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Free-Radical Scavenging Activities of 2-Benzoxazolinone Derivatives Containing Thiosemicarbazide, Triazole, Thiadiazole and Hydrazone Units

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Introduction

The development of an effective therapeutic agent for the treatment of inflammation continues to be a challenging problem in medicinal chemistry research. The therapeutic use of nonsteroidal antiinflammatory drugs (NSAIDs) is often limited by common side effects, such as gastrointestinal hemorrhage and ulceration[1]. Therefore, a major challenge of the pharmaceutical industry is to develop drugs that have antiinflammatory activities but lack the toxic side effects associated with currently used NSAIDs.

Reactive oxygen species (ROS) have gained a lot of importance because of their active role in many diseases[2]. The damage caused by ROS is called oxidative stress. It is caused by an imbalance between the production of ROS and a biological system's ability to detoxify the reactive intermediates or repair the resulting damage. A particularly negative side of oxidative stress is the production of ROS, which include free radicals. Human bodies are constantly exposed to ROS generated from endogenous and some exogenous sources. Antioxidants, both enzymatic

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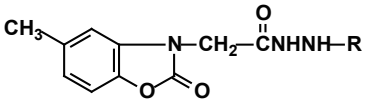
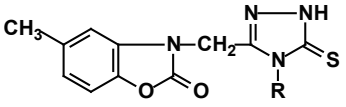
and nonenzymatic, prevent oxidative damage to biological molecules by various mechanisms[3].

It has been speculated that NSAIDs possess antioxidant activity and can act as the free radical scavengers. A considerable number of the benzoxazolinone derivatives have shown analgesic-antiinflammatory activity comparable to or higher than that of indomethacine[4-8]. Many reports indicate that acylthiosemicarbazides and their corresponding cyclized 1,2,4-triazoles and thiadiazoles derivatives possess analgesic-antiinflammatory activities[9-13]. Hydrazone derivatives possessing antiinflammatory and analgesic activities are also reported in the literature[14-17].

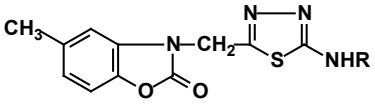
In a previous study, we had synthesized thiosemicarbazide, 1,2,4-triazole, 1,3,4-thiadiazoles and hidrazon derivatives[8] (Scheme 1) and demonstrated that they possess moderate antiinflammatory activities against carrageenan induced mice paw edema. In the light of this knowledge, all of these have made us thought that these compounds could possess antioxidant properties. Because of this, we have investigated the effects of these chemical moieties on free radical scavenging activity related to the antioxidant system.



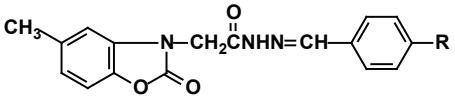
1: R:H; 2: R:CSNHCH$_3$; 3: R: CSNHC$_2$H$_5$
4: R:CSNHC$_3$H$_5$; 5: R: CSNHC$_6$H$_5$



6: R:CH$_3$; 7: R:C$_2$H$_5$; 8: R: C$_3$H$_5$; 9:C^{6}H$_5$



10: R:CH$_3$; 11: R:C$_2$H$_5$; 12: C$_6$H$_5$



13: R:H; 14: R:Br; 15: R:Cl; 16: R:CH$_3$; 17: R:OCH$_3$; 18: R:OH

Scheme 1: Chemical structures of the compounds

Material and Methods

Chemicals

The chemicals were purchased from Sigma-Aldrich™and Merck (Germany). Compounds were synthesized according to the method reported in our previous publication[8]. The purity of the novel compounds was checked by thin layer chromatography and elemental analyses. The structures of the compounds were assigned on the basis of spectral data such as UV, IR, ^{1}H-NMR, ^{13}C-NMR and MS. Chemical structures of the compounds were summarized in scheme 1.

Scavenging Capacity of DPPH

Hydrogen atom or electron-donation ability of the corresponding compounds were measured spectrophotometrically from the bleaching of the purple-colored methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH)[18]. 5 mL of various dilutions of the compounds in dimethyl sulfoxide (DMSO; final concentration 0-80 μM) were mixed with 0.5 mL of 0.004% methanol solution of DPPH. After an incubation period of 30 min at 37^{0}C, the absorbance of the samples was measured at 517 nm. Ascorbic acid was used as reference compound. Inhibition of DPPH free radical in percent (I%) was calculated in following way:

$$I\ \% = (A_{blank} - A_{sample}/A_{blank}) \times 100;$$

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 compound. Compound concentration providing 50% scavenging (EC_{50}) was calculated from the graph plotting inhibition percentage against compound concentration. Tests were carried out in triplicate.

Scavenging Capacity of Superoxide Anion Radical

Scavenging capacity of superoxide anion radical was evaluated by the method of Nagai et al.[19]. Incubation mixture contained 0.48 ml of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 ml of 3 mM xanthine, 0.02 ml of 3 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.02 ml of 0.15% bovine serum albumin, 0.02 ml of 0.75 mM nitroblue tetrazolium (NBT) and 0.02 ml of sample solution prepared as various

dilutions of the compounds in DMSO (final concentration 0, 10, 20, 40, 60, 80 μM). Under the present experimental conditions, DMSO did not interfere with the assay. After incubation at 25° for 10 min, the reaction was started by adding 6 mU xanthine oxidase (XOD) and carried out at 25° for 20 min. After 20 min the reaction was stopped by adding 0.02 ml of 6 mM CuCl. The absorbance of the reaction mixture was measured at 560 nm and the inhibition rate was calculated by measuring the amount of the formazan that was reduced from NBT by superoxide.

Dose dependent superoxide anion radical scavenging effects of the synthesized compounds were expressed as EC_{50} value (concentration required for 50% of scavenging capacity). Ascorbic acid and butylated hydroxytoluene (BHT) were used as the reference compounds.

