Full Paper

Synthesis and Evaluation of Antioxidant Properties of Novel 1,2,4-Triazole-Based Schiff Base Heterocycles

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A series of 1,2,4-triazole-based Schiff base heterocyclic compounds (**5a–f** and **8a–i**) and phenethylamines (**7a–h**) were synthesized and evaluated for antioxidant properties by free-radical scavenging, anti-hemolytic activity, lipid peroxidation, and their protective effects against DNA oxidative damage. Compounds **7c**, **7d**, **7h**, **8b**, and **8i** showed promising DPPH$^{\bullet}$ radical scavenging activity with the level of inhibition between 86.8% and 94%. Compounds **8a**, **8b**, **8d**, **8g**, and **8i** were effective against the oxidative hemolysis of human erythrocytes and lipid peroxidation, in a dose-dependent manner, with IC_{50} values in the range of 55.7–80.7 and 53.2–81.2 μg/mL, respectively. Compounds **8a** and **8b** were effective against oxidative damage on erythrocyte ghost membrane proteins, and **8g** and **8i** were able to protect against DNA oxidative damage.

Keywords: Anti-hemolytic / Antioxidant / Lipid peroxidation / Schiff base / 1,2,4-Triazole

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Introduction

The compounds containing 1,2,4-triazole nucleus represent an important class of heterocyclic compounds and their derivatives are characterized with a broad spectrum of biological activity. Structurally triazole nucleus forms a part of a wide variety of therapeutically active drug candidates, including H_1/H_2 histamine receptor blockers, cholinesterase active agents, CNS stimulants, antianxiety, and sedative agents [1]. Many 1,2,4-triazole and 1,3,4-thiadiazole derivatives have been used as "privileged" scaffolds to produce active pharmaceutical ingredients. Some of the modern-day drugs like ribavirin (antiviral agent), alprazolam (anxiolytic agent), fluconazole, itraconazole (anti-fungal agents), and rizatriptan (antimigrane agent) have triazole nucleus in their structure. The ambient nucleophilic centers present in 3-substituted-4-amino-5-mercapto-1,2,4-triazoles render them useful synthons for the synthesis of various N-bridged heterocycles. 1,2,4-Triazole, 1,3,4-thiadiazole, and thiadiazine are explored to the maximum extent owing to their wide spectrum of pharmacological activities such as antibacterial, anti-fungal [2, 3], antitubercular [4], anticancer [5], anticonvulsant [6], anti-inflammatory [7, 8], analgesic [9, 10], antitumor [11], molluscicidal [8, 12], and antiviral [11, 13] activities. Among the pharmacological profiles of 1,2,4-triazoles, their antimicrobial, anticonvulsant, and antidepressant properties have been best documented. Recently, some benzoxazoline, trisubstituted triazoles, and 4-benzylidenamino derivatives containing triazole and thiadiazole units have been found to be endowed with excellent free radical scavenging activities [14–16]. Prompted by these observations and in continuation of our research for biologically active heterocyclic compounds [17], it was contemplated to synthesize some N-bridged heterocycles containing 2-methylphenyl and 5-chloro-2-methylphenyl moieties with a view to explore their potency as better chemotherapeutic agents.

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Results and discussion

Chemistry

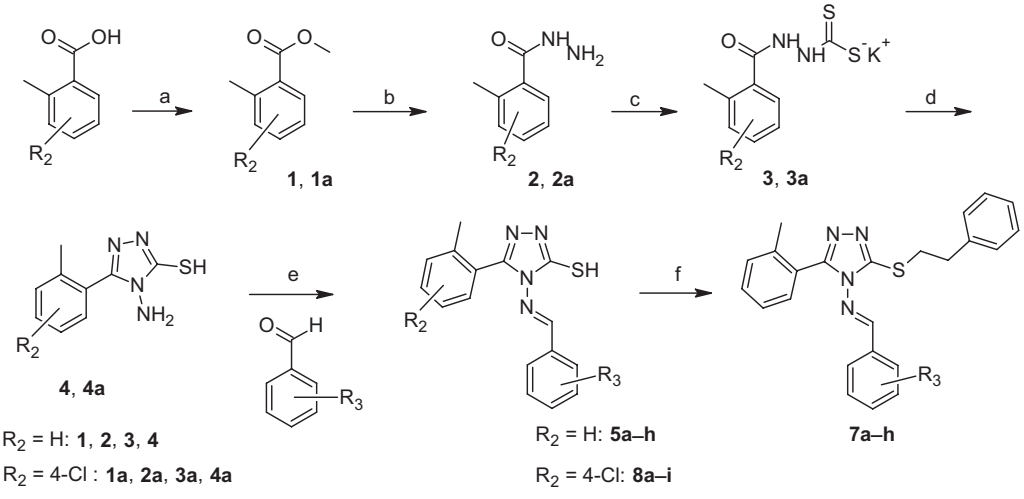
For the synthesis of title compounds, 4-amino-5-(2-methyl phenyl)-4*H*-1,2,4-triazole-3-thiol (**4**) or 4-amino-5-(5-chloro-2-methyl phenyl)-4*H*-1,2,4-triazole-3-thiol (**4a**) required as starting material was prepared according to a method that involves the condensation of 2-methylbenzohydrazide (**2**) or 5-chloro-2-methylbenzohydrazide (**2a**) with carbon disulfide and potassium hydroxide to yield potassium dithiocarbazate **3** or **3a**, which underwent ring closure with excess of hydrazine hydrate to produce aminothiol **4** or **4a** in good yield (79–87%). A new series of Schiff base 4-[(*E*)-benzylideneamino]-5-(2-methylphenyl)-4*H*-1,2,4-triazole-3-thiols (**5a–h**) and 4-[(*E*)-benzylideneamino]-5-(5-chloro-2-methylphenyl)-4*H*-1,2,4-triazole-3-thiols (**8a–i**) were prepared by treating compound **4** or **4a** with equimolar amounts of the appropriate benzaldehyde derivative in presence of catalytic quantity of sulfuric acid and ethanol under reflux conditions. The Schiff base (**5a–h**), upon further reaction with phenethyl bromide, in the presence of potassium hydroxide and ethanol under reflux conditions, resulted in (*E*)-*N*-(benzylidene)-3-(phenethylthio)-5-*o*-tolyl-4*H*-1,2,4-triazole-4-amines (**7a–h**) (Scheme 1). The compounds **5a**, **5c**, **5d**, **5e**, **5g**, and **5h** have been previously reported in the literature [18].

The structure of synthesized compounds was confirmed by analysis of ^{1}H and ^{13}C NMR spectra; signal for benzylideneamino CH proton of **5a–h**, **8a–i**, and **7a–h** was observed at around 10.17 ppm; triazolethiol SH proton of **5a–h** and **8a–i** was observed at around 14.25 ppm; signals for phenethyl CH_2 proton of **7a–h** were observed at around 3.0 ppm.

