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Synthesis and antioxidant properties of novel *N*-methyl-1,3,4-thiadiazol-2-amine and 4-methyl-2*H*-1,2,4-triazole-3(4*H*)-thione derivatives of benzimidazole class

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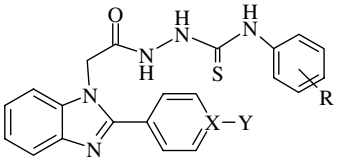
Abstract—Some novel 1-methyl-4-(2-(2-substitutedphenyl-1*H*-benzimidazol-1-yl)acetyl)thiosemicarbazides (**16a**–**20a**), 5-[(2-(substitutedphenyl)-1*H*-benzimidazol-1-yl)methyl]-*N*-methyl-1,3,4-thiadiazol-2-amines (**17b**–**20b**), and 5-[(2-(substitutedphenyl)-1*H*-benzimidazol-1-yl)methyl-4-methyl-2*H*-1,2,4-triazole-3(4*H*)-thiones (**16c**–**20c**) were synthesized and tested for antioxidant properties by using various in vitro systems. Compounds **16a**–**20a** were found to be a good scavenger of DPPH radical (IC_{50}, 26 μM; IC_{50}, 30 μM; IC_{50}, 43 μM; IC_{50}, 55 μM; IC_{50}, 74 μM, respectively) when compared to BHT (IC_{50}, 54 μM). Noteworthy results could not be found on superoxide radical. Compound **19b**, which is the most active derivative inhibited slightly lipid peroxidation (28%) at 10^{-3} M concentration. Compound **17c** inhibited the microsomal ethoxyresorufin *O*-deethylase (EROD) activity with an $IC_{50} = 4.5 \times 10^{-4}$ M which is similarly better than the specific inhibitor caffeine $IC_{50} = 5.2 \times 10^{-4}$ M.

1. Introduction

Nowadays antioxidants arouse researchers' interest in both medical plants and synthetic compounds. In our previous research,[1–3] we reported some novel benzimidazole derivatives in which substituted aminothiocarbamoylhydrazinecarbonyl, substituted 1,3,4-thiadiazole, and substituted 4*H*-1,2,4-triazole groups were attached to the 1st position of benzimidazole ring via a methyl chain. At the 2nd position of these derivatives, there are phenyl, *p*-chlorophenyl, *p*-methoxyphenyl, and pyridinyl rings. Antioxidant properties of these compounds were investigated by employing various in vitro systems interaction of 2,2-diphenyl-1-picrylhydrazyl (DPPH), and scavenging of superoxide radical, microsomal NADPH-dependent inhibition of lipid peroxidation (LP), microsomal ethoxyresorufin *O*-deethylase (EROD) activity. Among these compounds, thiosemicarbazide derivatives (Fig. 1) usually had stronger inhibitory effects on LP levels and more considerable scavenger effects on DPPH radical than those of their cyclic counterparts namely, 1,3,4-thiadiazoles and 2*H*-1,2,4-triazole-3(4*H*)-thiones.

In this study, we synthesized some novel benzimidazole derivatives (Fig. 2) bearing alkyl (methyl) group at the 4th position of triazole ring and at the 5th position of thiadiazole ring instead of aryl group. In addition to their IR, ^1H and ^{13}C NMR, Mass, and Elemental



X = C, N
Y = -H, -Cl, -OCH_3
R = -H, -Cl, di-Cl, -F, -Br, -CH_3, -OCH_3,

Figure 1. Structures of previously synthesized thiosemicarbazidobenzimidazoles.

Keywords: *N*-Methylthiosemicarbazides; *N*-Methyltriazolylbenzimidazoles; *N*-Methylthiadiazolyl-benzimidazoles; Antioxidant; X-ray structure analysis.
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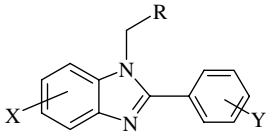


X = -H, 5,6-dichloro
Y = -H, 4-chloro, 4-methoxy, 3,4-dimethoxy, 4-benzyloxy, 4-hydroxy

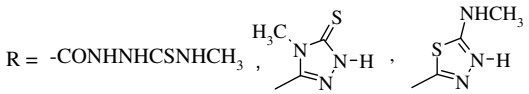


Figure 2. Structures of the synthesized compounds in this study.

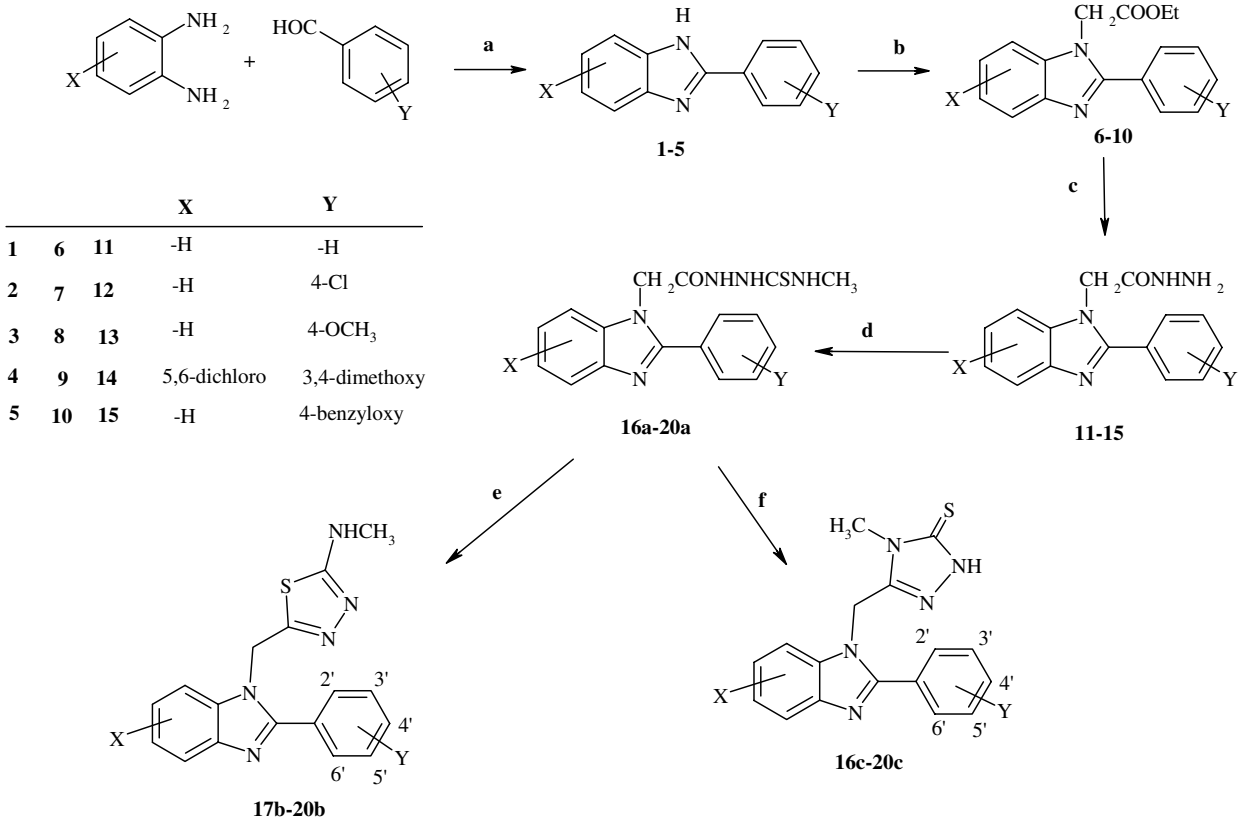
analysis, the structure of *5-[(2-phenyl-1H-benzimidazol-1-yl)methyl-4-methyl-2H-1,2,4-triazole-3(4H)-thione* **(16c)** was determined by X-ray analysis as well. All the synthesized compounds were evaluated by their scavenger effects on DPPH radical, superoxide radical, and rat liver microsomal NADPH-dependent lipid peroxidation levels by measuring the formation of 2-thiobarbituric acid reactive substances and also examined the inhibition capacity of their microsomal EROD activity.

