C %	H %	N %	C %	H %	N %
1	DMSO (+)	$C_{17}H_{11}ClN_6O_3$	53.34	2.90	21.96	53.12	2.83	22.10
2	DMSO (+)	$C_{18}H_{13}ClN_6O_4$	52.37	3.17	20.36	52.50	3.23	20.29
3	DMSO (+)	$C_{17}H_{11}FN_6O_3$	55.74	3.03	22.94	55.66	2.97	22.89
4	DMSO (+)	$C_{18}H_{14}N_6O_4$	57.14	3.73	22.21	57.18	3.68	22.24
5	DMSO (+)	$C_{17}H_{11}ClN_6O_2S$	51.20	2.78	21.07	51.05	2.83	21.02
6	DMSO (+)	$C_{18}H_{13}ClN_6O_3S$	50.41	3.06	19.60	50.51	2.98	19.55
7	DMSO (+)	$C_{17}H_{10}ClFN_6O_2S$	48.99	2.42	20.16	49.13	2.45	20.08
8	DMSO (+)	$C_{18}H_{13}FN_6O_3S$	52.42	3.18	20.38	52.39	3.09	20.42

Table 3
Experimental and theoretical IR vibration frequencies of the compounds.

	Comp.	ν N-H (ist)	ν N-H (crb)	ν C-H (Ar)	ν C=O/C=S (crb/tcrb)	ν C=O (ist)	ν C=N	ν C-N	Spec. peaks
Experimental	**1**	3287	3200	3064	1733	1697	1623	1208	C-Cl: 1144
	2	3296	3202	3071	1732	1697	1623	1207	C-Cl: 1143 C-O: 1101
	3	3283	3199	3055	1732	1695	1623	1210	C-F: 1144
	4	3275	3181	3062	1729	1695	1623	1210	C-O: 1101
	5	3264	3180	3043	1339	1695	1619	1196	C-Cl: 1122
	6	3248	3180	3012	1337	1693	1624	1283	C-Cl: 1129 C-O: 1189
	7	3271	3164	3036	1388	1698	1622	1230	C-F: 1155 C-Cl: 1127
	8	3254	3181	3048	1337	1692	1625	1189	C-F: 1128 C-O: 1030
Calculated	**1**	3646^a 3645^b	3444^b 3426^a	3204-3188	1795	1751^b 1748^a	1627^b 1616^a	1287	C-Cl: 1086
	2	3649^b 3647^a	3443^b 3430^a	3219-3186	1795	1749^b 1745^a	1629^b 1614^a	1287	C-Cl: 1086 C-O: 1050
	3	3648^a 3645^b	3445^b 3424^a	3204-3188	1794	1751^b 1747^a	1623^b 1617^a	1296	C-F: 1173
	4	3648^a 3645^b	3447^b 3423^a	3221-3185	1792	1750^b 1743^a	1626^b 1621^a	1261	C-O: 1058
	5	3646^a 3646^b	3435^b 3379^a	3203-3188	1320	1751^b 1747^a	1617^b 1613^a	1230	C-Cl: 1084
	6	3650^b 3647^a	3433^b 3385^a	3219-3186	1335	1748^b 1744^a	1618^b 1612^a	1289	C-Cl: 1084 C-O: 1051
	7	3646^b 3646^a	3435^b 3371^a	3212-3191	1327	1752^b 1746^a	1614^a 1613^b	1229	C-F: 1158 C-Cl: 1085
	8	3650^b 3648^a	3434^b 3383^a	3219-3186	1335	1748^b 1743^a	1618^b 1612^a	1235	C-F: 1128 C-O: 1030

ist: isatin, crb: carbohydrazide, tcrb: thiocarbohydrazide, Ar: Aromatic, a: isatin region bearing R^2, b: isatin region bearing R^1

1–8 resulting from the -C–Cl, -C–F, and -C–O functions, respectively. Compounds **1**, **2**, **5**, **6**, and **7**, the Ar-Cl stretching vibrations were observed at 1144–1122 cm^{-1}. The Ar–F stretching vibrations were observed at around 1155-1128 cm^{-1} for compounds **3**, **7** and **8**. Compounds **2**, **4**, **6**, and **8**, the -C–O stretching vibrations were observed at between 1189 and 1030 cm^{-1}. The –NH stretching vibrations of the carbohydrazide region was observed at 3181 cm^{-1} for compound **4**, while the –NH stretching vibration of the isatin ring was observed at 3275 cm^{-1}. At 1729 cm^{-1}, the –C=O stretching vibration was observed; at 1623 cm^{-1} the -C=N stretching vibration was showed; at 1210 cm^{-1}, the -C–N stretching vibration was observed; at 1101 cm^{-1}, the -C–O stretching vibration was showed (see at Figures S2-S9 in Supplementary information). These frequency results are highly consistent with similar compounds [13,38,39]. Experimental and theoretical frequency results are presented in Table 3. The reason for the difference between the experimental and the calculated values is that the calculations are performed for a single molecule in the gas phase, that is, the vibration modes are harmonic (anharmonicity is neglected) and the intermolecular effects do not exist in the calculations.