Pharmacology

Antioxidant activity

DPPH free radical scavenging activity: Newly synthesized compounds were evaluated for their antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical method as it is one of the most effective methods for evaluating the concentration of radical scavenging materials [19]. The DPPH radical is a stable free radical and its scavenging activity [20]



R3			
5a, 7a, 8a	2,4 - F	**5f, 7f, 8f**	3 - F - 4 - CF_3
5b, 7b, 8b	2,5 - OH	**5g, 7g, 8g**	4 - OCH_3
5c, 7c, 8c	2 - F	**5h, 7h**	4 - $CH(CH_3)_2$
5d, 7d, 8d	3 - F	**8h**	H -
5e, 7e, 8e	5 - Br - 2 - OH	**8i**	4 - OH

Reagents and conditions: (a) MeOH, H_2SO_4, reflux, 8–10 h; (b) hydrazine hydrate, EtOH, reflux, 8 h; (c) CS_2, KOH, EtOH, RT, 5 h; (d) hydrazine hydrate, reflux, 2–6 h; (e) aldehyde, EtOH, H_2SO_4, reflux, 5–6 h; (f) phenethyl bromide, EtOH, KOH, reflux, 4–6 h.

Scheme 1. Synthesis of 4-[(*E*)-benzylideneamino]-5-(2-methylphenyl)-4*H*-1,2,4-triazole-3-thiol, 4-[(*E*)-benzylideneamino]-5-(4-chloro-2-methylphenyl)-4*H*-1,2,4-triazole-3-thiol, and (*E*)-*N*-benzylidene-3-(phenethylthio)-5-*o*-tolyl-4*H*-1,2,4-triazol-4-amine derivatives.

was determined by the decrease in absorbance at 517 nm, due to reduction by the antioxidant or reaction with a radical species, as shown by the equation

$$DPPH^{\bullet} + R^{\bullet} \rightarrow DPPH - R$$

The results of free radical scavenging activity of the newly synthesized compounds are given in Table 1. Compounds **7c**, **7d**, **7h**, **8b**, and **8i** were the strongest free radical scavengers among the compounds tested, with the level of inhibition of 93.9%, 93.6%, 90.0%, 89.2% and 86.8% respectively, followed by **8c**, **8d**, **8g**, and **8h** with the level of inhibition of more than 70%. The structure–activity relationship studies reveal that the free radical scavenging activity of the triazole derivatives is due to the presence of fluoro group on 2nd or 3rd position of the benzylideneamino ring (**7c**, **7d**, **8c**, **8d**); methoxy or isopropyl group on the 4th position of benzylideneamino ring (**7g**, **7h**, **8g**); and hydroxyl group on 4th position of benzylideneamino ring (**8b**, **8i**). Compounds **7c**, **7d**, and **7h** with SH protected with phenethyl group, and fluoro or isopropyl group on benzylideneamino ring have shown the level of inhibition equal to or more than BHT.

In vitro inhibition of human erythrocyte hemolysis

The red blood cells (RBC) are nucleated with intrinsically poor repair mechanisms; this nature makes them good models to test the antioxidant capacity of synthetic and natural compounds in the presence of an oxidative stimulus [21]. Many synthetic and natural compounds have the capacity to prevent the hemolysis of erythrocyte by hydrogen peroxide (H_2O_2) [22]. We examined the ability of compounds **8a**, **8b**, **8d**, **8g**, and **8i** to inhibit the H_2O_2-induced hemolysis of human erythrocytes. The percentage of hemolysis by these compounds was found to be in the range of 4–5%, which was comparable with the control (2.3%). Figure 1 shows the inhibitory effect of different concentrations of these compounds (25–100 μg/mL) on H_2O_2-induced hemolysis of human erythrocytes. These compounds inhibited the hemolysis of human erythrocytes in a dose-dependent manner with 79.35% as maximum inhibition of erythrocyte hemolysis at 100 μg/mL and showed 50% hemolysis inhibition (IC_{50}) at concentrations ranging from 55.7 to 80.7 μg/mL (Table 2). The butylated hydroxytoluene (BHT) exhibited an IC_{50} value of 51 μg/mL, which is comparable to that of these compounds. The structure–activity relationship reveals that anti-hemolysis property of these compounds is due to the presence of 2,5-dihydroxy group on benzylideneamino ring (**8b**). The IC_{50} values of **8a** and **8g** are almost the same. The level of inhibition of **8d** and **8g** by DPPH method and hemolysis inhibition is almost equal in either of the cases.

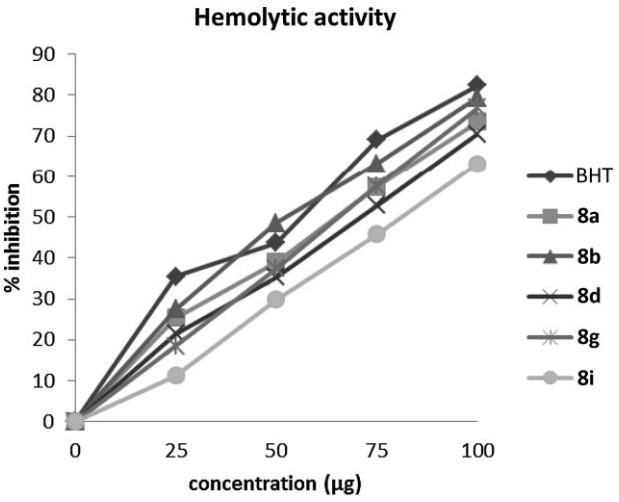


Figure 1. *In vitro* protective effects of 1,2,4-triazole-based Schiff base heterocycles against H_2O_2-induced hemolysis of human erythrocytes.

Table 1. DPPH assay in % of 1,2,4-triazole-based Schiff base heterocycles.

Compounds	Level of inhibition (%)	Compounds	Level of inhibition (%)
5a	16.5	**8a**	60.3
5b	19.8	**8b**	89.2
5f	15.3	**8c**	72.5
7a	30.19	**8d**	74.3
7b	53.53	**8e**	55.4
7c	93.94	**8f**	48.7
7d	93.58	**8g**	74.6
7e	6.46	**8h**	75.8
7f	18.74	**8i**	86.8
7g	65.13	BHT	90.42
7h	90.00		

In vitro lipid peroxidation

Lipid peroxides are the mixture of extremely reactive products of lipid peroxidation, which is a common process in all biological systems and has deleterious effects on the cell membrane and deoxyribonucleic acid (DNA) [23]. Malondialdehyde (MDA), one of the major products of lipid peroxidation has been extensively used as an index for lipid peroxidation and as a marker for oxidative stress. The reaction of MDA with thiobarbituric acid (TBA) has been widely adopted as a sensitive assay method for lipid peroxidation [24]. The results presented in Fig. 2 indicated that lipid peroxidation could be effectively inhibited by **8a**, **8b**, **8d**, **8g**, and **8i**. The IC_{50} value for lipid peroxidation inhibition on erythrocyte ghost membrane was found to be in the range of 53.2–81.2 μg/mL. As we can see from Table 2, compound **8a** showed higher inhibition (77.38%) at the concentration of 100 μg/mL. Compounds **8b**, **8d**, and **8g** showed relatively equal inhibition on lipid

Table 2. *In vitro* protective effects of 1,2,4-triazole-based Schiff base heterocycles against H_2O_2-induced haemolysis of human erythrocyte and lipid peroxidation on erythrocytes ghost membrane.