Scavenging Capacity of Hydrogen Peroxide

The ability of compounds to scavenge hydrogen peroxide was determined according to a previous method[20]. Hydrogen peroxide solution (2 mmol/l) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically at 230 nm with molar absorpivity 81 $M^{-1}cm^{-1}$. Compound dilutions was performed from compound stock solutions in DMSO (final concentration 0-200 μM). These prepared dilutions were added to 0.6 mL of hydrogen peroxide solution. Under the present experimental conditions, DMSO did not interfere with the assay. The absorbance of hydrogen peroxide was measured after 10 min of incubation against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background substraction. The activity was determined as a function of % inhibition which was calculated using the formula $[(A_0-A_1)/A_0]$ x100, where A_0 is the absorbance of the control and A_1 is the absorbance of the compound/standard. Ascorbic acid was used as the reference compound. Dose dependent hydrogen peroxide scavenging effects of the synthesized compounds were expressed as EC_{50} values.

Scavenging Capacity of Hydroxyl radical

The hydroxyl radical-scavenging ability of compounds were determined using 2-deoxy-D-ribose oxidative degradation mediated by hydroxyl radicals as described by Halliwell[21]. The reaction mixture contained various dilutions of the compounds in DMSO (final concentration 0-100

μM; 0.5 mL), 0.1 ml of 2.8 mM 2-deoxy-D-ribose, 0.2 ml of a premixed 100 μM $FeCl_3$, and 104 μM EDTA solution (1:1, v/v), 0.1 ml of 1 mM hydrogen peroxide and 0.1 ml of 0.1 mM ascorbic acid, was incubated at 37^0C for 60 min. Under the present experimental conditions, DMSO did not interfere with the assay. Thereafter, 1.0 ml of 0.5% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid was added and the mixtures were vortexed and heated in a water bath at 100^0C for 15 min. The reaction was stopped by a 5 min ice water bath. The mixtures were centrifuged at 12,000x*g* for 5 min at room temperature, and the absorbance of the supernatants were measured at 532 nm. The scavenging effect of hydroxyl radicals (%) was calculated using the following:

$$\text{Scavenging effect (\%)} = (1 - A_{samples}/A_{control}) \times 100,$$

where $A_{samples}$ is the absorbance in the presence of the tested samples, and $A_{control}$ is the absorbance of the control contained all the reaction reagents except the tested samples. Dose dependent hydroxyl radical scavenging effects of the synthesized compounds were expressed as EC_{50} values. Ascorbic acid was used as the reference compound.

Scavenging Capacity of Nitric Oxide

The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent[22]. Briefly, sodium nitroprusside (5mM) in phosphate buffer pH 7.4 was incubated with the novel compounds dissolved in DMSO (final concentration 0-100 μM) and the mixtures were incubated at 25°C for 120 min. Control experiment was conducted with equal amount of solvent in an identical manner. At intervals, 0.5ml of incubated solution was taken and diluted with 0.5 ml of griess reagent (1% sulfanilamide, 0.1% *N*-naphthylethylenediamine dihydrochloride and 2% orthophosphoric acid dissolved in distilled water). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent *N*-naphthylethylenediamine dihydrochloride was measured at 546 nm. Dose dependent nitric oxide scavenging effects of the synthesized compounds were expressed as EC_{50} values. Ascorbic acid and BHT were used as the reference compound.

Determination of Reducing Power

The reductive potential of the novel compounds was determined according to the method of Oyaizu[23]. Various dilutions of the compounds in DMSO (final concentration 0-100 μM) and ascorbic acid were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1% w/v). Under the present experimental conditions, DMSO did not interfere with the assay. The mixture was incubated at 50^0C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10% w/v) was added to the mixture, which was then centrifuged for 10 min at 1000xg. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1% w/v), and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. Dose dependent reducing power of the synthesized compounds were expressed as EC_{50} values Ascorbic acid was used as the reference compound.

Oxidative Hemolysis Inhibition Assay

Erythrocytes have been widely used as a model to investigate oxidative damage in biomembranes due to their high susceptibility to peroxidation. The *in vitro* inhibition of rat erythrocyte hemolysis with the novel compounds was assessed according to the procedure described by Yuan[24]. Blood was obtained from rats by cardiac puncture and collected in heparinized tubes. The Ethics Committee of Laboratory Animals at Hacettepe University, Turkey, approved the animal study. Erythrocytes were separated from plasma and the buffy coat and washed three times with 10 volumes of 0.15 M NaCI. During the last washing, the erythrocytes were centrifuged at 1500xg for 10 min to obtain a constantly packed cell preparation. Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system. A 10% suspension of erythrocytes in pH 7.4 phosphate buffered saline (PBS) was added to the same volume of 200 mM 2.2'-azobis(2-amidinopropane) dihydrochloride (AAPH) solution (in PBS) containing samples (final concentration range 0-100 μM). The reaction mixture was shaken gently while being incubated at 37°C for 2 h. The reaction mixture was then removed, diluted with 8 volumes of PBS and centrifuged at 2.500 rpm for 10 min. The absorbance (A_{sample}) of the resulting supernatant was measured at 540 nm. Similarly, the reaction mixture was treated with 8 ml of distilled water to obtain complete

hemolysis and the absorbance ($A_{control}$) of its supernatant in the same conditions was measured at 540 nm. The inhibition ratio (%) was calculated using the following formula:

Inhibition (%)= $(1-A_{sample}/ A_{control}) \times 100$

Ascorbic acid was used as a reference compound.

