Full Paper

Synthesis and Antioxidant Properties of (3,4-Dihydroxyphenyl)-(2,3,4-trihydroxyphenyl)methanone and Its Derivatives

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(3,4-Dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone (**5**) and its two derivatives with bromine were synthesized from reactions such as bromination and demethylation of (3,4-dimethoxyphenyl)(2,3,4-trimethoxyphenyl)methanone (**6**). The Wolf-Kishner reduction product (**9**) of **6** and its three derivatives with bromine were obtained. 4-(3,4-Dihydroxybenzyl)benzene-1,2,3-triol and its dibromide derivative (**16**) were also synthesized from **9** and the corresponding dibromide derivative. The *in vitro* antioxidant activities of nine new compounds synthesized in these reactions were determined by analyzing the radical scavenging activities of bromophenols for 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD), and the superoxide anion radical ($O_2^{\bullet -}$) and examining the total reducing power through Fe^{3+}-Fe^{2+} transformation, FRAP and CUPRAC assays and the ferrous ions (Fe^{2+}) chelating activities. Moreover, the results of these activities were compared to those of standard antioxidant compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol, and trolox. The results showed that the synthesized bromophenols had effective antioxidant power. The phenol **5** with two phenolic rings and five phenolic hydroxyl groups was the most potent antioxidant and radical scavenger. In conclusion, the new compounds are promising molecules to be used owing to their potential antioxidant properties.

Keywords: Antioxidant activity / Bromination / Bromophenols / Phenols / Radical scavenging

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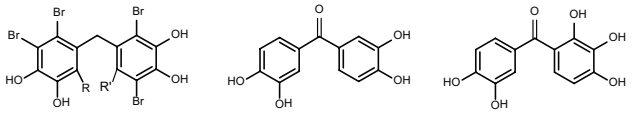
Introduction

Bromophenols, abundantly found in marine life, have some important biological activities [1, 2]. Bromophenols **1–3** are natural products [3–5] and some of them exhibit enzyme inhibition [6–7], cytotoxicity [8], feeding deterrent [3], antioxidant [9, 10] and microbial [11, 12] activities. Most derivatives of compound **4** with Br also exhibit enzyme inhibition [7] and antioxidant [10] activities. Compound **5**, similar to **4**, has five OH groups. The compound **5** and its derivatives, especially with Br, may exhibit important biological activities such as antioxidant activity.



1 R = R' = H
2 R = Br, R' =H
3 R = R' = Br

4 **5**

Oxidative stress is characterized by an increased concentration of intracellular oxidizing species, such as reactive oxygen species (ROS), and is often accompanied by the loss of antioxidant defense capacity. ROS are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. ROS include free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$), and nonfree radical species such as hydrogen

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peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [13, 14]. It is well known that excessive ROS attack many organs and induce oxidative damage directly to such critical biological molecules as lipoproteins, membrane lipids, polyunsaturated fatty acids, carbohydrates and nucleotides causing lipid peroxidation and protein oxidation [15, 16]. Metabolic oxidative stress has been implicated, directly or indirectly, in the development of diseases and degenerative processes, including inflammation, cancer, dementia and physiological aging [17]. Antioxidants have recently emerged as a way of minimizing the biomolecular damage caused by the attack of ROS to vital constituents of living organisms [18, 19]. Antioxidants protect the quality of foods by retarding oxidative breakdown of the lipid components [20]. Commercial antioxidants are generally synthetic compounds [21]. It is well known that bromophenols display antioxidant and radical scavenging activities [10]. Therefore, there is a great interest towards new synthetic [10, 22–25] and natural [26–34] antioxidants which can represent a good pharmacological alternative to counteract oxidative stress [35].

The aim of this study was to synthesize the compound **5** derivatives and investigate the effects of their biological activities including ferric ions (Fe^{3+}) and cupric ions (Cu^{2+}) reducing power, DPPH$^\bullet$, ABTS$^{\bullet+}$, DMPD$^{\bullet+}$, H_2O_2 and $O_2^{\bullet-}$ scavenging and ferrous ions (Fe^{2+}) chelating activity methods.

Results and discussion

Synthesis

(3,4-Dimethoxyphenyl)(2,3,4-trimethoxyphenyl)methanone (**6**) [36] was obtained from the reaction of 3,4-dimethoxybenzoic acid and 1,2,3-trimethoxybenzene in polyphosphoric acid (PPA) by the known method (Scheme 1) [7, 10, 37, 38]. According to literature method [36, 39], bromination reactions of **6** with 1.1 and 6.0 equivalents (equiv.) of ceric ammonium nitrate (CAN)/LiBr at room temperature (RT) were performed. A monobromide **7** and a dibromide **8** were obtained from these reactions as the sole products (Scheme 1).

To synthesize compounds without CO from **6**, the Wolff-Kishner reduction of ketone **6** was performed and diarylmethane derivative **9** was synthesized in high yield (Scheme 2). Bromination of aromatic compounds with ceric ammonium nitrate (CAN)/LiBr is selective bromination [39]. Brominations of **9** with different equivalents of LiBr/CAN were also performed at RT by applying the same procedure. Its reaction with 1.1 equivalents of LiBr/CAN gave a mixture of three products while its reaction with 2.2 equivalents of LiBr/CAN gave a sole product. The products were isolated and their structures were determined. The product produced in the reaction as sole product was dibromide **12**, and the products found in the mixture were bromides **10**, **11** and **12**. Brominations of **9** with 5.1 equivalents of LiBr/CAN were performed at RT so as to obtain products with more Br than two. Dibromide **12** was also obtained from the reaction as sole product (Scheme 2).

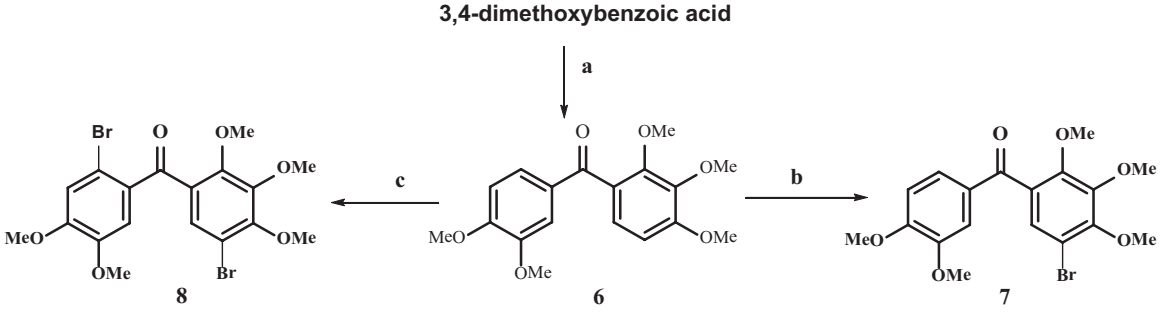
Reactions of **6–9** and **12** with BBr_3 in CH_2Cl_2 were performed at 0–25°C in order to synthesize the phenol and bromophenol derivatives and examine their antioxidant properties. Phenol **5** and bromophenol derivatives **13–16** were synthesized from these demethylation reactions (Scheme 2).

