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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Ultrasound promoted green synthesis of spiro[pyrano[2,3-*c*]pyrazoles] as antioxidant agents

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Abstract Ultrasound promoted, cerium ammonium nitrate catalyzed sustainable synthesis of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives (**4a**–**l**) is reported herein. The synthesized compounds were screened for their antioxidant activities as free radical scavenging effect on diphenylpicryl hydrazine ($DPPH^{\bullet}$), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) and nitric oxide (NO) radicals. The screened compounds showed potent scavenging activities against $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and NO radicals. In order to further extend on studies and to obtain a deep insight into structure activity relationship of this class of compounds, we designed *N*-substitution of indole moiety with the aim to study its antioxidant potential.

Keywords Indole-2,3-dione · 3-Methyl-1-phenyl-2-pyrazolin-5-one · Active methylene group · Antioxidant activity

Introduction

Macromolecules like proteins, lipids and DNA are major targets for free radical-induced damage. The oxidative damage to proteins has been found to be high in specific brain regions, and are elevated during ageing and in some types of neurodegenerative disorders (Foster *et al*., 1996; Floor and Wetzel, 1998). In particular, the damage to DNA is more erratic, and assail by free radicals can generate structural damage (i.e. strand breaks) and/or alteration of the bases. Unrepaired DNA lesions might impair transcription and protein synthesis (Hatahet *et al.*, 1994).

Thus, an increasing interest in antioxidants, such as free radicals and reactive oxygen species (ROS), has engrossed substantial interest. The free radicals are also believed to be associated with carcinogenesis, mutagenesis, arthritis, diabetes, inflammation, cancer and genotoxicity (Kourounakis *et al*., 1999; Buyukokuroglu *et al*., 2001) due to oxidative stress, which arises as a result of imbalance between free radical generations.

Moreover, ROS are continuously generated in very low amounts in active cells of aerobic organisms as byproducts of metabolic processes, and they have found to be the key players in the pathophysiological mechanisms associated with various inflammatory disorders (Trouba *et al.*, 2002). So the significance of ROS in the pathogenesis of multifarious diseases has attracted considerable attention. Disease progression may be retarded by administrating protective compounds, which can act in several different ways as inhibitors of ROS formation, free radical scavenging, chain breaking antioxidants or transition metal chelators, and therefore research on active antioxidant of natural or synthetic origin received a great attention (Delles *et al.*, 2008).

Moreover, the development of hybrid molecules having different pharmacophores in one frame may lead to compounds with interesting pharmacological profiles. In this regards, substituted pyrano[2,3-*c*]pyrazoles are much sought after class of heterocycles exhibiting wide range of biological activities like antimicrobial (Mityurina *et al*., 1981; Lakshmi *et al.*, 2010), anticancer (Wang *et al.*, 2009), anti-inflammatory (Zaki *et al.*, 2006), inhibitors of human Chk1 kinase (Foloppe *et al*. 2006) and also as biodegradable agrochemicals (Abdelrazek *et al*., 2007). Besides, indole derivatives constitute an important class of therapeutic agents in medicinal

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chemistry, including anticancer (Suzen and Buyukbingol, 2000; Lakshmi *et al.*, 2010), antioxidant (Lakshmi *et al.*, 2010), antirheumatoidal (Buyukbingol *et al.*, 1994), anti- HIV (Suzen and Buyukbingol, 1998), and also play a vital role in the immune system (Lieberman *et al.*, 1997; Page *et al.*, 2007) and as potent scavengers of free radicals (Chyan *et al.*, 1999).

Literature survey reveals that synthesis of pyran system has been accomplished by many workers (Saundane *et al.*, 2013; Mandha *et al.*, 2012) employing various catalysts as ammonium chloride (Dabiri *et al.*, 2009), ethylenediamine diacetate (Lee and Hari, 2010), triethylbenzyl ammonium (TEBA) salt (Zhu *et al.*, 2007), L-proline (Yuling *et al.*, 2010), surfactant metal carboxylates (Wang *et al.*, 2010), β-cyclodextrin (Sridhar *et al.*, 2009), ionic liquids (Moghadam and Miri, 2011) and reaction conditions (Shanthi *et al.*, 2007; Elinson *et al.*, 2008). We have also reported the synthesis of spirooxindole derivatives by a multi-step process catalyzed by Et_3N in ethanol under refluxing condition Dandia *et al.*, (2003a, b; Joshi *et al.*, 1989). Although most of the recent methods have their own merits, but some methods are weakened by at least one limitation such as low yield, (especially when bulky substituent on substrates lead to low solubility in water) complicated workup procedure, chromatographic separation and technical intricacy.

In the above regards and on the basis of pharmacological indications that show the existence of two or more different heterocyclic moieties in a single molecule often remarkably enhances the biocidal profile, we intended the synthesis of a series of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives (**4a–l**) through a three component reaction of substituted isatin, active methylene reagent, and 3-methyl-1-phenyl-2-pyrazolin-5-one in the presence of catalytic amount of cerium ammonium nitrate as a green and efficient catalyst in water, thus utilizing the multipurpose promoter efficacy of CAN (Sridharan and Menendez, 2010).

Results and discussion

Chemistry

We approached the synthesis of a library of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives (**4a–l**), bearing different substitutions and their evaluated for their antioxidant potential.

Initially, we tested the reaction of isatin, malanonitrile and 3-methyl-1-phenyl-2-pyrazolin-5-one as a simple model substrate under various reaction conditions (Scheme 1) for synthesis of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivative (**4a**). The results are shown in Table 1. With reference to the crucial utility of solvent in chemical transformation, a variety of solvents were employed for the synthesis of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives. As can be seen, that among all the solvents, water seems to be the solvent of choice both in terms of time and yields of the spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives (**4a–l**).

