Regular Article

Design, Synthesis, Biological Evaluation, and Antioxidant and Cytotoxic Activity of Heteroatom-Substituted 1,4-Naphtho- and Benzoquinones

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In the present paper, we report the synthesis, characterization, and biological evaluation as antifungal, antibacterial, antioxidant, and cytotoxic/anticancer agents of N-, S-, O-substituted-1,4-naphtho- and 2,5-bis(amino-substituted)-1,4-benzoquinone derivatives. In the synthesized compounds, antimicrobial activity at low concentrations against *Escherichia coli* B-906, *Staphylococcus aureus* 209-P, and *Mycobacterium luteum* B-917 bacteria and *Candida tenuis* VKM Y-70 and *Aspergillus niger* F-1119 fungi in comparison with controls was identified. 2-(*N*-Diphenylmethylpiperazin-1-yl)-3-chloro-1,4-naphthoquinone 9a was the most potent, with a minimum inhibitory concentration value of 3.9 μg/mL against test culture *M. luteum*. The synthesized compounds were screened for their antioxidant capacity using the cupric-reducing antioxidant capacity (CUPRAC) method. 2,2′-[1-(2-Aminoethyl)piperazin-1-yl]-3,3′-dichloro-bis(1,4-naphthoquinone) 10 showed the highest antioxidant capacity, with a 0.455 CUPRAC-trolox equivalent antioxidant capacity (TEAC) coefficient. Other parameters of antioxidant activity (scavenging effects on $OH^{\cdot}$, $O_2^{\cdot-}$, and H_2O_2) of these compounds were also determined. The cytotoxic activity of the compounds was investigated by employing the sulforhodamine B cell viability assay against A549 (lung), MCF-7 (breast), DU145 (prostate), and HT-29 (colon) cancer cell lines. Compound 10 exhibited the most powerful cytotoxic activity at a concentration of 20 μM against all cell lines. In addition to the strongest antioxidant activity of compound 10, it also had lowest IC_{50} values (<3 μM), warranting further *in vivo* studies due to its anticancer activity.

Key words quinone; antimicrobial activity; antioxidant activity; cupric-reducing antioxidant capacity method; reactive oxygen species-scavenging activity; cytotoxicity

Quinonic compounds are of great importance to understand different processes that are related to biology.[1)] The quinone structure is common in numerous natural products that are associated with antitumor, antibacterial, antimalarial and antifungal activities.[2)] Furthermore, several reports have appeared in literature about anticancer activities of quinones against various cancer cell lines.[3–5)]

1,4-Naphthoquinones are widely distributed in nature and there are many clinically important antitumor drugs containing a quinone nucleus, such as anthracyclines, mitoxantrones and saintopin, that show excellent anticancer activity. These anticancer agents are effective inhibitors of DNA topoisomerase and it is generally accepted that the cytotoxicity of quinone analogues results from the inhibition of DNA topoisomerase-II.[6)] Quinone analogues can also induce the formation of semiquinone radicals, which can transfer an electron to oxygen to produce superoxide. The radical process is catalyzed by flavoenzymes such as reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome-P-450 reductase. Both the superoxide and semiquinone radical anions of naphthoquinone analogues can generate the hydroxyl radical, which is known to cause DNA strand breaks.[7)] Structure–activity relationship studies from quinonoid compounds indicated that the number and position of nitrogen (N) atoms substituted in the heterocyclic ring were considerably important factors to affect the biological activities.[8,9)] The presence of amino, thio- or chloro-moiety on the quinones was considerably important factor to effect antifungal activity.[10)] Quinones, in particular naphthoquinone derivatives, have been repeatedly isolated from lower as well as higher species of plants, and are found frequently in animals. In addition to quinones possessing a biological function in cell metabolism as electron carriers, other compounds of this class have been found active against bacteria and fungi.[11,12)]

Antioxidants have received increased attention in the recent years from medical researchers and nutritionists for their potential activities in the prevention of several degenerative diseases such as cancer, cardiovascular disorder as well as aging.[13)] Because this activity is related with compounds capable of protecting a biological system against the potential harmful effects of oxidative processes. In recent years, several works have been published on structure–activity analysis on compounds with antioxidant activities. For examples: Kuwahara *et al.*[14)] also studied the antioxidant property of polyhydroxylated naphthoquinone pigment from shells of purple sea urchin anthocidarin, crassispina; Talcott *et al.*[15)] also evaluated antioxidant ability of Menadion against microsomal lipid peroxidation in the presence of physiologic reductase NADPH.

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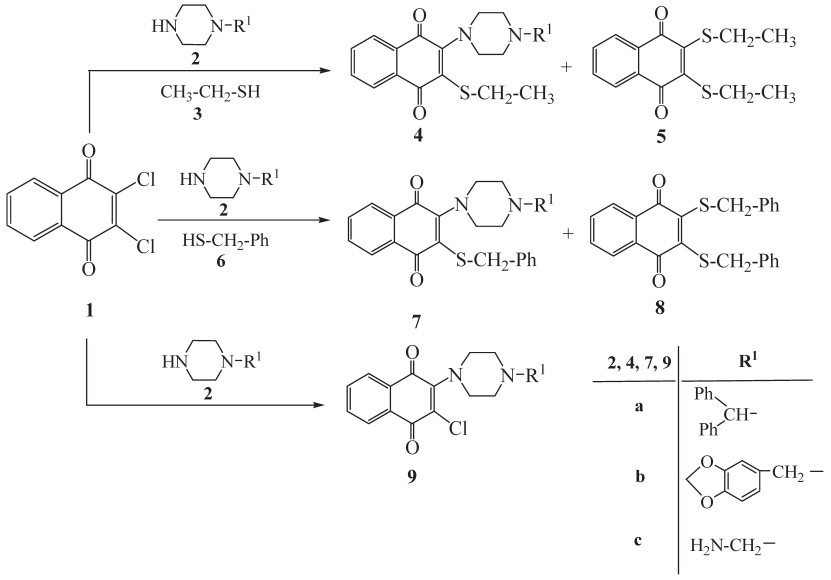

Chart 1

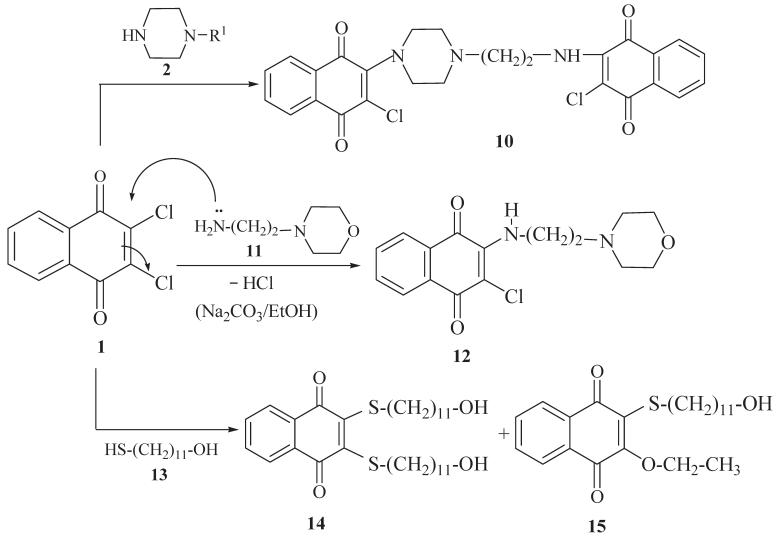

Chart 2

On the other hand, there are several reports available on antioxidant activity of synthetic compounds.[16)]

Consequently, the synthesis of new active derivatives with potential applications in this area and prepared by simple chemical procedures should be of increasing interest. Here we described the synthesis, characterization, antimicrobial, antioxidant and cytotoxic/anticancer activities of 1,4-naphtho- and benzoquinone derivatives. Their structures of synthesized compounds were characterizated by using micro analysis, Fourier transform (FT)-IR, ^{1}H-NMR, ^{13}C-NMR, MS, ultraviolet-visible spectroscopy (UV-Vis).

Chemistry

A series of N-, S-, O-substituted-1,4-naphthoquinones (**4a**, **5**, **7a**, **8**, **9a**, **b**, **10**, **12**) and 2,5-bis(aminosubstituted)-1,4-benzoquinones (**17**, **18**) are synthesized from the reactions of 2,3-dichloro-1,4-naphthoquinone **1** and *p*-chloranil **16** with different nucleophilic compounds (**2a**, **c**, **3**, **6**, **11**, **13**) under the aerobic condition as illustrated in Charts 1, 2 and 3. It is well known that the reactions of **1** and **16** with nucleophiles proceed by nucleophilic substitution whereas nucleophilic addition reactions of 1,4-naphtho- and benzoquinones are augmented by oxidative addition pathway.