3.3. ^{1}H-NMR Analysis

The experimental and theoretical ^{1}H-NMR spectra of the compounds were detected in DMSO-d_6 and the chemical shifts are given in Table 4.

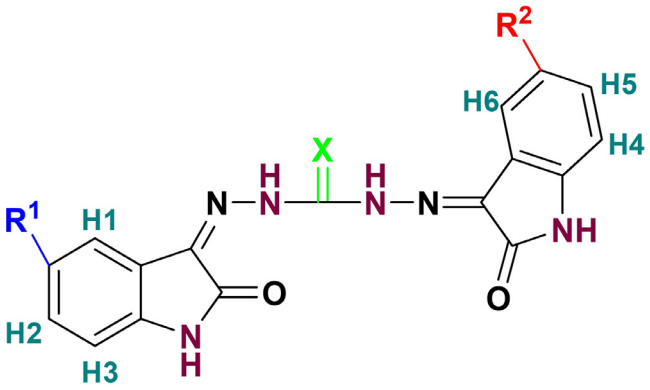
For compounds **1-8**, the aromatic protons (H1-H6) signals of the isatin ring were observed at between 7.60 and 6.75 ppm (see at Figures S10-S17 in Supplementary information). While the –NH signals of the isatin region were observed as a singlet at 11.43-10.99 ppm. The –NH signals of carbohydrazide region were showed as a singlet at 14.94-11.29 ppm. The methoxy group (-OCH_3) proton signals were detected as a singlet in the range of 3.78, 3.77, 3.76 and 3.75 ppm for compounds **2**, **4**, **6**, and **8**. DMSO-d_6 and water in DMSO (HOD, H_2O) signals are shown at around 2.00, 2.50 (quintet), and 3.30 (variable, based on the solvent and its concentration) ppm, respectively. These data are consistent with the values of those earlier for similar compounds [13,28,40].

3.4. ^{13}C-NMR Analysis

The experimental and theoretical ^{13}C-NMR spectra of the compounds are given in Tables 5(a-b), respectively.

For all compounds (**1-8**), the –C=O and –C=N peaks of the isatin region were observed at 165.37–162.88 and 154.23–139.27 ppm, respectively. While the –C=O peaks of the carbohydrazone region were observed at 162.88–155.08 ppm for compounds **1-4**, the –C=S peaks of the thiocarbohydrazone region were observed at 176.05–170.83 ppm for compounds **5-8** (see at Figure S18–S25 and the coupling constants J_{C-F} in Table S1 at Supplementary information). In compounds **2**, **4**, **6**, and **8**, the methoxy carbon (–OCH_3) resonated at 56.38, 56.00, 55.71, and 55.71 ppm, respectively. Moreover, the carbon atoms (for C7-C12) were also split into doublets caused by interacting with the atomic nucleus of F for compounds **3**, **7,** and **8**. The C10 atoms of compounds **3**, **4**, **7**, and **8** were resonated at down-field (~150–160 ppm) due to the presence of the electron-withdrawing groups (–F and –OCH_3). As far as chlorine atom (–Cl), the C10 atoms of compounds **1**, **2**, **5**, and **6** were shifted down-field (131–137 ppm) relative to the signal of benzene (128.5 ppm). The C1 atoms of compounds **2**, **6**, and **8** were observed at 155.66, 155.59, and 163.02 ppm, respectively. These carbon atoms shifted downfield (high values of δ), which was caused by the presence of the methoxy group. [13,28,40,41].

Table 4
Experimental and theoretical ^{1}H-NMR (δ, ppm, in DMSO-d_6) values related to synthesized compounds.