It was observed that when the reaction was carried out at room temperature stirring without any catalyst, the yield of product was very low (Table 1, 35 %, entry 1) even after prolong time. Increasing the temperature does not seem to affect the yields of the product. Afterwards, evaluation of various catalysts was carried out for the synthesis of spirooxindole derivatives in aqueous medium under ultrasonic irradiation (Table 1, entry 4–9). It can be seen that a mixture of isatin, ethylcyanoacetate and 3-methyl-1-phenyl-2-pyrazolin-5-one in the presence of catalytic amount of cerium ammonium nitrate afforded ethyl 6′-amino-3′-methyl-2-oxo-1′-phenyl-1′*H*-spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole]-5′-carboxylate (**4a**) in excellent yields (90 %), while with other catalysts, the product formed with yields ranging between 54 and 76 %. Although, ultrasound irradiation decreased the reaction time, but failed to increase the yield of the final product (Table 1, entry 3).

Further, the catalyst loading was optimized by using different concentration of CAN in the model reaction. It was found that with increasing the amount of CAN from 5 mol% to 10 and 20 mol%, the yields increased from 74 % to 90 and 92 %, respectively (Table 1, entries 10–12). Further increase in amount of catalyst does not seem to affect the overall yields of the product. 10 mol% CAN in water under ultrasonic irradiation, was found to the best combination for synthesis of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives (**4a–l**) in excellent yields.

Under the optimized reaction conditions, a series of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives

Scheme 1 Model reaction

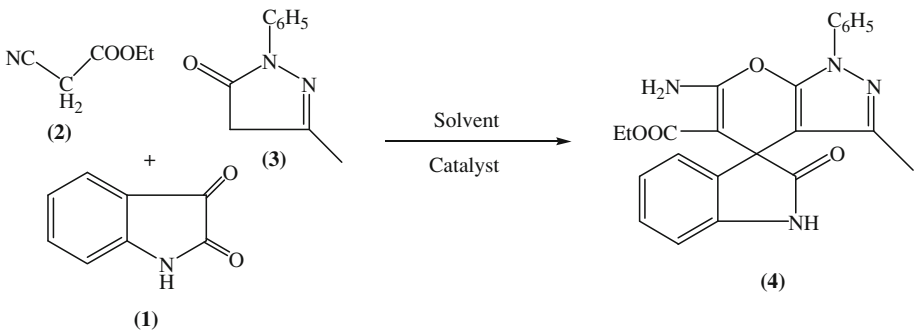


Table 1 Optimization of reaction conditions

Entry	Catalyst	Method	Time (min)	Yield (%)
1.	–	Stirr., r.t.	480	35
2.	–	Stirr., 80 °C	300	45
3.	–	))))	70	50
4.	K_2CO_3	))))	55	58
5.	$NaHCO_3$	))))	52	61
6.	TBAB	))))	60	63
7.	CTAC	))))	68	54
8.	P-TSA	))))	50	68
9.	L-Proline	))))	40	76
10.	CAN (5 mol%)	))))	15	74
11.	CAN (10 mol%)	))))	10	90
12.	CAN (20 mol%)	))))	14	92

Scheme 2 Synthesis of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole derivatives (**4a–l**)

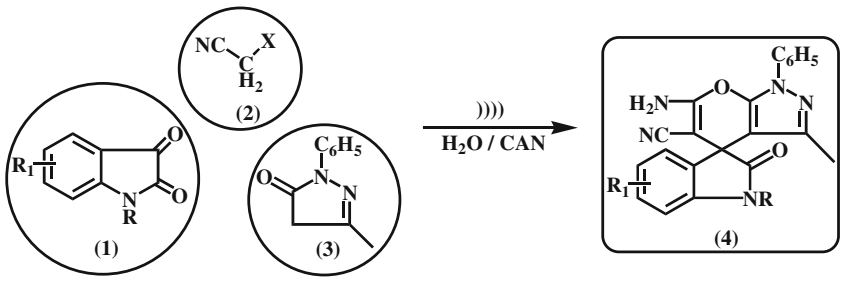
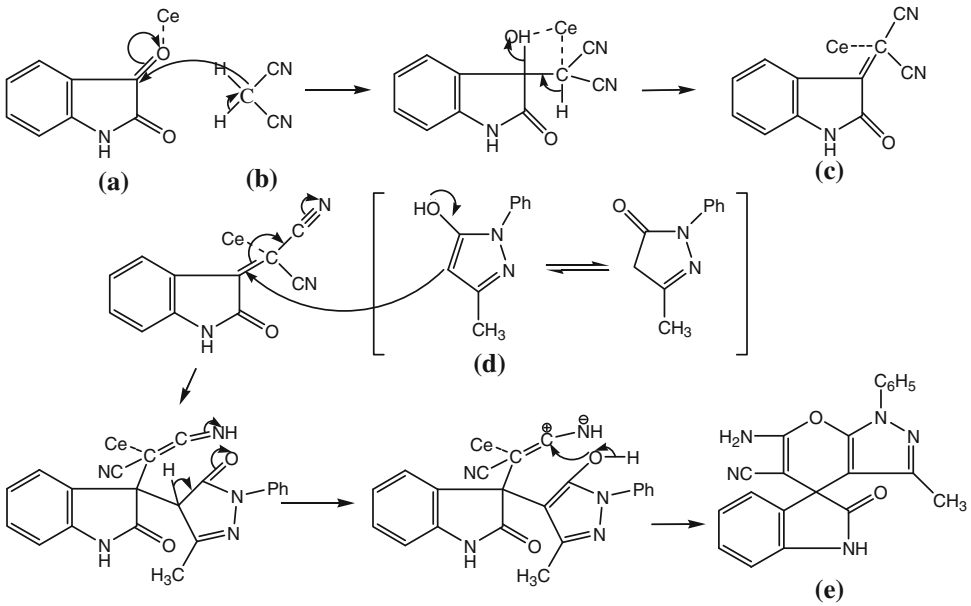