Determination of metal chelating activity

The chelation of ferrous ions by the novel compounds was estimated by the method of Dinis et al.[25] with slight modifications and compared with that of ascorbic acid. Sample solutions prepared as various dilutions of the compounds in DMSO (final concentration 0-120 μM) were added to a solution of 1 mM $FeCl_2$ (0.05 ml). Under the present experimental conditions, DMSO did not interfere with the assay. The reaction was initiated by the addition of 1 mM ferrozine (0.1 ml) and the mixture was finally quantified to 1 ml with methanol, shaken vigorously and left standing at room temperature for 10 min. The final concentrations of $FeCl_2$ and ferrozine in the reaction mixture were 0.05 and 0.10 mM respectively. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the formula of % Inhibition= $[(A_0-A_1)/A_0 x100]$, where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the compound. BHT was used as the reference compound.

Results and Discussion

1,2,4-Triazoles, 1,3,4-thiadiazoles and their condensed derivatives have been shown to exhibit analgesic-anti-inflammatory and antimicrobial activities[26-28]. The encouraging biological activities of these heterocycles prompted us to combine 2-benzoxazolinone with 1,2,4-triazole, 1,3,4-thiadiazoles. Our aim was to investigate the possible antioxidant power of the triazole, thiadiazole moieties and the para-phenyl substituent of the hydrazone subunit.

ROS, which are generated from many redox processes, are major free radicals in the human body that are capable of inducing damage to

biomolecules such as carbohydrates, proteins, lipids, and DNA. Intracellular oxidative damage by ROS causes many chronic diseases, including neurodegenerative and cardiovascular diseases and cancer[29-31]. Electron acceptors, such as molecular oxygen, react rapidly with free radicals to become radicals themselves, also referred to as reactive oxygen species (ROS). The ROS include superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$)[32-34]. Lipid peroxidation, which involves a series of free radical mediated chain reaction processes, is also associated with several types of biological damage[35,36]. Minimising the cellular redox imbalance may be one of the most important approaches to the prevention of these ageing-related diseases. Antioxidants scavenge free radicals initiating and propagating oxidative chain reactions, and thus they can delay or prevent intracellular oxidative damage[29].

The antioxidant capacity of the compounds were studied by invitro determination of DPPH scavenging capacity, superoxide anion radical scavenging capacity, hydrogen peroxide scavenging capacity, hydroxyl radical scavenging capacity, nitric oxide radical scavenging capacity, reducing power, metal chelating activity and oxidative hemolysis inhibition activity. The results are summarized in Table 1 and Figures 1-7. Ascorbic acid and/or BHT were used as reference compounds.

In order to probe structural requirements for optimal antioxidant activity in this series, the cyclization of thiosemicarbazide unit, the substituents attached to the nitrogen atom, and size of these substituents were examined

The model of the scavenging of the stable DPPH radical is extensively applied to evaluate antioxidant activities in less time than that is required by other methods. DPPH is a stable free radical that can accept an electron or hydrogen radical and must thus be converted to a stable, diamagnetic molecule. DPPH has an odd electron and so has a strong absorption band at 517 nm. When this electron becomes paired off, the absorption decreases stoichiometrically with respect to the number of electrons or hydrogen atoms taken up[37]. Such a change in the absorbance by this reaction has been extensively adopted to test the capacity of several molecules to act as free radical scavengers.

According to the *in vitro* data summarized in Table 1, $DPPH^{\cdot}$ scavenging capacity of hydrazone derivatives (compounds 13-18) were found to

TABLE I

Antioxidant capacity of the newly synthesized compounds*. EC_{50} is the concentration required for 50 % of scavenging capacity. IC_{50} is the concentration that reduces the activity by 50%.

Compound	R group	DPPH radical scavenging capacity EC_{50} (μM)	The superoxide anion radical scavenging capacity EC_{50} (μM)	hydrogen peroxide scavenging capacity EC_{50} (μM)	Hydroxyl radical-scavenging capacity EC_{50} (μM)	Nitric oxide scavenging capacity EC_{50} (μM)	Inhibition of oxidative hemolysis of erythrocytes IC_{50} (μM)	Reducing power (Absorbance of 50 μM of compound at 700 nM)	Metal chelating activity EC_{50} (μM)
1	H	57.40±4.20	37.05±2.55	30.55±1.30	69.00±4.30	90.48±6.28	90.55±7.28	0.24±0.01	75.00±6.10
2	$CSNHCH_3$	82.83±2.00	80.22±5.14	38.70±2.04	78.70±4.04	93.77±7.06	91.24±6.90	0.26±0.03	86.00±4.36
3	$CSNHC_2H_5$	62.54±3.50	65.50±4.00	33.55±2.16	84.60±3.70	90.13±6.83	90.10±7.72	0.25±0.01	73.25±5.00
4	$CSNHC_3H_5$	48.12±2.65	58.88±3.14	36.33±2.22	76.33±3.22	91.06±6.18	102.55±8.33	0.20±0.01	90.45±5.50
5	$CSNHC_6H_5$	32.93±5.05	25.50±2.00	25.68±1.90	60.23±2.50	52.12±3.35	34.20±2.13	0.60±0.05	53.90±4.15
6	CH_3	61.91±2.44	60.66±4.17	36.55±1.40	76.55±2.40	74.18±4.86	60.35±4.16	0.49±0.03	67.23±5.00
7	C_2H_5	83.82±1.66	88.00±5.56	40.22±1.30	70.22±5.76	72.08±4.77	80.36±6.40	0.40±0.02	74.00±4.90
8	C_3H_5	47.91±2.71	59.90±3.68	42.80±2.23	65.80±4.23	70.12±5.13	77.89±5.15	0.45±0.03	68.40±5.00
9	C_6H_5	33.68±4.20	28.70±1.99	57.80±2.41	79.00±5.50	66.16±5.01	32.20±2.05	0.66±0.04	61.20±4.00