2. Results and discussion

2.1. Synthesis

For the synthesis of the target compounds, the reaction sequences are outlined in Scheme 1. 1*H*-Benzimidazoles (**1**–**5**) were prepared via the oxidative condensation of related *o*-phenylenediamines with sodium metabisulfite adduct of appropriate benzaldehydes such as *p*-chloro, *p*-methoxy, 3,4-dimethoxy, and 4-benzyloxy in DMF.[4] Treatment of these compounds (**1**–**5**) with ethyl chloroacetate in KOH/DMSO yielded N-alkylated products (**6**–**10**).[5] Hydrazine hydrate and esters (**6**–**10**) in ethanol were refluxed for 4 h to give the desired hydrazide compounds (**11**–**15**).[6]

Thiosemicarbazides (**16a**–**20a**) were obtained upon the reaction of acid hydrazide with methylisothiocyanate in absolute ethanol. The cyclization of **17a**–**20a** with sulfuric acid resulted in the formation of thiadiazoles (**17b**–**20b**) and the cyclization of **16a**–**20a** with sodium hydroxide resulted in the formation of triazoles (**16c**–**20c**). Unexpectedly, **20b** was obtained from the



			X	Y
1	**6**	**11**	-H	-H
2	**7**	**12**	-H	4-Cl
3	**8**	**13**	-H	4-OCH_3
4	**9**	**14**	5,6-dichloro	3,4-dimethoxy
5	**10**	**15**	-H	4-benzyloxy

Scheme 1. Synthetic route for the preparation of compounds **1**–**15**, **16a**–**20a**, **17b**–**20b**, and **16c**–**20c**. Reagents: (a) $Na_2S_2O_5$; (b) $ClCH_2COOEt$/KOH–DMSO; (c) $NH_2NH_2{\cdot}H_2O$/EtOH; (d) methyl *iso*thiocyanate; (e) H_2SO_4; (f) NaOH.

Table 1. Physical and spectral data of **16a**–**20a**, **17b**–**20b**, and **16c**–**20c**