Antioxidant activity

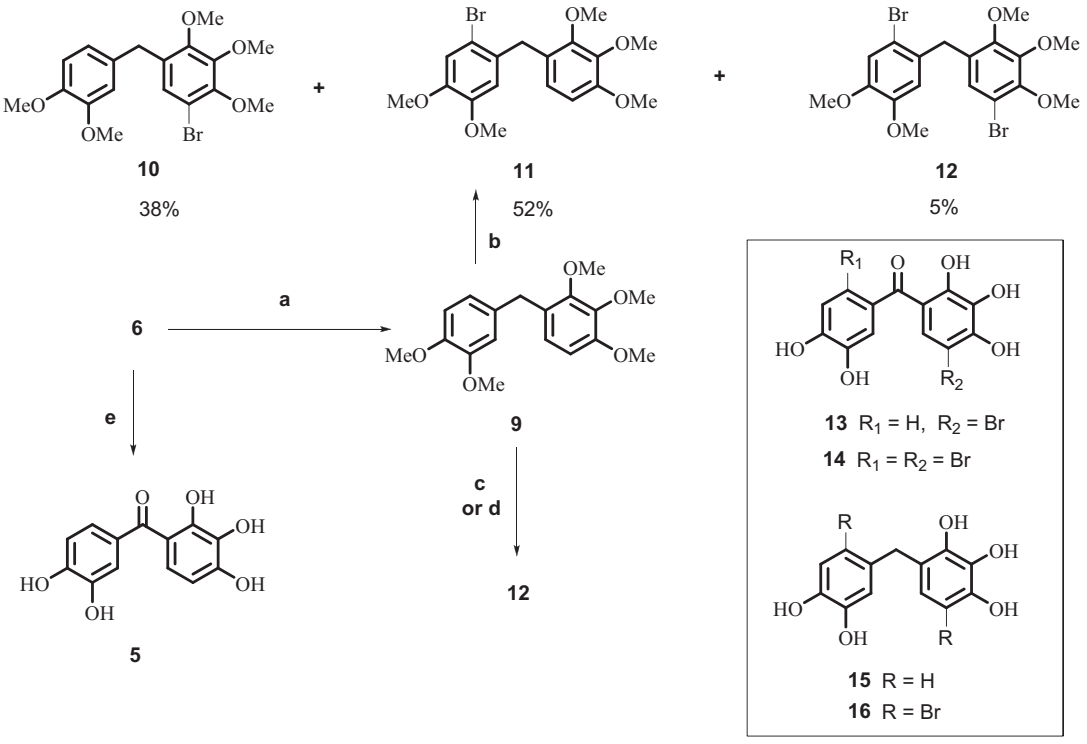
Antioxidant compounds play important roles in the scavenging and inhibition of free radicals. Therefore, investigating and finding new sources of antioxidants have gained importance. The antioxidant potential of bromophenols was analysed by DPPH$^\bullet$ scavenging, ABTS$^{\bullet+}$ scavenging, DMPD$^{\bullet+}$ scavenging, $O_2^{\bullet-}$ scavenging, Fe^{3+}-Fe^{2+} transformation, cupric ion (Cu^{2+}) reducing and ferrous ion (Fe^{2+}) chelating assays.

Radical scavenging activity

Many diseases such as Parkinson's disease, Alzheimer's disease, atherosclerosis, inflammation and reperfusion injury have been linked to ROS-mediated damage of biological



Scheme 1. Reagents and conditions: (a) 1,2,3-trimethoxybenzene-PPA, 80°C, 1.5 h, 94%; (b) LiBr (1.1 equiv.)/CAN (1.1 equiv.), CH_3CN, RT, 3 d, 97%; (c) LiBr (6.0 equiv.)/CAN (6.0 equiv.), CH_3CN, RT, 6 d, 95%.



Scheme 2. Reagents and conditions: (a) KOH-NH_2NH_2/$(OHCH_2)_2$, 110–190°C, 3 d, 83%; (b) LiBr (1.1 equiv.)/CAN (1.1 equiv.), CH_3CN, RT, 3 d; (c) LiBr (2.1 equiv.)/CAN (2.1 equiv.), CH_3CN, RT, 3 d, 95%; (d) LiBr (5.1 equiv.)/CAN (5.1 equiv.), CH_3CN, RT, 3 d, 98%; (e) BBr_3, CH_2Cl_2, 96%.

macromolecules which arises from an imbalance between radical-generating and radical-scavenging systems [40]. $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging assays are widely used to determine the radical scavenging abilities of various samples [41–43]. The radical scavenging activity of bromophenols was determined by $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ scavenging assays in the present study. The efficient concentration EC_{50} or concentration necessary to decrease the initial $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ concentration by 50% (in μM) was then obtained for each compound. The results are summarized in Table 1. When an antioxidant is added to the radicals, there is a degree of decolorization owing to the presence of the antioxidants, which reverse the formation of the $DPPH^{\bullet}$ radical, $ABTS^{\bullet+}$ and $DMPD^{\bullet+}$ cation:

$$DPPH^{\bullet} + AH \rightarrow DPPH-H + A^{\bullet}$$

$$ABTS^{\bullet+} + AH \rightarrow ABTS^{+} + A^{\bullet}$$

$$DMPD^{\bullet+} + AH \rightarrow DMPD^{+} + A^{\bullet}$$

These methods are rapid; a sample analysis takes 15 min in total and little manpower, no expensive reagents or sophisticated instrumentation is required. These chromogens are easy to use, have a high sensitivity, and allow for rapid analysis of the antioxidant activity of a large number of samples. These assays have been applied to determine the antioxidant activity of food components [40, 44].

The antiradical activity of bromophenols or standard compounds can be determined by assessing the scavenging activity on DPPH radicals. In this assay, the purple chromogen radical $DPPH^{\bullet}$ is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine [45, 46]. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule, and convert them to a colourless product. In this assay, we measure the DPPH initial absorbance, and the absorbance once the potential antioxidant has been added. The reduction of absorbance is a measure of the free DPPH due to the action of the antioxidant. The scavenging capacity is generally evaluated in organic media by monitoring the absorbance decrease at 517 nm until the absorbance remains constant [47]. This method has been widely used to evaluate

Table 1. EC_{50} values (μM) for DPPH•, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging assays of bromophenols and standard antioxidants (ABTS: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), DPPH: 1,1-diphenyl-2-picryl-hydrazyl, DMPD: *N,N*-dimethyl-*p*-phenylenediamine, and $O_2^{\bullet-}$: superoxide anion radicals, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene; EC_{50}: the antioxidant concentration (μM) necessary to decrease the initial radical concentration by 50%).

	DPPH• scavenging	$ABTS^{\bullet+}$ scavenging	$DMPD^{\bullet+}$ scavenging	$O_2^{\bullet-}$ scavenging	Metal chelating	
					Ferrozine	Bipyridyl
	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
BHA	400	84	160	90	97	84
BHT	171	423	87	129	67	29
α-Tocopherol	109	144	50	73	26	25
Trolox	233	191	232	57	85	24
5	87	47	80	227	68	29
9	162	265	68	103	53	18
10	77	161	55	70	50	27
11	99	195	78	91	51	25
12	198	193	56	116	31	9
13	71	20	62	38	70	36
14	85	108	54	42	38	18
15	36	19	52	47	98	67
16	66	93	46	3	73	23

the free radical scavenging activity of various antioxidant substances [48, 49]. This method is based on the reduction of DPPH in alcoholic solutions in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H in the reaction [30]. The dark colour of the DPPH radical becomes lighter due to the antioxidant compound, causing a decrease in the absorbance at 517 nm. The DPPH free radical scavenging activities of bromophenols and standard antioxidants (BHA, BHT, α-tocopherol and trolox) were investigated.