N-, S-Substituted-1,4-naphthoquinone **4a** and bis(thio)-substituted-1,4-naphthoqinone **5** were obtained from reaction of **1** with *N*-(diphenylmethyl)piperazine **2a** and ethanethiol **3**. Compounds **7a** and **8** were synthesized from the reaction of **1** with **2a** and benzylthiol **6**. The synthesis and spectral characterization of compound **5** were previously reported.[17)] Result of micro analysis and melting point for compound **8** were given in the releated literature.[18)] However, there is no spectroscopic data for compound **8**. The piperazine ring (2-position) and chlorine atom (3-position) substituted compounds **9a**, **b** were obtained from reactions of **1** with *N*-(diphenylmethyl)piperazine **2a** or 1-piperonylpiperazine **2b** (Chart 1). The reaction of 2,3-dichloro-1,4-naphthoquinone **1** with (2-aminoethyl)piperazine **2c** resulted in the formation of intramolecular cyclization to yield heterocyclic diquinone

2,2′-[1-(2-aminoethyl)piperazin-1-yl]-3,3′-dichloro-bis(1,4-naphthoquinone) **10** (Chart 2). In the mass spectrum of compounds **4a**, **7a**, **8** and **9a**, **b**, the accurate mass measurements of the molecular ion peaks were noticed at *m/z* 469 $[M]^+$, 531 $[M]^+$, 403 $[M]^+$, 443 $[M]^+$ and 412 $[M]^+$, respectively.

The reaction of 2,3-dichloro-1,4-naphthoquinone **1** with (2-aminoethyl)piperazine **2c** resulted in the formation of intramolecular cyclization to yield heterocyclic diquinone 2,2′-[1-(2-aminoethyl)piperazin-1-yl]-3,3′-dichloro-bis(1,4-naphthoquinone) **10** (Chart 2). In the positive ion mode of electrospray ionization (ESI) mass spectrum for compound **10** the respective molecular ion peak was observed at *m/z* (%) 510 (100) $[M]^+$. The cleavage of the chlorine ion from compound **10** of the molecular ion gave to rise fragment F_1 at *m/z* (%) 473 (100) $[M-37]^+$ which was the base peak.

Brun *et al.*[19] and Xu *et al.*[20] synthesized the compound **12** entitled 2-chloro-3-[2-morpholin-4-yl)ethylamino][1,4]-naphthoquinone in the presence of triethylammonium acetate (0.6 mL) in ether (40 mL) and the presence of anhydrous ethanol, respectively. We synthesized the compound **12** by the different procedure according to *general method 2* as given in Experimental. Brun *et al.* reported the CDC25 phosphatase inhibitory activity of naphthoquinone derivatives in literature.[19] Hsu *et al.* showed that the compound **12** was the most potent to induce cell death in human A549 lung cancer cells.[21] Xu *et al.*[20] investigated the biological properties of compound **12** as antiproliferative agents and 20S proteasome inhibitors[20] showed features that biological activity properties of compound **12** encouraged us to synthesize this compound again and to investigate antifungal, antibacterial properties and antioxidant capacity of compound **12**. It is confirmed that the product formed between the reaction of compounds **1** and 4-(2-aminoethyl)morpholine **11** was compound **12**. The proposed mechanism for the reaction of **1** with **11** and the synthesis pathway of **12** were illustrated in Chart 2. Alkenes generally undergo nucleophilic addition reactions. However, alkenes undergo nucleophilic addition reactions in the presence of electron withdrawing groups (EWG's) bound to sp^2-hybrided C atoms and this is known as the Michael reaction. First, an addition of the attacking reagent to the C, C double bond occurs, and in a second step the intermediate product is stabilized by elimination of hydrogen chloride as illustrated in Chart 2.

UV-Vis electronic absorption spectra of **4a**, **7a**, **8**, **9a**, **b** showed the expected benzene and naphthoquinone bands in the UV region around at 204–210, 214–248 and 276–291 nm (π–π* electronic transitions). In addition, a third lower energy transition appeared as a broad band in the visible region between 455 and 512 nm for **4a**, **7a**, **9a**, **b**, **10** and **12**. This absorption is typical of N-substituted quinones[22,23] and is assigned to charge-transfer transitions and weak n–π* transitions of the carbonyl group in the quinone unit.[24]

Mono(thio)-substituted naphthoquinone compounds containing chlorine atom were not observed potentially due to the decreased thiol amount in the medium of the reaction, while mono(thio)-substituted naphthoquinone containing ethoxy group (3-position) compounds were obtained successfully. The synthesis and characterization of compounds **14** and **15** were previously reported.[25] In present study, properties of biological activity and antioxidant capacity of compounds **14** and **15** have been investigated.

Brun *et al.*,[19] Xu *et al.*[20] and Hsu *et al.*[21] reported that the high biological activities of the compound **12** which was obtained by the reaction of 2,3-dichloro-1,4-napthoquinone **1** with **11**. We want to investigate the biological activity properties of the target compound [(2-aminoethyl)morpholin-1-yl)]-substituted-1,4-benzoquinone **17**. For this reason, 2,5-bis[4-(2-aminoethyl)morpholin-1-yl]-3,6-dichloro-1,4-benzoquinone **17** and 2,5-bis[1-piperonyl-piperazin-1-yl]-3,6-dichloro-1,4-benzoquinone **18** were synthesized from reactions of *p*-chloranil **16** with **11** and **2b**, respectively in Chart 3.

The reactions were found to be exceptionally selective and leads to only 2,5-bis(aminosubstituted)-3,6-dichloro-1,4-benzoquinones of the corresponding amine. From these reactions we could not obtain 2,6-bis(aminosubstituted)-1,4-benzoquinone derivatives. The steric factors arising from the substituent effect predominates in these reactions. The result of selective formation of 2,5-isomer may be assumed to be due to attack of two amines to 1,4-benzoquinone. For such attack to give exclusive product of one isomer would require approach of two amines from the furthest possible distance. Thus, exclusively 2,5-isomer were formed due to electrostatic reasons for compounds **17** and **18**. The results agree well with the corresponding mechanism in the similar compounds.[24,26,27] The ^{13}C-NMR spectra of benzoquinone unit gave one carbonyl signals spectra at 171.29 and 175.00 ppm in compounds **17** and **18**, respectively. If 2,6-isomer was obtained from these reactions, we must determine two different carbonyl signals in the ^{13}C-NMR spectra for **17** and **18**. FT-IR spectrum in

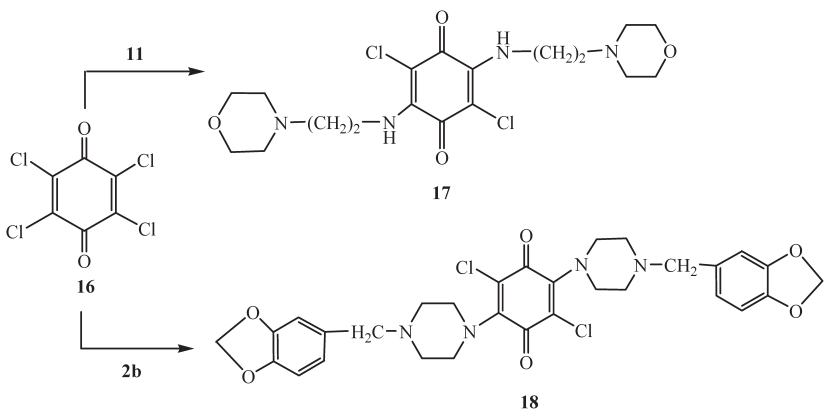

Chart 3

KBr showed the following important absorption bands for compounds **17** and **18**. In the FT-IR spectra of compounds **17** and **18**; two typical strong quinonic carbonyl absorptions were observed between at 1614 and 1676 cm^{-1} for compounds **17** and **18**. The (–NH) absorption appeared at 3245 cm^{-1} for compound **17**. For compound **18**, no bands were observed in the region 3200–3450 cm^{-1} attributable to the streching vibration of the bonded (–NH) group. In the mass spectrum of compounds **17** and **18**, the accurate mass measurements of the molecular ion peaks were noticed at *m/z* 433 $[M]^+$ and 613 $[M]^+$, respectively.