	Comp.	H1	R^1	H2	H3	H4	H5	H6	NH(ist)	NH(crb)	R^2
Experimental	**1**	7.13-7.08 (m)	7.56-7.52 (t)	7.39-7.36 (t)	6.96-6.93 (m)	6.92-6.91 (d)	7.36-7.35 (d)	7.43 (s)	11.26, 11.21 (s)	11.36, 11.31 (s)	-
	2	7.08 (s)	3.78:OCH_3 (s)	6.84-6.82 (d)	6.94-6.92, 2H, (d)		7.38-7.36, (d)	7.43 (s)	11.01, 10.99 (s)	11.31, 11.29 (s)	-
	3	7.13-7.08 (m)	7.55-7.52 (t)	7.39-7.36 (t)	6.96-6.94, (m)	6.93-6.92, (d)	7.36-7.35, (d)	7.28 (s)	11.25, 11.23 (s)	13.86, 12.16 (s)	-
	4	7.14-7.09 (m)	7.56-7.53 (t)	7.40-7.36, 2H, (m)		6.97-6.93, 2H, (m)		6.87-6.85 (d)	11.24, 11.06 (s)	13.86, 12.15 (s)	3.77: OCH_3 (s)
	5	7.15-7.13 (m)	7.45-7.39, 3H, (m)			6.98-6.96, (d)	7.60-7.58, (d)	7.50 (s)	11.42, 11.32 (s)	14.94, 12.99, (s)	-
	6	7.10 (s)	3.76:OCH_3 (s)	6.94-6.91, 2H, (dd)		6.82-6.80, (d)	6.94-6.91, (dd)	7.01 (s)	11.15, 11.07 (s)	14.93, 12.87, (s)	-
	7	7.40 (s)	-	7.36-7.33, (d)		6.89-6.87, (d)	7.36-7.33, (d)	7.40 (s)	11.43, 11.33 (s)	14.79, 12.78 (s)	-
	8	7.06 (s)	3.75:OCH_3 (s)	6.89-6.86 2H, (dd)		6.77-6.75, (d)	6.89-6.86, (dd)	6.96 (s)	11.11, 11.03 (s)	14.88, 12.83, (s)	-
Calculated	**1**	8.14	7.58	7.77	7.32	7.22	7.72	8.00	7.52^a 7.47^b	14.09^a 12.78^b	-
	2	7.61	4.19-3.96	7.33	7.20	7.21	7.72	8.06	7.46^a 7.30^b	14.13^a 12.74^b	
	3	8.16	7.57	7.74	7.30	7.24	7.47	7.75	7.51^a 7.47^b	14.15^a 12.75^b	-
	4	8.13	7.57	7.74	7.31	7.18	7.21	7.59	7.34^{a}7.46^b	14.15^a 12.73^b	4.17-3.83
	5	8.19	7.57	7.77	7.30	7.22	7.75	8.06	7.34^a 7.46^b	14.97^a 13.72^b	-
	6	7.63	4.20-3.96	7.33	7.20	7.24	7.74	8.14	7.47^{a}7.28^b	15.01^a 13.72^b	-
	7	8.10	-	7.73	7.23	7.25	7.51	7.84	7.47^{a}7.47^b	15.06^a 13.58^b	-
	8	7.63	4.20-3.96	7.29	7.19	7.24	7.51	7.83	7.43^{a}7.27^b	15.01^{a}13.70^b	-

ist: isatin, crb: carbohydrazide, s: (singlet), d: (doublet), dd: (doublet of doublet), t: (triplet), m: (multiplied); a: isatin region bearing R^2, b: isatin region bearing R^1.

3.5. Evaluation of antioxidant activity

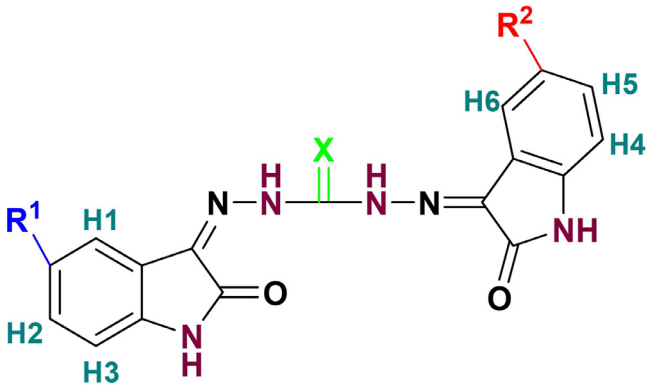
The results of ABTS and CUPRAC activity of synthesized compounds are given in Table 6. IC_{50} values of the synthesized compounds ranged from 18.44 μM to 27.38 μM for $ABTS^{\bullet+}$ activity and $A_{0.50}$ values of them were between 0.600 μM and 0.810 μM for CUPRAC activity (Table 6).

It was observed that bis-isatins containing thiourea moiety (compounds **5-8**) showed moderate ABTS activity, whereas bis-isatins containing urea moiety (compounds **1-4**) did not exhibit any ABTS activity. It is considered that this difference is due to the S atom contained in the thiourea compounds. The ABTS method is based on the ability of antioxidants to donate hydrogen or electrons [4]. The raising polarizability stabilizes the cationic radicals formed after donating electrons. The presence of S atom increased the polarizability of the molecules (from Table 7, while the polarizability (α) of the synthesized ureas (compounds **1-4**) ranged from 305.42 to 341.43, the α of the synthesized thioureas (compounds **5-8**) ranged from 363.28 to 384.39). Also, the thioureas have higher local electron affinity via S atom than the ureas (from Fig. S27). Consequently, thioureas show better ABTS activity than ureas due to all these effects.

Compound **5** had the strongest ABTS activity with the IC_{50} values of 18.44 μM. The presence of $-OCH_3$ or –Cl as R^1 substituent decreased the ABTS activity (compare **5** (R^1=H; IC_{50}=18.44 μM) with **6** (R^1=OCH_3; IC_{50}=23.54 μM), **7** (R^1=Cl; IC_{50}=27.38 μM) and **8** (R^1=OCH_3; IC_{50}=23.81 μM)). The changing –Cl with –F as R^2 substituent did not significantly affect the ABTS activity (compare **6** (R^2=Cl; IC_{50}=23.54 μM) with **8** (R^2=F; IC_{50}=23.81 μM)).