As can be seen in Table 1, the DPPH•, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging activities of the new synthesized compounds (**5** and **9–16**) were evaluated. Lower IC_{50} values indicate higher DPPH• radical scavenging ability. In this respect, all of the newly synthesized compounds (**5** and **9–16**) exhibited radical scavenging abilities on DPPH• when compared to standard antioxidants such as BAH, BHT, α-tocopherol and trolox. As can seen in Table 1, the most effective IC_{50} value was found in phenol compound **15** as 36 μM (r^2: 0.961). On the other hand, IC_{50} values of four standard compounds were changed from 109 to 400 μM.

Another effective method to measure the radical scavenging activity is the ABTS radical cation decolourisation assay, which showed similar results to those obtained in the DPPH reaction. Similar to the DPPH radical scavenging activity, the new compounds (**5** and **9–16**) had effective ABTS radical scavenging activity. The ABTS radical scavenging activities of the new compounds (**5** and **9–16**) and standard antioxidants decreased in the following order: **15** (19 μM) ≈ **13** (20 μM) < **5** (47 μM) < BHA (84 μM) < **16** (93 μM) < **14** (108 μM) < α-tocopherol (144 μM) < **10** (161 μM) < trolox (191 μM) ≤ **12** (193 μM) ≤ **11** (195 μM) < **9** (265 μM) < BHT (423 μM), respectively. These results show that the newly synthesized compounds have effective ABTS radical scavenging activity (Table 1).

Another assay used to measure the radical scavenging activity involves the decolourisation of the $DMPD^{\bullet+}$ cation radical, similar to the DPPH scavenging and ABTS cation radical decolourisation assay. $DMPD^{\bullet+}$ has a maximum absorbance at 505 nm. Antioxidant compounds or radical scavengers, which can act as hydrogen donors for $DMPD^{\bullet+}$, decrease the absorbance at 505 nm of $DMPD^{\bullet+}$ [50]. As it is seen in Table 1, the new compounds (**5** and **9–16**) have effective $DMPD^{\bullet+}$ radical scavenging activity and their $DMPD^{\bullet+}$ scavenging capacity decreased in the following order: **16** (46 μM) < α-tocopherol (50 μM) ≤ **15** (52 μM) ≤ **14** (54 μM) ≤ **10** (55 μM) ≤ **12** (56 μM) < **13** (62 μM) < **9** (68 μM) < **11** (78 μM) < **5** (80 μM) < BHT (87 μM) < BHA (160 μM) < trolox (191 μM). These results show that the newly synthesised compounds have marked $DMPD^{\bullet+}$ scavenging activity.

Superoxide radical anion ($O_2^{\bullet-}$) is produced as a result of the donation of one electron to oxygen. This radical arises either from several metabolic processes or following oxygen activation by irradiation. This process can generate other more harmful ROS, such as H_2O_2, OH•, HOCl and $^1O_2$ [40, 51]. Two different *in vitro* superoxide-generating systems are commonly used. The first one is a xanthine oxidase–hypoxanthine system and the other one is a riboflavin–methionine–illuminate system. In this study, we preferred the riboflavin–methionine–illuminate system to generate superoxide anion radicals.

The greatest inhibition of superoxide radical formation was observed in bromophenol compound **16** (Table 1). IC_{50} value of this compound (3 μM) was lower than that of the used standard and bromophenol compounds.

The riboflavin–methionine–illuminate system was used to generate superoxide anion radicals ($O_2^{\bullet -}$) in this study. Then, superoxide anion radicals reduced NBT^{2+} to produce formazan which was blue in colour. Antioxidants inhibit the formation of blue NBT [52]. Superoxide anion radicals appear indirectly when the assay is performed under aerobic conditions. In the presence of an antioxidant molecule that can donate an electron to NBT, the typical purple colour of formazan decays and this change can be monitored spectrophotometrically at 560 nm. Antioxidants have the ability to inhibit the formation of NBT and scavenge superoxide anion radicals. The decrease observed in absorbance at 560 nm in the presence of antioxidants indicates the scavenging of superoxide anions in the reaction mixture [53].

Antioxidants inhibit the formation of blue NBT [52]. The two principal reactions are involved in this assay are [54]:

$$2NBTH^{\bullet} \rightarrow NBT + NBTH_2 \quad (\text{Formazan}) \quad (a)$$

$$NBTH^{\bullet} + O_2 \leftrightarrow NBT + O_2^{\bullet -} \ (\text{A quasi equilibrium}) \quad (b)$$

When riboflavin is photochemically activated, it reacts with NBT to generate $NBTH^{\bullet}$ [55], which leads to formazan according to reaction (a). In the presence of oxygen, radical species are controlled by quasi-equilibrium (b). Thus, superoxide anion radicals appear indirectly when the assay is performed under aerobic conditions. In the presence of an antioxidant molecule that can donate an electron to NBT, the typical purple colour of formazan decays, which can be followed spectrophotometrically at 560 nm. Antioxidants are able to inhibit the formation of NBT and scavenge superoxide anion radicals. The decrease in absorbance at 560 nm in the presence of antioxidants indicates the scavenging of superoxide anions in the reaction mixture [43].

Metal ion chelating activity

One measurement of the metal-chelating activity of an antioxidant is based on the absorbance measurement of the Fe^{2+}-ferrozine complex after the prior treatment of a ferrous ion solution with test material. The metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential [43]. The data obtained from Table 1 reveal that bromophenol compounds possess marked capacity for iron binding, suggesting that their main action as a peroxidation inhibitor may be related to their iron binding capacity. In this assay, the new compounds interfered with the formation of the ferrous-ferrozine complex. It suggests that bromophenol compounds have chelating activity and are able to capture ferrous ion before ferrozine [14]. The present study demonstrated that the new compounds bound ferrous ions (Fe^{2+}). Metal-binding capacity was investigated by assessing the ability of the antioxidants to compete with the indicator ferrozine to complex with ferrous ions (Fe^{2+}) in solution [13]. Bromophenol compounds had strong chelating effect on ferrous ions (Fe^{2+}). All new compounds exhibited effective chelation of ferrous ion (Fe^{2+}). As can be seen in Table 1, the IC_{50} values of the ferrous ion (Fe^{2+}) chelating effects of the new compounds ranged between 30–100 μM.

A significant drawback of this complexation reaction in measuring the presence of antioxidant chelator is that the reaction is affected by both the antioxidant-Fe^{2+} and ferrozine-Fe^{2+} complex formation constants, and the competition between the two chelators for binding to iron. Thus, a weak antioxidant iron chelator would be seriously underestimated in quantitative determination. From a nutritional point of view, it is not yet possible to assess the role of a weak antioxidant iron chelator in preventing the Fenton reaction *in vivo*. Nonetheless, this reaction serves as a convenient assay to assess the iron chelating activity of antioxidants.

$$3\,\text{Ferrozine} + Fe(H_2O)_6^{2+} \rightarrow \text{Fe-(Ferrozine)}_3^{4-} + 6H_2O$$

The metal chelating capacity was significant since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ions [56].