Results and Discussion

Antibacterial and Antifungal Activities The profound antifungal and antibacterial activity exhibited by quinone compounds has prompted us to synthesize new N,S,O-substituted-1,4-naphtho- and 2,5-bis(aminosubstituted)-1,4-benzoquinones. In our new endeavors, we have synthesized new 1,4-naphtho- and benzoquinones and evaluated their antibacterial and antifungal activity by diffusion method[28] and serial dilution method[29] with a view to search new perspective compounds having broad spectrum of biological activity. Antibacterial and antifungal activity of compounds **4a**, **7a**, **9a**, **b**, **10**, **12**, **14** and **15** were elucidated against *Escherichia coli* B-906, *Staphylococcus aureus* 209-P, and *Mycobacterium luteum* B-917, *Candida tenuis* VKM Y-70 and *Aspergillus niger* F-1119 by diffusion method in Table 1 and by serial dilution method as shown in Tables 2 and 3. Activities of quinone compounds were compared with those of the known antibacterial agent Vancomycin and antifungal agent Nystatin (control C).

The test-cultures *E. coli*, *S. aureus*, *C. tenuis* and *A. niger* appeared not to be sensitive to all compounds (by diffusion method, see Table 1). Compound **15** was sensitive at a concentration of 0.1%, and the diameter of the inhibition zone was 13.4 mm (Table 1). The *M. luteum* bacteria strain was sensitive to compound **15** at a concentration of 0.5% and the diameter of the inhibition zone was 14.4 mm. Compound **10** has moderate activity against *C. tenuis* (12.3 mm at 0.5% concentration). Compounds **4a**, **7a**, **9a**, **b**, **10**, **12** and **14** (at 0.1% concentration) have no antibacterial and antifungal activity against *E. coli*, *S. aureus*, *M. luteum*, *C. tenuis* and *A. niger* at 0.5 and 0.1% evaluated concentrations by diffusion method (Table 1).

The biological activity results of the synthesized compounds were classified as follows: the antimicrobial activities were considered as significant when the minimum inhibition concentration (MIC) was 100 μg/mL or less; moderate, when the MIC was 100.0–500.0 μg/mL; weak, when the MIC was 500.0–1.000 μg/mL; and inactive when the MIC was above 1.000 μg/mL. According to the literature[30] the compounds containing *N*-phenyl piperazine ring similiar the compound **9a** showed more significant antibacterial activity among the other similiar compounds. Evaluation of the antibacterial activity of the synthesized compound showed that **9a** was the most potent with MIC=3.9 μg/mL for *M. luteum* (for control MIC=7.8 μg/mL). Compounds **4a**, **7a** and **9b** showed significant anti-Mycobacterium activity with MIC value in the range of 15.6 μg/mL in serial dilution assay against *M. luteum*. Evaluation of antibacterial activity of synthesized compounds showed that **4a** and **9b** have MIC=62.5 μg/mL; **7a** and **12** have MIC=125.0 μg/mL; **14** has MIC=250.0 μg/mL for *S. aureus*. Compounds **10** and **12** have MIC=250.0 and 125.0 μg/mL,

Table 1. Antibacterial and Antifungal Activities of Compounds by Diffusion Method

Compound	Concentration (%)	Inhibition diameter of microorganism growth (mm)				
		Antibacterial activity			Antifungal activity	
		E. coli	*S. aureus*	*M. luteum*	*C. tenuis*	*A. niger*
4a	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
7a	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
9a	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
9b	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
10	0.5	0	0	12.3	0	0
	0.1	0	0	0	0	0
12	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
14	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
15	0.5	0	0	14.4	0	0
	0.1	0	0	13.4	0	0
17	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
18	0.5	0	0	0	0	0
	0.1	0	0	0	0	0
Control*	0.5	14	15	18	19	20

* Vancomycin was used as a control in the tests of antibacterial activity of the synthesized compounds, and Nystatin was used in the tests of antifungal activity of the synthesized compounds.

Table 2. Antibacterial Activities of Compounds by Serial Dilution Method

Compound	MIC (μg/mL)		
	E. coli	*S. aureus*	*M. luteum*
4a	*	62.5	15.6
7a	*	125.0	15.6
9a	*	*	3.9
9b	*	62.5	15.6
10	+	+	125.0
12	*	125.0	125.0
14	+	250.0	+
15	+	+	*
17	250.0	250.0	125.0
18	+	+	+
Control	31.2±0.8	3.9±0.2	7.8±0.2

+: Growth of microorganisms. *: In the investigated concentrations the indexes of biocidic effect were not determined.

Table 3. Antifungal Activities of Compounds by Serial Dilution Method

Compound	MIC (μg/mL)	
	C. tenuis	*A. niger*
4a	250.0	500.0
7a	500.0	*
9a	62.5	500.0
9b	31.2	125.0
10	+	+
12	125.0	125.0
14	+	+
15	+	+
17	500.0	500.0
18	500.0	500.0
Control	7.8±0.2	15.6±0.8

+: Growth of microorganisms. *: In the investigated concentrations the indexes of biocidic effect were not determined.

Table 4. Calibration Equations of Synthesized Compounds, Linear Ranges and TEAC Coefficients (N=3)

Compound	Linear range (mol L^{-1})	Calibration equation	r	TEAC*
4a	0.012–0.049	y=5220c+0.021	0.9981	0.313
5	0.025–0.098	y=3850c+0.221	0.9978	0.231
7a	0.006–0.049	y=4900c+3.58×10^{-3}	0.9919	0.293
8	0.070–0.143	y=3726c+0.267	0.9928	0.223
9a	0.023–0.095	y=3822c+2.0×10^{-3}	0.9951	0.229
9b	0.049–0.172	y=5822c+0.032	0.9928	0.349
10	0.051–0.123	y=7605c+0.113	0.9980	0.455
12	0.024–0.097	y=2735c+0.031	0.9929	0.164
14	N.D.	N.D.	N.D.	N.D.
15	0.046–0.120	y=5340c+0.134	0.9951	0.320
17	0.065–0.132	y=2400c+0.048	0.9961	0.144
18	0.026–0.100	y=5665c+0.065	0.9950	0.340

* TEAC=$\varepsilon_{compound}/\varepsilon_{TR}$ (TR: trolox); ε_{TR}=1.67×10^4 L mol^{-1} cm^{-1}. N.D.: Not defined.

respectively, for *M. luteum* in Table 2.

Significant antifungal activity for **9b** was observed against *C. tenuis* fungi at 31.2 μg/mL. Evaluation of antifungal activity of compounds **4a**, **7a**, **9a**, **10**, **12**, **14** and **15** showed their activity in concentrations 62.5–500.0 μg/mL against test-culture *C. tenuis*. Compounds **4a**, **7a**, **9a**, **b**, **10**, **12**, **14** and **15** showed moderate antifungal activity with MIC value in the range of 125.0–500.0 μg/mL against *A. niger* in Table 3.

Summary of obtained data of antibacterial activity in Table 2 showed that the compounds **4a**, **7a**, **9a**, **b** and **12** were sensitive to bacteria *S. aureus* and especially **9a** to *M. luteum*, **9a**, **b** were sensitive to fungies *C. tenuis* and *A. niger* in Table 3.

Antioxidant Capacity The newly synthesized compounds **4a**, **5**, **7a**, **8**, **9a**, **b**, **10**, **12**, **14, 15**, **17, 18** were screened for their antioxidant capacity by using the cupric-reducing antioxidant capacity (CUPRAC) methods[31,32] against trolox as the standard reference compound. The linear calibration equations of these compounds (as absorbance in a 1 cm cell *vs.* molar concentration) gave the molar absorption coefficient (ε) as the slope. The CUPRAC molar absorption coefficient of the tested compound divided by that of trolox under the same conditions gave the trolox equivalent antioxidant capacity (TEAC), or TEAC coefficient of that compound tested for antioxidant capacity (Table 4). Among the synthesized compounds, **10** showed the highest antioxidant capacity, and CUPRAC-TEAC coefficients (in parentheses) decreased in the following order: **10** (0.455)>**9b** (0.349)>**18** (0.340)≥**15** (0.320)>**4a** (0.313)>**7a** (0.293)>**5** (0.231)>**9a** (0.229). Compound **10**, showed the highest antioxidant capacity, possibly due to its dimeric structure, similar to the observation that polymeric polyphenols had higher antioxidant activity than their monomeric analogues.