Compound	Formulas	% Yield	Mp (°C)	^{1}H NMR (400 MHz, DMSO-d_6); (δ ppm)	ESI-MS (M+H)
16a	$C_{17}H_{17}N_5OS$	78	210–212	2.91 (d, 3H, $NHCH_3$, J = 4), 4.97 (s, 2H, CH_2), 7.25–7.76 (m, 9H, Ar-H), 8.11, 9.43, 10.31 (3H, CO*NHNH*CS*NH*)	340 (100%)
17a	$C_{17}H_{16}ClN_5OS{\cdot}0.3H_2O$	62	237–239	2.90 (d, 3H, $NHCH_3$, J = 4), 4.94 (s, 2H, CH_2), 7.25–7.31 (m, 2H, H-5,6), 7.52–7.79 (m, 6H, other Ar-H), 8.08, 9.39, 10.30 (3H, CO*NHNH*CS*NH*)	374 (100%), 376 (33%)
18a	$C_{18}H_{19}N_5O_2S$	59	257	2.90 (d, 3H, $NHCH_3$, J = 4), 3.84 (s, 3H, OCH_3), 4.93 (s, 2H, CH_2), 7.10 (d, 2H, J_o = 8.99, H-2′,6′), 7.22–7.28 (m, 2H, H-5,6), 7.48–7.69 (m, 4H, H-4,7,3′,5′), 8.11, 9.42, 10.31 (3H, CO*NHNH*CS*NH*) ^{13}C NMR (DMSO-d_6) δ: 31.6, 46.5, 56.0, 111.4, 114.9, 119.5, 122.7, 122.9, 131.4, 137.1, 143.1, 154.1, 161.1, 167.8, 171.2, 182.9	370 (100%)
19a	$C_{19}H_{19}Cl_2N_5O_3S{\cdot}1.1H_2O$	63	229	2.89 (d, 3H, $NHCH_3$, J = 4), 3.78 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.97 (s, 2H, CH_2), 7.08–7.28 (m, 3H, H-2′,5′,6′), 7.82 (s, 1H, H-4), 7.95 (s, 1H, H-7), 8.09, 9.42, 10.29 (3H, CO*NHNH*CS*NH*)	468 (100%), 470 (68%), 472 (12%)
20a	$C_{24}H_{23}N_5O_2S$	71	206	2.90 (d, 3H, N-CH_3, J = 4), 4.93 (s, 2H, OCH_2), 5.20 (s,2H, CH_2), 7.16–7.69 (m,13H, Ar-H), 8.09, 9.41, 10.29 (s, 3H, CO*NHNH*CS*NH*)	446 (100%)
17b	$C_{17}H_{14}N_5ClS{\cdot}0.9H_2O{\cdot}0.6\ C_3H_8O$	61	275	2.76 (d, 3H, $NHCH_3$, J = 4), 5.73 (s, 2H, CH_2), 7.26–7.29 (m, 2H, H-5,6), 7.63–7.64 (m, 4H, H-4,2′,6′, NH), 7.68 (d, 1H, H-7, J_o = 7.76 Hz), 7.85 (d, 2H, H-3′,5′, J_o = 8.20 Hz). ^{13}C NMR (DMSO-d_6) δ: 31.9, 43.9, 111.8, 120.1, 123.3, 123.8, 129.4, 129.7, 131.8, 135.6, 136.3, 143.2, 152.5, 153.3, 170.7	356 (100%), 358 (34%)
18b	$C_{18}H_{17}N_5OS$	52	187–188	2.78 (d, 3H, $NHCH_3$, J = 4), 3.84 (s, 3H, O-CH_3), 5.70 (s, 2H, CH_2), 7.12 (d, 2H, J_o = 8.10 Hz, H-2′,6′), 7.22–7.28 (m, 2H, H-5,6), 7.59–7.68 (m, 3H, H-4,7, NH), 7.78 (d, 2H, *Jo* = 8.11 Hz, H-3′,5′); ^{13}C NMR (DMSO-d_6) δ: 31.8, 43.9, 56.0, 111.5, 114.9, 119.7, 122.6, 123.1, 123.3, 131.4, 136.2, 143.3, 153.6, 161.2, 170.7	352 (100%)
19b	$C_{19}H_{17}Cl_2N_5O_2S{\cdot}0.4\ H_2O$	49	194–196	2.78 (d, 3H, $NHCH_3$, J = 4.8), 3.78 (s, 3H, O-CH_3), 3.82 (s, 3H, O-CH_3), 5.76 (s, 2H, CH_2), 7.11 (d, 1H, *Jo* = 8.98 Hz, H-5′), 7.31–7.42 (m, 2H, H-2′,6′), 7.66 (q, 1H, NH), 7.96 (s, 1H, H-4), 8.04 (s, 1H, H-7); ^{13}C NMR (DMSO-d_6) δ: 31.9, 44.2, 56.3, 112.5, 113.2, 113.4, 120.9, 121.8, 122.9, 125.6, 125.7, 136.0, 142.8, 149.4, 151.3, 153.0, 156.1, 170.8	450 (100%)
20b	$C_{17}H_{15}N_5OS{\cdot}0.7H_2O$	37	262	2.78 (d, 3H, $NHCH_3$, J = 4.8), 5.69 (s, 2H, CH_2), 6.92 (d, 2H, J_o = 8.6, H-3′, 5′), 7.23–7.25 (m, 2H, H-5,6), 7.57–7.59 (m, 1H, H-4), 7.64-7.67 (m, 4H, H-7,2′,6′,NH), 10.01 (s, 1H, OH); ^{13}C NMR (DMSO-d_6) δ: 31.8, 43.9, 111.4, 116.3, 119.6, 120.9, 122.9, 123.0, 131.4, 136.1, 143.3, 153.6, 153.9, 159.7, 170.6	338 (100%)
16c	$C_{17}H_{15}N_5S$	64	258–261	3.39 (s, 3H, N-CH_3), 5.70 (s, 2H, CH_2), 7.25–7.29 (m, 2H, H-5,6), 7.55–7.75 (m, 7H, H-4,7,2′,3′,4’,5′,6′), 13.64 (s, 1H, NH)	322 (100%)
17c	$C_{17}H_{14}ClN_5S{\cdot}0.75H_2O$	51	279–280	3.41 (s, 3H, N-CH_3), 7.27–7.30 (m, 2H, H-5,6), 7.58–7.74 (m, 6H, H-4,7,2′,3′,5′,6′), 13.62 (s, 1H, NH)	356 (100%), 358 (32%)

18c	$C_{18}H_{17}N_5OS$	53	268	3.39 (s, 3H, N-CH_3), 3.81 (s, 3H, O-CH_3), 5.66 (s, 2H, CH_2), 7.10 (d, 2H, J_o = 8.99, H-2′,6′), 7.22–7.67 (m, 6H, H-4,5,6,7,3′,5′), 13.62 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 30.6, 41.0, 56.1, 112.1, 115.2, 118.7, 120.9, 123.9, 123.9, 131.4, 135.9, 140.8, 148.9, 153.4, 161.7, 168.3	352 (100%)
19c	$C_{19}H_{17}Cl_2N_5O_2S{\cdot}1.6H_2O$	55	250–252	3.32 (s, 3H, N-CH_3), 3.71 (s, 3H, O-CH_3), 3.79 (s, 3H, O-CH_3), 5.65 (s, 2H, CH_2), 7.10 (d, 1H, J_o = 8.50, H-5′), 7.18 (dd, 1H, J_o = 8.50, J_m = 1.81, H-6′), 7.24 (d, 1H, J_m = 1.81, H-2′), 7.96 (s, 1H, H-4), 8.05 (s, 1H, H-7), 13.62 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ: 30.7, 41.4, 56.1, 56.3, 112.5, 112.9, 113.5, 120.8, 121.6, 122.4, 125.6, 125.7, 136.5, 142.7, 149.1, 149.4, 151.3, 156.4, 168.5	450 (100%), 452 (68%), 454 (13%)
20c	$C_{24}H_{21}N_5OS$	58	251–254	3.39 (s, 3H, N-CH_3), 5.18 (s, 2H, O-CH_2), 5.67 (s, 2H, CH_2), 7.18–7.68 (m, 13H, Ar-H), 13.6 (s, NH)	428 (100%)

cyclization reaction of **20a** in the cold H_2SO_4 by missing the benzyl group.

All physical and spectral data of **16a**–**20a**, **17b**–**20b**, and **16c**–**20c** were seen in Table 1. In the IR spectra of compounds **16c**–**20c** no absorption bands were detected about 1671–1696 cm^{-1} indicating the absence of CO group of acylthiosemicarbazides (**16a**–**20a**) which is an evidence for the conversion of thiosemicarbazides to triazoles. Although two types of tautomers (thione or thiole) could be expected from the cyclization of compounds **16a**–**20a** under alkaline conditions, only the thione type compounds (**16c**–**20c**) were observed. This cyclization and formation of thione tautomer also demonstrated both by the presence of two absorption maxima at 1310–1322 and 1265–1290 cm^{-1} belonging to the C=S group and X-ray data of compound **16c**. X-ray analysis results show that compound **16c** exists in the thione tautomeric form in the solid state. The location of the H-atom on the atom N2 rather than the atom S, and the C1–S1, N2–C1, and C1–N1 bond lengths support this idea. The IR spectra of novel esters and hydrazides revealed sharp bands at around 1731–1752 and 1660–1698 cm^{-1}, respectively. As expected, in the mass spectra of **14**, **17a**–**17c**, **19a**, and **19c** both mono and two chlorine isotopes of this atom were seen. The structures of the synthesized compounds were consistent with the ^{1}H and ^{13}C NMR spectra. In the ^{1}H NMR spectra, NH–CH_3 coupling constants (J) are seen as 4 Hz (**16a**–**20a**, **17b**, **18b**) and 4.8 Hz (**19b**, **20b**). In the ^{13}C NMR spectra of the triazolethione derivatives (**18c**, **19c**), CH_2– peak was observed at about 41.0–41.5 ppm which was close to the DMSO-d_6 peaks. In the ^{13}C NMR spectra of thiadiazole derivatives (**17b**, **18b**, and **19b**), these peaks were seen at 43.85, 43.96, and 44.22 ppm, respectively. The crystal data, intensity data collection parameters, and final refinement results for compound **16c** are summarized in Table 2.