All of the compounds showed cupric reducing antioxidant activity. Compound **2** had the highest CUPRAC activity with the $A_{0.50}$ value of 0.600 μM. Most of the synthesized compounds (except **6** and **8**) exhibited slightly better CUPRAC activity than BHT ($A_{0.50}$ = 0.634 μM) used as a standard. The synthesized bis-isatins containing urea moiety (compounds **1-4**; $A_{0.50}$ values of 0.600 μM-0.626 μM) showed higher CUPRAC activity than bis-isatins containing thiourea moiety (compounds **5-8**; $A_{0.50}$ values of 0.629

Table 5a
Experimental ^{13}C-NMR (δ, ppm, in DMSO-d_6) values of the compounds.



	1	2	3	4	5	6	7	8
C1	123.01	155.66	123.07	121.05	120.45	155.59	126.80	163.02
C2	131.26	112.44	139.05	123.03	127.25	112.49	123.02	129.88
C3	120.32	120.37	111.68	117.84	120.00	120.80	120.86	132.99
C4	142.75	146.86	142.79	136.43	134.31	139.20	135.81	140.62
C5	111.63	113.21	107.95	112.42	112.18	117.93	113.14	131.53
C6	120.92	106.14	120.90	120.86	121.72	106.66	110.43	120.93
C7	141.40	136.61	132.05 131.99	131.93	132.61	137.19	131.93 131.24	137.19 136.84
C8	120.85	121.06	121.84 121.54	120.27	120.07	128.02	127.50 127.12	118.89 118.80
C9	127.13	127.21	118.41 118.17	106.13	123.19	136.81	120.57 120.26	112.59 112.43
C10	131.91	131.31	159.94 157.58	150.77	131.87	136.86	150.76 150.58	155.83 155.74
C11	122.04	121.96	112.87 112.80	103.35	121.81	134.18	111.62 111.51	106.69 106.55
C12	113.16	117.89	120.28 120.22	111.64	117.91	118.96	121.97 121.89	118.12 117.93
C=O (ist)	163.19	165.37	163.23	163.24	163.31	163.41	163.14	163.52
C=N	150.75	150.62	150.79	142.76	143.61	146.96	142.75	154.23
C=X	162.88	157.08	161.14	155.79	176.05	175.64	170.83	175.69
C=N	150.58	150.50	150.66	139.79	143.08	139.27	141.40	153.65
C=O (ist)	163.14	163.67	163.19	163.15	163.15	163.25	162.88	163.19
OCH_3	-	56.38	-	56.00	-	55.71	-	55.71

Table 5b. Theoretical ^{13}C-NMR (δ, ppm, in DMSO-d_6) values computed by B3LYP/6-311++g(2d,2p).

Compounds								
Atom	1	2	3	4	5	6	7	8
C1	128.75	164.67	128.75	128.71	128.89	164.77	142.02	164.84
C2	138.15	126.33	138.14	137.98	139.06	127.37	138.25	127.33
C3	127.59	127.56	127.83	127.82	127.32	127.18	128.29	127.08
C4	149.56	142.31	149.57	149.45	150.47	143.39	148.59	143.43
C5	116.10	117.27	116.12	116.07	116.35	117.54	117.56	117.58
C6	127.39	107.19	127.29	127.31	128.02	107.91	127.42	108.13
C7	147.33	147.54	144.70	141.07	148.09	148.01	145.72	145.68
C8	129.18	129.29	129.15	129.46	128.87	128.91	128.42	128.56
C9	137.10	137.10	123.55	117.01	138.09	138.09	124.99	124.81
C10	142.35	142.27	169.42	164.39	142.54	142.54	169.56	169.50
C11	126.40	126.57	113.13	114.63	127.34	127.55	114.14	113.98
C12	117.11	116.95	117.10	116.11	117.31	117.15	117.10	117.03
C=O (ist)[a]	168.38	168.46	168.75	168.58	168.29	168.49	168.77	168.69
C=N[a]	140.46	140.44	141.31	142.14	143.31	143.57	144.53	143.96
C=X	157.82	157.77	157.84	157.96	186.23	186.16	186.25	186.18
C=N[b]	139.73	139.35	140.15	139.56	139.80	139.29	137.16	139.49
C=O (ist)[b]	169.05	169.44	168.93	168.98	168.64	168.96	168.46	168.96
OCH_3	-	57.48	-	57.34	-	57.54	-	57.54

a: isatin region bearing R^2, b: isatin region bearing R^1.

Table 6
The results of ABTS and CUPRAC activity of synthesized compounds.

Comp.	ABTS(IC_{50}, μM)	CUPRAC($A_{0.50}$, μM)
1	na	0.619
2	na	0.600
3	na	0.605
4	na	0.626
5	18.44	0.629
6	23.54	0.680
7	27.38	0.631
8	23.81	0.810
BHA	3.55	0.634

na: not available.