Reactive Oxygen Species (ROS) Scavenging Activity Hydroxyl radical ($OH^{\cdot}$) is the most reactive free radical that can be formed from superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), in the presence of metal ions.[33] In the present study, the hydroxyl radical scavenging (HRS)-CUPRAC method was used for determining the HRS activity of substituted quinonic compounds. The results are shown in Table 5. The % inhibition ratio values of compounds in this assay were in the range of 60.6 to 76.7. The compounds **10** and **18** were found to show better inhibition compared to other compounds with % inhibition values 75.10 and 76.70, respectively. The compounds **12**, **17** and **9b** showed poor ($OH^{\cdot}$) scavenging activity.

Hydrogen peroxide is thought to be the major precursor

of highly reactive free radicals, and it has been reported to induce apoptosis in cells of the central nervous system.[34] The ability of the compounds to scavenge H_2O_2 was determined according to the method of literatures.[35,36] A concentration-dependent assay was carried out with newly synthesized substituted quinonic compounds. Thus, the free radical scavenging activities of newly synthesized compounds decreased in the order of **10**>**4a**>**15**>**18**>**5**, showing a parallelism with that of CUPRAC-TEAC. It can be seen that the leading hydrogen peroxide scavenging (HPS) compounds like **10** and **4a** are also the ones with high HPS activity, as measured in this work.

Superoxide anion radical ($O_2^{\cdot -}$) have gained great attention due to their important role in the progression of a number of human diseases and carcinogenesis. So it is important to eliminate excessive ($O_2^{\cdot -}$) *in vivo* to prevent important diseases.[37] In this study, superoxide anion radical scavenging (SARS) activity of newly synthesized compounds was evaluated according to the method of Yu *et al.*[38] Superoxide anion ($O_2^{\cdot -}$) can be formed from dissolved oxygen by PMS-reduced nicotinamide adenine dinucleotide (NADH) coupling reaction, and ($O_2^{\cdot -}$) reduces the yellow dye (NBT^{2+}) to produce a blue formazan, of which the absorbance value is measured at 560 nm. Antioxidants are able to inhibit formazan formation. The decrease of absorbance with antioxidants indicates the consumption of ($O_2^{\cdot -}$) in the reaction mixture.

The order of SARS activity of the synthesized compounds in terms of % inhibition ratio was: **10**>**4a**>**9a**>**8**>**15**.

Table 5. ROS Scavenging Activity of Synthesized Compounds (N=3)

Compound	HRS-CUPRAC (% Inh.)	HPS-CUPRAC (% Inh.)	SARS activity (% Inh.)
4a	74.10	35.15	76.24
5	62.15	30.21	N.D.
7a	70.60	8.86	N.D.
8	65.75	15.60	23.45
9a	68.20	11.48	42.10
9b	61.25	15.73	14.15
10	75.10	39.85	78.45
12	60.70	8.07	N.D.
14	65.50	17.44	14.92
15	62.70	35.65	19.45
17	60.60	8.58	N.D.
18	76.70	30.51	N.D.

N.D.: Not defined.

Table 6. IC_{50} Values after 48 h of Treatment with Compounds **9b** and **10** in MCF-7 and DU145 Cell Lines Were Determined by ATP Assay

Cell lines	Compound **9b***	Compound **10***
MCF-7	9.42 μM	2.16 μM
DU145	15.96 μM	2.52 μM

* IC_{50} is defined as the dose inhibiting 50% of viability.

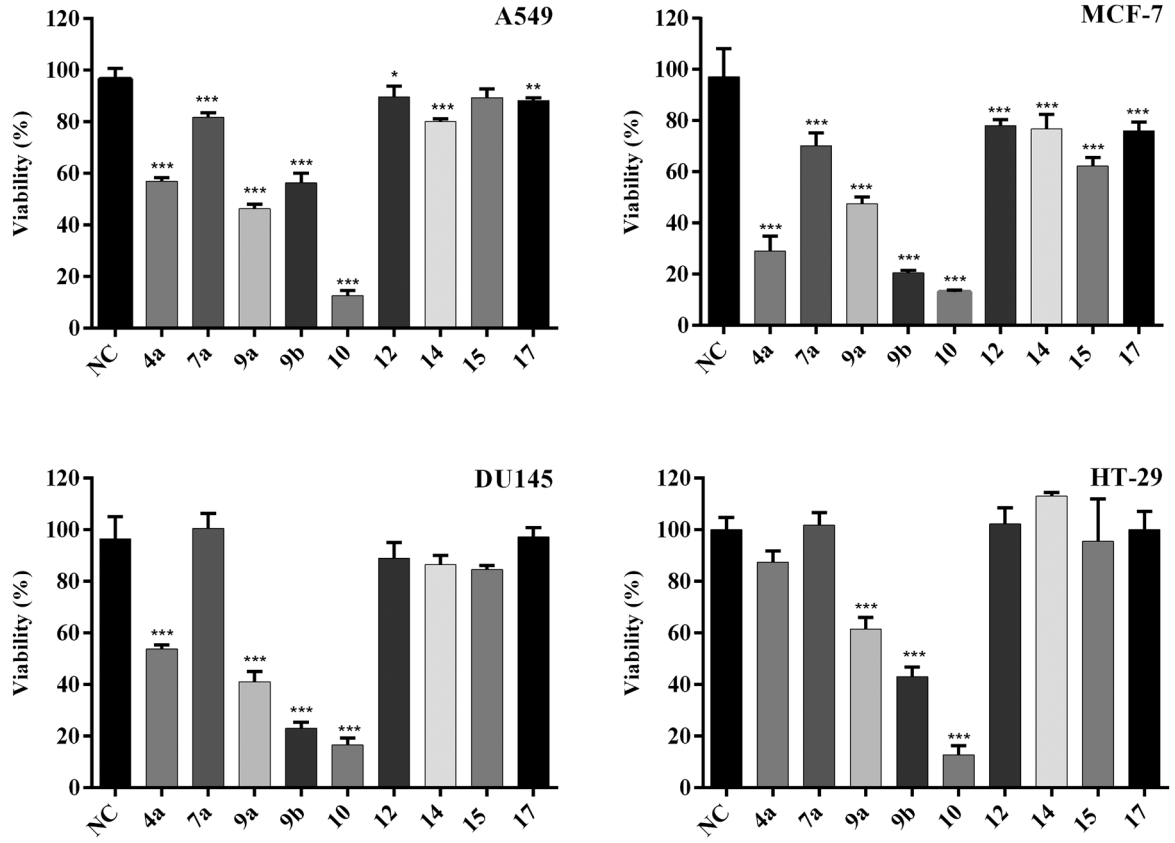

Fig. 1. Viability (%) of A549, MCF-7, DU145 and HT-29 Cells Treated with 20 μM Concentration of 11 Different Compounds (**4a**, **7a**, **9a**, **b**, **10**, **12**, **14**, **15, 17**) for 48 h

After treatment period, the SRB assay was performed to measure cell viability. ANOVA was used to demonstrate statistical significance between different doses with a Tukey's multiple comparison post test. * denotes statistically significant differences in comparison with negative control (NC). *$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.001$.

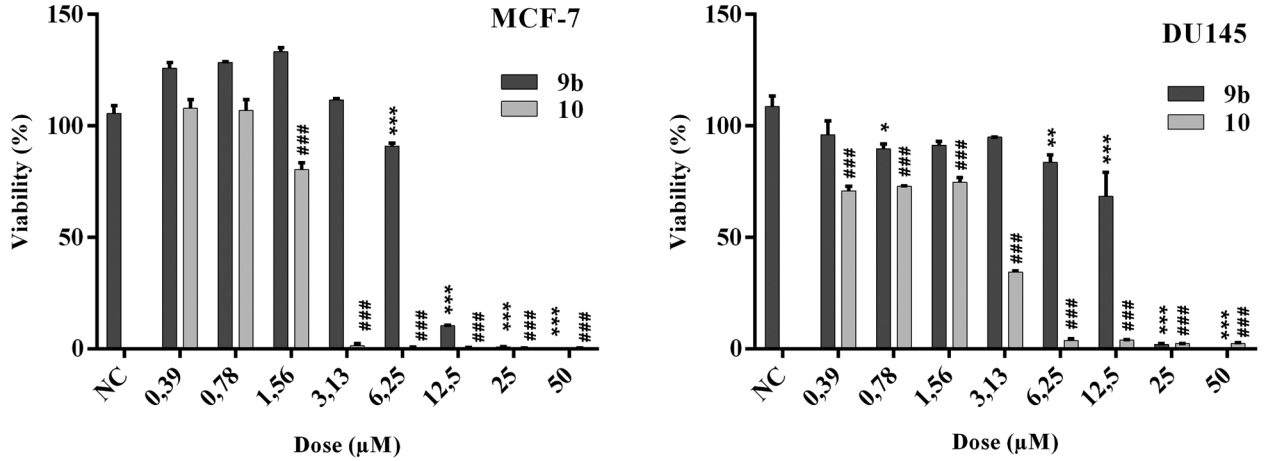

Fig. 2. Viability of MCF-7 and DU145 Cells after the Treatment with Varying Concentrations of **9b** and **10** for 48 h Was Measured by ATP Viability Assay

* (for **9b**) and # (for **10**) denotes statistically significant differences in comparison with negative control (NC) *$p \leq 0.05$; **$p \leq 0.01$; and ***$p \leq 0.001$.