Hydrogen bonding geometry (Å, °) for **16c** is shown in Table 3. In all essential details, the geometry of the molecule in terms of bond lengths and angles is in good agreement within experimental errors, with those observed in other benzimidazole derivatives. As expected, the benzimidazole moiety of the molecule is planar due to the wide electronic delocalization effect [maximum deviations 0.032(2) Å for N8]. The dihedral angle between the imidazole and benzene ring planes is 1.9(1)°.

Selected bond lengths and angles are listed in Table 4. The molecular structure with atom numbering scheme and the packing arrangement of the molecules are presented in Figures 3 and 4.

The phenyl and triazole groups at C16 and N8 are essentially planar, and twisted out of the plane of the benzimidazole ring with torsion angles of −54.6(3)° and −85.4(2)(3)° for N15–C16–C17–C18 and C16–N8–C7–C1, respectively. The dihedral angle between the 2,4-dihydro-1,2,4-triazole ring and the benzimidazole nucleus is 76.9(1)°. The methyl and thione groups attached to triazole ring almost lie linearly in the triazole ring

Table 2. Crystal data and details of the structure determination of **16c**

Crystal formula	$C_{17}H_{15}N_5S$	Z; D_{calc} [g cm^{-3}]	8; 1.303
Formula weight	321.4	θ_{max} [°]	74.27
Crystal dimensions [mm]	$0.33 \times 0.48 \times 0.52$	μ (Mo Kα) [cm^{-1}]	1.8
Temperature [K]	295	Reflections collected	3323
Crystal system	Monoclinic	Reflections used in refinement	3225
Space group	*C*2/*c*	No. of refined parameters	213
a [Å]	17.3529(14)	R/R_w values	0.0388/0.1124
b [Å]	9.6909(13)	GOF	1.049
c [Å]	19.6297(9)	Final shift	0.000
β [°]	97.036(5)	$(\Delta\rho)_{min}$, $(\Delta\rho)_{max}$ [e Å^{-3}]	−0.227, 0.232

Table 3. Hydrogen bonding geometry (Å, °) for **16c**

D–H ⋯ A	D–H	H ⋯ A	D ⋯ A	D–H ⋯ A
C6–H6A ⋯ S1	0.96	2.77	3.253	112
N3–H3 ⋯ N15^{i}	0.91(3)	1.96(3)	2.831(2)	159
C18–H18 ⋯ N2^{ii}	0.93	2.56	3.389	148
C22–H22 ⋯ N15^{iii}	0.93	2.55	3.423	156

Symmetry code: (i) $1/2 - x$, $-1/2 + y$, $1/2 - z$, (ii) $1/2 - x$, $1/2 + y$, $1/2 - z$, (iii) $1 - x$,y, $1/2 - z$.

Table 4. Selected bond distances and bond angles (Å, °) for **16c**

C1–N2	1.295(2)	C12–C13	1.373(3)
C1–N5	1.376(2)	C13–C14	1.397(3)
C1–C7	1.497(3)	C14–N15	1.395(2)
C4–N3	1.341(2)	C16–N15	1.321(2)
C4–N5	1.372(2)	C16–N8	1.371(2)
C4–S1	1.6751(19)	C16–C17	1.474(2)
C6–N5	1.459(2)	C17–C18	1.381(3)
C7–N8	1.462(2)	C17–C22	1.399(3)
C9–N8	1.387(2)	C18–C19	1.384(3)
C9–C14	1.391(3)	C19–C20	1.376(3)
C9–C10	1.393(3)	C20–C21	1.375(3)
C10–C11	1.378(3)	C21–C22	1.381(3)
C11–C12	1.393(4)	N2–N3	1.372(2)
C4–N5–C1	107.74(15)	N5–C4–S1	128.65(14)
C4–N5–C6	124.74(16)	N15–C16–N8	112.18(15)
C1–N5–C6	127.52(16)	C16–N8–C7	127.15(15)
N2–C1–N5	111.19(16)	C16–N8–C9	107.09(14)
N2–C1–C7	122.56(17)	C9–N8–C7	124.49(16)
N5–C1–C7	126.24(16)	N8–C7–C1	110.89(14)
N3–C4–N5	103.54(16)	C1–N2–N3	104.29(15)
N3–C4–S1	127.76(15)	C4–N3–N2	113.20(16)

plane, with the greatest deviation being −0.100(1) and 0.051(2) Å for S1 and C6 atoms, respectively.

The phenyl group makes a dihedral angle of 56.2(1)° and 48.3(1)° with the benzimidazole and triazole rings, respectively. Two types of intermolecular hydrogen bonds, N–H…N and C–H…N, and one intramolecular interaction, C–H…S, are observed in the structure (Fig. 4 and Table 3). Screw related molecules are interconnected by hydrogen bonds between triazole and benzimidazole groups and phenyl C18 and C22 hydrogen forming infinite chains along a direction (Fig. 4).

The antioxidant properties of the targeted compounds (**16a**–**20a**, **17b**–**20b**, and **16c**–**20c**) were evaluated by the interaction of DPPH, scavenging of superoxide radical

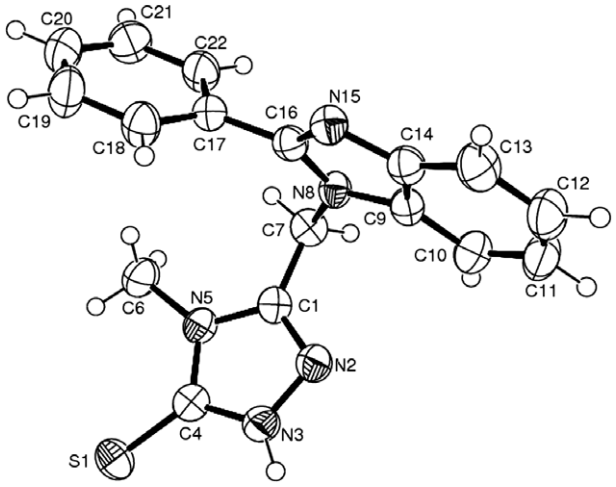


Figure 3. Molecular structure of **16c**, showing the atom-numbering scheme. Displacement ellipsoids are drawn at the 50% probability level.

