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SOME AZO DYES CONTAINING EUGENOL AND GUAIACOL, SYNTHESIS, ANTIOXIDANT CAPACITY, UREASE INHIBITORY PROPERTIES AND ANTI-*HELICOBACTER PYLORI* ACTIVITY

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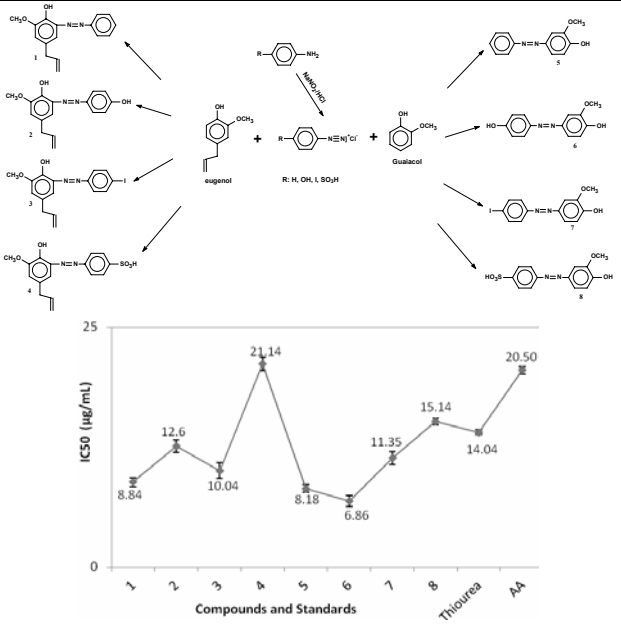
There are many studies about azo compounds at literature. However, antioxidant capacity, urease inhibitory and anti-*Helicobacter pylori* activity properties are not well known. Therefore, some azo dyes containing eugenol and guaiacol were synthesized by coupling reactions of different amines with eugenol and guaiacol, respectively. The newly synthesized compounds were screened for their antioxidant properties by employing three *in vitro* assays like 2,2–diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and CUPric Reducing Antioxidant Capacity (CUPRAC). All compounds were assayed for inhibitory effect against urease and *Helicobacter pylori* growth *in vitro*. These results suggest that compounds **1**, **5**, **6** and **7** can be novel urease inhibitory and anti-*Helicobacter pylori* agent.



INTRODUCTION

Urease enzymes catalyze urea hydrolysis to ammonia (NH_3) and carbon dioxide (CO_2).[1, 2] The increase of medium pH by the accumulation of NH_3 creates a friendly environment for *Helicobacter pylori* survival.[3, 4] *Helicobacter pylori* colonizes the stomachs of about 50% of the world's human population. This organism is the main risk factor for peptic ulceration as well as gastric mucosal-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma.[5] The inhibitors of urease and *Helicobacter pylori* have been studied extensively during the past 20 years.[6] Current treatments for *Helicobacter pylori* infections are based on the combination of a proton pump inhibitor (omeprazole) and two antibiotics (amoxicillin and clarithromycin).[7] However, antibiotic resistance and secondary effects are the major causes of treatment failure. Therefore,

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researches focused on the potential of natural products as urease inhibitors and *Helicobacter pylori* treatments.[6, 8-11] The natural products we focus on are eugenol and guaiacol. Eugenol (2-methoxy-4-allyl phenol) is a phytochemical obtained from *Eugenia caryophillis, Ocimum Sanctum*, which finds wide applications of use ranging from perfumeries, flavourings, and in medicines.[12] Eugenol affects the peripheral aspects of the cardiovascular system. It shows antidepressant-like activity[13], antibacterial[14,15] and antioxidant activity.[16-18] Also, there are many reports about anti-*Helicobacter pylori* activity of eugenol, *in vitro*.[5, 7, 19-23] Guaiacol (2-methoxy phenol) is a phenolic compound that widely distributed in various plants and it can also be found in common foods and plant origins.[24] The guaiacol derivatives have definitely a positive effect on human health thanks to their anti–allergenic, anti–inflammatory, anti–artherogenic, antimutagens, antioxidant and antimicrobial activity.[15, 16, 25] There are few reports about anti-*Helicobacter pylori* activity of guaiacol in the literature.[26]

Although there are many manuscripts on the antimicrobial effect of azo dyes[27-31] only one of natural azo dyes (calvatic acid) has been studied as anti-*Helicobacter pylori* agent.[32, 33] The aim of this work was to synthesize some azo compounds containing natural phenols like eugenol and guaiacol and investigate antioxidant capacity, urease inhibitory and anti-*Helicobacter pylori* activity.

The results indicate that azo compounds containing eugenol and guaiacol (especially guaiacol) may be candidate for the development of new drugs for prevention or treatment of peptic ulceration.