Cytotoxic Activity A549, MCF-7, DU145 and HT-29 cells were treated with different compounds (**4a, 7a, 9a, b, 10, 12, 14, 15, 17**) at 20 μM concentration (the arbitrary dose of our laboratory for cytotoxicity screening studies) for 48 h. After the treatment period, cytotoxic effects of compounds were investigated by employing sulforhodamine B (SRB) cell viability assay (Table 6).

Based on SRB viability results, it was found that compound **10** exhibited the most powerful cytotoxic activity against all cell lines. In addition, the SRB results showed that compound **9b** had strong cytotoxic effect on MCF-7 and DU145 cell lines (Fig. 1).

Based on the SRB results, two cell lines (MCF-7 and DU145) and two compounds (**9b**, **10**) to were selected to perform ATP viability assay. Although SRB and ATP assays measure cell viability, ATP assay is considered to be more sensitive than SRB assay due to its being luminescence-based assay.[39] For this purpose, MCF-7 and DU145 cells were treated with different concentrations (0.39–50 μM) of compounds **9b** and **10** for 48 h and then ATP cell viability assay was performed (Fig. 2). It was found that both compounds exhibited statistically significant anti-growth effects in a dose-dependent manner compared to negative control (NC). IC_{50} values were calculated on the basis of the results of the ATP assay and were shown in Table 6. IC_{50} values of compound **9b** were 9.42 and 15.96 μM for MCF-7 and DU145 cell lines, respectively. It was found that compound **10** had lower IC_{50} values when compared to compound **9b** (2.16 μM for MCF-7 and 2.52 μM for DU145 cells). These results demonstrated that compound **10** had more strong cytotoxic activity, compared to compound **9b**.

In the literature, IC_{50} values of quinones have a broad range. Dolan *et al.*, reported that IC_{50} values of different naphthoquinones was ranging from 1.1 to 10.8 μM for MCF-7 cells.[3] In the other study, IC_{50} values were determined between 0.88 and 43.5 μM for MCF-7 cells.[40] Copeland *et al.* determined IC_{50} values of different quinones as 1, 3, 1.5, 3 and 10 μM for LNCaP, CWR-22, PC-3, DU-145 and HS-5 cells, respectively.[41]

Conclusion

The quinone compounds deserve the great prominence by exhibiting a broad spectrum of biological activities and forming the charge transfer complexes that have enormous applications ranging from sensors, magnetic materials to chemistry of drugs. The aim of this study was synthesis, characterization, evaluation of biological properties, antioxidant capacity and anticancer activity of some naphtho- and benzoquinone compounds. All new compounds were characterized on the basis of nuclear magnetic resonance spectroscopy (^{1}H- and ^{13}C-NMR), MS, FT-IR and UV-Vis. Among the synthesized compounds with antimicrobial activity at low concentrations against *S. aureus, M. luteum* bacteria and *C. tenuis* and *A. niger* fungi in comparison with control were identified. The *M. luteum* bacteria strain was sensitive to compound **15** at a concentration of 0.5% and the diameter of the inhibition zone was 14.4 mm. Antibacterial activity showed that compounds **4a**, **7a**, **9a**, **b** and **12** are sensitive to bacteria, *S. aureus* and especially to *M. luteum*, **9a** and **b** are sensitive to fungies *C. tenuis* and *A. niger*. 2-(*N*-Diphenylmethylpiperazin-1-yl)-3-chloro-1,4-naphthoquinone **9a** was the most potent with MICs (minimum inhibition concentrations)=3.9 μg/mL against test culture *M. luteum* bacteria. It has been observed that naphthoquinones **9** containing chlorine atom in the position 3 of 1,4-naphthoquinone moiety show more significant antibacterial activity against *M. luteum* in comparison with thiosubstituted-1,4-naphthoqinones **4** and **7** and control. The antioxidant properties of the synthesized compounds have also been tested using CUPRAC method in which 2,2′-[1-(2-aminoethyl)piperazin-1-yl]-3,3′-dichloro-bis(1,4-naphthoquinone) **10** exhibited better antioxidant capacity (CUPRAC-TEAC: 0.455) than the other compounds. Moreover, the related compound exhibited high ROS scavenging properties (depending on the type of ROS) compared to the other compounds. Cytotoxicity assay (SRB assay) showed that compound **10** exhibited the most powerful cytotoxic activity against four different cancer cell lines (A549, MCF-7, DU145, HT-29). Interestingly, it has also the most powerful antioxidant capacity. IC_{50} values of compounds **9b** and **10** found 9.42 and 15.96 μM for MCF-7 cells and DU145 cell lines, respectively. In addition, compound **10** had lower IC_{50} values when compared to compound **9b** (2.16 μM for MCF-7 and 2.52 μM for DU145 cells).

Consequently, the synthesis of new active derivatives with potential applications in this area and prepared by simple chemical procedures should be of increasing interest. In

conclusion, we have synthesized a series of 1,4-naphtho- and benzoquinone derivatives that are the promising candidates with respect to biological activity as potential antibacterial, antifungal, antioxidant and anticancer agents.

Experimental

Melting points were measured on a Buchi B-540 melting point apparatus. TLC plates silica $60F_{254}$ (Merck, Darmstadt), detection with ultraviolet light (254 nm). Elemental analyses were performed on a Thermo Finnigan Flash EA 1112 Elemental analyser. IR spectra were recorded in KBr pellets in Nujol mulls on a PerkinElmer, Inc. Precisely Spectrum One FT-IR spectrometry. UV spectra in $CHCl_3$ were recorded on PerkinElmer, Inc. Lambda 35 UV/VIS Spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on VarianUNITYINOVA operating at 500 MHz. Mass spectra were obtained on a Thermo Finnigan LCQ Advantage MAX LC-MS/MS spectrometer according to ESI probe. Products were isolated by column chromatography on Silica gel (Fluka Silica gel 60, particle size 63–200 μm). All chemicals were reagent grade and used without further purification. Moisture was excluded from the glass apparatus using $CaCl_2$ drying tubes. Solvents, unless otherwise specified, were of reagent grade and distilled once prior to use.

General Methods for the Synthesis of 1,4-Naphtho- and Benzoquinones

General Method 1

Sodium carbonate (1.52 g) was dissolved (60 mL) in ethanol. 2,3-Dichloro-1,4-naphthoquinone **1** and nuchleophilies (**2a**, **3**, **6**) were added slowly to this solution. Without heating, the mixture was stirred for 6 h. The colour of the solution quickly changed and the extent of the reaction was monitored by TLC. Chloroform (30 mL) was added to the reaction mixture. The organic layer was washed with water (4×30 mL), and dried with Na_2SO_4. After the solvent was evaporated the residue was purified by column chromatography on silica gel.

General Method 2

2,3-Dichloro-1,4-naphthoquinone **1** and nuchleophilies (**2b**, **c**, **11**) were stirred in chloroform (25 mL) for 8 h. Chloroform (30 mL) was added to the reaction mixture. The organic layer was washed with water (4×30 mL), and dried with Na_2SO_4. After the solvent was evaporated the residue was purified by column chromatography on silica gel.