10	CH_3	62.62±2.60	60.40±4.70	26.77±2.12	86.77±5.00	122.2±8.16	75.14±5.00	0.48±0.03	69.00±3.20
11	C_2H_5	98.67±4.70	66.30±4.70	30.76±3.12	70.88±4.80	129.05±9.00	78.34±4.88	0.46±0.02	70.50±5.35
12	C_6H_5	45.95±2.30	36.30±2.45	30.40±2.45	65.33±4.21	60.41±3.41	35.23±2.56	0. 63±0.04	70.00±4.00
13	H	38.74±2.61	40.50±2.22	53.45±2.40	39.80±2.20	98.28±8.05	55.00±2.90	0.50±0.03	64.00±4.05
14	Br	13.18±1.65	23.18±2.06	29.30±1.05	29.08±2.00	52.05±5.03	30.27±3.53	0.80±0.06	49.90±3.00
15	Cl	14.32±1.07	24.50±1.75	30.55±2.00	20.55±1.15	53.00±4.90	32.15±2.90	0.79±0.05	38.55±3.12
16	CH_3	36.95±2.13	33.27±3.00	33.50±2.13	33.10±1.80	93.07±8.15	60.23±4.37	0.50±0.05	29.50±4.35
17	OCH_3	14.54±1.10	18.22±1.30	27.40±1.67	29.00±1.90	61.20±4.20	24.60±1.99	0.96±0.07	25.00±4.00
18	OH	11.85±0.98	16.02±1.13	24.90±1.00	17.56±1.05	50.25±4.05	21.12±1.80	1.16±0.10	21.40±1.10
Ascorbic acid		14.82±2.30	23.80±1.90	56.16±2.30	58.16±2.30	59.20±3.26	34.20±2.36	0.47±0.02	
BHT**			21.03±1.72			75.20±5.00			53.44±4.70

* Results are the mean±SEM of three independent experiments.
** BHT: Butylated Hydroxy Toluene

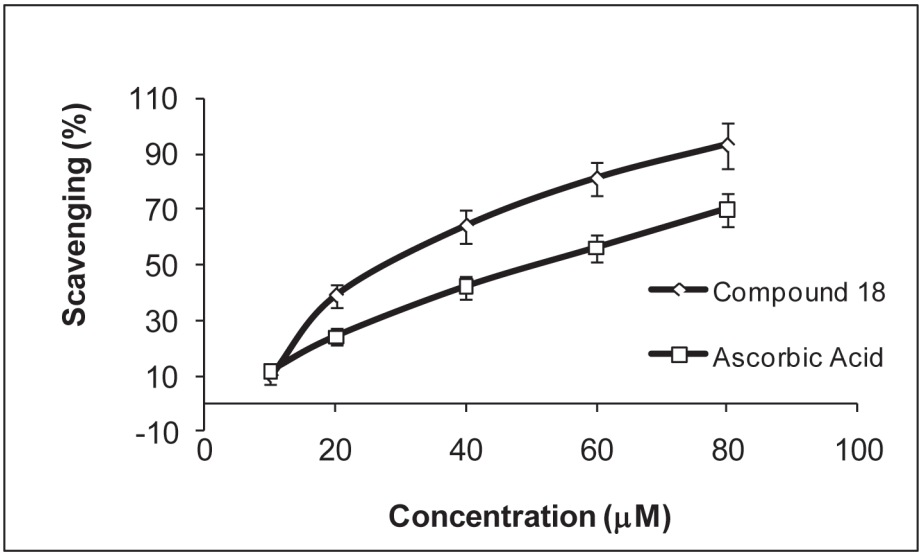


Figure 1
Dose dependent DPPH radical scavenging activity of compound 18. Results are mean ± SEM of triplicate measurements.

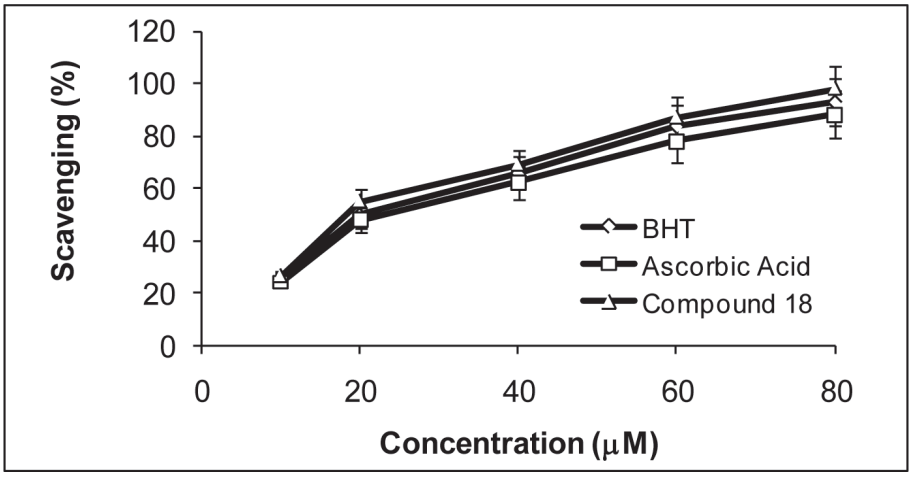


Figure 2
Dose dependent superoxide anion radical scavenging activity of compound 18. Results are mean ± SEM of triplicate measurements.