Another method that is widely used in measuring the metal chelating activity is 2,2′-bipyridine-ferrous ion (Fe^{2+}) chelating activity. Metal ions can cause lipid peroxidation which, in turn, can produce free radicals and lipid peroxides [45]. Therefore, metal chelating activity indicates the antioxidant property. The decreased absorbance in the reaction mixture indicates higher metal chelating ability. 2,2′-Bipyridine forms a complex with free Fe^{2+} but not with Fe^{2+} that is bound to other chelators. Thus, a decrease in the amount of 2,2′-bipyridine-Fe^{2+} complex formed after treatment indicates the presence of antioxidant chelators. The 2,2′-bipyridine-Fe^{2+} complex produced a chromophore with absorbance that could be measured at 522 nm. According to the results, bromophenols had effective ferrous ion (Fe^{2+}) binding afinity. The IC_{50} values of metal chelating by 2,2′-bipyridine ranged between 9–84 μM. The results obtained from both metal chelating methods clearly showed that compound **12** had the most powerful ferrous ions metal chelating effect (Table 1). The compound **12** had five methoxy

groups (–OCH_3). This compound may chelate the ferrous ion (Fe^{2+}) with its five methoxy groups (–OCH_3). The compounds with structures containing two or more of these functional groups in a favorable structure–function configuration can show ferrous ion (Fe^{2+}) chelation activity [32].

Reducing power determination

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Antioxidant compounds are able to donate electrons to reactive radicals converting them into more stable and unreactive species [57]. Reducing powers of the newly synthesized compounds (**5** and **9–16**) were investigated by FRAP and CUPRAC assays.

Antioxidant compounds reduce Fe^{3+}-ferricyanide complexes to the ferrous (Fe^{2+}) form. The Prussian blue coloured complex is formed by adding $FeCl_3$ to the ferrous (Fe^{2+}) form. Therefore, the amount of reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm [58]. In this assay, the yellow colour of the test solution turned to green or blue depending on the reducing power of the antioxidant. A higher absorbance means a higher ferric reducing power.

$$Fe(CN)_6^{3-} \xrightarrow{\text{Reductant}} Fe(CN)_6^{4-}$$

$$Fe(CN)_6^{4-} + FeCl_3 \longrightarrow Fe_4[Fe(CN)_6]_3$$

As shown in Table 2, the synthesized new compounds (**5** and **9–16**) demonstrated effective Fe^{3+}-Fe^{2+} reducing power. Especially, phenol **5** had the best activity (2.029 ± 0.199) when compared to the other synthesized bromophenols and standard compounds. The presence of different substituents in the phenol backbone structure modulates their antioxidant property, in particular their hydrogen-donating capacity. In general, unsubstituted phenol is inactive as hydrogen donor and monophenol is a less efficient antioxidant than polyphenol. The introduction of an electron-donating group such as a hydroxyl group in the ortho or para position increases the antioxidant activity of phenol. In addition, the reducing power of a phenolic molecule increases with the presence of an extra hydroxyl group on the phenolic ring. When the structure of compound **5** was compared to compound **4**, it is clearly seen that compound **5** had an extra phenolic hydroxyl group (–OH) than compound **4**. Because of this phenolic hydroxyl group (–OH), compound **5** (2.029) demonstrated higher ferrous (Fe^{2+}) reducing activity than compound **4** (1.737). Ferric ion (Fe^{3+}) reduction is often used as an indicator of electron-donating activity which is an important mechanism of phenolic antioxidants [59].

Table 2. Reducing power of synthesised bromophenols and standard compounds by Fe^{3+}-Fe^{2+} transformation ability, Cu^{2+}-Cu^{+} reducing ability and FRAP assay (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene).

	Fe^{3+}-Fe^{2+} reducing power	Cu^{2+}-Cu^{+} reducing power	FRAP assay
BHA	1.900 ± 0.178	0.728 ± 0.091	0.508 ± 0.367
BHT	0.789 ± 0.265	0.519 ± 0.233	2.402 ± 0.259
Trolox	0.283 ± 0.072	0.260 ± 0.039	1.510 ± 0.165
α-Tocopherol	0.532 ± 0.021	0.345 ± 0.073	1.159 ± 0.346
5	2.029 ± 0.199	0.794 ± 0.061	0.538 ± 0.381
9	0.300 ± 0.283	0.129 ± 0.007	0.807 ± 0.320
10	0.118 ± 0.013	0.202 ± 0.032	2.108 ± 0.027
11	0.126 ± 0.039	0.139 ± 0.025	0.574 ± 0.348
12	0.122 ± 0.019	0.162 ± 0.041	0.588 ± 0.239
13	1.971 ± 0.163	0.667 ± 0.026	0.952 ± 0.286
14	0.490 ± 0.099	0.306 ± 0.057	2.398 ± 0.262
15	1.768 ± 0.214	0.743 ± 0.066	1.080 ± 0.189
16	1.556 ± 0.345	0.638 ± 0.015	2.590 ± 0.237

The CUPRAC method is also used to determine the reducing powers of antioxidant compounds [60]. This method is based on the reduction of Cu^{2+} to Cu^{+} by antioxidants in the presence of neocuproine [61]. In this assay, a higher absorbance indicates higher cupric ion (Cu^{2+}) reducing ability. Of all compounds, bromophenol compound **5** (0.794 ± 0.061) has the highest cupric ion (Cu^{2+}) reducing activity (Table 2). Also, the cupric ion (Cu^{2+}) reducing activity absorbance values of the synthesized new compounds (**5** and **9–16**) ranged from 0.129 to 0.794 at the concentration of 10 μg/mL. These values were found to be 0.759 for resveratrol [14], 1.085 for cepharanthine, 1.336 for fangchinoline [45], 0.350 for eugenol [56], 0.568 for propolis [16], 0.190–1.009 for some 5,10-dihydroindeno[1,2-*b*]indole derivatives [62]. Reducing power of bioactive compounds or food componenets reflects the electron donating capacity and is associated with antioxidant activity. Bioactive compounds with antioxidant effects can be reductants and inactivate oxidants [63]. The reducing capacity of a bioactive compound can be measured by the direct reduction of $Fe[(CN)_6]_3$ to $Fe[(CN)_6]_2$. Addition of free Fe^{3+} to the reduced product leads to the formation of the intense Perl's Prussian blue complex, $Fe_4[Fe(CN)_6]_3$, which has a strong absorbance at 700 nm [43].

$$Fe(CN)_6^{3-} \xrightarrow{\text{Reductant}} Fe(CN)_6^{4-}$$

$$Fe(CN)_6^{4-} + Fe^{3+} \longrightarrow Fe_4[Fe(CN)_6]_3$$

An increase in absorbance of the reaction mixture would indicate an increase in the reducing capacity due to an increase in the formation of the complex. There are a number of assays designed to measure overall antioxidant activity, or

reducing potential, as an indication of a host's total capacity to withstand free radical stress. The ferric ions (Fe^{3+}) reducing antioxidant power assay takes advantage of a single electron transfer in which a ferric salt is used as an oxidant [33]. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [26]. This method is not only cost-effective and rapid, it is also robust, selective and suitable for a variety of antioxidants regardless of chemical type or hydrophilicity [19].

The FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-*s*-triazine complex $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ to the intensely blue coloured ferrous complex $[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ in acidic medium. As can be seen in Scheme 3, the reaction measures reduction of ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ) to a colored product [64].

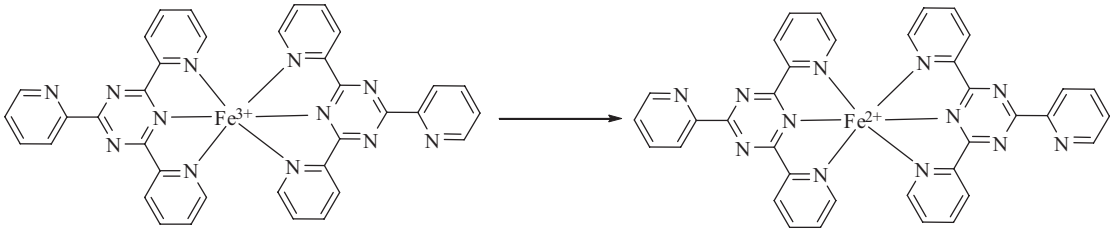
FRAP values are calculated by measuring the absorbance increase at 593 nm and relating it to a ferrous ions standard solution or to an antioxidant standard solution such as ascorbic acid. This method has also better reproducibility and higher sample throughput [65]. As the FRAP assay measures the reducing capacity based upon reduction of ferric ion, antioxidants acting by radical quenching such as thiols and carotenoids in particular will not be determined [66, 67]. In this respect, this method is very suitable in order to determine the reducing capacity of the new compounds. We selected the FRAP assay to evaluate the antioxidant activities of the new phenolic compounds for the following reasons. Firstly, the FRAP assay treats the antioxidants in the sample as reductants in a redox-linked colorimetric reaction. Secondly, the procedure of FRAP assay is relatively simple and easy to be standardized. This method has been frequently used to conduct rapid evaluations on the total antioxidant capacities of various food and beverages as well as pure compounds [68]. Furthermore, it has been applied to measure the antioxidant activity of polyphenols [66]. One probable disadvantage of this assay is the fact that this assay does not react rapidly with some antioxidants such as glutathione. However, we are of the opinion that the FRAP assay is still suitable for assessment of antioxidant activities of new compounds because only a limited amount of plant glutathione is absorbed by humans [69]. As can be seen in Table 2, all compounds showed marked $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$-$[Fe^{2+}\text{-}(TPTZ)_2]^{2+}$ reducing abilities. However, the most powerful $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$ reducing power was observed in bromophenol compound **16** (2.590 ± 0.237). This activity was greater than that of BHA (0.508 ± 0.367), BHT (2.402 ± 0.259), α-tocopherol (1.159 ± 0.346) and trolox (1.510 ± 0.165).

Conclusion

Syntheses of phenols (**5** and **15**) and bromophenols (**13**, **14** and **16)** were performed in two steps. The first step was the synthesis of their derivatives with methoxides **6–12** because phenolic OH groups in reactions were usually protected as arylmethylethers [5, 10, 15, 17, 70–73]. The second step was the demethylation of the corresponding compounds.

(3,4-Dihydroxyphenyl)(2,3,4-trihydroxyphenyl) methanone (**5**), its monobromide **13** and dibromide **14** derivatives were synthesized from corresponding reactions such as bromination and demethylation of (3,4-dimethoxyphenyl)(2,3,4-trimethoxyphenyl) methanone (**6**). The Wolf-Kishner reduction product (**9**) of **6** reacted with LiBr/CAN to produce three bromides derivatives **10–12**. 4-(3,4-Dihydroxybenzyl)-benzene-1,2,3-triol (**15**) and its dibromide derivative **16** were also synthesized by demethylation of **9** and dibromide **12**, respectively. Nine new compounds were synthesized in these reactions and their structures were determined.

In the context of our study, we have determined antioxidant, antiradical and metal chelating effects of the newly synthesized compounds (**5** and **9–16**) and four standard antioxidant molecules (BHA, BHT, α-tocopherol and trolox). The results clearly indicate that the most effective $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $DMPD^{\bullet+}$ and $O_2^{\bullet-}$ radical scavenging activities were found in phenol **15**. This synthesized phenol was found as the most potent radical scavenger. On the other hand, phenol **5**



Fe^{3+}-TPTZ **Fe^{2+}-TPTZ**

Scheme 3. $[Fe^{3+}\text{-}(TPTZ)_2]^{3+}$-$[Fe^{3+}\text{-}(TPTZ)_2]^{2+}$ reduction reaction for the FRAP assay.

obtained maximum reducing power in three different assays.

Experimental section

General information

All chemicals and solvents are commercially available and were used after distillation or treatment with drying agents. Melting points were determined on a capillary melting apparatus (BUCHI 530) and are uncorrected. IR spectra were obtained from solutions in 0.1 mm cells with a Perkin-Elmer spectrophotometer. The ^{1}H- and ^{13}C-NMR spectra were recorded on a 200 (50) and 400 (100)-MHz Varian spectrometer; δ in ppm, Me_4Si as the internal standard. Elemental analyses were performed on a Leco CHNS-932 apparatus. All column chromatography was performed on silica gel (60-mesh, Merck). PLC is preparative thick-layer chromatography: 1 mm of silica gel 60 PF (Merck) on glass plates.

The compounds **6–8** were synthesized according to [36].

*Synthesis of 1-(3,4-dimethoxybenzyl)-2,3,4-trimethoxybenzene (**9**)*

To a solution of the ketone **6** (2.1 g, 6.3 mmol) in $OHCH_2CH_2OH$ (4.0 mL) were added KOH (227 mg, 5.9 mmol) and hydrazine hydrate (0.6 mL, 619 mg, 12 mmol), consecutively, at RT and under N_2(g). After the mixture was heated to 110°C and stirred at this temperature for 1 h, it was stirred at 190°C for 3 d. The mixture was cooled to RT, acidified with HCl (10%), and extracted with ethyl acetate (EtOAc) (3 × 40 mL). The combined organic phases were dried over Na_2SO_4 and the solvent was evaporated. The residue with EtOAc/hexane (3:7) was filtrated from silica gel column (SiO_2, 80 g) and **9** (83%, 1.67 g) was obtained and crystallized from methanol as white crystals in the refrigerator at 4°C. Mp 58–60°C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 6.79 (d, A part of AB-system, J = 8.1 Hz, aromatic, 1H), 6.78 (d, A part of AB-system, J = 8.5 Hz, aromatic, 1H), 6.75 (d, J = 1.7 Hz, aromatic, 1H), 6.72 (dd, B part of AB-system, J = 8.1, 1.8 Hz aromatic, 1H), 6.60 (d, B part of AB-system, J = 8.5 Hz, aromatic, 1H), 3.88 (s, OCH_3, 3H), 3.87 (s, OCH_3, 3H), 3.85 (s, CH_2, 2H), 3.84 (s, OCH_3, 6H), 3.77 (s, OCH_3, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 152.5 (C), 152.0 (C), 149.1 (C), 147.5 (C), 142.6 (C), 134.3 (C), 127.7 (C), 124.5 (CH), 120.9 (CH), 112.5 (CH), 111.4 (CH), 107.4 (CH), 61.0 (OCH_3), 60.9 (OCH_3), 56.2 (OCH_3), 56.1 (OCH_3), 56.0 (OCH_3), 35.5 (CH_2). IR (CH_2Cl_2, cm^{-1}) 2991, 2940, 2836, 1601, 1513, 1494, 1465, 1442, 1417, 1311, 1275, 1260, 1204, 1164, 1136, 1100, 1094, 1027, 917. Anal. Calcd. for ($C_{18}H_{22}O_5$): C 67.91; H 6.97. Found C 67.97; H 6.99. ($C_{18}H_{22}O_5$): C 67.91 MS *m/z* (CI, methane) 318.1 (M^+).