	Level of inhibition of hemolysis of human erythrocyte (%)					Level of lipid peroxidation inhibition (%)				
Concentration (μg/mL)	25	50	75	100	IC_{50}	25	50	75	100	IC_{50}
BHT	35.43	43.74	69.06	82.37	51	43.65	57.82	69.43	79.37	36.1
8a	25.32	39.02	57.45	73.45	64.3	27.18	51.32	62.17	77.38	55.5
8b	27.52	48.52	63.17	79.35	55.7	24.21	42.81	61.23	76.61	60.8
8d	21.48	35.34	52.79	70.53	70	26.31	39.03	57.38	72.13	64.6
8g	18.43	37.43	57.65	76.84	65.6	31.28	51.32	63.62	75.31	53.2
8i	11.3	29.76	45.83	63.17	80.7	15.14	32.16	47.26	60.36	81.2

peroxidation than **8i**. Increased inhibition of **8a** is due to the presence of 2,4-difluoro on benzylideneamino ring.

Evaluation of oxidative damage on erythrocyte ghost membrane proteins by SDS–PAGE

The change in the protein pattern of membrane proteins produced by the oxidative stress was observed by the SDS–PAGE. The ghost membrane was prepared from the hypotonic lysis of normal human erythrocytes. Membranes were treated with H_2O_2 and with and without the test compounds and analyzed on SDS–PAGE. The membrane protein bands diminished after 1 h of incubation with H_2O_2, and very light new bands of lower molecular weight were observed in the SDS–PAGE (Fig. 3, lane 1). The protein bands of lanes 2–6, which were treated with the test compound, were still clearly evident in the membranes even after 1 h incubation with H_2O_2. The compounds **8a** and **8b** were able to protect better than the other tested compounds. Increased inhibition of **8b** is due to the presence of hydroxy group on 2nd and 5th positions of the benzylideneamino ring.

DNA damage protective activity

The efficiency of newly synthesized compounds in preventing the oxidative damage of DNA induced by H_2O_2 was evaluated. The hydroxyl radical generated by the Fenton's reaction damages the plasmid DNA, resulting in cleavage of one of the phosphodiester chains, and produces a relaxed open circular or relaxed form of DNA. The open circular form of DNA indicates the formation of linear form of DNA, which in turn indicates the double-strand breaking into plasmid DNA [25]. The pUC18 plasmid is mainly in supercoiled form (Fig. 4, lane 7), and when this plasmid is reacted with Fe^{2+} and H_2O_2, the supercoiled form of DNA is converted into an open circular and a linear form (lane 1). The open circular

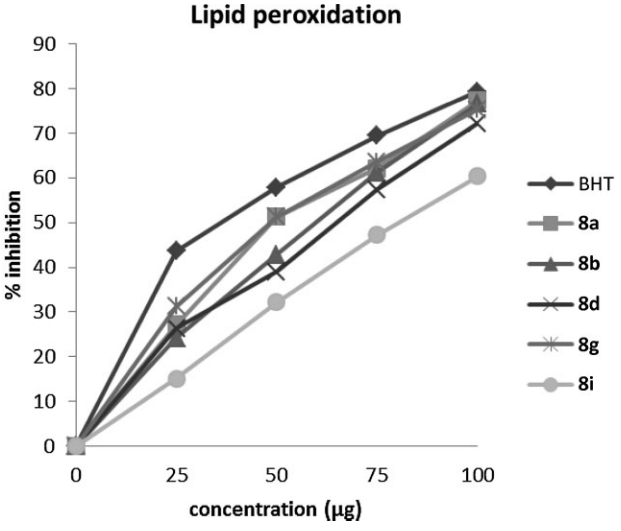


Figure 2. *In vitro* protective effects of 1,2,4-triazole-based Schiff base heterocycles against H_2O_2-induced lipid peroxidation on erythrocyte ghost membrane.

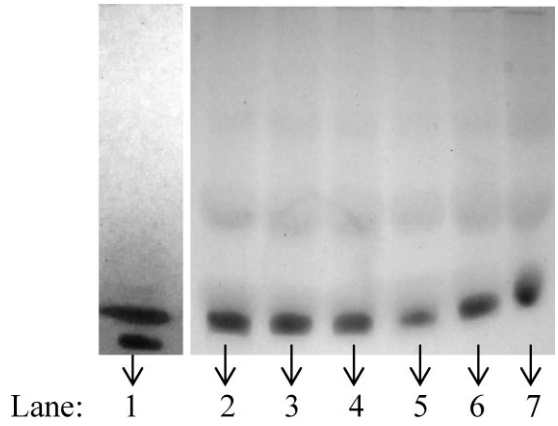


Figure 3. SDS–PAGE of RBC membranes showing protective effects of 1,2,4-triazole-based Schiff base heterocycles against H_2O_2-induced oxidative damage on erythrocyte ghost membrane. Lane 1: membrane proteins treated with H_2O_2; Lane 2: membrane proteins, H_2O_2 and **8a**; Lane 3: membrane proteins, H_2O_2 and **8d**; Lane 4: membrane proteins, H_2O_2 and **8g**; Lane 5: membrane proteins, H_2O_2 and **8i**; Lane 6: membrane proteins, H_2O_2 and **8b**; Lane 7: membrane proteins (untreated).

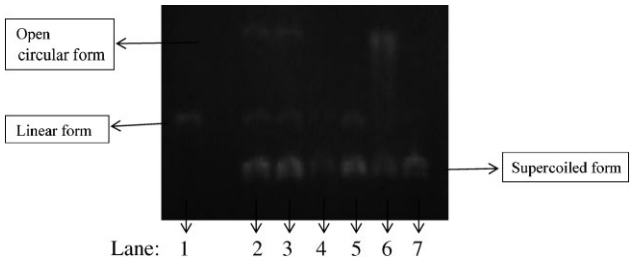


Figure 4. DNA damage protective activity of 1,2,4-triazole-based Schiff base heterocycles. Lane 1: DNA + Fenton's reagent; Lane 2: DNA + **8a** + Fenton's reagent; Lane 3: DNA + **8d** + Fenton's reagent; Lane 4: DNA + **8g** + Fenton's reagent; Lane 5: DNA + **8i** + Fenton's reagent; Lane 6: DNA + **8b** + Fenton's reagent, and Lane 7: DNA.

and linear forms indicate that the $OH^{\cdot}$ free radical is generated from iron-mediated decomposition of H_2O_2, which produces both single-strand and double-strand DNA breaks. No further fragmentation of linear form was observed in the presence of test compounds (lanes 2–6). From the figure, it is evident that compound **8g** has higher protective effect than **8i** and is comparable to the blank. These compounds prevent the reaction of Fe^{2+} ions with H_2O_2 and directly quench $OH^{\cdot}$ free radical by donating hydrogen atom or electron and therefore protecting the supercoiled plasmid DNA from $OH^{\cdot}$ radical-dependent strand breaks. The increased inhibition of **8g** and **8i** is due to the presence of hydroxyl or methoxy group on the *para* position of benzylideneamino ring. The compounds **8a**, **8b**, and **8d** have less ability to scavenge the $OH^{\cdot}$ free radicals, and circular, linear, and supercoiled forms of DNA are observed (lanes 2, 3, and 6).