μM-0.810 μM). The binding the -OCH_3 group as R^1 substituent in urea series increased the CUPRAC activity (compare **1** (R^1=H; $A_{0.50}$ =0.619 μM) with **2** (R^1=OCH_3; $A_{0.50}$ =0.600 μM)), while R^2 is –Cl atom, conversely, it decreased the CUPRAC activity in thiourea series (compare **5** (R^1=H; $A_{0.50}$ =0.629 μM) with **6** (R^1=OCH_3; $A_{0.50}$ =0.680 μM)). The changing –F with –Cl or –OCH_3 as R^2 substituent in urea series reduced the CUPRAC activity (compare **3** (R_2=F; $A_{0.50}$ =0.605 μM) with **1** (R^2=Cl; $A_{0.50}$ =0.619 μM) and **4** (R^2=OCH_3; $A_{0.50}$ =0.626 μM)). In thiourea series, the changing –F with –Cl as R^2 substituent significantly enhanced the CUPRAC activity (compare **8** (R^2=F; $A_{0.50}$ =0.810 μM) with **6** (R^2=Cl; $A_{0.50}$ =0.680 μM)). Furthermore, the changing –OCH_3 with –Cl as R^1 substituent re-

Table 7
Calculated electronic parameters of the compounds.

Comp.	E (a.u)	E_{HOMO} (eV)	E_{LUMO} (eV)	$\Delta E_g(eV)$	η	χ (eV)	μ (Debye)	α (a.u)
1	-1669.21	-6.409	-2.918	3.491	1.746	4.664	7.681357	323.122333
2	-1783.77	-6.057	-2.912	3.145	1.572	4.484	7.569417	341.430667
3	-1308.86	-6.395	-2.897	3.498	1.749	4.646	7.507341	305.423333
4	-1324.15	-5.968	-2.742	3.225	1.613	4.355	6.431093	328.698667
5	-1992.17	-6.193	-3.146	3.047	1.524	4.669	8.034362	365.922667
6	-2106.73	-6.016	-3.143	2.873	1.437	4.580	7.959715	384.386333
7	-2091.44	-6.285	-3.249	3.036	1.518	4.767	6.090474	363.284000
8	-1746.37	-6.005	-3.123	2.882	1.441	4.564	7.866436	366.634000

E: Molecular energy, $\Delta E_g = E_{LUMO} - E_{HOMO}$, χ: Electronegativity, η: Chemical hardness, μ : Dipol moment, α: Polarizability.

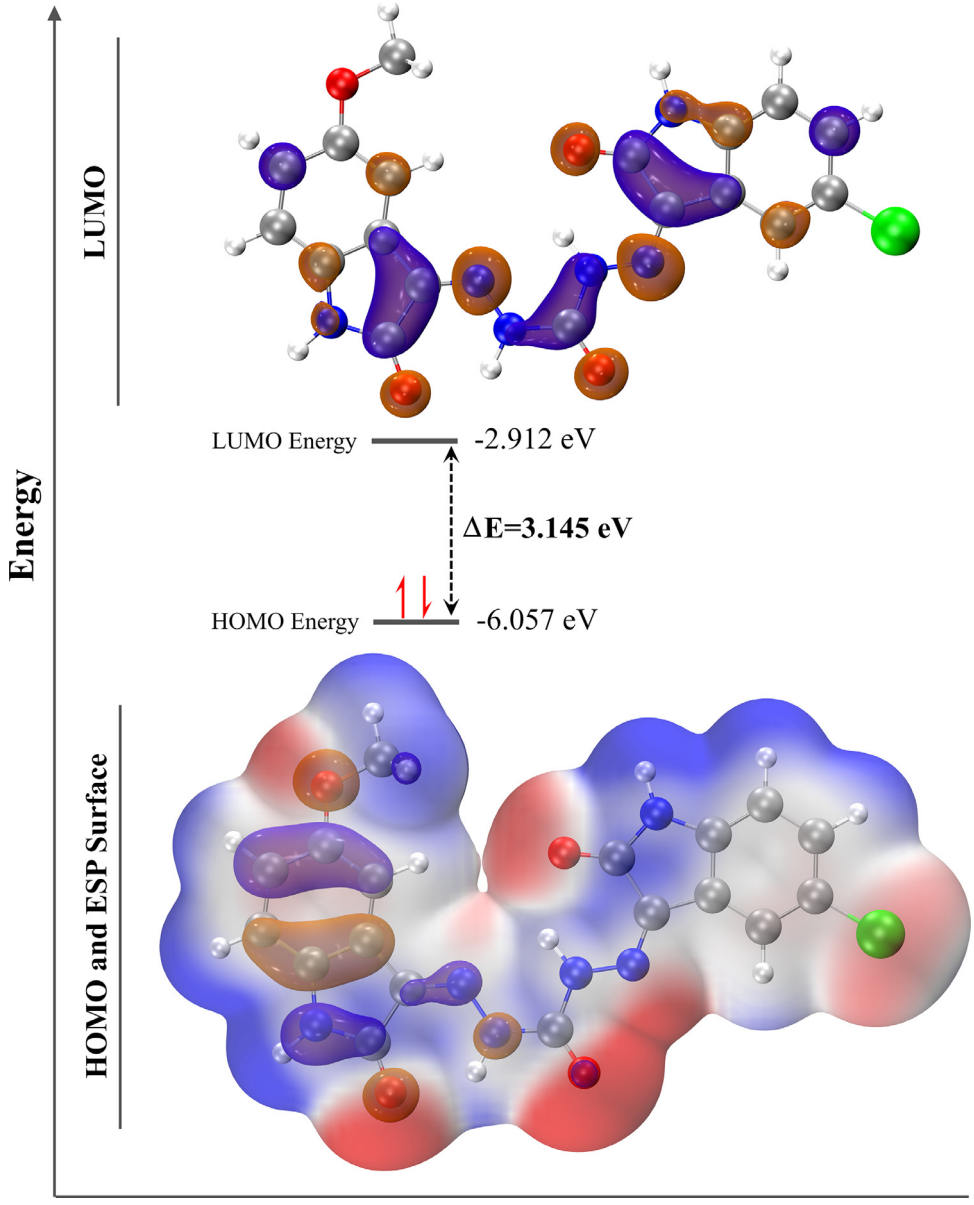


Fig. 1. ESP-HOMO and LUMO surface of the compound **2**.