(data are not shown), NADPH-dependent lipid peroxidation levels, and EROD enzyme activity (Table 5).

Each method relates to the generation of a different radical, acting through a variety of mechanisms.

The inhibitory effects of different concentrations of synthesized compounds on DPPH radical are presented in Table 5. It appears that compounds **16a**–**20a** were found to be a good scavenger of DPPH radical (IC_{50}, 26 μM; IC_{50}, 30 μM; IC_{50}, 43 μM; IC_{50}, 55 μM; IC_{50}, 74 μM, respectively) when compared to BHT (IC_{50}, 54 μM). Compounds **18c**, **16c**, **19c**, **17c**, and **20c** exhibited moderate effect on DPPH radical with IC_{50} values of 140, 271, 271, and 462 μM, respectively. All thiadiazole series of compounds (**17b**–**20b**) have the weakest effect on DPPH radical with IC_{50} values of >1000 μM, when compared to BHT.

The superoxide radical scavenging activities of the compounds were also investigated by using the xanthine/xanthine oxidase system, and there was no significant activity pattern obtained from this experiment (data are not shown). Only compounds **17a**, **19b**, **19a**, **16a**, and **17b** showed moderate inhibitory effect on superoxide radical at 0.1 mM concentration and inhibition rates were 38%, 31%, 26%, 22%, and 21%, respectively. The scavenging rates of the rest of the compounds were in

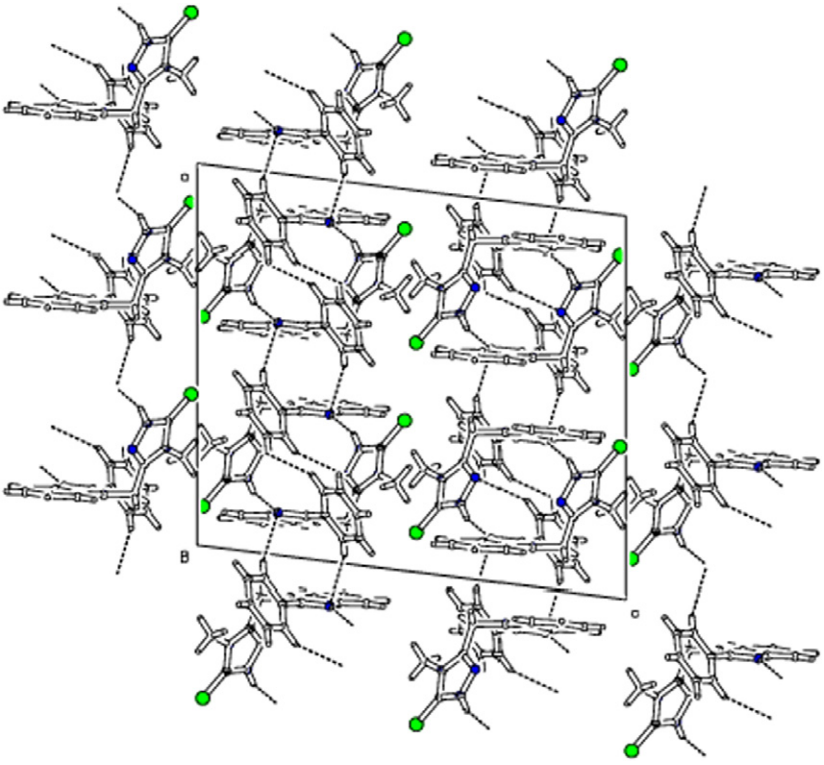
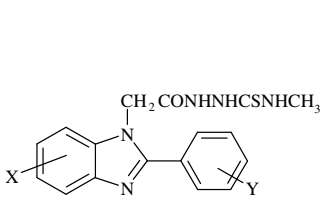
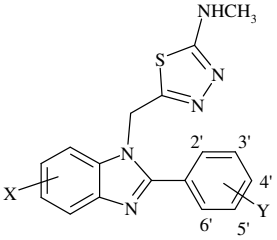


Figure 4. Crystal packing of **16c** projected onto ac plane. The dashed lines indicated the intermolecular hydrogen bonds.

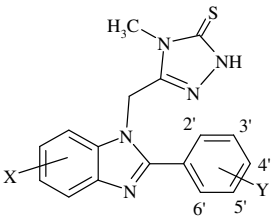
Table 5. Effects of the compounds on liver LP levels, on EROD and on DPPH free radical scavenging activities in vitro[a]



16a-20a



17b-20b



16c-20c

Compound[b]	X	Y	DPPH IC_{50} (μM)	LP (nmol/mg per min)	% of control	EROD (pmol/mg per min)	% of control
16a	–H	–H	26 ± 4.2	19.19 ± 0.27	118	50.98 ± 2.10	128
17a	–H	4-Cl	30 ± 1.4	13.08 ± 0.14	80	28.26 ± 0.31	68
18a	–H	4-OCH_3	43 ± 2.8	16.12 ± 0.98	99	34.55 ± 0.45	83
19a	5,6-Dichloro	3,4-Dimethoxy	55 ± 3.5	14.81 ± 0.09	91	12.31 ± 0.11	30
20a	–H	4-Benzyloxy	74 ± 4.2	96.56 ± 0.38	594	28.58 ± 0.27	69
17b	–H	4-Cl	—	16.12 ± 0.15	99	2.46 ± 0.05	60
18b	–H	4-OCH_3	>1000	14.39 ± 0.11	89	34.29 ± 0.47	83
19b	5,6-Dichloro	3,4-Dimethoxy	>1000	11.78 ± 0.27	72	12.20 ± 0.18	34
20b	–H	4-Hydroxy	>1000	129.22 ± 0.74	795	36.08 ± 0.56	87
16c	–H	–H	271 ± 14	20.95 ± 1.27	129	13.13 ± 0.91	32
17c	–H	4-Cl	462 ± 21	26.60 ± 1.50	164	7.89 ± 0.26	19
18c	–H	4-OCH_3	140 ± 7.0	23.10 ± 1.76	142	15.85 ± 0.16	38
19c	5,6-Dichloro	3,4-Dimethoxy	271 ± 28	27.48 ± 0.71	169	18.32 ± 0.27	44
20c	–H	4-Benzyloxy	605 ± 35	69.58 ± 0.56	428	17.96 ± 0.19	43
BHT	—	—	54 ± 4.9	5.68 ± 0.22	35	—	—
Caffeine	—	—		—	—	8.31 ± 0.42	20
Control[c]	—	—		16.25 ± 1.45	100	41.53 ± 0.99	100

[a] Each value represents mean ± SD of 2–4 independent experiments.
[b] Concentration in incubation medium (10^{-3} M).
[c] DMSO only, control for compounds.

the range of 5–10%. Compounds **17c**, **18a**, and **19c** had no effect on superoxide radical.