Conclusion

To summarize, a series of new substituted 4-[(*E*)-benzylideneamino]-5-(2-methylphenyl)-4*H*-1,2,4-triazole-3-thiol (**5a–h**), 4-[(*E*)-benzylideneamino]-5-(5-chloro-2-methylphenyl)-4*H*-1,2,4-triazole-3-thiol (**8a–i**), and (*E*)-*N*-(benzylidene)-3-(phenethylthio)-5-*o*-tolyl-4*H*-1,2,4-triazole-4-amines (**7a–7h**) were synthesized and evaluated for antioxidant property by free radical scavenging method, anti-hemolytic activity, lipid peroxidation, and protective effect against DNA oxidative damage. Some of the substituted (*E*)-*N*-(benzylidene)-3-(phenethylthio)-5-*o*-tolyl-4*H*-1,2,4-triazole-4-amines showed significant DPPH free radical scavenging activity, compounds **7c**, **7d**, and **7h** comparable to the standard (BHT). The compound **8b** showed the protective effect against H_2O_2-induced hemolysis of human erythrocytes, **8g** showed an ability to inhibit lipid peroxidation effectively, and **8g** and **8i** were able to protect against DNA oxidative damage. The results exhibit that some of the synthesized compounds **7c**, **7d**, **7h**, **8b**, **8g**, and **8i** are potential antioxidant agents.

Experimental

Chemistry

^1H and ^{13}C NMR spectra were recorded with a Bruker Avance DPX400 spectrometer operating at 400 MHz, with DMSO-d_6 as solvent and Me_4Si as internal standard. The chemical shifts are expressed as δ values in parts per million (ppm) and the coupling constants (*J*) are given in Hertz (Hz). Purity of the synthesized compounds was analyzed by HPLC or UPLC method. Mass spectra were determined with Agilent LC/MS instrument. Flash column chromatography was performed with silica gel (230–400 mesh; Merck), and TLC was carried out on precoated silica plates (kiesel gel 60 F_{254}, BDH). Melting points were determined in open capillaries on Buchi melting point apparatus and are uncorrected. All reagents involved in the experiments are commercially available and used without further purification. The yields are of purified compounds and are not optimized.

*Synthesis of methyl-2-methyl benzoate (**1**) or 5-chloro-2-methylphenyl benzoate (**1a**)*

To a stirred solution of 2-methyl benzoic acid (73.45 mmol) in dry methanol (100 mL), three to four drops of con. H_2SO_4 were added. The reaction mixture was refluxed for 8–10 h under nitrogen atmosphere. The reaction was monitored by TLC. After the reaction completion, methanol was distilled off and the residue was dissolved in ethyl acetate (200 mL). The organic layer was washed with 10% sodium bicarbonate solution (50 mL) and water (50 mL) followed by saturated sodium chloride solution. The organic layer was treated with anhydrous sodium sulfate, filtered, and concentrated under vacuum to afford title compound. Methyl-2-methyl benzoate, pale yellow oil, % yield: 81.74, MF: $C_9H_{10}O_2$, MW: 150.17; 5-chloro-2-methylphenyl benzoate, % yield: 76.2, MF: $C_9H_9ClO_2$, MW: 183.67.

*Synthesis of 2-methylbenzohydrazide (**2**) or 5-chloro-2-methylbenzohydrazide (**2a**)*

To a stirred solution of 2-methyl benzoate (**1**) or 5-chloro-2-methylphenyl benzoate (**1a**) (59.93 mmol) in absolute ethanol (90 mL) hydrazine hydrate (269.68 mmol) was added. The reaction mixture was refluxed for about 8 h. The reaction was monitored by TLC. After the reaction completion, the solvent was distilled under vacuum, ice water (50 mL) was added, and the mixture was stirred for 15 min. The solid obtained was filtered and dried under vacuum to yield 2-methylbenzohydrazide (**2**) as white solid or 5-chloro-2-methylbenzohydrazide (**2a**) as off-white solid. **2**: % yield: 88.9. m.p.: 152–154.5°C; MF: $C_8H_{10}N_2O$; MW: 150.17; $[m/z]^+$: 151.2.

*Synthesis of 2-(2-methylbenzoyl)hydrazinecarbodithiol acid potassium salt (**3**) or 2-(5-chloro-2-methylbenzoyl)-hydrazinecarbodithiol acid potassium salt (**3a**)*

Potassium hydroxide pellets (106.54 mmol) were dissolved in 40 mL of absolute ethanol. To this solution, 2-methylbenzohydrazide (**2**) or 5-chloro-2-methylbenzohydrazide (**2a**) (53.27 mmol) was added followed by carbon disulfide (117.19 mmol) and the contents were stirred at room temperature for 5 h. The reaction was monitored by TLC. After the reaction completion, diethylether (100 mL) was added and stirred for 10 min. The solid form was filtered and dried under vacuum to obtain 2-(2-methylbenzoyl)hydrazinecarbodithiol acid potassium salt (**3**) as white solid or 2-(5-chloro-2-methylbenzoyl)hydrazinecarbodithiol

acid potassium salt (**3a**) as off-white solid. **3**: % yield: 85.10; m.p.: 167–170°C; MF: $C_9H_9KN_2OS_2$; MW: 264.42; $[m/z]^+$: 265.3.

*Synthesis of 4-amino-5-(2-methyl phenyl)-4H-1,2,4-triazole-3-thiol (**4**) or 4-amino-5-(5-chloro-2-methyl phenyl)-4H-1,2,4-triazole-3-thiol (**4a**)*

Hydrazine hydrate (45.38 mmol) was added to compound **3** or **3a** (45.38 mmol) and the contents were refluxed for 2 h. The reaction was monitored by TLC. After the reaction completion, the reaction mixture was acidified with con. HCl. The precipitate was filtered and dried under vacuum to obtain compound **4** or **4a**. 4-Amino-5-(2-methyl phenyl)-4*H*-1,2,4-triazole-3-thiol (**4**), 8.2 g. White solid, yield: 87.7%, m.p.: 167–170°C; ^{1}H NMR: 2.27 (s, 3H), 5.49 (bs, 2H), 7.18 (t, $J = 7.6$ Hz, 1H), 7.30 (t, $J = 7.2$ Hz, 1H), 7.37 (d, $J = 7.2$ Hz, 2H), 14.25 (s, 1H); MF: $C_9H_{10}N_4S$; MW: 206.3, $[m/z]^+$: 207.4.