Table 2 Synthesis of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives (**4a–l**)

Entry	R	R_1	X	Time (min)	Yield (%)	Mp (°C)
4a	H	H	COOEt	20	94	238–240
4b	H	5-Cl	COOEt	18	92	246–248
4c	H	5,7-diCH_3	COOEt	18	91	260–262
4d	CH_2–CH=CH_2	H	COOEt	20	84	182–184
4e	C_2H_5	H	COOEt	15	92	208–210
4f	CH_2–C_6H_5	H	COOEt	10	97	244–246
4g	H	H	CN	10	95	237–238
4h	H	5-Br	CN	12	92	242–244
4i	H	5-CH_3	CN	10	98	288–290
4j	CH_2–CH=CH_2	H	CN	18	90	218–220
4k	C_2H_5	H	CN	10	84	210–212
4l	CH_2–C_6H_5	H	CN	8	97	232–234

(**4a–l**) were synthesized (Scheme 2). The results are summarized in Table 2.

The multicomponent synthesis of spiro[indoline3,4′-pyrano[2,3-*c*]pyrazole] derivatives can be explained by a plausible mechanism presented in Scheme 3. The ultrasonic cavitation induced shear forces and the jets produced near the surface of the vessel, and the catalyst may activate malanonitrile through sonolysis of the C–H bond. The reaction between the activated malanonitrile and the isatin (activated by CAN) facilitates the formations of the corresponding isatiylidene malanonitrile intermediate (**c**) under sonic condition, which in turn reacts with the active methylene site of 3-methyl-1-phenyl-2-pyrazolin-5-one. This C-4 alkylation of 3-methyl-1-phenyl-2-pyrazolin-5-one with electrophilic C=C of intermediate (**c**) followed by nucleophilic addition of the −OH group on the cyano moiety subsequently results in formation of the desired product (**e**).



Scheme 3 A probable mechanism of formation of spiro[indoline3,4′-pyrano [2,3-*c*]pyrazole derivatives

Antioxidant activity

The antioxidant activities of spiroindolinones were determined as an index of pharmacological efficacy. Three model systems were used namely DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and NO scavenging activity. In the assessment of antioxidant activity, only synthetic relevant free radicals were used. The synthetic nitrogen-centred DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and NO radicals were used as indicator compounds in testing hydrogen transfer capacity that are related to the antioxidant activity. Antioxidant activities data were compared with standard drug ascorbic acid. In results, we have found correlation between substitution in indole ring and substitution of pyran ring at 5th position. Overall, all compounds exhibited good DPPH$^{\bullet}$ and NO scavenging activity whereas, moderate ABTS$^{\bullet+}$ scavenging activity. The antioxidant properties were expressed as EC_{50} values.

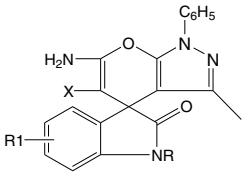
DPPH radical scavenging activity

Although the DPPH radical scavenging abilities of all the spiroindoline derivatives were moderately lower than those of ascorbic acid (409.75 ± 0.288) μg/ml, it was evident that they show reducing ability (possibly by hydrogen transfer) and could serve as free radical scavengers. It was anticipated that compounds possessing carboxylate group (an electron donating group) at 5th position of pyran ring showed higher activity except compound **4b** and **4c**. The combination of carboxylate group with *N*-benzyl substitution in indole ring (i.e. compound **4f**) showed good activity. This revealed to be important for activity, since the *N*-benzyl substituted indole ring with carbonitrile group (i.e. compound **4l**) showed decreased potency. The compound **4a**, i.e. without any substitution in indole ring and carboxylate group at 5th position of pyran ring showed good activity, while compound **4g** without any substitution in indole ring and carbonitrile group showed least activity. In compound **4d**, with *N*-allyl substitution and carboxylate group showed moderate activity, while same indole ring with carbonitrile group, i.e. compound **4j**, showed least activity. In compound **4i**, incorporation of CH_3 group at 5th position of isatin showed electron donating effect, but carbonitrile group with electron withdrawing effect turned it to give moderate activity, while in compound **4c** two electron donating groups are present at 5th and 7th position of isatin with carboxylate group, but this compound is only moderately active whereas, compound **4b** and **4h**, i.e. Cl and Br substitution in indole ring, have not shown any effect and gives least activity. The *N*-ethyl substitution in indole ring with carboxylate group, i.e. compound **4e**, showed good activity and with carbonitrile group showed least activity. The results are shown in Table 3 and Fig. 1. The results show that compound **4a** and **4f** showed highest activity. In compound **4a**, there is presence of an electron donating groups (hydrogen in form of NH of indole ring and carboxylate group at 5th position of pyran ring), which stabilized the compound after donating the hydrogen to DPPH$^{\bullet}$ radical. In compound **4f**, there is presence of an electron donating groups (*N*-benzyl, a bulky group substitution on indole ring and carboxylate group at 5th position of pyran ring) which stabilized the compound after donating the hydrogen to DPPH$^{\bullet}$ radical.