The effect of compounds (**16a–20a**, **17b–20b**, and **16c–20c**) were evaluated on lipid peroxidation levels. The NADPH-dependent lipid peroxidation inhibition produced by all new compounds in rat liver microsomes was examined by measuring the formation of 2-thiobarbituric acid reactive substance for their antioxidant capacity. As can be seen from Table 5, the BHT decreased the LP level by about 65% at 10^{-3} M concentration. However, the synthesized compounds have no significant effect on lipid peroxidation level in rat liver microsomes at the same concentration.

Compounds **17a** (20%) and **18b** (11%) showed rather limited lipid peroxidation at 10^{-3} M concentration but compounds **16a**, **16c**, **17c**, **18c**, **19c**, **20a**, **20b**, and **20c** enhanced LP levels. The most active compound in this series is **19b**, which caused only 28% inhibition.

The in vitro effects of compounds and caffeine on liver microsomal EROD activity are also shown in Table 5. The concentrations required for 50% inhibition (IC_{50}) of liver microsomal EROD activity were 8.4×10^{-4}, 7.4×10^{-4}, and 4.5×10^{-4} μg/ml for **19a**, **16c**, and **17c**, respectively. Compound **17c** inhibited the microsomal EROD activity (81%) which was similarly better than the specific inhibitor caffeine (80%) at 10^{-3} M concentration. Significant inhibitory activities were observed also by compounds **19a** (70%), **19b** (66%), and **16c** (68%). In this series of compounds, particularly the triazole derivatives (**16c–20c**) were the most active according to the method.

3. Conclusion

In this study, a series of new benzimidazole derivatives **16a–20a**, **17b–20b**, and **16c–20c** having [*N*-methylthiosemicarbazide], [2-methylamino-1,3,4-thiadiazole], and [4-methyl-2*H*-1,2,4-triazole-3(4*H*)-thione] moieties, respectively, at 1st position was synthesized, and their antioxidant activities were evaluated. It was observed that the effects of the compounds on DPPH radical, superoxide radical, lipid peroxidation, and EROD activity levels were variable. The strong scavenging effects of compounds **16a–20a** were noted on the level of DPPH radical but not superoxide radical. These and previous published results[2] clearly indicate that both *N*-aryl and *N*-methyl thiadiazole derivatives were found to have no interaction with DPPH. If we compare with *N*-methyl and *N*-aryl series reported before,[2,3] in all the series, triazole derivatives have moderate activity and thiosemicarbazides have the best effect on DPPH. IC_{50} values of *N*-methylthiosemicarbazides **16a–20a** (26–74 μM) are similar to *N*-aryl analogues 1-[(phenylthiocarbamoylhydrazinecarbonyl)methyl]-2-(4-pyridinyl)-1*H*-benzimidazole (13 μM) and 1-[(*p*-methoxyphenylthiocarbamoylhydrazinecarbonyl)-methyl]-2-(4-pyridinyl)-1*H*-benzimidazole (12 μM) so it can be said that there is no noteworthy difference between their scavenger effects on DPPH radical.

As we reported before, It is well known that there exists two mechanisms for an antioxidant to scavenge DPPH. The first one is a direct H-atom abstraction process (Eq. 1), and the second one is a proton concerted electron-transfer process (Eq. 2).[7]

$$\mathrm{DPPH^{\cdot}} + \mathrm{RXH} \rightarrow \mathrm{DPPHH} + \mathrm{RXH^{\cdot}} \quad (1)$$

$$\mathrm{DPPH^{\cdot}} + \mathrm{RXH} \rightarrow \mathrm{DPPH^{-}} + \mathrm{RXH^{\cdot +}} \rightarrow \mathrm{DPPHH} + \mathrm{RX^{\cdot}} \quad (2)$$

DPPH-scavenging mechanism of our compounds could not clarified yet but our effort on this way is going on.[8]

Compound **19b** which is the most active derivative slightly inhibited lipid peroxidation (28%) at 10^{-3} M concentration. Bearing *N*-phenyl and *N*-substitutedphenyl derivatives (triazoles, thiadiazoles and especially thiosemicarbazides) it had stronger inhibitory effects on LP levels than those of *N*-methyl analogues.[2,3]

Compound **17c** inhibited the EROD activity with an $IC_{50} = 4.5 \times 10^{-4}$ M which is similarly better than the specific inhibitor caffeine $IC_{50} = 5.2 \times 10^{-4}$ M.

Since the mechanisms of these methods are different, it is quite difficult to explain the observed different effects of synthesized compounds. Different effects of compounds in these systems have been noticed previously.[3,9,10] Therefore, the observation of distinct effects of synthetic compounds on DPPH radical, superoxide radical, LP levels, and EROD is not surprising since the mechanisms of production of oxidative stress using these methods are different.[11–13] The differences in the kinetic behavior of the radicals and substrates should also be considered when comparing the results of different free radical scavenging methods to determine antioxidant capacity.[14] Therefore, it is extremely difficult to compare the results from different assays.

As a result, data obtained from all our researches associated with this field guide us for the development of novel antioxidant compounds.

4. Experimental

4.1. Synthesis

Melting points were determined with an Electrothermal and a Büchi SMP-20 melting point apparatus and are uncorrected. IR spectra were recorded on a Jasco FT/IR 420 spectrometer as potassium bromide discs. ^{1}H and ^{13}C NMR spectra were measured with a Varian Mercury 400, 400 MHz instrument using TMS internal standard and DMSO-d_6, coupling constants (*J*) are reported in Hertz. All chemical shifts were reported as δ (ppm) values. ES-MS were obtained with a Waters ZQ Micromass LC–MS spectrometer with Positive Electrospray Ionization method. Elemental analyses (C, H, N, and S) were determined on a Leco CHNS 932 instrument, and were within ±0.4% of the theoretical values. All instrumental analyses were performed at Ankara

University, Faculty of Pharmacy. The chemical reagents used in synthesis were purchased from E. Merck and Aldrich. BHT and caffeine were obtained from Sigma. Analytical thin-layer chromatography was performed with Merck precoated TLC plates and spots were visualized with ultraviolet light.