*General procedure for the synthesis of benzylideneamine derivatives (**5a**, **5b**, and **5f**; and **8a–i**)*

To a stirred solution of compound **4** or **4a** (4.84 mmol) in ethanol (10 mL), benzaldehyde (1 eq.) and two to three drops of con. H_2SO_4 were added and the contents were refluxed for 5 h. The reaction was monitored by TLC. After the reaction completion, the solvent was removed under vacuum. To the residue, 5 mL of ice water was added, stirred for 5 min, and the precipitated solid was filtered and dried under vacuum. The compounds were purified by column chromatography using ethyl acetate and petroleum ether.

*4-[(E)-(2,4-Difluorobenzylidene)amino]-5-(2-methylphenyl)-4H-1,2,4-triazole-3-thiol (**5a**):* Yellow solid, 76%; m.p.: 119–121.5°C; ^{1}H NMR: 2.27 (s, 3H), 7.18 (t, $J = 7.6$ Hz, 1H), 7.30 (t, $J = 7.2$ Hz, 1H), 7.37 (d, $J = 7.2$ Hz, 1H), 7.45 (q, $J = 8$ Hz, 3H), 7.78 (q, $J = 7.6$ Hz, 1H), 10.17 (s, 1H), 14.27 (s, 1H); ^{13}C NMR: 156.87, 138.28, 131.40, 131.15, 130.91, 129.49, 129.39, 126.16, 113.83, 113.60, 105.57, 105.31, 20.14. Anal. calcd. for $C_{16}H_{12}F_2N_4S$: C, 58.12; H, 3.63; N, 8.47%; found: C, 58.30; H, 3.65; N, 8.49%. MW: 330.35, $[m/z]^+$: 331.6.

*2-[(E)-{[3-(2-Methylphenyl)-5-sulfanyl-4H-1,2,4-triazol-4-yl]imino}methyl]benzene-1,4-diol (**5b**):* Pale yellow solid, 72%; m.p.: 145–147°C; ^{1}H NMR: 2.24 (s, 3H), 6.91–6.97 (m, 1H), 7.34 (t, $J = 7.2$ Hz, 1H), 7.38 (d, $J = 7.6$ Hz, 1H), 7.41–7.46 (m, 2H), 7.51 (dd, $J_1 = 2.4$ Hz, $J_2 = 6.2$ Hz, 1H), 7.61 (d, $J = 2.8$ Hz, 1H), 10.78 (s, 1H), 10.83 (s, 1H), 11.2 (s, 1H), 14.25 (s, 1H). Anal. calcd. for $C_{16}H_{14}N_4O_2S$: C, 58.82; H, 4.28; N, 17.15%; found: C, 58.84; H, 4.30; N, 17.18%. MW: 326.37, $[m/z]^+$: 327.5.

*4-{(E)-[3-Fluoro-4-(trifluoromethyl)benzylidene]amino}-5-(2-methylphenyl)-4H-1,2,4-triazole-3-thiol (**5f**):* Pale yellow solid, 71%; m.p.: 131.5–134°C; ^{1}H NMR: 2.21 (s, 3H), 7.31 (t, $J = 7.6$ Hz, 1H), 7.37 (d, $J = 7.6$ Hz, 1H), 7.43–7.48 (m, 2H), 7.73–7.80 (m, 2H), 7.91 (t, $J = 8$ Hz, 1H), 10.13 (s, 1H), 14.34 (s, 1H); ^{13}C NMR: 162.19, 161.47, 149.80, 138.29, 131.34, 131.25, 130.98, 128.98, 128.93, 126.23, 125.29, 116.79, 116.57, 20.14. Anal. calcd. for $C_{17}H_{12}F_4N_4S$: C, 53.63; H, 3.15; N, 14.72%; found: C, 53.65; H, 3.18; N, 14.73%. MW: 380.36, $[m/z]^+$ H_2O: 399.5.

*5-(4-Chloro-2-methylphenyl)-4-[(E)-(2,4-difluorobenzylidene)amino]-4H-1,2,4-triazole-3-thiol (**8a**):* White solid, 76%; m.p.: 155–158°C; ^{1}H NMR: 2.37 (s, 3H), 7.28 (t, $J_1 = 1.6$ Hz, 1H), 7.55 (t, $J = 1.9$ Hz, 1H), 7.57 (d, $J = 8.4$ Hz, 1H), 7.70 (dd, $J_1 = 1.6$ Hz, $J_2 = 8.0$ Hz, 1H), 7.85 (s, 1H) 8.03 (d, $J = 6.8$ Hz, 1H), 10.07 (s, 1H), 14.3 (s, 1H); ^{13}C NMR: 168.97, 160.45, 151.67, 151.55, 149.44, 137.93, 133.98, 131.42, 129.87, 128.29, 122.39, 120.49, 112.88, 104.54, 103.84, 22.82; IR cm^{-1}, –SH: 2575, –C=N–: 1670, Ar–Me: 2925, C–F: 1200. Anal. calcd. for $C_{16}H_{11}ClF_2N_4S$: C, 52.50; H, 3.0; N, 15.31%; found: C, 52.52; H, 3.03; N, 15.34%. MW: 364.80, $[m/z]^+$: 365.7.

*2-((E)-{[3-(4-Chloro-2-methylphenyl)-5-sulfanyl-4H-1,2,4-triazol-4-yl]imino}methyl)benzene-1,4-diol (**8b**):* Pale brown solid, 73%; m.p.: 140–144°C; ^{1}H NMR: 2.36 (s, 3H), 7.19–7.22 (m, 3H), 7.39 (s, 1H), 7.49 (d, $J = 2.2$ Hz, 1H), 7.62–7.69 (m, 1H), 7.91 (s, 1H), 8.86 (s, 1H), 9.85 (s, 1H), 14.17 (s, 1H); ^{13}C NMR: 159.07, 157.45, 155.97, 153.59, 146.48, 142.93, 141.98, 139.40, 136.07, 135.20, 125.37, 119.49, 116.88, 111.49, 107.54, 21.82; IR cm^{-1}, –SH: 2950, –C=N–: 1690, Ar–Me: 2895, Ar–OH: 3365. Anal. calcd. for $C_{16}H_{13}ClN_4O_2S$: C, 53.21; H, 3.60; N, 15.52%; found: C, 53.24; H, 3.63; N, 15.55%. MW: 360.81, $[m/z]^+$: 361.7.

*5-(4-Chloro-2-methylphenyl)-4-[(E)-(2-fluorobenzylidene)-amino]-4H-1,2,4-triazole-3-thiol (**8c**):* Off-white solid, 81%; m.p.: 158–164°C; ^{1}H NMR: 2.37 (s, 3H), 7.36–7.44 (m, 2H), 7.58 (d, $J = 8.4$ Hz, 1H), 7.67–7.72 (m, 2H), 7.86 (d, $J = 1.6$ Hz, 1H), 7.95–7.99 (m, 1H), 10.12 (s, 1H), 14.32 (s, 1H); ^{13}C NMR: 169.97, 168.45, 161.67, 159.59, 149.48, 137.93, 133.98, 130.40, 129.87, 128.29, 126.39, 120.49, 119.88, 104.54, 103.84, 22.52; IR cm^{-1}, –SH: 2572, –C=N–: 1665, Ar–Me: 2920; C–F: 1250. Anal. calcd. for $C_{16}H_{12}ClFN_4S$: C, 55.18; H, 3.44; N, 16.09%; found: C, 55.21; H, 3.41; N, 16.11%. MW: 346.81, $[m/z]^+$: 347.9.