markably increased the CUPRAC activity (compare **8** (R^1=OCH_3; $A_{0.50}$ =0.810 μM) with **7** (R^1=Cl; $A_{0.50}$ =0.631 μM)).

The CUPRAC activities of the compounds **1-7** were almost same but compound **8** showed lower activity. As the reason for this result, it can be said that the presences of both fluorine and oxygen, which are the strongest electronegative atoms, in the phenyl rings of the compound **8** shift the electron density from the thiourea moiety to the phenyl rings and decrease electron density of the thiourea moiety. Therefore, the oxidation ability through the thiourea moiety of the molecule required to reduce copper for CUPRAC assay decreases.

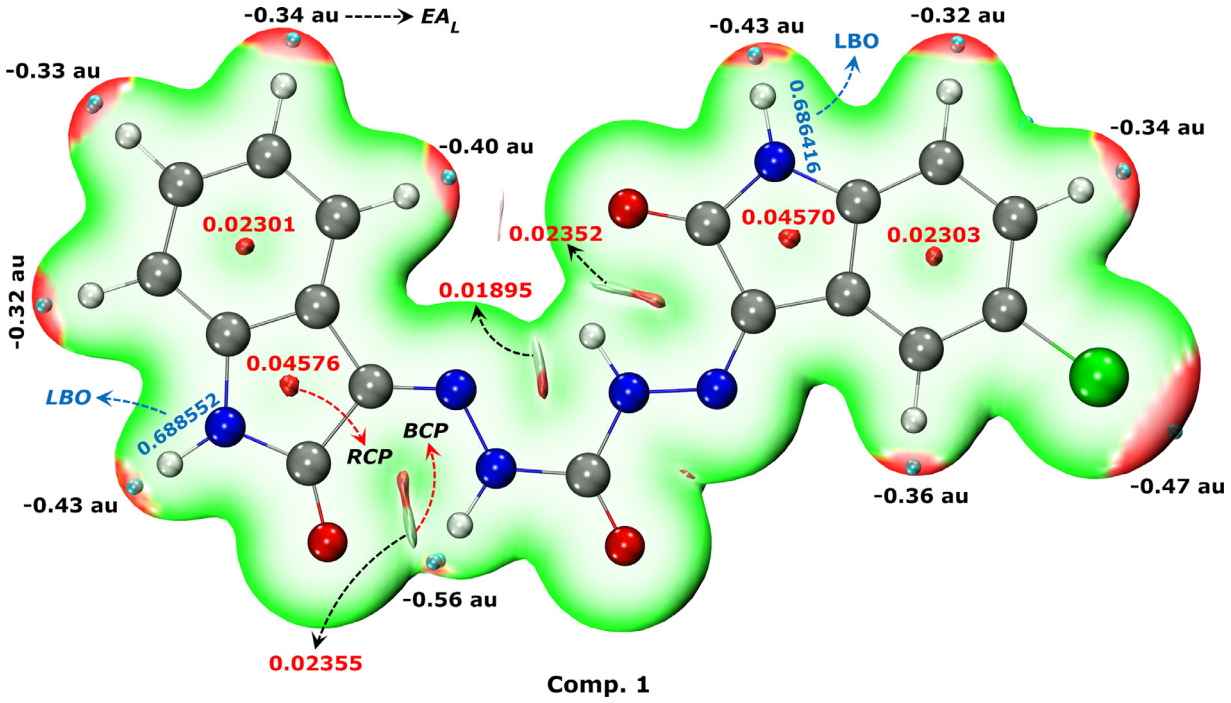


Fig. 2. Local electron affinity sites of the compound **1**.

3.6. Computational results

Compounds **1-4** and **5-8** with oxygen and sulfur in the center of asymmetry, that is, Oxygen-centered compounds **1-4** (OCCs **1-4**) and Sulfur-centered compounds **5-8** (SSCs **5-8**) respectively, were examined separately in two groups. Calculated electronic data are given in Table 7.

The substituents R^1 and R^2 on the compounds are a combination of Cl, F and methoxy substitute groups. In the OCCs and SSCs, the compounds with higher electronegative substituents showed more antioxidant activity than other compounds. In the OCCs, for 5-Cl, 5-F and 5-OCH_3 substituted compounds **1**, **3,** and **4**, respectively, the substituent 5-F caused greater ΔE_g (3.498 eV) compared to 5-Cl and 5-OCH_3, while decreasing the polarizability (305.42 au) of the compound. It was observed that bis-isatins containing urea moiety showed higher CUPRAC activity while having lower polarizability than bis-isatins containing thiourea moiety. The binding of the methoxy group as the R^1 substituent in the urea series decreased the ΔE_g value and accordingly it was observed that the CUPRAC activity increased. A similar result was partially seen with the thiourea series of compounds.