Standard procedure for bromination with LiBr/CAN

*Bromination of 1-(3,4-dimethoxybenzyl)-2,3,4-trimethoxybenzene (**9**) with LiBr/CAN (1 equiv.)*

To a solution of **6** (630 mg, 1.98 mmol) and LiBr (172 mg, 1,98 mmol) in CH_3CN (15 mL) was added a solution of CAN (1.086 g, 1.98 mmol) in CH_3CN (15 mL) dropwise at RT and under N_2 over 10 min. After the solution was stirred at RT and under N_2 for 3 d, water (15 mL) was added and the mixture was extracted with EtOAc (3 × 40 mL). The combined organic phases were washed with solutions (5%, 2 × 20 mL) of $NaHCO_3$, water (2 × 20 mL), and brine (20 mL). Then it was dried over Na_2SO_4 and the solvents were evaporated. The residue was subjected to column chromatography on silica gel (SiO_2, 110 g) and eluted using EtOAc/hexane (1:9) to give dibromide **12** (47 mg, 5%), monobromide **10** (299 mg, 38%) and monobromide **11** (409 mg, 52%), respectively.

*1-Bromo-5-(3,4-dimethoxybenzyl)-2,3,4-trimethoxybenzene (**10**)*

Liquid as pale yellow. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 7.00 (bs, aromatic, 1H), 6.79 (d, A part of AB-system, J = 8.1 Hz, aromatic, 1H), 6.73 (s, aromatic, 1H), 6.71 (dd, B part of AB-system, J = 8.2, 1.83 Hz, aromatic, 1H), 3.91 (s, OCH_3, 3H), 3.87 (s, OCH_3, 3H), 3.85 (s, OCH_3, 6H), 3.83 (s, CH_2, 2H), 3.76 (s, OCH_3, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 151.55 (C), 149.91 (C), 149.14 (C), 147.73 (C), 133.04 (C), 132.20 (C), 127.93 (CH), 127.59 (C), 121.06 (CH), 112.52 (CH), 111.51 (CH), 111.47 (C), 61.19 (OCH_3), 61.15 (OCH_3), 61.04 (OCH_3), 56.12 (OCH_3), 56.10 (OCH_3), 35.44 (-CH_2–). IR (CH_2Cl_2, cm^{-1}) 2928, 2850, 1590, 1513, 1460, 1418, 1403, 1292, 1236, 1139, 1082, 1037, 1006, 962. Anal. Calcd. for ($C_{18}H_{21}BrO_5$): C 54.42; H 5.33 Found C 54.47; H 5.34.

*1-(2-Bromo-4,5-dimethoxybenzyl)-2,3,4-trimethoxybenzene (**11**)*

Amorphous as pale yellow. Mp 75–77°C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 7.03 (s, aromatic, 1H), 6.68 (d, A part of AB-system, J = 8.6 Hz, aromatic, 1H), 6.63 (s, aromatic, 1H), 6.58 (d, B part of AB-system, J = 8.6 Hz, aromatic, 1H), 3.97 (s, CH_2, 2H), 3.87 (s, OCH_3, 3H), 3.85 (s, OCH_3, 3H), 3.832 (s, OCH_3, 3H), 3.828 (s, OCH_3, 3H), 3.75 (s, OCH_3, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 152.62 (C), 152.04 (C), 148.58 (C), 148.15 (C), 132.56 (C), 126.19 (C), 124.31 (CH), 115.67 (CH), 114.71 (C), 113.86 (CH), 113.77 (C), 107.34 (CH), 60.97 (OCH_3), 60.95 (OCH_3), 56.36, (OCH_3), 56.15 (2 OCH_3), 35.44 (CH_2). IR (CH_2Cl_2, cm^{-1}) 3072, 3008, 2937, 2834, 2602, 2021, 1723, 1601, 1510, 1491, 1467, 1443, 1428, 1415, 1385, 1343, 1329, 1257, 1228, 1200, 1193, 1170, 1095, 1036, 972, 937, 905. Anal. Calcd. for ($C_{18}H_{21}BrO_5$): C 54.42; H 5.33 Found C 54.50; H 5.36.

*1-Bromo-5-(2-bromo-4,5-dimethoxybenzyl)-2,3,4-trimethoxybenzene (**12**)*

Pale yellow crystals from CH_2Cl_2/hexane. Mp 97–100°C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 7.03 (s, aromatic, 1H), 6.87 (s, aromatic, 1H), 6.64 (s, aromatic, 1H), 3.95 (s, CH_2, 2H), 3.91 (s, OCH_3, 3H), 3.88 (s, OCH_3, 3H), 3.86 (s, OCH_3, 3H), 3.84 (s, OCH_3, 3H), 3.78 (s, OCH_3, 3H).

^{13}C-NMR (100 MHz, $CDCl_3$) δ 151.56 (C), 150.00 (C), 148.67 (C), 148.44 (C), 147.64 (C), 131.31 (C), 130.75 (CH), 127.57 (CH), 115.78 (CH), 114.85 (CH), 113.94 (CH), 111.55 (C), 61.21, 61.00, 56.37, 56.26 (OCH_3), 35.35 (CH_2). IR (CH_2Cl_2, cm^{-1}) 3074, 2957, 2052, 2010, 1935, 1715, 1610, 1592, 1522. Anal. Calcd. for ($C_{18}H_{21}BrO_5$): C 45.40; H 4.23 Found C 45.58; H 4.22.

*1-(3,4-Dimethoxybenzyl)-2,3,4-trimethoxybenzene (**9**) with different equivalents (2.1 and 6.0.) of LiBr/CAN*

Only dibromide **12** (287 mg, 95%) was obtained when the reaction of **6** (200 mg, 0,6 mmol, 1.0 equiv.) with LiBr (114 mg, 1.32 mmol, 2.1 equiv.) and CAN (724 mg, 1.32 mmol, 2.1 equiv.) was performed according to the standard procedure described above. The reaction of **6** (200 mg, 0.6 mmol, 1.0 equiv.)

with LiBr (273 mg, 3.14 mmol, 5.1 equiv.) and CAN (1.72 g, 3.14 mmol, 5.1 equiv.) at the same condition also gave dibromide **12** (293 mg, 98%) as sole product.