ABTS radical scavenging activity

In ABTS assay, among the tested compounds, compound **4f** with combination of carboxylate group at 5th position of

Table 3 EC_{50} values of compounds in antioxidant properties



Compound	R	R_1	X	EC_{50}		
				DPPH	ABTS	NO
4a	H	H	COOEt	539.53 ± 0.345	1010.20 ± 0.346	671.95 ± 0.230
4b	H	5-Cl	COOEt	811.20 ± 0.236	3678.57 ± 0.246	1372.50 ± 0.345
4c	H	5,7-diCH_3	COOEt	920.20 ± 0.257	2763.61 ± 0.269	1122.33 ± 0.236
4d	CH_2–CH=CH_2	H	COOEt	619.45 ± 0.347	1597.09 ± 0.257	891.09 ± 0.185
4e	C_2H_5	H	COOEt	600.66 ± 0.191	1076.53 ± 0.242	730.86 ± 0.202
4f	CH_2–C_6H_5	H	COOEt	456.91 ± 0.148	488.60 ± 0.358	560.49 ± 0.173
4g	H	H	CN	1054.45 ± 0.259	3324.66 ± 0.256	1188.57 ± 0.132
4h	H	5-Br	CN	959.28 ± 0.239	2941.47 ± 0.358	1131.47 ± 0.218
4i	H	5-CH_3	CN	727.42 ± 0.282	1147.95 ± 0.159	793.73 ± 0.239
4j	CH_2–CH=CH_2	H	CN	974.25 ± 0.311	3345.33 ± 0.512	898.09 ± 0.198
4k	C_2H_5	H	CN	969.48 ± 0.178	3340.00 ± 0.347	877.28 ± 0.241
4l	CH_2–C_6H_5	H	CN	922.98 ± 0.193	4167.91 ± 0.567	892.67 ± 0.188
Ascorbic acid				409.75 ± 0.288	550.00 ± 0.134	544.21 ± 0.132

EC_{50} value: The effective concentration at which the antioxidant activity was 50 %; $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and NO radicals were scavenged by 50 %, respectively. EC_{50} values were obtained by interpolation from linear regression analysis. Values were the mean of three replicates ± SE

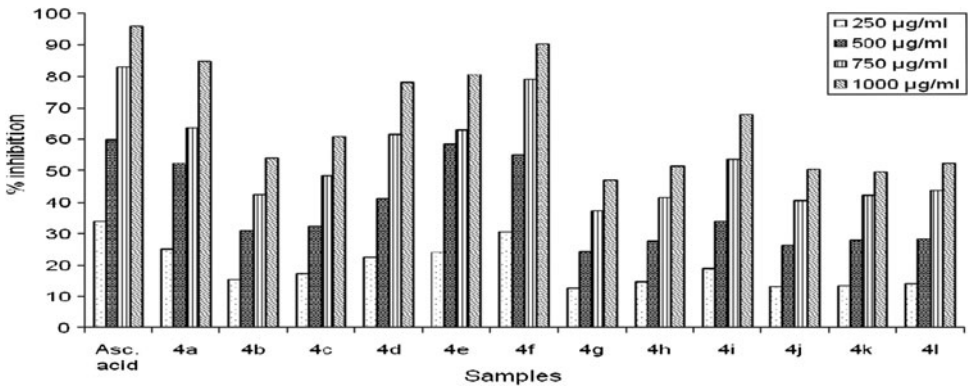


Fig. 1 DPPH radical scavenging activity of indole derivatives at different concentrations

pyran ring and *N*-benzyl substitution at indole ring, showed good activity which is equipotent to ascorbic acid, which we took as standard drug. Besides, with *N*-substitution and carboxylate group at 5th position of pyran ring (i.e. compound **4d**) and with carbonitrile group (i.e. compounds **4j–l**) showed least activity, but compound **4e**, having *N*-ethyl substitution at indole ring and carboxylate group at 5th position of pyran ring showed moderate activity. Incorporation of Cl, H and Br (i.e. compound **4b**, **4g** and **4h**) have not showed any effect and shows least activity. It was observed that unsubstituted in indole ring with carboxylate group (i.e. compound **4a**) shown moderate activity. Whereas, incorporation of CH-3 group at 5th position in indole ring with carbonitrile group (i.e. compound **4i**) also gave moderate activity, but the 5,7-diCH_3 substitution with carboxylate group (i.e. compound **4c**) displayed least activity (Table 3; Fig. 2). The results show that compound **4f** showed highest activity. In compound **4f**, there is presence of an electron donating groups (in form of benzyl group substituted on nitrogen of indole ring and carboxylate at 5th position of pyran ring) which stabilized the compound after donating the hydrogen or lone pair to $ABTS^{\bullet+}$ radical cation.

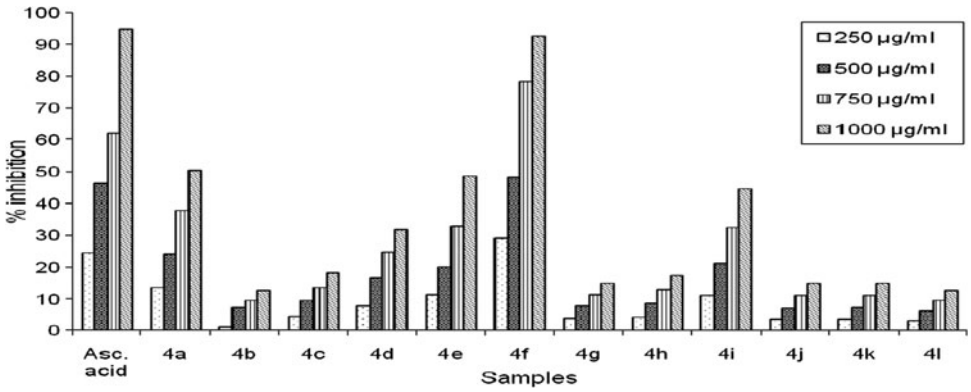


Fig. 2 ABTS scavenging activity of indole derivatives at different concentrations