General Method 3

p-Chloranil **16** and nuchleophilies (**2b**, **11**) were stirred in triethylamine (1 mL) in chloroform (25 mL) for 6 h. The extent of the reaction was monitored by TLC. Chloroform (30 mL) was added to the reaction mixture. The organic layer was washed with water (4×30 mL), and dried with Na_2SO_4. After solvent recovery, the crude product was purified by chromatography.

2-(1-Ethylsulfanyl)-3-(1-*N*-diphenylmethylpiperazin-1-yl)-1,4-naphthoquinone (4a) Compound **4a** was synthesized from the reaction of **1** (1.0 g, 4.4 mmol) with **2a** (1.1 g, 4.4 mmol) and **3** (0.27 g, 4.4 mmol) according to general method 1. Red solid, Yield: 1.2 g, 58%, mp: 124–125°C, *Rf*=0.45 ($CHCl_3$), FT-IR (in KBR pellet, cm^{-1}): 3050 (Ar-H), 2957, 2923 (C-H), 1664 (C=O), 1592, 1537 (C=C), 1279 (C-N). UV-Vis [$CHCl_3$, λ (log ε)]: 206 (3.5), 226 (3.4), 248 (3.5), 286 (3.4), 498 (2.5). ^{1}H-NMR (499.74 MHz, $CDCl_3$): 1.2 (t, *J*=7.32 Hz, 3H, CH_3), 3.00 (q, 2H, S-CH_2), 2.5 (4H, brs, H_{piper}), 3.6 (4H, brs, H_{piper}), 4.3 (1H, s, –CH<), 7.0–8.0 ppm (14H, m, H_{arom}). ^{13}C-NMR (125.66 Hz, $CDCl_3$) δ: 13.76 (CH_3), 33.87 (S-CH_2), 44.62, 51.61 (N-CH_2), 73.83 (–CH<), 125.55, 126.53, 126.76, 126.91, 127.26, 127.47, 127.57, 127.81, 127.85, 128.00, 128.69, 129.03, 131.08, 131.38, 131.87, 132.65, 133.17, 133.61, 136.64 (CH_{arom}, C_{arom}), 146.09 (=C-S), 162.60 (=C-N), 180.68, 181.10 ppm (C=O). MS [+ESI]: *m/z* 469 [M]$^+$. *Anal.* Calcd for $C_{29}H_{23}N_2S_1O_2$ (M=468.62 g/mol) C, 74.32; H, 6.02; N, 5.97; S, 6.48. Found C, 74.30; H, 6.06; N, 5.98; S, 6.43%.

2-(*N*-Diphenylmethylpiperazin-1-yl)-3-benzylsulfanyl-1,4-naphthoquinone (7a) Compound **7a** was synthesized from the reaction of **1** (1.0 g, 4.4 mmol) with **2a** (1.1 g, 4.4 mmol) and **6** (0.54 g, 4.4 mmol) and according to general method 1. Red solid, Yield: 1.8 g, 81%, mp: 126–127°C, *Rf*=0.42 ($CHCl_3$), FT-IR (in KBR pellet, cm^{-1}): 3026 (Ar-H), 2894, 2816 (C-H), 1666 (C=O), 1593, 1521 (C=C), 1281 (C-N). UV-Vis [$CHCl_3$, λ (log ε)]: 206 (4.1), 220 (4.1), 241 (4.2), 291 (4.1), 512 (3.5). ^{1}H-NMR (499.74 MHz, $CDCl_3$): 2.4 (4H, brs, H_{piper}), 3.4 (t, *J*=4.88 Hz, 4H, H_{piper}), 3.9 (s, 2H, S-CH_2), 4.2 (1H, s, –CH<), 7.0–8.1 ppm (m, 24H, H_{arom}). ^{13}C-NMR (125.66 Hz, $CDCl_3$) δ: 38.15 (S-CH_2), 51.53, 51.98 (N-CH_2), 75.14 (–CH<), 120.95, 125.21, 125.47, 125.53, 125.95, 126.07, 126.51, 126.95, 127.08, 127.20, 127.24, 127.34, 127.38, 127.45, 127.54, 127.74, 127.97, 128.16, 131.05, 131.52, 131.99, 132.62, 137.06 (CH_{arom}, C_{arom}), 141.32 (=C-S), 154.87 (=C-N), 180.68, 181.01 ppm (C=O). MS [+ESI]: *m/z* 531 [M]$^+$, MS/MS [+ESI]: *m/z* 363 [M−168]$^+$. *Anal.* Calcd for $C_{34}H_{30}N_2O_2S_1$ (M=530.69 g/mol) C, 76.95; H, 5.69; N, 5.27. Found C, 76.97; H, 5.64; N, 5.65%.

2,3-Bis(Benzylsulfanyl)-1,4-naphthoquinone (8) Compound **8** was synthesized from the reaction of **1** (1.0 g, 4.4 mmol) with **2a** (1.1 g, 4.4 mmol) and **6** (0.54 g, 4.4 mmol) according to general method 1. Red crystal, Yield: 0.3 g, 17%, mp: 183–184°C (183°C[14]), *Rf*=0.52 ($CHCl_3$), FT-IR (in KBR pellet, cm^{-1}): 3020 (Ar-H), 2892, 2805 (C-H), 1651 (C=O), 1591, 1463 (C=C). UV-Vis [$CHCl_3$, λ (log ε)]: 208 (4.8), 214 (4.8), 241 (4.8), 284 (4.5), 468 (3.9). ^{1}H-NMR (499.74 MHz, $CDCl_3$): 4.2 (s, 4H, S-CH_2), 7.1–8.1 ppm (m, 14H, H_{arom}). ^{13}C-NMR (125.66 Hz, $CDCl_3$) δ: 38.23 (S-CH_2), 125.76, 126.39, 127.46, 127.53, 128.17, 128.39, 131.88, 132.40, 136.18 (CH_{arom}, C_{arom}), 146.69 (=C-S), 178.09 ppm (C=O). MS [+ESI]: *m/z* 403 [M]$^+$, 425 [M+Na]$^+$, MS/MS [+ESI]: *m/z* 303 [(M+Na)−123]$^+$, 281 [M−123]$^+$. *Anal.* Calcd for $C_{24}H_{18}S_2O_2$ (M=402.538 g/mol). C, 71.61; H, 4.51; S, 15.93. Found C, 71.55; H, 4.52; S, 15.95% (C, 71.30; H, 4.50%[14]).

2-(*N*-Diphenylmethylpiperazin-1-yl)-3-chloro-1,4-naphthoquinone (9a) Compound **9a** was synthesized from the reaction of **1** (1.0 g, 4.4 mmol) with **2a** (2.2 g, 8.8 mmol) according to general method 1. Brown oil, Yield: 2.8 g, 72%. *Rf*=0.41 ($CHCl_3$), FT-IR (in KBR pellet, cm^{-1}): 3025 (Ar-H), 2959, 2813 (C-H), 1648 (C=O), 1592, 1556 (C=C), 1282 (C-N). UV-Vis [$CHCl_3$, λ (log ε)]: 204 (4.6), 225 (4.0), 237 (4.0), 276 (4.0), 480 (3.4). ^{1}H-NMR (499.74 MHz, $CDCl_3$): 2.5 (4H, brs, H_{piper}), 3.6 (4H, brs, H_{piper}), 4.2 (1H, s, –CH<), 7.0–8.0 ppm (14H, m, H_{arom}). ^{13}C-NMR (125.66 Hz, $CDCl_3$) δ: 50.66, 51.74 (N-CH_2), 75.21 (–CH<), 121.59, 125.48, 125.83, 126.14, 126.49, 126.93, 127.19, 127.24, 127.62, 127.83, 129.01, 130.45, 130.63, 131.35, 131.95, 132.98 (CH_{arom}, C_{arom}), 141.28 (=C-N), 148.9 (=C-Cl), 176.94, 180.81 ppm (C=O). MS [+ESI]: *m/z* 443 [M]$^+$. *Anal.* Calcd for $C_{27}H_{23}N_2O_2Cl_1$ (M=442.94 g/mol) C, 73.21; H, 5.23; N, 6.32. Found C, 73.20; H, 5.26; N, 6.31%.