Standard procedure for demethylation of compounds with OMe by ether cleavage

*Synthesis of (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl) methanone (**5**)*

A solution of ketone **6** (400 mg, 1.2 mmol) in CH_2Cl_2 (15 mL) was cooled to 0°C and then a solution of BBr_3 (0.8 mL, 8.30 mmol) in CH_2Cl_2 (12 mL) was added dropwise under N_2(g) over 5 min. After the cold bath was removed, the mixture was stirred at RT and under N_2 for 1 day. After the reaction mixture was cooled to 0°C, methanol (25 mL) was slowly added over 15 min and then the solvent was evaporated. After water (40 mL) and EtOAc (50 mL) were added, the mixture was shaken. The organic phase was separated and the water phase was extracted with EtOAc (2 × 40 mL). The combined organic phases were washed with $Na_2S_2O_3$ (saturated, 2 × 50 mL), $NaHCO_3$ (5%, 2 × 50 mL) and water (2 × 100 mL), and then it was dried over Na_2SO_4 and the solvent was evaporated. The phenol **5** (302 mg, 96%) was obtained from EtOAc/hexane as yellow solid. Mp 206–208°C. ^{1}H-NMR (400 MHz, CD_3COCD_3) δ (ppm): 12.65 (s, OH, 1H), 8.42 (s, OH, 4H), 7.25 (d, J = 2.1 Hz, aromatic, 1H), 7.20 (d, A part of AB-system, J = 8.9 Hz, aromatic, 1H), 7.15 (dd, A part of AB-system, J = 2.1, 8.2 Hz, aromatic, 1H), 6.96 (d, B part of AB-system, J = 8.2 Hz, aromatic, 1H), 6.48 (d, B part of AB-system, J = 8.9 Hz, aromatic, 1H). ^{13}C-NMR (100 MHz, CD_3COCD_3) δ 194.34 (CO), 155.88 (C), 153.48 (C), 152.52 (C), 149.14 (C), 131.32 (C), 127.03 (C), 125.79 (CH), 124.66 (CH), 111.51 (CH), 111.40 (CH), 109.95 (C), 106.95 (CH). IR (acetone, cm^{-1}): 2929, 2683, 1637, 1563, 1496, 1217, 1106, 1073, 1000, 930. Anal. Calcd. for ($C_{13}H_{10}BrO_6$): C 59.55; H 3.84 Found C 59.39; H 3.85.

*Synthesis of phenols **5**, **13–16** from **6–9** and **12***

The standard procedure described above was applied. Phenols **5**, **13–16** were obtained from these reactions.

*(5-Bromo-3,4-trihydroxyphenyl)(3,4-dihydroxyphenyl) methanone (**13**)*

It was obtained as yellow solid (97%). Mp 232–235°C. ^{1}H-NMR (400 MHz, CD_3COCD_3) δ (ppm): 7.43 (s, aromatic, 1H), 7.26 (d, J = 1.7 Hz, aromatic, 1H), 7.17 (dd, A part of AB-system, J = 8.2, 1.7 Hz, aromatic, 1H), 6.99 (d, B part of AB-system, J = 8.2 Hz, aromatic, 1H). ^{13}C-NMR (100 MHz, CD_3COCD_3) δ 198.35 (CO), 151.63 (C), 149.81 (C), 149.11 (C), 145.16 (CH), 133.76 (C), 129.84 (C), 127.69 (CH), 123.00 (CH), 116.63 (CH), 115.09 (CH), 113.79 (C), 99.41 (C). IR (acetone, cm^{-1}): 2695, 1926, 1706, 1625, 1520, 1487, 1440, 1365, 1123, 1001, 964, 946. Anal. Calcd. for ($C_{13}H_9BrO_6$): C 45.77; H 2.66 Found C 45.61; H 2.65.

*(5-Bromo-2,3,4-trihydroxyphenyl)(2-bromo-4,5-dihydroxyphenyl) methanone (**14**)*

It was filtered from silica gel (50 g) with EtOAc/CH_2Cl_2 (1:1) and obtained as brown solid (95%). Mp 204–207°C. ^{1}H-NMR (400 MHz, CD_3COCD_3) δ (ppm): 7.16 (s, aromatic, 1H), 7.07 (s, aromatic, 1H), 6.96 (s, aromatic, 1H). ^{13}C-NMR (100 MHz, CD_3COCD_3) δ 198.35 (CO), 151.60 (C), 149.95 (C), 148.05 (C), 144.82 (C), 133.37 (C), 130.76 (C), 128.14 (CH), 119.62 (CH), 115.96 (CH), 114.10 (C), 108.25 (CH), 99.91 (C). IR (acetone, cm^{-1}): 3467, 1970, 1739, 1630, 1514, 1474, 1434, 1373, 1190, 1099, 1019, 960. Anal. Calcd. for ($C_{13}H_8Br_2O_6$): C 37.18; H 1.92 Found C 37.07; H 1.91.

*4-(3,4-Dihydroxybenzyl)benzene-1,2,3-triol (**15**)*

It was filtered from silica gel (50 g) with EtOAc/CH_2Cl_2 (1:1) and obtained as pale brown solid (94%). Mp 127–129°C. ^{1}H-NMR (400 MHz, CD_3COCD_3) δ (ppm): 6.70 (d, A part of AB-system, J = 8.0 Hz, aromatic, 1H), 6.70 (d, J = 2.0 Hz, aromatic, 1H), 6.57 (dd, B part of AB-system, J = 8.0, 2.0 Hz, aromatic, 1H), 6.39 (d, A part of AB-system, J = 8.3 Hz, aromatic, 1H), 6.32 (d, B part of AB-system, J = 8.3 Hz, aromatic, 1H), 3.85 (s, methylenic, 2H). ^{13}C-NMR (100 MHz, CD_3COCD_3) δ 144.88 (C), 144.04 (C), 143.97 (C), 143.10 (C), 133.85 (C), 132.84 (C), 120.52 (CH), 120.44 (C), 120.21 (CH), 116.06 (CH), 115.10 (CH), 106.80 (CH), 34.56 (CH_2). IR (acetone, cm^{-1}): 3335, 1804, 1693, 1620, 1500, 1432, 1339, 1279, 1222, 1184, 1143, 1050, 964. ($C_{13}H_{12}O_5$): C 62.90; H 4.87 Found C 62.74; H 4.88.

*4-Bromo-6-(2-bromo-4,5-dihydroxybenzyl)benzene-1,2,3-triol (**16**)*

It was filtered from silica gel (50 g) with EtOAc/CH_2Cl_2 (1:1) and obtained as brown solid (95%). Mp 114–116°C. ^{1}H-NMR (400 MHz, CD_3COCD_3) δ (ppm): 8.08 (OH, 1H), 8.07 (OH, 1H), 7.86 (OH, 1H), 7.79 (OH, 1H), 7.62 (OH, 1H), 7.04 (s, aromatic, 1H), 6.62 (s, aromatic, 1H), 6.61 (s, aromatic, 1H), 3.85 (s, methylenic, 2H). ^{13}C-NMR (100 MHz, CD_3COCD_3) δ 144.98 (C), 144.75 (C), 143.91 (C), 141.74 (C), 134.43 (C), 131.08 (C), 123.12 (CH), 120.64 (C), 119.11 (CH), 117.43 (CH), 112.73 (C), 100.06 (C), 34.29 (CH_2). IR (acetone, cm^{-1}): 3335, 1809, 1693, 1620, 1500, 1432, 1339, 1279, 1222, 1185, 1143, 1050, 963. ($C_{13}H_{10}Br_2O_5$): C 38.46; H 2.48 Found C 38.53; H 2.47.