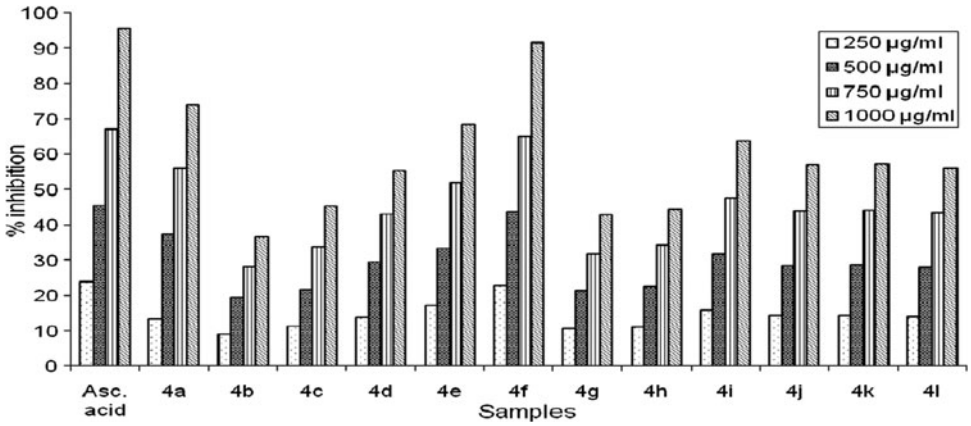


Fig. 3 NO scavenging activity of indole derivatives at different concentrations

Nitric oxide scavenging activity

In NO assay, among the tested compounds, compounds **4e** and **4f** having *N*-substituted indole ring with carboxylate group at 5th position of pyran ring, showed good activity. Other *N*-substituted indole with carboxylate group or with carbonitrile group (i.e. compounds **4d**, **4j**, **4k**, **4l**, respectively) showed moderate activity. Free NH functionality of indole ring with carboxylate group (i.e. compound **4a**) showed moderate activity, while with carbonitirle group (i.e. compound **4g**) showed least activity. The incorporation of CH-3 group at 5th position in indole ring with carbonitrile group (i.e. compound **4i)** gives moderate activity, but the incorporation of 5,7-diCH_3, Cl with carboxylate group (i.e. compounds **4b** and **4c**), and Br with carbonitrile group (i.e. compound **4h**) have not shown any effect and gives least activity (Table 3; Fig. 3). The results show that compound **4f** possess highest activity. In compound **4f**, there is presence of an electron donating groups (in form of benzyl group substituted on nitrogen of indole ring and carboxylate at 5th position of pyran ring) which stabilized the compound after donating the hydrogen or lone pair to NO radical.

Conclusion

This work describes for the first time the in vitro antioxidant activities of spirooxindole derivatives, as a new class of potential antioxidants. The results showed that all the spirooxindole derivatives had demonstrated strong activity in $DPPH^{\bullet}$ and NO scavenging method and moderate activity in $ABTS^{\bullet+}$ scavenging procedure. The compound **4f** (Ethyl6′-amino-3′-methyl-2-oxo-1′-phenyl-1′*H*-spiro[1-benzylindoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carboxylate) has shown strong activity in $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and NO scavenging screening. Overall, compound **4a** having electron donating carboxylate group and compound **4f** having both *N*-benzyl and carboxylate group having electron donating effect were found to be the most potent antioxidant described in this study.

Experimental

Chemistry

The melting points of all the compounds were determined on a Toshniwal apparatus. The purity of compounds was

checked on thin layers of silica gel G-coated glass plates with *n*-hexane ethyl acetate (7:3) as eluent. Infrared (IR) spectra were recorded on a Shimadzu Fourier transform (FT)-IR 8400 S spectrophotometer using KBr pellets.

Sonication was carried out with the help of a standard ultrasonic irradiation instrument SonaprosPR-1000 MP (Oscar Ultrasonics Pvt. Ltd.) operating at 750 W and generating 23 KHz output frequency. It has the following characteristics: Standard Titanium horn with a diameter of 6 mm/12 mm, replaceable flat stain less steel tip and digital thermometer to determine temperature. The glass reactor was designed and made from borosil glass.

*Synthesis of spiro[indoline3,4′-pyrano[2,3-c]pyrazole derivatives (**4a–l**)*

An equimolar mixture of isatin (1 mmol, 0.147 g), ethylcyanoacetate (1 mmol, 0.113 g), 3-methyl-1-phenyl-2-pyrazolin-5-one (1 mmol, 0.174 g) and 10 mol% CAN in 5 ml water was introduced in a 20 mL, heavy-walled, pear-shaped, two-necked flask with non standard taper outer joint. The flask was attached to a 12 mm tip diameter probe, and the reaction mixture was sonicated at ambient temperature for the specified period at 50 % power of the processor and 230 W output in a 4 s pulse mode. At the end of the reaction period, thin-layer chromatography (TLC) was checked, and the flask was detached from the probe. The contents were transferred to a beaker. The formed solid was filtered off, washed thoroughly with warm water (2 × 20 ml), and then dried to obtain crude products which were purified by crystallization from mixture of methanol acetone (6:4) to give compound **4**, giving satisfactory spectral and elemental analysis.