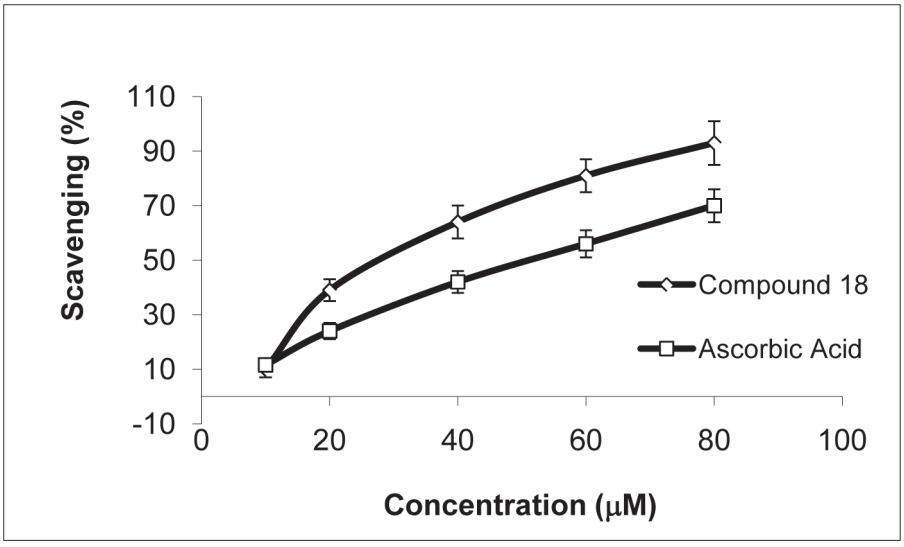


Figure 3
Dose dependent hydrogen peroxide scavenging activity of compound 18. Results are mean ± SEM of triplicate measurements

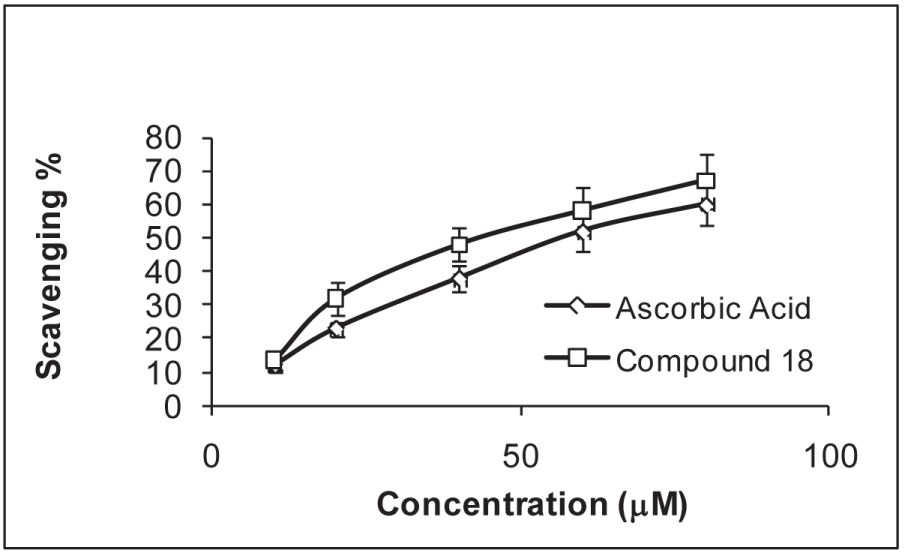


Figure 4
Dose dependent hydroxyl radical scavenging activity of compound 18. Results are mean ± SEM of triplicate measurements

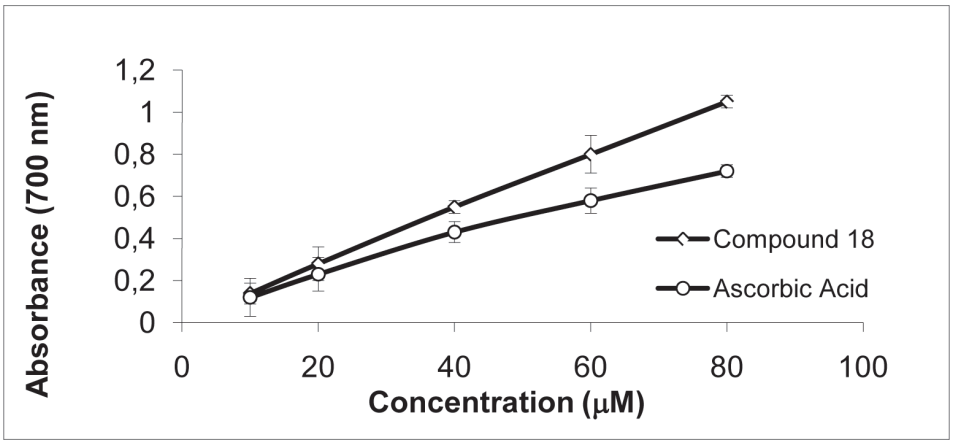


Figure 5
The reducing power of compound 18. Results are mean ± SEM of triplicate measurements.

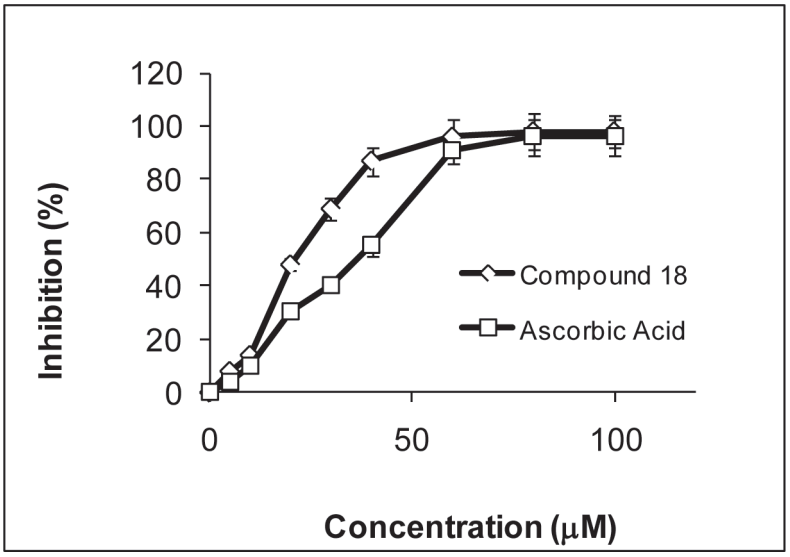


Figure 6
Inhibitory effect of compound 18 on hemolysis. Results are mean ± SEM of triplicate measurements.