*5-(4-Chloro-2-methylphenyl)-4-[(E)-(3-fluorobenzylidene)-amino]-4H-1,2,4-triazole-3-thiol (**8d**):* Yellow solid, 75%; m.p.: 147–152°C; ^{1}H NMR: 2.36 (s, 3H), 7.46–7.50 (m, 1H), 7.57–7.64 (m, 2H), 7.68–7.73 (m, 3H), 7.85 (s, 1H), 9.80 (s, 1H), 14.31 (s, 1H); ^{13}C NMR: 163.67, 162.45, 160.67, 159.59, 146.48, 137.93, 135.98, 130.40, 129.87, 127.29, 126.39, 120.49, 116.88, 104.54, 103.84, 22.12; IR cm^{-1}, –SH: 2542, –C=N–: 1675, Ar–Me: 2930, C–F: 1300. Anal. calcd. for $C_{16}H_{12}ClFN_4S$: C, 55.18; H, 3.44; N, 16.09%; found: C, 55.20; H, 3.42; N, 16.10%. MW: 346.81, $[m/z]^+$: 347.8.

*4-Bromo-2-[(E)-{[3-(4-chloro-2-methylphenyl)-5-sulfanyl-4H-1,2,4-triazol-4-yl]imino}methyl]phenol (**8e**):* Pale yellow solid, 80%; m.p.: 139–143°C; ^{1}H NMR: 2.37 (s, 3H), 6.93 (t, $J = 5.2$ Hz, 1H), 7.51–7.61 (m, 3H), 7.87–7.90 (m, 2H), 10.05 (s, 1H), 10.90 (s, 1H), 14.22 (s, 1H); ^{13}C NMR: 167.07, 165.45, 164.97, 157.59, 156.48, 142.93, 141.98, 139.40, 136.07, 127.29, 125.37, 119.49, 113.88, 111.44, 107.54, 22.42; IR cm^{-1}, –SH: 2740, –C=N–: 1595, Ar–Me: 2893, Ar–OH:3350; C–Br: 580. Anal. calcd. for $C_{16}H_{12}BrClN_4OS$: C, 45.32; H, 2.83; N, 13.21%; found: C, 45.35; H, 2.86; N, 13.25%. MW: 423.71, $[m/z]^+$: 424.65.

*5-(4-Chloro-2-methylphenyl)-4-{(E)-[2-fluoro-4-(trifluoromethyl)benzylidene]amino}-4H-1,2,4-triazole-3-thiol (**8f**):* Pale yellow solid, 77%; m.p.: 120–123°C; ^{1}H NMR: 2.37 (s, 3H), 7.56–7.60 (m, 1H), 7.70 (dd, $J_1 = 1.7$ Hz, $J_2 = 8.2$ Hz, 1H), 7.78

(d, $J = 1.6$ Hz, 1H), 7.83–8.06 (m, 3H), 10.06 (s, 1H), 14.37 (s, 1H); ^{13}C NMR: 160.67, 155.45, 149.67, 137.59, 135.48, 132.93, 131.98, 128.40, 128.07, 127.29, 126.39, 120.49, 112.88, 104.54, 103.84, 102.11, 21.42; IR cm^{-1}, –SH: 2540; –C=N–: 1575; Ar–Me: 2830; C–F: 1270. Anal. calcd. for $C_{17}H_{11}ClF_4N_4S$: C, 49.07; H, 2.64; N, 13.47%; found: C, 49.10; H, 2.67; N, 13.50%. MW: 414.80, $[m/z]^+$: 415.7.

*5-(4-Chloro-2-methylphenyl)-4-[(E)-(4-methoxybenzylidene)amino]-4H-1,2,4-triazole-3-thiol (**8g**):* Pale brown solid, 76%; m.p.: 159–163°C; ^{1}H NMR: 2.37 (s, 3H), 3.81 (s, 3H), 7.03–7.12 (m, 3H), 7.56 (d, $J = 8.4$ Hz, 1H), 7.68 (d, $J = 0.8$ Hz, 1H), 7.78–7.86 (m, 2H), 9.5 (s, 1H), 14.22 (s, 1H); ^{13}C NMR: 168.57, 165.45, 164.67, 157.59, 156.48, 142.93, 141.98, 137.40, 129.07, 127.29, 125.39, 119.49, 112.88, 110.44, 107.54, 55.84, 22.42; IR cm^{-1}, –SH: 2640, –C=N–: 1565, Ar–Me: 2813, Ar–MeO: 1150. Anal. calcd. for $C_{17}H_{15}ClN_4OS$: C, 56.72; H, 4.17; N, 15.57%; found: C, 56.75; H, 4.20; N, 15.60%. MW: 358.84, $[m/z]^+$: 359.6.

*4-[(E)-Benzylideneamino]-5-(4-chloro-2-methylphenyl)-4H-1,2,4-triazole-3-thiol (**8h**):* Off-white solid, 70%; m.p.: 125–129°C; ^{1}H NMR: 2.36 (s, 3H), 7.57 (dd, $J_1 = 1.6$ Hz, $J_2 = 8.2$ Hz, 2H), 7.61–7.63 (m, 1H), 7.70 (dd, $J_1 = 2$ Hz, $J_2 = 8.4$ Hz, 2H), 7.87 (d, $J = 0.8$ Hz, 2H), 7.89 (s, 1H), 9.71 (s, 1H), 14.28 (s, 1H); ^{13}C NMR: 167.57, 155.45, 149.67, 137.59, 135.48, 133.93, 131.98, 128.40, 129.07, 127.29, 126.39, 119.49, 112.88, 107.54, 102.84, 22.42; IR cm^{-1}, –SH: 2640, –C=N–: 1565, Ar–Me: 2813. Anal. calcd. for $C_{16}H_{13}ClN_4S$: C, 58.23; H, 3.94; N, 6.98%; found: C, 58.25; H, 3.97; N, 6.70%. MW: 328.82, $[m/z]^+$: 329.7.