*Ethyl 6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[indoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carboxylate (**4a**)*

White crystalline solid; YIELD: 94 %; m.p. 238–240 °C; IR (KBr) : 3392, 3230, 3172, 1716, 1652, 1600, 1554, 1160 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 0.71 (t, 3H, CH_3), 1.55 (s, 3H, CH_3), 3.72 (q, $J = 6.80$ Hz, 2H, CH_2), 6.84–6.93 (m, 3H), 7.18 (t, $J = 5.40$ Hz, 1H), 7.32 (t, $J = 07.5$ Hz, 1H), 7.49(t, $J = 8.4$ Hz, 2H), 7.75 (d, $J = 7.5$ Hz, 2H), 8.14 (brs, 2H, NH_2, D_2O exchangeable), 10.53(s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 12.0, 13.4, 48.0, 59.6, 75.1, 98.7, 109.4, 120.5, 122.3, 123.5, 126.9, 128.3, 129.9, 136.1, 137.9, 142.4, 144.7, 161.7, 168.3, 179.9; MS (*m/z*): 416 (M+). Anal. calcd. for $C_{23}H_{20}N_4O_4$: C, 66.34, H, 4.84, N, 13.45. Found: C, 66.30, H, 4.81, N, 13.38.

*Ethyl 6′-amino-5-chloro-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[indoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carboxylate (**4b**)*

White crystalline solid; YIELD: 92 %; m.p. 246–248 °C; IR (KBr): 3396, 3228, 3172, 1698, 1644, 1608, 1566, 1160 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) : δ 0.77 (t, 3H, CH_3), 1.61 (s, 3H, CH_3), 3.75 (q, $J = 6.6$ Hz, 2H, CH_2), 6.89 (d, $J = 8.4$ Hz, 1H), 7.08 (s, 1H), 7.23 (d, $J = 8.1$ Hz, 1H), 7.33 (t, $J = 7.2$ Hz, 1H), 7.50 (t, $J = 7.8$ Hz, 2H), 7.80 (d, $J = 8.1$ Hz, 2H), 8.25 (brs, 2H, NH_2, D_2O exchangeable), 10.68 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 11.8, 13.2, 47.8, 59.2, 74.1, 97.6, 110.4, 120.2, 123.5, 125.9, 126.5, 127.7, 129.5, 137.3, 137.9, 141.1, 144.1, 161.5, 167.8, 179.2; MS (*m/z*) : 450 (M+); Anal. calcd. for $C_{23}H_{19}ClN_4O_4$: C, 61.27, H, 4.25, N, 12.43. Found : C, 61.30, H, 4.31, N, 12.42.

*Ethyl 6′-amino-3′,5,7-trimethyl-2-oxo-1′-phenyl-1′H-spiro[indoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carboxylate (**4c**)*

White crystalline solid; YIELD: 91 %; m.p. 260–262 °C; IR (KBr): 3398, 3228, 3170, 1702, 1648, 1611, 1570, 1168 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) : δ 0.75 (t, 3H, CH_3), 1.58 (s, 3H, CH_3), 2.12 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 3.73 (q, $J = 6.90$ Hz, 2H, CH_2), 6.57 (s, 1H), 6.78 (s, 1H), 7.32 (t, $J = 7.2$ Hz, 1H), 7.50 (t, $J = 7.8$ Hz, 2H), 7.80 (d, $J = 7.8$ Hz, 2H), 8.17 (brs, 2H, NH_2, D_2O exchangeable), 10.47 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 11.8, 12.9, 16.3, 18.6, 20.6, 47.8, 56.2, 59.2, 75.0, 98.6, 117.9, 120.0, 121.2, 126.4, 129.4, 129.5, 130.6, 135.5, 137.4, 138.3, 144.0, 144.4, 161.3, 168.1, 179.9; MS (*m/z*) : 444 (M+); Anal. calcd. for $C_{25}H_{24}N_4O_4$: C, 67.55, H, 5.44, N, 12.60. Found: C, 67.49, H, 5.39, N, 12.63.

*Ethyl 6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[1-allylindoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carboxylate (**4d**)*

White crystalline solid; YIELD: 84 %; m.p. 182–184 °C; IR (KBr): 3398, 3228, 3170, 1702, 1648, 1611, 1570, 1168 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) : δ 0.67 (t, 3H, CH_3), 1.50 (s, 3H, CH_3), 3.73 (q, $J = 6.90$ Hz, 2H, CH_2), 4.29 (dd, 1H, CH_2), 4.58 (dd, 1H, CH_2), 5.25 (d, 1H, CH_2), 5.43 (d, 1H, CH_2), 5.84 (m, 1H, CH), 6.95–7.83 (m, 9H, ArH), 8.24 (brs, 2H, NH_2, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, 75 MHz): δ 11.7, 13.4, 47.0, 58.9, 74.6, 97.6, 118.7, 119.8, 119.8, 122.2, 126.0, 127.4, 129.0, 131.4, 134.7, 137.4, 142.3, 143.9, 144.0, 167.6, 177.1; MS

(*m/z*) : 457 (M+). Anal. calcd. for $C_{26}H_{24}N_4O_4$: C, 68.40, H, 5.32, N, 12.10. Found: C, 68.49, H, 5.39, N, 12.13.