In the SSCs, considering the **5** and **6** compounds, the 5-OCH_3 substituted compound **6** has lower ΔE_g (2.873 eV), χ (4.580 eV) and μ (7.96 Debye) values, while a higher polarizability (384.39 au) was calculated. It was also observed in compounds **7** and **8** that the 5-OCH_3 substituent showed the same effect as compared to the 5-Cl substituent. In addition, compounds **6** and **8** were compared and it was observed that the C1 substituent decreased the ΔE_g value and increased the electronegativity of the compound relative to the fluorine substituent, and experimentally increased the CUPRAC activity.

ESP-HOMO and LUMO surface of compound **3** is given Fig. 1 (All HOMO-LUMO and ESP maps of the compounds are given in Supplementary file 1). In methoxy substituted compounds **2**, **4**, **6,** and **8**, HOMOs concentrated around methoxy, while other compounds showed a more common distribution. In methoxy-free SSCs, it was observed that HOMO concentrates around the sulfur atom and N−NH structures.

Although the HOMO-LUMO energy gap, ΔE_g, is simply a measure of kinetic stability, and molecules with small ΔE_g can be said to be more chemically reactive, it is not the only factor that determines reactivity. The OCCs and SSCs have multiple reactive sites that can perform the antioxidant reaction, and the distribution of molecular orbitals is directly affected by both intramolecular interactions and the environment. To better analyze the electronic properties and antioxidant behavior of the compounds, LEA calculation was made to determine the electrophilic attack sites (Fig. 2; all of the LEA surfaces of the compounds are given in Supplementary file). EA_L values of the reactive N−H bonds, which did not show intramolecular interaction, were calculated as -0.43 or -0.44 au in all compounds. LBO has a direct correlation with bond polarity and bond dissociation energy [42], and can be used to determine the antioxidant properties of the compounds in the CUPRAC reaction. The LBOs of reactive N−H bonds were also calculated, and a correlation was observed between the antioxidant properties of the compounds and the LBO. LBO values of $N-H^{(b)}$ bonds in OCCs for compounds **1-4** were calculated as 0.688552, 0.687748, 0.688521 and 0.690669, respectively. Compounds with small LBO showed higher antioxidant properties, and a similar relationship was observed between LBO and antioxidant properties for $N-H^{(a)}$ (except compound **7**).

Intrinsic bond strength index (IBSI) is dimensionless, and expressed as (normalized to 1 for the H_2):

$$IBSI = \Delta g^{pair} = \frac{\int_V \frac{\delta g^{pair}}{d^2}}{\int_V \frac{\delta g^{H_2}}{d_{H_2}^2}}$$

where d is the distance between two atoms whose interactions are studied. Descriptor δg is given as [43].

$$\delta g = \left|\nabla \rho^{IGM}\right| - |\nabla \rho|,$$

and is a quantity dependent on the norm of the electron density gradient. δg^{IGMH} and $IBSI^{IGMH}$ descriptors based on the Hirshfeld approach were used to analyze the reactive N−H bonds in the reduction reaction of Cu(II)-Nc. In OCCs, δg^{IGMH} for $N-H^{(b)}$ was calculated as 0.23735, 0.23696, 0.23733, and 0.23833, while in SSCs,