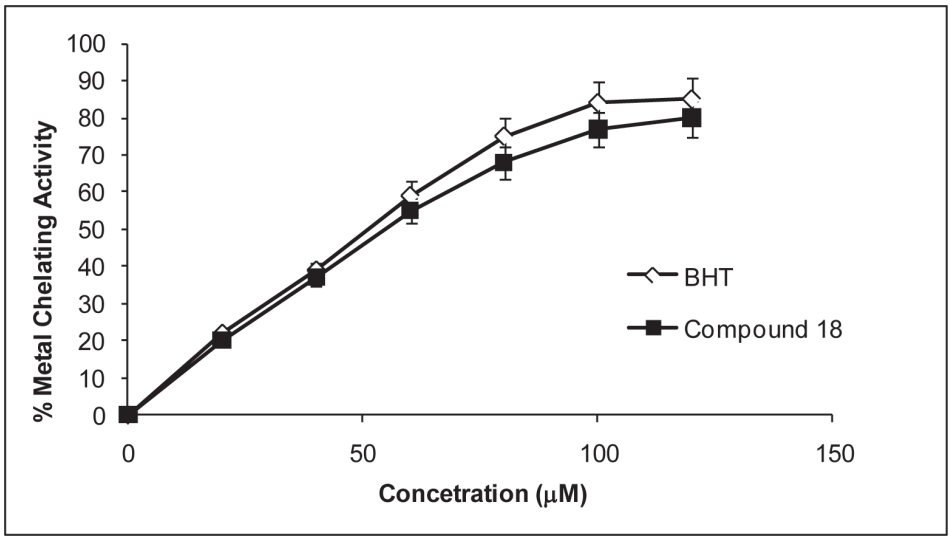


Figure 7
Metal chelating activity of compound 18. Results are mean ± SEM of triplicate measurements.

be greater than that of the other derivatives ($p<0.01$). It seems that thiosemicarbazide moiety and the cyclization of the thiosemicarbazide moiety to 1,2,4-triazole and thiadiazole do not produce more active antioxidant compounds. The scavenging activity of compounds **13-18** are possibly due to the presence of an N-H group in the hydrazino moiety, which can donate a hydrogen atom to be DPPH radical After donating a hydrogen atom, compounds **13-18** may exist in a radical form, and this radical may delocalize to the phenyl ring to produce a stable resonance hybride shown in Figure 8. The electron conjugation in the structure is suggested to stabilize the radical, and prevent it from participating in a destructive biochemical reaction.

As shown in Table 1, the electronic properties of substituent size produced noticeable differences in activity of the compounds. An increase in antioxidant activity was observed with replacement of alkyl chains such as methyl, ethyl, allyl to phenyl ring (compounds **5, 9, 12**) due to the electron resonance effect of phenyl group.

When compared the effect of substituents on the phenyl ring of hydrazone derivatives (compounds **13-18**), hydroxyl substituent (**18**)

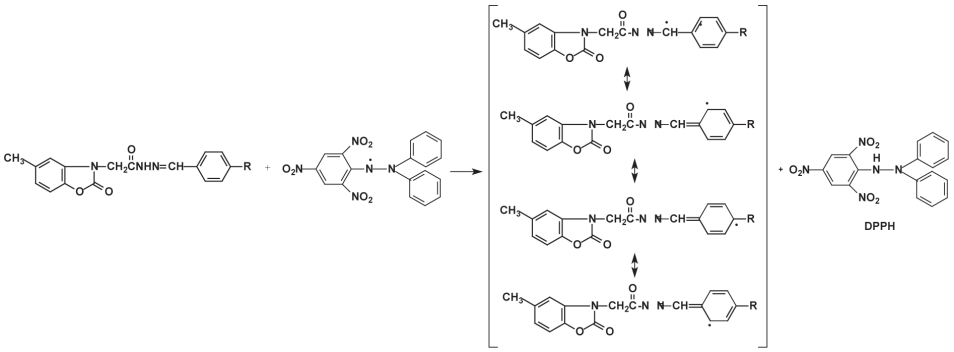


Figure 8
Purposed reaction of DPPH with benzylidenhydrazine containing benzoxazolinone ring

resulted in significant antioxidant activity. This significant scavenging power was thought to be resulted from the presence of the free OH group which can donate hydrogen atoms. After donating the hydrogen atom, compound **18** possibly exists in its radical form, and the electron conjugation effect in the structure stabilizes the radical which favors the reaction occur. Figure 1 illustrates the concentration dependent DPPH radical scavenging activity of compound **18**. The scavenging activity increased with increasing concentration of the compound to 80 μM and then almost levelled off with further increase in concentration. The EC_{50} value corresponding the DPPH radical scavenging activity of compound **18** was found to be smaller than the one of ascorbic acid indicating that compound **18** appears as a powerful DPPH radical scavenger.

Compound **17**, which carries a methoxy group on the phenyl ring of the hydrazone moiety also scavenged the radicals stronger than ascorbic acid and its strong radical scavenging capacity was suggested to be resulted from its electron donating ability enhancing the stabilization of the phenoxy radicals. Compounds carry a bromide and a chloride on their hydrazone moieties at para position also showed a remarkable scavenging activity possibly due to the electron donating capacities of these groups.

Compounds **14, 15, 17** and **18** caused instantaneous decrease in the absorbance of DPPH, in a way similar to that of ascorbic acid, suggesting a high radical scavenging activity, whereas compounds **13** and **16** caused a more gradual decrease in absorbance, indicating a slower reactivity as compared with the compounds **14, 15, 17, 18** and the

reference compound. Therefore, compounds **13** and **16** behave slightly worse DPPH radical scavenging ability than the other compounds.