Ethyl 6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[1-ethylindoline-3,4′-pyrano[2, 3-c]pyrazole]-5′-carboxylate ***(4e)***

White crystalline solid; YIELD: 92 %; m.p. 208–210 °C; IR (KBr): 3398, 3228, 3170, 1702, 1648, 1611, 1570, 1168 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) : δ 0.63 (t, 3H, CH_3), 1.33 (t, 3H, $J = 6.8$ Hz), 1.50 (s, 3H, CH_3), 3.73 (q, 2H, $J = 6.8$ Hz), 6.39–7.24 (m, 9H, ArH), 8.29 (brs, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 10.2, 11.7, 47.5, 58.7, 72.6, 95.6, 114.2, 117.9, 118.2, 120.0, 121.2, 124.4, 127.4, 129.5, 130.6, 134.3, 137.4, 142.5, 143.3, 144.0, 166.1, 176.9; MS (*m/z*) : 445 (M+); Anal. calcd. For $C_{25}H_{24}N_4O_4$: C, 67.50, H, 5.41, N, 12.57. Found: C, 67.55, H, 5.44, N, 12.60.

Ethyl 6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[1-benzylindoline-3,4′-pyrano[2, 3-c]pyrazole]-5′-carboxylate ***(4f)***

White crystalline solid; YIELD: 97 %; m.p. 244–246 °C; IR (KBr): 3398, 3228, 3170, 1702, 1648, 1611, 1570, 1168 cm^{-1}. ^1H NMR (DMSO-d_6, 300 MHz) : δ 0.63 (t, 3H, CH_3), 1.50 (s, 3H, CH_3), 3.73 (q, $J = 6.90$ Hz, 2H, CH_2), 4.91 and 5.13 (2H, AB system $J = 15.5$ Hz), 6.57–7.89 (m, 14H, ArH), 8.20 (brs, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 11.5, 13.6, 46.9, 58.9, 73.6, 96.6, 117.9, 118.2, 120.0, 122.2, 126.4, 127.4, 129.5, 131.6, 134.3, 137.4, 142.3, 143.8, 144.0, 161.6, 165.5, 176.9; MS (*m/z*) : 507 (M+); Anal. calcd. for $C_{30}H_{26}N_4O_4$: C, 71.04, H, 5.13, N, 11.01. Found: C, 71.13, H, 5.17, N, 11.06.

6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[indoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carboxylate ***(4g)***

White crystalline solid; YIELD: 95 %; m.p. 237–238 °C; IR (KBr): 3412, 3280, 3174, 2200, 1692, 1650, 1526, 1132 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) : δ 1.55 (s, 3H, CH_3), 6.94 (d, $J = 7.4$ Hz, 1H), 7.03 (t, $J = 7.6$ Hz, 1H), 7.18 (d, $J = 7.2$ Hz, 1H), 7.28 (t, $J = 7.5$ Hz, 1H), 7.36 (t, $J = 7.6$ Hz, 1H), 7.52 (t, $J = 7.8$ Hz, 2H), 7.58 (brs, 2H, NH_2, D_2O exchangeable), 7.79 (d, $J = 7.9$ Hz, 2H), 10.76 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 12.3, 48.3, 56.6, 96.9, 110.4, 118.6, 120.7, 123.2, 125.4, 127.1, 129.8, 130.8, 132.6, 138.2, 142.1, 144.5, 145.5, 162.3, 178.8; MS (*m/z*) : 369 (M+); Anal. calcd. for $C_{21}H_{15}N_5O_2$: C, 68.28, H, 4.09, N, 18.96. Found: C, 68.26, H, 4.04, N, 18.88.

6′-Amino-5-bromo-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[indoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carbonitrile ***(4h)***

White crystalline solid; YIELD: 92 %; m.p. 242–244 °C; IR (KBr): 3436, 3269, 3168, 2198, 1706, 1650, 1576, 1168 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 1.58 (s, 3H, CH_3), 6.91 (d, $J = 7.6$ Hz, 1H), 7.42–7.54(4H, m), 7.28 (t, $J = 7.9$ Hz, 1H), 7.64 (brs, 2H, NH_2, D_2O exchangeable), 7.79 (d, $J = 7.8$ Hz, 2H), 10.90 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 12.1, 47.9, 56.4, 96.7, 110.6, 118.2, 120.9, 125.8, 126.6, 129.5, 129.6, 131.7, 132.3, 137.3, 139.2, 144.1, 145.0, 161.0, 178.5; MS (*m/z*): 447(M+); Anal. calcd. for $C_{21}H_{14}BrN_5O_2$: C, 56.27, H, 3.15, N, 15.62. Found: C, 56.17, H, 3.21, N, 15.60.

6′-Amino-3′,5-dimethyl-2-oxo-1′-phenyl-1′H-spiro[indoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carbonitrile ***(4i)***

White crystalline solid; YIELD: 98 %; m.p. 288–290 °C; IR (KBr): 3426, 3269, 3178, 2192, 1696, 1650, 1576, 1174 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 1.56 (s, 3H, CH_3), 2.26 (s, 3H, CH_3),6.84 (d, $J = 7.8$ Hz, 1H), 7.08 (s, 1H), 7.12 (d, $J = 7.6$ Hz, 1H), 7.35 (t, $J = 8$ Hz, 1H), 7.52 (t, $J = 8.4$ Hz, 2H), 7.55 (brs, 2H, NH_2, D_2O exchangeable), 7.78 (d, $J = 8.3$ Hz, 2H), 10.64 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6,75 MHz): 11.9, 20.4, 48.2, 56.7, 96.6, 110.1, 118.4, 119.9, 125.3, 126.8, 129.9, 130.5, 131.9, 132.6, 137.4, 139.8, 144.3, 145.7, 161.2, 177; MS(*m/z*):383(M+); Anal. calcd. for $C_{22}H_{17}N_5O_2$: C, 68.92, H, 4.47, N, 18.27. Found: C, 68.82, H, 4.51, N, 18.11.