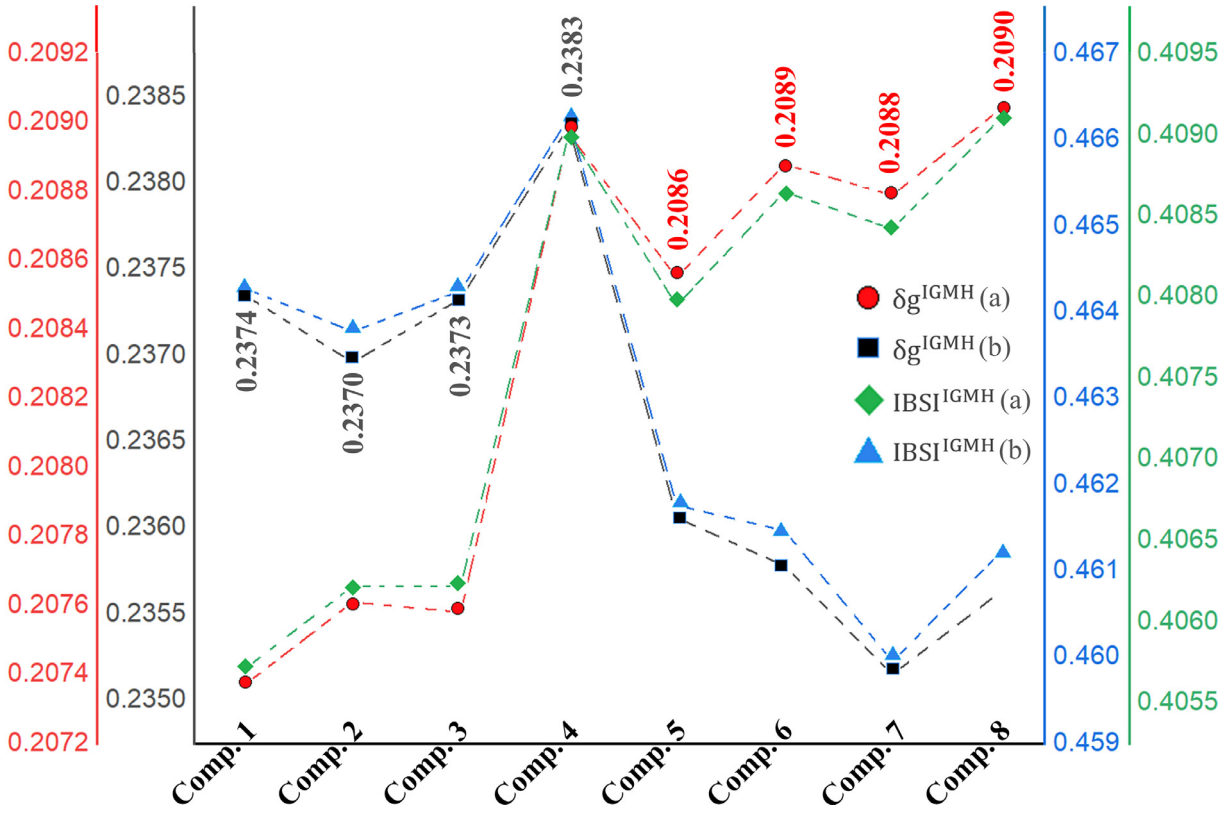


Fig. 3. δg^{IGMH} and $IBSI^{IGMH}$ values of reactive N-H$^{(a)}$ and N-H$^{(b)}$ bonds. a: isatin region bearing R^2, b: isatin region bearing R^1.

Table 8
Calculated IBSI values of the compounds.

Comp.	δg^{IGMH}		$IBSI^{IGMH}$	
	N−H$^{(a)}$	N−H$^{(b)}$	N−H$^{(a)}$	N−H$^{(b)}$
1	0.20737	0.23735	0.40571	0.46428
2	0.20761	0.23696	0.40622	0.46378
3	0.20758	0.23733	0.40622	0.46425
4	0.20897	0.23833	0.40901	0.46626
5	0.20855	0.23606	0.40795	0.46176
6	0.20888	0.23578	0.40865	0.46147
7	0.20879	0.23515	0.40842	0.45995
8	0.20904	0.23563	0.40910	0.46122

δg^{IGMH} for N−H$^{(a)}$ was calculated as 0.20855, 0.20888, 0.20879, and 0.20904, respectively (Table 8). The Pearson correlation coefficient between the δg^{IGMH} and $IBSI^{IGMH}$ values of N−H$^{(a)}$ and N−H$^{(b)}$ bonds is 0.99797 and 0.997852, respectively (see Fig. 3). Compared to experimental results, in the Cu(II)-Nc reaction, it can be said that the hydrogen of OCCs on the N−H$^{(b)}$ bond is more reactive, while in SSCs, the hydrogen on the N−H$^{(a)}$ bond is more reactive (Fig. 3).

Conclusions

In this study, novel asymmetric bis-isatin compounds have been designed, obtained, and elucidated by using FT-IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopic methods and elemental analyses. Spectral data were compared with theoretical calculation results, and DFT calculations were performed to analyze the antioxidant properties of the compounds. The results of antioxidant assays showed that all the synthesized compounds exhibited CUPRAC activity on the low micromolar level, whereas only the thiourea series of bis-isatins had ABTS activity on a moderate micromolar level. While compound **5** showed the highest ABTS activity with IC_{50} value of 18.44 μM, compound **2** had the strongest CUPRAC activity with $A_{0.50}$ value of 0.600 μM. Most of the synthesized compounds (except **6** and **8**) exhibited slightly better CUPRAC activity than BHT ($A_{0.50}$ = 0.634 μM) used as a standard. The changing substituents on the isatin skeleton slightly affected the antioxidant activities. Various theoretical calculations were performed in order to determine reactive hydrogens and local electron affinity regions, and to investigate intramolecular interactions. It was observed that compounds with lower LBOs showed higher antioxidant properties. In addition, the δg^{IGMH} and $IBSI^{IGMH}$ values of reactive hydrogen bonds were calculated and it was concluded that N-H bonds with lower bond strength in the reaction with Cu(II)-Nc can be broken more easily, therefore compounds with lower δg^{IGMH} and $IBSI^{IGMH}$ values for reactive hydrogens can exhibit higher antioxidant properties.

Author Contributions

Hasan Yakan: Synthesis, Characterization, Writing–Review. M. Serdar Çavuş: Theoretical Calculations, Writing–Review. Belma Zengin Kurt: Biological Studies, Writing–Review. Halit Muğlu: Synthesis, Characterization, Writing–Review. Fatih Sönmez: Biological Studies, Writing–Review. Emre Güzel: Writing–Review, Visualization & Editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2021.130495.

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