Antioxidant activity determination

DPPH$^\bullet$ scavenging activity

The DPPH free radical scavenging activity of bromophenols was evaluated by the method of Blois [46] as previously described by Gülçin [72]. Briefly, different concentrations (66–400 μg/mL) of bromophenols were prepared and diluted to 3 mL with ethanol. Then, 1 mL of ethanolic DPPH solution (0.1 mM) was added to the samples. These samples were vortexed and incubated in the dark at 30°C for 30 min. The absorbance was measured at 517 nm against blank samples. A decrease in absorbance indicates DPPH free radical scavenging activity.

ABTS$^{\bullet+}$ scavenging activity

The ABTS$^{\bullet+}$ radical scavenging activity of bromophenols was evaluated according to the method of Re et al. [73] with minor modifications [14]. ABTS$^{\bullet+}$ is blue-green in colour with a characteristic absorbance at 734 nm. ABTS$^{\bullet+}$ cation radical was produced by reacting ABTS (2 mM) in H_2O and potassium persulphate (2.45 mM) at room temperature for 12 h. The ABTS$^{\bullet+}$ solution was diluted with phosphate buffer (0.1 M, pH 7.4) to achieve an absorbance of 0.750 ± 0.025 at 734 nm. Then, 1 mL of ABTS$^{\bullet+}$ solution was added to 3 mL of bromophenols solution in methanol at different concentrations (10–30 μg/mL). These samples were vortexed and incubated in the dark for

30 min. After 30 min, the absorbance at 734 nm was measured for each concentration relative to a blank. Decreased absorbance of the samples indicates $ABTS^{\bullet+}$ cation radical scavenging activity.

$DMPD^{\bullet+}$ scavenging activity

The DMPD radical scavenging ability of bromophenols was determined by the method of Fogliano et al. [74] with slight modifications by Gülçin [75]. This assay is based on the capacity of the extract to inhibit $DMPD^{\bullet+}$ cation radical formation. Briefly, 105 mg of DMPD was dissolved in 5 mL of distilled water. Then, 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.3). $DMPD^{\bullet+}$ was produced by adding 0.3 mL ferric chloride (0.05 M) to this solution. Different concentrations of standard antioxidants or bromophenols (10–30 μg/mL) were added, and the total volume was adjusted to 0.5 mL with distilled water. One millilitre of the $DMPD^{\bullet+}$ solution was directly added to the reaction mixture. The reaction mixtures were vortexed and incubated in the dark for 15 min. The absorbance was measured at 505 nm.

Superoxide anion radical scavenging activity

The superoxide radical scavenging activity of bromophenols was determined by the riboflavin–methionine–illuminate assay [62]. This assay is based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The total volume of the reaction mixture was 3 mL, and the concentration of bromophenols was 30 μg/mL. The concentrations of riboflavin, methionine and NBT were 1.33×10^{-5}, 4.46×10^{-5} and 8.15×10^{-8} M, respectively. The photo-induced reactions were performed using fluorescent lamps (20 W). The reaction mixture was illuminated for 40 min at 25°C. The photochemically reduced riboflavin generates $O_2^{\bullet-}$, which reduces NBT to form blue formazan. The absorbance was measured at 560 nm. A decrease in absorbance indicates increased superoxide anion scavenging activity. The un-illuminated reaction mixture was used as a blank.

Fe^{3+} reducing activity

The reducing power of bromophenols was measured according to the Fe^{3+}-Fe^{2+} transfomation with slight modifications [13]. According to this method, the reduction of Fe^{3+} to Fe^{2+} is determined by measuring the absorbance of Perl's Prussian blue complex. Briefly, different concentrations (10–30 μg/mL) of bromophenols in distilled water (0.75 mL) were mixed with 1 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 1 mL (1%) of potassium ferricyanide [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 min. Afterwards, the reaction mixture was acidified with 1 mL of trichloroacetic acid (10%). Finally, 0.25 mL of $FeCl_3$ (0.1%) was added to this solution. The absorbance of the mixture at 700 nm was measured. A decrease in absorbance indicates increased ferric reducing power.

Cupric ion (Cu^{2+}) reducing-CUPRAC assay

The cupric ion (Cu^{2+}) reducing power of bromophenols was determined by the method proposed by Apak et al. [60] with minor modifications [10]. Briefly, 0.25 mL of 10 mM $CuCl_2$ solution, 0.25 mL of 7.5 mM ethanolic neocuproine solution and 0.25 mL of ammonium acetate buffer solution (1 M) were added to a test tube and mixed with 0.25 mL of different concentrations (10–30 μg/mL) of bromophenols. The total volume was adjusted to 2 mL with distilled water, and the reaction was mixed well. The tubes were kept at room temperature. After 30 min of incubation, the absorbance was measured at 450 nm against a blank. Increased absorbance indicates increased Cu^{2+}-Cu^{+} reduction.

Ferrous ion (Fe^{2+}) chelating activity by 2,2′-bipyridine reagent

For determination of ferrous ion (Fe^{2+}) chelating activity, two distinct methods were used: ferrous ion (Fe^{2+}) chelating activity by ferrozine reagent and ferrous ion (Fe^{2+}) chelating activity by 2,2′-bipyridine reagent.

Firstly, the ferrous ion (Fe^{2+}) chelating activity of bromophenol compounds was measured according to the method of Re et al. [73] as previously explained [75, 76]. Briefly, different concentrations (10–30 μg/mL) of bromophenols in 0.25 mL methanol were added to 0.25 mL of ferrous sulphate (0.2 mM). The reaction was initiated by the addition of 1 mL Tris-HCl buffer (pH 7.4) and 1 mL 2,2′-bipyridine (0.2% in 0.2 M HCl) dissolved in 2.5 mL methanol. The total volume was adjusted to 5 mL with distilled water. The mixture was shaken vigorously and kept at room temperature for 10 min. Then absorbance was measured at 522 nm.

Ferrous ion (Fe^{2+}) chelating activity by ferrozine reagent

Ferrous ions (Fe^{2+}) chelating activity was measured by inhibiting the formation of Fe^{2+}-ferrozine complex after treatment of test material with Fe^{2+}, following the method of Dinis et al. [77]. Fe^{2+}-chelating ability of bromophenol compounds was monitored by the absorbance of the ferrous iron-ferrozine complex at 562 nm. Briefly, different concentrations of bromophenol compunds (10–30 μg/mL) in 0.4 mL methanol were added to a solution of 0.6 mM $FeCl_2$ (0.1 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.1 mL) dissolved in methanol. Then, the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm [29]. The percentage of inhibition of ferrozine-Fe^{2+} or 2,2′-bipyridine-Fe^{2+} complexes formation was calculated by using the formula given below:

$$\text{Bound ferrous ions}(\%) = \left(1 - \frac{\lambda_S}{\lambda_C}\right) \times 100$$

where λ_C is the absorbance of control and λ_S is the absorbance in the presence of bromophenols or standards. The control contains only Fe^{2+} and ferrozine or 2,2′-bipyridine [13, 58].

Statistical analysis

Each experiment was performed in triplicate. The data are reported as the mean ± standard deviation and were analysed by SPSS (version 17.0 SPSS Inc.). One-way analysis of variance was performed by ANOVA procedures. Significant differences between the means were determined by Duncan's multiple range tests. $P < 0.05$ was considered significant.

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