2-Phenyl-1*H*-benzimidazole (**1**), 2-(4-chlorophenyl)-1*H*-benzimidazole **(2)**, 2-(4-methoxy-phenyl)-1*H*-benzimidazole (**3**), (2-phenyl-1*H*-benzimidazol-1-yl)-acetic acid ethyl ester (**6**), (2-(4-chlorophenyl)-1*H*-benzimidazol-1-yl)-acetic acid ethyl ester (**7**), (2-phenyl-1*H*-benzimidazol-1-yl)-acetic acid hydrazide (**11**), and 2-(4-chlorophenyl-1*H*-benzimidazol-1-yl)-acetic acid hydrazide (**12**) were previously prepared in our laboratory.[1–3]

4.1.1. General procedure for the preparation of 2-substituted benzimidazoles (1–5). For the synthesis of compounds **1**–**5**, the mixture of appropriate *o*-phenylendiamine (1 mmol) and sodium metabisulfite adduct of substituted benzaldehyde (1.25 mmol) in DMF was heated at 110 °C for 5 h.[4] Water was added to the reaction medium and the solid product was collected by filtration and washed with water. The crude product was crystallized from EtOH.

4.1.1.1. 5,6-Dichloro-2-(3,4-dimethoxyphenyl)-1*H*-benzimidazole (4). Yield: 87%; mp 269 °C; ES (+) (M+H): 323 (100%); ^{1}H NMR (DMSO-d_6) δ: 3.85 (s, 3H, O-CH_3), 3.88 (s, 3H, O-CH_3), 7.16 (s, 1H), 7.75 (3H), 7.89 (s, 1H), 13.01 (s, 1H, NH).

4.1.1.2. 2-(4-Benzyloxyphenyl)-1*H*-benzimidazole (5). Yield: 79%; mp 276 °C; ES (+) (M+H): 301 (100%); ^{1}H NMR (DMSO-d_6) δ: 5.20 (s, 2H, O-CH_2), 7.17–7.20 (m, 4H), 7.33–7.61 (7H), 8.13 (d, 2H, J = 8), 12.70 (br s, 1H, NH).

4.1.2. General procedure for the preparation of [2-(substitutedphenyl)-1*H*-benzimidazolyl]-acetic acid ethyl esters (6–10). Dimethylsulfoxide (15 ml) was added to potassium hydroxide (27 mmol) (crushed pellets) and the mixture was stirred 15 min and related benzimidazole derivative (6.7 mmol) was added and then the mixture was stirred for 2 h. Ethylchloroacetate (27 mmol) was added and the mixture was cooled briefly and stirred for further 2 h. Water was added and the mixture was extracted with ether. Ether layers were washed with water, dried, and solvent and excess of ethylchloroacetate was removed under reduced pressure. The residue was recrystallized from ethanol and gave the desired ester compounds (**6**–**10**).

4.1.2.1. [2-(4-Methoxyphenyl)-1*H*-benzimidazol-1-yl]-acetic acid ethyl ester (8). Yield: 68%; mp 96–97 °C; ES (+) (M+H): 311 (100%); IR (KBr cm^{-1}) 1752 (C=O); ^{1}H NMR (DMSO-d_6) δ: 1.17 (t, 3H, H_2C–CH_3), 3.85 (s, 3H, O-CH_3), 4.15 (q, 2H, H_2C–CH_3), 5.28 (s, 2H, N-CH_2), 7.11–7.69 (m, 8H, Ar-*H*).

4.1.2.2. [5,6-Dichloro-2-(3,4-dimethoxyphenyl)-1*H*-benzimidazol-1-yl]-acetic acid ethyl ester (9). Yield: 76%; mp 207–208 °C; ES (+) (M+H): 409 (100%); IR (KBr cm^{-1}) 1731 (C=O); ^{1}H NMR (DMSO-d_6) δ: 1.13 (t, 3H, H_2C–CH_3), 3.80 (s, 3H, O-CH_3), 3.83 (s, 3H, O-CH_3), 4.09–4.15 (q, 2H, H_2C–CH_3), 5.35 (s, 2H, N-CH_2), 7.16 (d, 1H, J_o = 8.2), 7.28 (d, 1H, J_o = 8.2), 7.3 (s, 1H), 7.99 (s, 1H), 8.24 (s, 1H).

4.1.2.3. 2-(4-Benzyloxyphenyl-1*H*-benzimidazol-1-yl)-acetic acid ethyl ester (10). Mp 127 °C; ES (+) (M+H): 387 (100%); IR (KBr cm^{-1}) 1749 (C=O); ^{1}H NMR (DMSO-d_6) δ: 1.13 (t, 3H, H_2C–CH_3), 4.12 (q, 2H, H_2C–CH_3), 5.2 (s, 4H, N-CH_2, O-CH_2), 7.15–7.68 (m, 13H, Ar-H).

4.1.3. General procedure for the preparation of [2-(substitutedphenyl)-1*H*-benzimidazol-1-yl]-acetic acid hydrazides (11–15). Hydrazine hydrate (4 ml) and related 1*H*-benzimidazole acetic acid ethyl esters (**6**–**10**) (1.5 mmol) in ethanol (5 ml) were refluxed for 4 h. The reaction mixture was cooled and poured into water. The crude product was filtered off and recrystallized from ethanol to give the desired hydrazide compounds (**11**–**15**).

4.1.3.1. [2-(4-Methoxyphenyl)-1*H*-benzimidazol-1-yl]-acetic acid hydrazide (13). Yield: 73%; mp 197–198 °C; ES (+) (M+H): 297 (100%); IR (KBr cm^{-1}) 1694 (C=O); ^{1}H NMR (DMSO-d_6) δ: 3.8 (s, 3H, O-CH_3), 4.43 (s, 2H, –NH_2–), 4.79 (s, 2H, –CH_2–), 7.07–7.77 (m, 8H, Ar-H), 9.59 (s, 1H, NH).

4.1.3.2. [5,6-Dichloro-2-(3,4-dimethoxyphenyl)-1*H*-benzimidazol-1-yl]-acetic acid hydrazide (14). Yield: 68%; mp 265–266 °C; ES (+) (M+H): 395 (100%), 397 (67%), 399 (11%); IR (KBr cm^{-1}) 1698 (C=O); ^{1}H NMR (DMSO-d_6) δ: 3.81 (s, 3H, O-CH_3), 3.85 (s, 3H, O-CH_3), 4.40 (br s, 2H, –NH_2), 4.90 (s, 2H, CH_2–), 7.10 (d, 1H, J_o = 8.0), 7.31–7.35 (2H), 7.83 (s, 1H), 7.93 (s, 1H), 9.54 (s, 1H).