Hydrazone derivatives also showed a strong superoxide anion radical scavenging activity with EC_{50} values of 16.02–40.50 μM, with the exception of compounds **13** and **16**, bearing no substituent and 4-methylphenyl, respectively (Table 1). Compounds **14, 17** and **18** appeared as the most potent superoxide anion radical scavenger among the newly synthesized hydrazones. Superoxide anion radical scavenging capacities of compounds **17** and **18**, which are the hydrazone carrying methoxy and hydroxyl substituent respectively, were found greater than the capacity of ascorbic acid and BHT. Figure 2 shows the concentration dependent superoxide anion radical scavenging activity of compound **18**. There is a steady increase in the superoxide anion radical scavenging capacity of compound **18** up to 80 μM followed by a relatively low increase to 200 μM.

All novel compounds, except compound **9**, exhibited more potent hydrogen peroxide scavenging activity than that of ascorbic acid (Table 1, Figure 3). The EC_{50} value of compound **18** was found to be 24.90±1.00 μM while the EC_{50} value of ascorbic acid was 56.16±2.30 μM suggesting that compound **18** scavenged hydrogen peroxide much more stronger than ascorbic acid. This strong scavenging power was thought to be resulted from the presence of the free OH group which can donate hydrogen atoms:

$$H_2O_2 + 2H^+ + 2e \rightarrow 2H_2O$$

The novel compounds exhibited hydroxyl radical scavenging activity in a dose dependent manner in the EC_{50} range of 17.56-.77 μM (Table 1. Figure 4). Hydroxyl radical scavenging capacity of hydrazone derivatives (compounds **13-18**) were found to be greater than that of the other derivatives. It seems that thiosemicarbazide moiety and the cyclization of the thiosemicarbazide moiety to 1,2,4-triazole and thiadiazole do not produce more active antioxidant compounds. Compound **18** was found to be approximately 3 times more efficient than ascorbic acid. Generally molecules that inhibit deoxyribose degradadation are those that can chelate the iron ions and there by prevent them from complexing with the deoxyribose and render them inactive in a Fenton reaction[38]. The hydroxyl scavenging activities of phenolic substances might be due to the active

hydrogen donating ability of hydroxyl substitutions. Since electron donor groups are present in the compounds, they were suggested to accelerate the conversion of H_2O_2 in to H_2O. As shown in Fig. 7, compound **18** exhibited chelating effect on ferrous ions, suggesting that they minimize the concentration of metal in the Fenton reaction, though to a smaller extent compared to EDTA. The antioxidant effect of several polyphenols that acts as inhibitors of hydroxyl radical formation and lipid peroxidation has been correlated with iron chelating properties[39].

Significant activity pattern was not obtained from nitric oxide scavenging activity experiment. Only compounds **18, 15**, **14**, and **5** showed significant inhibitory effect on nitric oxide radical at 75 μM concentration. EC_{50} values of these compounds were determined as 50.25±4.05, 53.00±4.90, 52.05±5.03 and 52.12±3.35 μM, respectively (Table 1). It appeared that for hydrazone derivatives bromide (compound **14**), chloride (compound **15**) or methoxy (compound **18**) at the para position on the phenyl ring and for 2-benzoxazolinone derivatives phenyl substitution (compound **5**) of the thiocarbomoyl moiety were beneficial for the enhanced NO scavenging activity.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The novel compounds having a hydrazone moiety had a significant reducing power (Table 1). The reducing power of the compound **18**, which carries a free hydroxyl group on the phenyl ring of the hydrozone moiety, was found much higher than that of ascorbic acid. The reducing power of compound **18** increased steadily with the increasing concentration (Figure 5). The reducing power of this compound may be due to the hydroxyl substitution in the aromatic ring, which possess potent hydrogen donating abilities as described by Shimada et al[40]. The reducing properties are recently suggested to be associated with the presence of reductones[41], which have been shown to exert antioxidant activity by breaking the free radical chain by donating a hydrogen atom. Compound **17**, which carries a methoxy group on the phenyl ring of the hydrozone moiety had also a remarkable reducing effect. Compounds **6-9**, the 1,2,4 triazole derivatives, also showed a significant reducing activity. Among these derivatives, compound **9**, which carries a phenyl ring was the most effective compound. It appeared that either a bromine or chloride at the para position on the phenyl ring was beneficial for the reducing activity.

The *in vitro* inhibitory effect of compounds on oxidative hemolysis of human erythrocytes are shown in Table 1. The concentrations required for 50% inhibition (IC_{50}) of this activity were found as 21.12±1.80, 24.60±1.99, 30.27±3.53, 32.15±2.90 μM for compounds **18, 17, 14** and **15** in a dose-dependent manner, respectively (inhibitory activity of compound **18** on hemolysis was shown in Figure 6. This effect was suggested to be resulted from its hydrogen-donating ability. Inhibitory effects of these compounds on hemolysis of human erythrocytes were better than the specific inhibitor ascorbic acid (IC_{50} 34.20±2.36 μM). The other significant inhibitory activities were observed also by compounds **5** (34.20±2.13μM), **9** (32.20±2.05 μM), and **12** (35.23±2.56 μM). In this series of compounds, particularly the hydrazone derivatives (**13-18**) were the most active compounds according to the method. It is known that polyphenolics enhance red blood cell resistance to oxidative stress both *in vitro* and *in vivo*[42]. The compounds contain one or more aromatic hydroxyl groups that actively scavenge free radicals possess strong antioxidant activity. So, compound **18** may quench the chain propagating peroxyl radicals in the aqueous phase to stop the peroxidation and inhibit hemolysis. However, the molecular mechanisms of the antioxidant action of phenolic compounds have not been fully elucidated and are still a matter of considerable debate. Recent studies have suggested that the ability of certain polyphenols to partition in cell membranes and the resulting restriction of their fluidity could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions[43].