*4-[(E)-{[3-(4-Chloro-2-methylphenyl)-5-sulfanyl-4H-1,2,4-triazol-4-yl]imino}methyl]phenol (**8i**):* Yellow solid, 81%; m.p.: 135–139°C; ^{1}H NMR: 2.35 (s, 3H), 6.83–6.93 (m, 3H), 7.54 (d, $J = 4.8$ Hz, 1H), 7.66–7.75 (m, 3H), 7.86 (s, 1H), 9.38 (s, 1H), 14.19 (s, 1H); ^{13}C NMR: 157.07, 155.45, 154.97, 147.59, 146.48, 142.93, 140.98, 139.40, 136.07, 127.20, 125.37, 119.49, 116.88, 111.49, 107.54, 21.72; IR cm^{-1}, –SH: 2940; –C=N–: 1695; Ar–Me: 2890; Ar–OH: 3370. Anal. calcd. for $C_{16}H_{13}ClN_4OS$: C, 55.68; H, 3.77; N, 16.24%; found: C, 55.70; H, 3.80; N, 16.25%. MW: 344.81, $[m/z]^+$: 345.75.

*General procedure for the synthesis of (E)-N-(2-benzylidene)-3-(phenethylthio)-5-o-tolyl-4H-1,2,4-triazole-4-amine derivatives (**7a–7h**)*

To a stirred solution of **5** (1 eq.) in absolute ethanol (10 mL), potassium hydroxide (2.5 eq.) was added followed by phenethyl-bromide (1 eq.). The reaction mixture was refluxed for 4 h and the reaction was monitored by TLC. After the completion of reaction, solvent was distilled off and the product from the residue was extracted using ethyl acetate (25 mL). The organic layer was washed with water and saturated sodium chloride solution, dried with anhydrous sodium sulfate, and concentrated under vacuum to afford title compounds. These compounds were purified by column chromatography using ethyl acetate and petroleum ether.

*(E)-N-(2,4-Difluorobenzylidene)-3-(phenethylthio)-5-o-tolyl-4H-1,2,4-triazole-4-amine (**7a**):* Pale yellow solid, 57%; m.p.: 209–211°C; ^{1}H NMR: 2.39 (s, 3H), 3.07 (t, $J = 7.2$ Hz, 2H), 3.44 (t, $J = 7.3$ Hz, 2H), 6.90–7.81 (m, 11H), 8.02 (s, 1H), 8.49 (s, 1H). Anal. calcd. for $C_{24}H_{20}F_2N_4S$: C, 66.28; H, 4.60; N, 12.88%; found: C, 66.32; H, 4.65; N, 12.92%. MW: 434.50, $[m/z]^+$: 435.0.

*(E)-2-((3-(Phenethylthio)-5-o-tolyl-4H-1,2,4-triazol-4-ylimino)methyl)benzene-1,4-diol (**7b**):* Pale yellow solid, 64%; m.p.: 215–217°C; ^{1}H NMR: 2.40 (s, 3H), 3.1 (t, $J = 7.4$ Hz, 2H), 3.28 (t, $J = 7.2$ Hz, 2H), 7.10–7.93 (m, 11H), 8.12 (s, 1H), 8.5 (s, 1H), 10.26 (s, 1H), 10.29 (s, 1H). Anal. calcd. for $C_{24}H_{22}N_4O_2S$: C, 66.89; H, 5.11; N, 13.00%; found: C, 66.93; H, 5.15; N, 13.04%. MW: 430.52, $[m/z]^+$: 431.8.

*(E)-N-(2-Fluorobenzylidene)-3-(phenethylthio)-5-o-tolyl-4H-1,2,4-triazole-4-amine (**7c**):* Off-white solid, 61%; m.p.: 198–201°C; ^{1}H NMR: 2.38 (s, 3H), 3.08 (t, $J = 7.3$ Hz, 2H), 3.31 (t, $J = 7.3$ Hz, 2H), 7.06–7.84 (m, 12H), 8.12 (s, 1H), 8.42 (s, 1H). Anal. calcd. for $C_{24}H_{21}FN_4S$: C, 69.14; H, 5.04; N, 13.44%; found: C, 69.18; H, 5.08; N, 13.47%. MW: 416.51, $[m/z]^+$: 417.3.

*(E)-N-(3-Fluorobenzylidene)-3-(phenethylthio)-5-o-tolyl-4H-1,2,4-triazole-4-amine (**7d**):* Off-white solid, 67%; m.p.: 171–173.5°C; ^{1}H NMR: 2.41 (s, 3H), 3.12 (t, $J = 7.2$ Hz, 2H), 3.34 (t, $J = 7.4$ Hz, 2H), 7.07–7.92 (m, 12H), 8.09 (s, 1H), 8.39 (s, 1H); ^{13}C NMR: 164.00, 163.65, 161.22, 161.15, 131.92, 131.83, 131.67, 131.57, 131.49, 130.87, 130.89, 130.79, 130.73, 129.29, 129.21, 129.02, 128.84, 128.54, 127.69, 118.46, 114.57, 36.21, 28.34, 19.8. Anal. calcd. for $C_{24}H_{21}FN_4S$: C, 69.14; H, 5.04; N, 13.44%; found: C, 69.19; H, 5.08; N, 13.48%. MW: 416.51, $[m/z]^+$: 417.1.

*(E)-2-((3-(Benzylthio)-5-o-tolyl-4H-1,2,4-triazol-4-ylimino) methyl)-4-bromo-phenol (**7e**):* Pale yellow solid, 58%; m.p.: 129–133°C; ^{1}H NMR: 2.27 (s, 3H), 3.01 (t, $J = 7.3$ Hz, 2H), 4.84 (t, $J = 7.3$ Hz, 2H), 6.70–7.94 (m, 11H), 7.98 (s, 1H), 8.32 (s, 1H), 10.26 (s, 1H); ^{13}C NMR: 162.55, 156.01, 137.71, 137.25, 130.91, 130.24, 129.29, 129.18, 128.78, 128.72, 128.67, 128.53, 127.99, 127.75, 127.51, 126.42, 126.34, 126.11, 122.34, 119.68, 114.45, 37.01, 19.88. Anal. calcd. for $C_{23}H_{19}BrN_4OS$: C, 57.57; H, 3.96; N, 11.68%; found: C, 57.60; H, 3.99; N, 11.70%. MW: 479.39, $[m/z]^+$: 480.9.

*(E)-N-(3-Fluoro-4-(trifluoromethyl)benzylidene)-3-(phenethylthio)-5-o-tolyl-4H-1,2,4triazole-4-amine (**7f**):* Off-white solid, 65%; m.p.: 204–206°C; ^{1}H NMR: 2.41 (s, 3H), 3.02 (t, $J = 7.4$ Hz, 2H), 3.33 (t, $J = 7.4$ Hz, 2H), 6.94–7.84 (m, 11H), 7.96 (s, 1H), 8.39 (s, 1H). Anal. calcd. for $C_{25}H_{20}F_4N_4S$: C, 61.91; H, 4.12; N, 11.55%; found: C, 61.95; H, 4.15; N, 11.59%. MW: 484.51, $[m/z]^+$: 485.8.