4.1.3.3. [2-(4-Benzyloxyphenyl)-1*H*-benzimidazol-1-yl]-acetic acid hydrazide (15). Yield: 61%; mp 227–229 °C; ES (+) (M+H): 373 (100%); IR (KBr cm^{-1}) 1660 (C=O); ^{1}H NMR (DMSO-d_6) δ: 4.39 (s, 2H, NH_2), 4.80 (s, 2H, –CH_2–), 5.20 (s, 2H, –CH_2–), 7.17–7.79 (m, 13H, Ar-H), 9.59 (s, 1H, NH).

4.1.4. General procedure for the preparation of thiosemicarbazides [(1-(methylaminothio carbamoylhydrazinecarbonyl)-methyl-2-substitutedphenyl-1*H*-benzimidazoles)] (16a–20a). Appropriate acid hydrazides (**11**–**15**) 0.54 g (2.03 mmol) in absolute ethanol (20 ml) and methyl isothiocyanate (3.05 mmol) were heated under reflux for 30 min. Precipitate formed was cooled, filtered, and recrystallized from ethanol.

4.1.5. General procedure for the preparation of 5-[(2-(4-substitutedphenyl)-1*H*-benzimidazol-1-yl)methyl*N*-methyl-1,3,4-thiadiazol-2-amines (17b–20b). Appropriate thiosemicarbazides **17a**–**20a** (3.4 mmol) in 10 ml ice-cold concentrated sulfuric acid were stirred for 10 min, and then left for another 10 min at room temperature. The resulting solution was poured slowly into ice-cold water, made alkaline to pH 8 with aqueous ammonia and the precipitated product was filtered, washed with water,

and crystallized from ethanol or ethanol/isopropanol (10:1).

4.1.6. General procedure for the preparation of the 5-[(2-(substitutedphenyl)-1*H*-benzimidazol-1-yl)methyl]-4-methyl-2*H*-1,2,4-triazole-3(4*H*)-thiones (16c–20c). Appropriate thiosemicarbazides (3.4 mmol) **16a–20a** in 10 ml 1 N sodium hydroxide were refluxed for 1 h. The reaction mixture was cooled and then acidified to pH 6 with 1 N hydrochloric acid. The precipitate was filtered, washed with water, and recrystallized from ethanol.

4.2. Single crystal X-ray structure determinations

Single crystals suitable for X-ray diffraction analysis were obtained by crystallization from ethanol. Measurements were performed on a Enraf-Nonius CAD4 four circle diffractometer equipped with graphite monochromator Mo K_α radiation.[15] Intensity of peaks was corrected for standard reflections automatically. Structure was solved by direct methods using SHELXS[16] and, refinement was performed with SHELXL.[17] Non-hydrogen atom parameters were refined anisotropically. All hydrogen atoms, except for N3, were placed in idealized positions and refined using a riding model with $U_{eq}(H) = 1.3U_{eq}(C)$, and fixed distances of C–H = 0.93 Å (aromatic), C–H = 0.96 Å (methyl), and C–H = 0.97 Å (ethyl). Hydrogen atom of N3 was found from a difference Fourier map and refined isotropically. The geometric calculations were performed using the program Platon.[18]

4.3. Antioxidant properties of novel benzimidazole derivatives

4.3.1. DPPH free radical scavenging activity. The free radical scavenging activities of these compounds were tested by their ability to bleach the stable radical 2,2,diphenyl-1-picrylhydrazyl (DPPH) as described by Blois.[19] This assay has often been used to estimate the antiradical activity of antioxidants. Because of its odd electra, DPPH gives a strong absorption bound at 517 nm in visible spectroscopy. DPPH was dissolved in methanol to give a 100 μM solution. To 1.0 ml of the methanolic solution of DPPH was added 0.1 ml of the test compounds and BHT dissolved in dimethylsulfoxide (DMSO). Absorbance at 517 nm was determined after 30 min at room temperature and the scavenging activity was calculated as a percentage of the radical reduction. Each experiment was performed in triplicate. DMSO was used as a control solution and BHT as a reference compound. The radical scavenging activity was expressed as IC_{50} which was determined from a calibration curve for each compound.

4.3.2. Superoxide radical scavenging activity. The capacity of compounds to scavenge superoxide anion formation was determined spectrophotometrically on the basis of inhibition of cytochrome *c* reduction according to the modified method of McCord et al.[20]

Superoxide anion was generated in the xanthine/xanthine oxidase system. The reaction mixture contained in a final volume of 1 ml, 0.05 M phosphate buffer, pH 7.8, 0.32 U xanthine oxidase, 50 μM xanthine, 60 mM cytochrome *c*, and different concentration of synthesized compounds at 100 μl. The absorbance was measured spectrophotometrically at 550 nm for cytochrome *c* reduction.

4.3.3. Assay of lipid peroxidation. Male albino Wistar rats (200–225 g) were used in the experiments. Animals were fed with standard laboratory rat chow and tab water ad libitum. The animals were starved for 24 h prior to sacrifice and then killed by decapitation under anesthesia. The livers were removed immediately and washed in ice-cold water and the microsomes were prepared as described previously.[21]

NADPH-dependent LP was determined using the optimum conditions determined and described previously.[21] NADPH-dependent was measured spectrophotometrically by the estimation of thiobarbituric acid reactant substances (TBARS). Amounts of TBARS were expressed in terms of nanomole malondialdehyde (MDA)/mg protein. The assay was essentially derived from the methods of Wills[22,23] as modified by Bishayee.[24] A typical optimized assay mixture contained 0.2 nM Fe^{++}, 90 mM KCl, 62.5 mM potassium-phosphate buffer, pH 7.4, NADPH generating system consisting of 0.25 mM $NADP^+$, 2.5 mM $MgCl_2$, 2.5 mM glucose-6-phosphate, 1.0 U glucose-6-phosphate dehydrogenase, and 14.2 mM potassium phosphate buffer pH 7.8 and 0.2 mg microsomal protein in a final volume of 1.0 ml.

4.3.4. Assay of EROD. EROD activity was measured by the spectrofluorometric method of Burke et al.[25] A typical optimized assay mixture contained 1.0 mM ethoxyresorufin, 100 mM Tris–HCl buffer, pH 7.8, NADPH generating system consisting of 0.25 mM $NADP^+$, 2.5 mM $MgCl_2$, 2.5 mM glucose-6-phosphate, 1.0 U glucose-6-phosphate dehydrogenase, and 14.2 mM potassium phosphate buffer pH 7.8 and 0.2 mg liver microsomal protein in a final volume of 1.0 ml. EROD activity of compounds was expressed as IC_{50} which determined from a calibration curve.

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