Transition metals have been previously proposed to catalyse the formation of the radicals to start the propagation of radical chain reaction in lipid peroxidation. Chelating agents may inhibit lipid oxidation by stabilising transition metals. Ferrozine can quantitatively form complexes with Fe^{+2}. In the presence of other chelating agents, the complex formation is disrupts with the result that the red color of the complex is decreases. The metal chelating activity of the novel compounds was evaluated against Fe^{+2} and expressed as EDTA equivalents. Hydrazones (compounds **13-18**) had greater metal chelating ability than that of BHT (Table 1). Compound **18** exhibited the most potent metal chelating activity with EC_{50} value of 21.40±1.10 μM in a dose dependent manner (Figure 7) among the newly synthesized hydrazones.

As a result, hydrazone derivatives and compounds bearing electron donating aromatic rings on the thiosemicarbazide, triazolo and thidiazole

systems showed significantly good antioxidant activities. Since our findings are preliminary results; further studies need to be carried out to investigate the other specifications, such as in vitro assays, toxicological studies or side effect-activity profiles of these compounds.

Conclusion

In conclusion, the present data has shown that the novel compounds effectively scavenged ROS under *in vitro* conditions and hydrogen donating ability of hydrazones carrying hydroxyl substituent at aromatic ring has been proven to be most effective through the assessment of reducing power and DPPH scavenging activity. Since hydrazones also were appeared as potent metal chelators and exhibit relatively high reducing power, their free radical scavenging capacity and ability to inhibit lipid peroxidation is of considerable interest. Compound **18** possessed remarkable dose-dependent antioxidant properties. Different relative scavenging capacities of the compounds against different radicals may be due to the different mechanisms involved in the radical-antioxidant reactions. The stoichiometry of reactions between the synthesized compounds and the DPPH is possibly different and this may be one reason for the difference in their scavenging potential. Other factors like stereoselectivity of the radicals or the compounds may also affect the capacity of the compounds to react and quench different radicals.

Summary

It has been reported that some benzoxazolinone, triazole, thiadiazole and hydrazone derivatives showed significant analgesic and antiinflammatory activity. It has been speculated that non-steroidal antiinflammatory agents (NSAIDs) can act as the free radical scavengers and show antioxidant activity. It is also well documented that oxidative stres can play an important role in the side effects of many xenobiotics including NSAIDs. Therefore, in the present study, it was thought that the combination of different pharmacophores in one frame may lead to compounds with interesting pharmacological profiles and the effects of

thiosemicarbazide, triazole, thiadiazole and hydrazone bearing 2-benzoxazolinone were investigated on free radical scavenging activity. Antioxidant activities of the novel compounds were evaluated by the determinations of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, superoxide radical scavenging activity, hydrogen peroxide scavenging activity, nitric oxide scavenging activity and reducing power. The various antioxidant activities were compared to standard antioxidants such as butylated hydroxytoluen and ascorbic acid and the results were expressed as IC_{50} values. All of the newly synthesized compounds exhibited varying degrees of scavenging capacity on different active radicals; the compounds **13-18** (hydrazone derivatives) proved to be most active. Among those, compound **17** and **18**, having p-methoxy and -hydroxyl groups on their phenyl rings, respectively showed the highest scavenging capacity of active radical species. These preliminary *in vitro* results may contribute to explain the potency of antiinflammatory activity of the compounds.

Keywords: Free-Radical Scavenging Activity, 2-Benzoxazolinone Derivatives, 2,2-diphenyl-1-picrylhydrazyl, superoxide radical, hydrogen peroxide, nitric oxide

Özet

Tiyosemikarbazit, Triazol, Tiyadiazol ve Hidrazon içeren 2-Benzoksazolinon Türevlerinin Serbest Radikal Süpürme Aktiviteleri

Bazı benzoksazolinonil triazol, tiyadiazol ve hidrazon türevlerinin belirgin analjezik ve antienflamatuar aktivite gösterdikleri rapor edilmiştir. Non steroidal antienflamatuar ajanların (NSAIDler) serbest radikal süpürücüleri olarak görev yaparak antioksidan aktivite gösterdikleri düşünülmüştür. Oksidatif stresin NSAIDler dahil bir çok ksenobiyotiğin yan etkilerinde önemli rol oynadığı açıkça belirlenmiştir. Dolayısıyla, bu çalışmada farklı farmakoforların kombinasyonlarının bir çerçevede toplanmasıyla ilginç farmakolojik profillere sahip bileşikler meydana gelebileceği düşünülmüş ve tiyosemikarbazit, triazol, tiyadiazol ve hidrazon taşıyan 2 benzoksazolinon türevlerinin serbest radikal süpürücü aktiviteleri incelenmiştir. Yeni sentezlenen bu türevlerin antioksidan aktiviteleri 2,2-difenil-1-pikrilhidrazil (DPPH) radikali, süperoksit radika-

li, hidrojen peroksit, nitrik oksit süpürücü aktivitelerinin ve indirgeyici güçlerinin belirlenmesiyle değerlendirilmiştir. Çeşitli antioksidan aktiviteler bütillenmiş hidroksitoluen ve askorbik asit gibi standart antioksidanlarla kıyaslanmış ve sonuçlar IC_{50} değeri şeklinde ifade edilmiştir. Yeni sentezlenen tüm bileşikler çeşitli derecelerde farklı aktif radikaller üzerinde süpürücü etki göstermişlerdir; bileşik 13-18'in (hidrazon türevleri) en aktif bileşikler olduğu saptanmıştır. Bunlar arasında fenil halkasında sırasıyla p-metoksi ve p-hidroksi grupları taşıyan bileşik **17** ve **18** aktif radikal türleri üzerinde en yüksek süpürme kapasitesine sahip oldukları saptanmıştır. Bu öncül *in vitro* sonuçlar bu bileşiklerin antienflamatuar aktivitelerinin derecelerini açıklamaya katkıda bulunmaktadır.

Anahtar Kelimeler: Serbest radikal süpürücü aktivite, 2-benzoksazolinon türevleri, 2,2-difenil-1-pikrilhidrazil, süperoksit radikali, hidrojen peroksit, nitrik oksit

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