*(E)-N-(4-Methoxybenzylidene)-3-(phenethylthio)-5-o-tolyl-4H-1,2,4-triazol-4-amine (**7g**):* Pale yellow solid, 61%; m.p.: 199–201°C; ^{1}H NMR: 2.39 (s, 3H), 3.09 (t, $J = 7.0$ Hz, 2H), 3.41 (t, $J = 7.1$ Hz, 2H), 3.96 (s, 3H), 7.01–7.67 (m, 12H), 8.02 (s, 1H), 8.46 (s, 1H); ^{13}C NMR: 160.79, 155.34, 145.45, 140.53, 139.86, 132.23, 132.09, 132.01, 131.12, 131.02, 129.54, 128.43, 127.78, 126.34, 125.65, 124.54, 123.87, 122.65, 120.98, 115.98, 114.87, 55.26, 36.21, 28.34, 19.89. Anal. calcd. for $C_{25}H_{24}N_4OS$: C, 70.0; H, 5.60; N, 13.06%; found: C, 70.05; H, 5.64; N, 13.10%. MW: 428.55, $[m/z]^+$: 429.6.

*(E)-N-(4-Isopropoxybenzylidene)-3-(phenethylthio)-5-o-tolyl-4H-1,2,4-triazol-4-amine (**7h**):* Off-white solid, 67%; m.p.: 162–166°C; ^{1}H NMR: 1.1 (d, $J = 2.4$ Hz, 6H), 2.38 (s, 3H), 3.07 (t, $J = 4.6$ Hz, 2H), 3.36 (q, $J = 2$ Hz, 2H), 4.1 (m, 1H), 7.21–7.91 (m, 12H), 8.09 (s, 1H), 8.41 (s, 1H). Anal. calcd. for $C_{27}H_{28}N_4S$: C, 73.53; H, 6.35; N, 12.70%; found: C, 73.57; H, 6.39; N, 12.75%. MW: 440.60, $[m/z]^+$: 441.6.

Pharmacology

Antioxidant activity

DPPH free radical scavenging activity: Free radical-scavenging capacities of test compounds were determined according to the previously reported procedure [26], using the stable DPPH radical. This is the most commonly used method for screening of antioxidant activity of newly synthesized organic compounds. This method is based on the reduction of free radical DPPH by free radical scavengers. The procedure involves the measurement of decrease in absorbance of DPPH [27] at 517 nm, which is proportional to the activity of free radical scavenger added to DPPH reagent solution. A stock solution of test compounds (1 mg/mL) and DPPH (0.004%) was prepared in 95:5 methanol/water. To 3 mL of freshly prepared DPPH solution in a test tube, stock solution of test compound (100 μL) was added and reacted for 15 min, and the absorbance was measured at 517 nm using UV–Visible spectrophotometer (Shimadzu UV-1800, Japan). BHT was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (1 mg/mL). Ascorbic acid was used as control sample, and 95% methanol served as blank. Free radical inhibition in % (I%) was calculated as

$$I\% = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test sample.

Hemolytic activity

RBC suspension preparation: Blood was collected through vein puncture from fully informed and consenting healthy volunteers, into tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. Samples were centrifuged at 2000 rpm, for 5 min at 4°C. Plasma and buffy coat was then carefully removed and discarded; RBCs were washed three times with saline phosphate buffer (PBS: 125 mM NaCl and 10 mM sodium phosphate, pH 7.4), and finally re-suspended in PBS to produce an RBC suspension at 2% v/v hematocrit.

In vitro inhibition of human erythrocyte hemolysis: The inhibition of human erythrocyte hemolysis by newly synthesized compounds was evaluated according to the procedure described by Tedesco et al. [22] with due modifications. The human erythrocyte hemolysis was performed with H_2O_2 as a free radical initiator. To 100 μL of 2% v/v suspension of erythrocytes in PBS, 100 μL of test compound with different concentrations (25, 50, 75, and 100 μg/mL in PBS, pH 7.4) was added followed by 100 μM H_2O_2 (in PBS, pH 7.4). The reaction mixture was shaken gently while being incubated at 37°C for 3 h. Then, it was diluted with 8 mL of PBS and centrifuged at 3000 rpm for 10 min. The absorbance of the resulting supernatant liquid was measured at 540 nm by spectrophotometer to determine the hemolysis. Likewise, the erythrocytes were treated with 100 μM H_2O_2 without inhibitors (test compounds) to obtain a complete hemolysis. The absorbance of the supernatant was measured under the same condition. The inhibitory effect of these compounds was compared with that of standard antioxidant BHT. To evaluate the hemolysis induced by the test compounds, erythrocytes were pre-incubated with 50 μL of test compound for 1 h and the hemolysis was determined. The percentage of hemolysis was calculated by taking hemolysis caused by 100 μM H_2O_2 as 100%. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% hemolysis.

In vitro assay for the inhibition of lipid peroxidation on erythrocyte ghost membrane

The erythrocyte ghost membranes were prepared following the procedure of Fairbanks et al. [28] by hypotonic lysis of erythrocytes in 5 mM phosphate buffer (pH 8.0) with the addition of 1 mM EDTA to the lysis buffer. Protein content in the membrane was determined according to the method described by Lowry et al. [29] using bovine serum albumin as standard.

The erythrocyte ghost membrane lipid peroxidation was performed according to the method of Stocks and Dormandy [30] with slight modifications. The test compound (25–100 μg concentration) was added to 1 mL of erythrocyte ghost membrane suspension (200 μg protein) followed by 100 μL of 100 μM H_2O_2. After incubation for 1 h at 37°C, the reaction was stopped by adding 2 mL of 0.25 M HCl containing 15% trichloroacetic acid and 0.375% TBA. The reaction mixture was boiled for 15 min, cooled, and centrifuged, and the absorbance of the supernatant was measured at 532 nm. The appropriate blanks and controls were run along with the test samples. The percentage of inhibition was calculated and plotted against the concentration of the samples. The IC_{50} values were calculated from the plots as the antioxidant concentration required for the inhibition of 50% lipid peroxidation.

Evaluation of oxidative damage on erythrocyte ghost membrane proteins by SDS–PAGE

The oxidative modification on erythrocyte ghost membrane proteins was determined by SDS–PAGE according to the method described by Carini et al. [31] and Ajila et al. [32] with slight modifications. The oxidation of membrane protein was induced by addition of 100 μL of 200 μM H_2O_2 to 1 mL of solution containing 200 μg of protein and with or without test compound (100 μg of AAE) and incubated for 1 h. Appropriate controls were run along with the test samples, SDS–PAGE was performed on 10% discontinuous gel according to the method of Laemmli [33]. The protein bands were visualized by staining with Coomassie brilliant blue.

DNA damage protective activity

Induction of DNA scission by Fenton's reagent was measured on pUC18 DNA according to the procedure described by Lee et al. [34] with slight modifications. A mixture of 10 μL of synthesized compound (concentration 1 μg/mL) and 0.5 μg of DNA was incubated for 10 min at 37°C followed by addition of 10 μL of Fenton's reagent (30 mM of H_2O_2, 50 μM of ascorbic acid, and 80 μM of $FeCl_3$). The final volume of mixture was made up to 20 μL and the mixture was incubated for 30 min at 37°C. The DNA

was analyzed on 1% agarose gel followed by ethidium bromide staining.

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