2-[1-Piperonylpiperazin-1-yl]-3-chloro-1,4-naphthoquinone (9b) Compound **9b** was synthesized from the reaction of **1** (1.0 g, 4.4 mmol) with **2b** (2.9 g, 13.2 mmol) according to general method 2. Red solid, Yield: 1.2 g, 66%. mp: 169–170°C, *Rf*=0.41 (CH_2Cl_2), FT-IR (in KBR pellet, cm^{-1}): 3030 (Ar-H), 2908, 2887, 2809, 2768 (C-H), 1676, 1637 (C=O), 1592, 1558 (C=C). UV-Vis [$CHCl_3$, λ (log ε)]: 210 (4.0), 221 (4.6), 246 (4.4), 282 (4.6), 491 (3.8). ^{1}H-NMR (499.74 MHz, $CDCl_3$): 2.6 (br s, 4H, H_{piper}), 3.4 (s, 2H, N-CH_2), 3.6 (4H, br s, H_{piper}), 5.84 (s, 2H, O-CH_2-O), 6.6–8.1 ppm (7H, m, H_{arom}). ^{13}C-NMR (125.66 Hz, $CDCl_3$) δ: 51.33, 61.05 (N-CH_2), 53.68 (N-CH_2), 101.19 (O-CH_2-O), 108.18, 109.82, 122.77, 123.16, 126.71, 127.05, 130.95, 131.63, 131.80, 133.25, 134.25, 147.16 (CH_{arom}, C_{arom}), 147.99 (=C-N), 150.09 (=C-Cl), 178.14, 181.98 ppm (C=O). MS [+ESI]: *m/z* 412 $[M+H]^+$. *Anal.* Calcd for $C_{22}H_{19}N_2O_4Cl_1$ (M=410.86 g/mol) C, 64.31; H, 4.66; N, 6.81. Found C, 64.10; H, 4.68; N, 6.78%.

2,2′-[1-(2-Aminoethyl)piperazin-1-yl]-3,3′-dichlorobis(1,4-naphthoquinone) (10) Compound **10** was synthesized from the reaction of **1** (1.0 g, 4.4 mmol) with **2c** (0.57 g, 4.4 mmol) according to general method 2. Red solid. mp: 167–168°C, Yield: 1.9 g, 86%. *Rf*=0.42 ($CHCl_3$), FT-IR (in KBR pellet, cm^{-1}): 3330 (N-H), 3067 (Ar-H), 2947, 2815 (C-H), 1670 (C=O), 1574, 1524 (C=C). UV-Vis [$CHCl_3$, λ (log ε)]: 207 (4.5), 219 (4.5), 237 (4.5), 257 (4.4), 268 (4.5), 472 (3.8). ^{1}H-NMR (499.74 MHz, $CDCl_3$): 2.9 (s, H_{piper}, 4H), 3.4 (t, *J*=6.84 Hz, 2H, NH-CH_2-C***H***$_2$-), 3.6 (q, NH-CH_2-, 2H), 3.7 (s, H_{piper}, 4H), 4.2 (t, *J*=5.37 Hz, NH), 7.4–8.4 ppm (m, 4H, H_{arom}). ^{13}C-NMR (125.66 Hz, $CDCl_3$) δ: 47.87 (NH-CH_2-***C***H_2-), 49.35 (NH-CH_2-), 52.43, 56.27 (CH_{2piper}), 121.64, 123.71, 125.70, 125.76, 125.96, 128.97, 130.36, 130.53, 131.50, 132.30, 133.22, 133.84 (CH_{arom}, C_{arom}), 143.51 (=C-N), 148.52 (=C-Cl), 179.49, 180.75 ppm (C=O). MS [+ESI]: *m/z* 510 $[M]^+$, MS/MS [+ESI]: *m/z* 473 [M−37]. *Anal.* Calcd for $C_{26}H_{21}N_3O_4Cl_2$ (M=510.38 g/mol). C, 61.18; H, 4.14; N, 8.23. Found C, 61.19; H, 4.15; N, 8.28%.

2-[4-(2-Aminoethyl)morpholin-1-yl]-3-chloro-1,4-naphthoquinone (12) Compound **12** was synthesized from the reaction of **1** (1.0 g, 4.4 mmol) with **11** (1.72 g, 13.2 mmol) according to general method 2. Dark red solid, Yield: 0.9 g, 64%, mp: 140–141°C, *Rf*=0.50 (EtAc), FT-IR (in KBR pellet, cm^{-1}): 3342 (N-H), 3210 (Ar-H), 2966, 2849 (C-H), 1672 (C=O), 1571, 1519 (C=C). UV-Vis [$CHCl_3$, λ (log ε)]: 205 (4.5), 212 (4.5), 223 (4.5), 238 (4.5), 272 (4.3), 455 (3.5). *Anal.* Calcd for $C_{16}H_{17}N_2O_3Cl_1$ (M=320.77 g/mol) C, 59.91; H, 5.34; N, 8.73. Found C, 59.89; H, 5.35; N, 8.75%).

2,5-Bis[4-(2-aminoethyl)morpholin-1-yl]-3,6-dichloro-1,4-benzoquinone (17) Compound **17** was synthesized from the reaction of **16** (1.0 g, 4.06 mmol) with **11** (2.1 g, 16.13 mmol) according to general method 3. Yellow solid. mp: 205–206°C. Yield: 1.5 g (85%). *Rf*=0.52 (EtAc). FT-IR (in KBR pellet, cm^{-1}): 3245 (N-H), 3013 (Ar-H), 2980, 2956, 2929, 2894, 2855, 2814 (C-H), 1614 (C=O), 1560, 1492 (C=C). UV-Vis [$CHCl_3$, λ (log ε)]: 202 (3.7), 213 (3.8), 226 (3.8), 234 (3.9), 243 (4.2), 361 (4.4), 536 (2.5). ^{1}H-NMR (499.74 MHz, $CDCl_3$): δ=2.4 (t, *J*=8.3 Hz, 8H, H_{morph}), 3.6 (t, *J*=7.32 Hz, 8H, H_{morph}), 2.58 (t, *J*=6.35 Hz, 4H, NH-CH_2-C***H***$_2$), 3.9 (q, 4H, NH-CH_2), 7.73 (s, 2H, NH) ^{13}C-NMR (125.66 MHz, $CDCl_3$): δ=39.76 (NH-CH_2-***C***H_2-), 55.47 (NH-CH_2-), 52.02, 65.93 (CH_{2morph}), 139.49 (=C-N), 144.41 (=C-Cl), 171.29 ppm (C=O). MS [+ESI]: *m/z* 433 $[M]^+$, MS/MS [+ESI]: *m/z* 396 $[M-37]^+$, *Anal.* Calcd for $C_{18}H_{26}N_4O_4Cl_2$ (M=433.33 g/mol): C, 49.89; H, 6.04; N, 12.92%. Found: C, 49.90; H, 6.06; N, 12.83%.

2,5-Bis[1-piperonylpiperazin-1-yl]-3,6-dichloro-1,4-benzoquinone (18) Compound **18** was synthesized from the reaction of **16** (1.0 g, 4.06 mmol) with **2b** (3.58 g, 13.2 mmol) according to general method 3. Brown solid, mp: 197–198°C. Yield: 2.1 g, 84%. *Rf*=0.32 [$CHCl_3$: EtAc (3 : 1)], FT-IR (in KBR pellet, cm^{-1}): 3013 (Ar-H), 2946, 2910, 2895, 2853, 2814, 2765 (C-H), 1656 (C=O), 1577, 1499 (C=C). UV-Vis [$CHCl_3$, λ (log ε)]: 204 (4.0), 220 (4.0), 228 (4.6), 233 (4.4), 244 (4.4), 283 (4.1), 384 (3.8), 438 (4.1). ^{1}H-NMR (499.74 MHz, $CDCl_3$): 2.5 (s, 8H, H_{piper}), 3.4 (s, 4H, N-CH_2), 3.5 (t, *J*=4.8 Hz, 8H, H_{piper}), 5.87 (s, 4H, O-CH_2-O), 6.6–6.8 ppm (m, 6H, H_{arom}). ^{13}C-NMR (125.66 Hz, $CDCl_3$) δ: 50.80, 61.66 (N-CH_2), 52.58 (N-CH_2), 99.91 (O-CH_2-O), 106.90, 108.40, 114.81, 121.21, 130.66, 145.75 (CH_{arom}, C_{arom}), 146.73 (=C-N), 147.45 (=C-Cl), 175.00 ppm (C=O). MS [+ESI]: *m/z* 613 $[M]^+$, MS/MS [+ESI]: *m/z* 577 $[M-37]^+$. *Anal.* Calcd for $C_{30}H_{30}N_4O_6Cl_2$ (M=613.502 g/mol). C, 58.73; H, 4.92; N, 9.13%. Found C, 58.74; H, 4.98; N, 9.28%.