6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[1-allylindoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carbonitrile ***(4j)***

White crystalline solid; YIELD: 90 %; m.p. 218–220 °C; IR (KBr): 3408, 1708, 1660, 1605, 1540, 1166 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): 1.42 (s, 3H, CH_3), 4.29 (dd, 1H, CH_2), 4.58 (dd, 1H, CH_2), 5.25 (d, 1H, CH), 5.43 (d, 1H, CH), 5.84 (m, 1H, CH), 6.94–7.50 (m, ArH, 14H), 8.28 (brs, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): 11.7, 43.4, 55.9, 109.2, 117.8, 120.0, 123.2, 124.5, 126.2, 127.3, 128.3, 129.0, 131.2, 135.5, 137.2, 141.9, 143.8, 144.9, 161.1, 176.0; MS (*m/z*): 410 (M+); Anal. calcd. for $C_{24}H_{19}N_5O_2$: C, 70.35, H, 4.59, N, 17.15. Found: C, 70.40, H, 4.68, N, 17.10.

*6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[1-ethylindoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carbonitrile (**4k**)*

White crystalline solid; YIELD: 84 %; m.p. 210–212 °C; IR (KBr): 3406, 1705, 1656, 1608, 1542, 1162 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 1.33 (t, 3H, $J = 6.8$ Hz), 1.42 (s, 3H, CH_3), 3.85 (q, 2H, $J = 6.8$ Hz), 6.92–7.52 (m, ArH, 14H), 8.23 (brs, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): 11.7, 43.4, 55.9, 109.2, 117.8, 120.0, 123.2, 124.5, 126.2, 127.3, 128.3, 129.0, 131.2, 135.5, 137.2, 141.9, 143.8, 144.9, 161.1, 176.0; MS (*m/z*): 398 (M+); Anal. calcd. for $C_{23}H_{19}N_5O_2$: C, 73.29, H, 4.67, N, 15.27. Found: C, 73.19, H, 4.61, N, 15.24.

*6′-amino-3′-methyl-2-oxo-1′-phenyl-1′H-spiro[1-benzylindoline-3,4′-pyrano[2,3-c]pyrazole]-5′-carbonitrile (**4l**)*

White crystalline solid; YIELD: 97 %; m.p. 232–234 °C; IR (KBr): 3406, 1705, 1656, 1608, 1542, 1162 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 1.42 (s, 3H, CH_3), 4.89 (d, 1H, $J = 15.6$ Hz, benzylic proton), 5.09 (d, 1H, $J = 15.6$ Hz, benzylic proton), 6.97–7.42 (m, ArH, 14H), 8.13 (brs, 2H, NH_2, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 75 MHz): 11.7, 43.4, 55.9, 109.2, 117.8, 120.0, 123.2, 124.5, 126.2, 127.3, 128.3, 129.0, 131.2, 135.5, 137.2, 141.9, 143.8, 144.9, 161.1, 176.0; MS (*m/z*): 460 (M+); Anal. calcd. for $C_{28}H_{21}N_5O_2$: C, 68.92, H, 4.47, N, 18.27. Found: C, 68.82, H, 4.51, N, 18.11.

Antioxidant activity

Antioxidant activities of test compounds were measured by estimating DPPH$^\bullet$, ABTS$^{\bullet+}$ and NO scavenging activity in vitro using ascorbic acid as standard drug.

DPPH$^\bullet$ scavenging activity

Ability of synthesized compounds to scavenge the stable free radical, DPPH$^\bullet$ is measured by the method, appeared in the literature (Mensor *et al*., 2001). To 1 ml methanolic solution of DPPH$^\bullet$ (0.25 mM), 1 ml of ethanolic solution of synthesized compounds was added. To prepare control, 1 ml of methanol was added to the 1 ml methanolic solution of DPPH$^\bullet$ (0.25 mM). After 20 min, absorbance was recorded at 517 nm in a UV–Vis double beam spectrophotometer. The inhibition (%) of free radicals was calculated by using the following formula:

$$\text{Inhibition } (\%) = \frac{(\text{AC} - \text{AA})}{(\text{AC})} \times 100$$

where AC absorbance of control and AA absorbance of tested compounds.

ABTS$^{\bullet+}$ scavenging activity

ABTS$^{\bullet+}$ scavenging activity of synthesized compounds were measured by the method, appeared in the literature (Re *et al.*, 1999). First, ABTS$^{\bullet+}$ free radicals were generated through the oxidation of ABTS with potassium persulphate. For this purpose, ABTS was dissolved in deionized water to 7 mM concentration, and potassium persulphate was added to a concentration of 2.45 mM. The reaction mixture was kept in dark at room temperature for 12–16 h before final use. Lastly, the ABTS$^{\bullet+}$ solution was diluted with absolute ethanol till the absorbance was read 0.700 ± 0.020 at 734 nm. Synthesized compounds in ethanol were added to 3 ml of ABTS$^{\bullet+}$ solution and the absorbance was read after 6 min.

Nitric oxide scavenging activity

The interaction of synthesized compounds with nitric oxide (NO) was assessed by the nitrate ion detection method (Sreejayan, 1997). Sodium nitropruside (5 mM) in phosphate buffer spontaneously generates NO in an aqueous solution. NO interacts with oxygen and produces nitrate ions, which can be estimated by the use of Greiss reagent (1 % sulphanilamide, 2 % H_3PO_4 and 0.1 % napthylethylene diamine dihydrochloride). Sodium nitroprusside (5 mM) in phosphate buffer was mixed with synthesized compounds and incubated at 25 °C for 150 min. Prepared samples were allowed to react with Greiss reagent. The absorbance of chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylene diamine was read at 546 nm. The same reaction mixture without the synthesized compound, but with equal amount of distilled water served as control.

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