Antibacterial and Antifungal Evaluation

Diffusion Method[25] Antibacterial activity of compounds was evaluated by diffusion in peptone on nutrient medium (meat-extract agar for bacteria; wort agar for fungi). The microbial loading was 109 cells (spores)/1 mL. The required incubation periods were as: 24 h at 35°C for bacteria and 48–72 h at 28–30°C for fungi. The results were recorded by measuring the zones surrounding the disk. Control disk contained Vancomicine (for bacteria), Oxacilinum (for bacteria), or Nistatine (for fungi) as a standard.

Serial Dilution Method[26] Testing was performed in a flat-bottomed 96-well tissue culture plate. The tested compounds were dissolved in dimethyl sulfoxide (DMSO) to the necessary concentration. The exact volume of solution of compounds is brought in nutrient medium. The inoculum of bacteria and fungi was in nutrient medium (meat-extract agar for bacteria; wort agar for fungi). The duration of incubation was at 37°C for bacteria and 30°C for fungi over 24–72 h. The results were estimated according to the presence or absence of microorganism growth.

The microorganisms that were tested included the following: bacteria *E. coli* B-906, *S. aureus* 209-P, and *M. luteum* B-917 and fungi *C. tenuis* VKM Y-70, and *A. niger* F-1119.

Antioxidant Capacity The CUPRAC reagent solutions were prepared as follows: $CuCl_2$ solution (10 mM) was prepared in distilled water, ammonium acetate solution (1.0 M, pH=7) and neocuproine solution (7.5 mM) were prepared in pure ethanol for the CUPRAC assay, as a difference from the original CUPRAC method[31] where ammonium acetate buffer is prepared in distilled water. The solutions of all other compounds were freshly prepared in DMSO.

One milliliter $CuCl_2$, 1 mL Nc solution, and 1 mL NH_4Ac solution were added to *x* mL of the tested compound, followed by (1.1−*x*) mL DMSO. The absorbance of the final solution (of 4.1 mL total volume) at 450 nm was read against a reagent blank after 30 min standing at room temperature.[31] The absorbance of the emerging cuprous neocuproine chromophore was correlated to tested compound concentration.

ROS Scavenging Activity

HRS Activity

$OH^{\cdot}$ in aqueous media were generated through the Fenton system and spectrophotometrically determined—*via* hydroxylation of a probe—by the modified CUPRAC method.[35] To a test tube were added 1.5 mL of phosphate buffer (pH 7.0), 0.5 mL of 10 mM sodium salicylate, 0.25 mL of 20 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.25 mL of 20 mM $FeCl_2$ solution, 18 mL H_2O, (*x*) mL sample solution (*x* varying between 0.1 and 0.5 mL) at a concentration of 3.0×10^{-5} M, and 0.5 mL of 10 mM H_2O_2 rapidly in this order. The mixture in a total volume of 5 mL was incubated for 10 min in a water bath kept at 37°C. After incubation, the reaction was stopped with adding 0.5 mL of 268 U mL^{-1} catalase solution, and mixed for 30 s. Final mixtures (0.5 mL of the incubation solution) were subjected to the HRS-CUPRAC method. The HRS activity (%) of samples was calculated using the equation:

$$\text{HRS}\,(\%) = [(A_0 - A)/A_0]\times100$$

where A_0 and A are the CUPRAC absorbances of the system in the absence and presence of sample, respectively.

HPS Activity

The ability of compounds to scavenge H_2O_2 was determined according to the method of Özyürek *et al*.[35] To a test tube were added 0.7 mL of phosphate buffer (pH 7.4), 0.4 mL of 1 mM H_2O_2, 0.4 mL of 0.1 mM $CuCl_2{\cdot}2H_2O$ in this order (H_2O_2 incubation solution, used as reference). To the other two test tubes were added 0.5 mL of phosphate buffer (pH 7.4), 0.4 mL of 1.0 mM H_2O_2, 0.2 mL sample (3.0×10^{-5} M), and 0.4 mL of 0.1 mM $CuCl_2$. $2H_2O$ solution rapidly in this order (named as scavenger solutions-I, -II). The mixtures in a total volume of 1.5 mL were incubated for 30 min in a water bath kept at 37°C. At the end of this period, to both reference and scavenger solution-I was added 0.4 mL H_2O, and to scavenger solution-II was added 0.4 mL of 268 U mL^{-1} catalase solution, and mixed for 30 s. Final mixtures (1.0 mL of the incubation solution) were subjected to the HPS-CUPRAC method. The HPS activity (%) of sample was calculated using the equation:

$$\text{HPS}\,(\%) = [(A_0 - (A_1 - A_2))/A_0]\times100$$

where A_0 is the CUPRAC absorbance of reference H_2O_2 incubation solution, A_1 and A_2 are the CUPRAC absorbances of scavenger solutions-I and -II, respectively.

SARS Activity

The superoxide anion radicals ($O_2^{\cdot-}$) were generated *in vitro* in a non-enzymatic system (PMS-NADH) and determined spectrophotometrically by nitroblue tetrazolium (NBT) reduction method described by Yu *et al*.[38] To a test tube were added 2.3 mL DMSO, 0.2 mL of sample (3.0×10^{-5} M), 2 mL of 468 μM NADH, 1 mL of 300 μM NBT, in this order. The reaction was started by adding 1 mL of 60 μM PMS solution to the incubation mixture. The mixture in a total volume of 6.5 mL was incubated for 5 min in a water bath kept at 25°C, and the absorbance was read at 560 nm against DMSO. Decreased absorbance of the incubation reaction mixture indicated increased superoxide anion radical scavenging activity. The SARS (%) of sample was calculated using the equation:

$$\text{SARS}\,(\%) = [(A_0 - A)/A_0]\times100$$

where A_0 and A are the absorbances of the incubation reaction mixture in the absence and presence of scavenger, respectively.

Cytotoxic Activity

Chemicals and Cell Culture

All compounds were dissolved in DMSO at a concentration of 25 mM as a stock solution. Further dilutions were made in culture medium. DMSO final concentration was 0.1%. All tested cells, A549 (lung), MCF-7 (breast), DU145 (prostate) and HT-29 (colon), were cultured in RPMI 1640 medium supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), L-glutamine, and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

The SRB Viability Assay

To detect the cytotoxicity of compounds against tested cell lines, firstly all compounds were used at 20 μM concentrations. Cells were seeded at a density of 5×10^3 cells per well of 96-well culture plate in 100 μL medium in triplicate. After 24 h, compounds were added at 20 μM concentrations and cells were incubated with these different compounds for 48 h.

At the end of treatment, 50 μL of 50% trichloroacetic acid (TCA) was added and fixation was allowed to proceed for 1 h at 4°C. After fixation, the supernatant was discarded and the plate was washed with deionized water five times. TCA-fixed cells were stained with 50 μL of SRB solution (0.4% in 1% acetic acid) for 30 min at room temperature. After staining, the unbound SRB was washed out with 1% acetic acid and air-dried. Bound SRB was solubilized with 150 μL of Tris base (10 mM, pH 10.0) and then plate was shaken for 10 min at 150 rpm. The 96-well plate was read by a spectrophotometer at 570 nm.

The ATP Viability Assay

MCF-7 and DU145 were seeded as exactly the same as done in the SRB assay. After 24 h, compounds (**9b** and **10**) were added at different concentrations (0.39–50 μM) and cells were incubated with these compounds for 48 h.

In order to carry out the ATP assay, 150 μL of medium was removed from each well and 50 μL of somatic cell ATP releasing agent (ATP Bioluminescent Somatic Cell Assay Kit, Sigma-Aldrich, St. Louis, MO, U.S.A.) was added. After mixing thoroughly, the microplate was allowed to stand on the bench for 20–30 min at room temperature. At the end of the incubation 50 μL of mixture from each well was transferred to a white non-translucent plate. To each well of the plate, 50 μL luciferin–luciferase reagent (ATP Bioluminescent Somatic Cell Assay Kit, Sigma-Aldrich) was added and the 96-well plate was measured using a count integration time of 1 s at luminometer (Bio-Tek, VT, U.S.A.).

Statistical Analyses All of the statistical analyses of cell viability assays were performed by using the Graphpad Prism 6.0 statistical software for Windows. All results were expressed as mean±standard deviation (S.D.).

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Conflict of Interest The authors declare no conflict of interest.

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