EXPERIMENTAL

5-Allyl-2-hydroxy-3-methoxyazobenzene (**1**), 4,2'-dihydroxy-5'-allyl-3'methoxyazobenzene (**2**), 4-[(2-hydroxy-3-methoxyphenyl)-azo] benzene sodiumsulfonate (**4**), 2-methoxy-4-(phenylazo)phenol (**5**), 4-[(4-hydroxy,3-methoxyphenyl)-azo]benzene sodiumsulfonate (**8**) were obtained according to literature.[34-37] FTIR spectra were recorded by Perkin-Elmer Spectrum 100 Infrared Spectrometer. ^{1}H NMR and ^{13}C NMR studies were performed by Agilent 400 FT-NMR. Mass spectra were performed by ADVION Expression CMS.

General synthesis method of new azo compounds

In order to synthesize compounds **3**, **6** and **7**; the corresponding amine compounds (1 000 mg) were converted to diazonium salts by addition of $HCl/NaNO_2$ and then coupled to eugenol or guaiacol. Finally, pure products were obtained by column chromatography (silica gel, MeOH : $CHCl_3$, 2,5:100). All new azo compounds were soluble in DMSO and ethanol. Physical and spectral analysis data were as follows

4-Iodo-2'-hydroxy-5'-allyl-3'-methoxyazobenzene (**3**)

Yield: 1200 mg (67 %.); m.p. 105-106 ^{o}C. FTIRν_{max}/cm^{-1} 3357, 3071, 3000, 2932, 2834, 1577, 1486 (N=N), 1375, 1261, 1142, 1095, 1050, 1000, 911, 819. ^{1}H NMR (DMSO-d_6) δ, ppm: 10.54 (1H, s, OH), 7.92-7.90 (2H, d, ArCH), 7.76-7.74 (2H, d, ArCH), 7.11 (1H, s, ArCH), 6.95 (1H, s, ArCH), 5.97-5.92 (1H, m, =CH), 5.11-5.03 (2H, m, CH_2), 3.82 (3H, s, OCH_3), 3.33-3.31 (2H, d, =CH_2). ^{13}C NMR (DMSO-d_6) δ, ppm: 151.43, 149.53, 144.60, 138.81, 138.69, 137.97, 130.80, 124.98, 116.44, 116.34, 112.16, 98.90, 56.47, 39.39. MS: m/z 395.4 (M+1).

2-Methoxy-4-[(4-hydroxyphenyl)azo]phenol (**6**)

Yield: 1500 mg (61 %.); m.p. 72-73^{o}C. FTIRν_{max}/cm^{-1} 3321, 2936, 2837, 1585, 1501, 1462 (N=N), 1372, 1261, 1201, 1145, 1102, 1025, 837, 793. ^{1}H NMR (DMSO-d_6) δ, ppm: 10.09 (1H, s, OH), 9.73 (1H, s, OH), 7.71-7.69 (2H, d, ArCH), 7.37 (1H, s, ArCH), 7.356-7.353 (1H, d, ArCH), 6.927-6.926 (1H, d, ArCH), 6.89-6.87 (2H, d, ArCH), 3.83 (3H, s, OCH_3). ^{13}C NMR (DMSO-d_6) δ, ppm: 160.40, 150.02, 148.68, 145.82, 145.64, 124.29, 119.61, 116.23, 115.62, 103.46, 55.96. MS: m/z 245.3 (M+1).

2-Methoxy-4-[(4-iodophenyl)azo]phenol (**7**)

Yield: 1500 mg (93 %.); m.p. 74-75^{o}C. FTIRν_{max}/cm^{-1} 3488, 3075, 3007, 2935, 2838, 1590, 1566, 1499 (N=N), 1454, 1416, 1389, 1260, 1202, 1135, 1103, 1001, 825. ^{1}H NMR (DMSO-d_6) δ, ppm: 10.02 (1H, s, OH), 7.90-7.88 (2H, d, ArCH), 7.58-7.56 (2H, d, ArCH), 7.48-7.46 (1H, d, ArCH), 7.43 (1H, s, ArCH), 6.97-6.94 (1H, d, ArCH), 3.84 (3H, s, OCH_3). ^{13}C NMR (DMSO-d_6) δ, ppm: 151.82, 151.46, 148.85, 145.65, 138.68, 124.45, 121.35, 115.72, 103.44, 97.78, 56.01. MS: m/z 355.3 (M+1).

CUPric reducing antioxidant capacity assay (CUPRAC)

In order to determine the cupric ions (Cu^{2+}) reducing ability of the synthesized compounds was determined according to the literature.[38, 39] The standard curve was linear between 8 mg/mL and 0.03125 mg/mL trolox ($r^2 = 0.9989$). CUPRAC values were expressed as mM Trolox equivalent of 1 mg synthesized compound.

DPPH-Free radical scavenging assay

The DPPH radical scavenging activity of the synthesized compounds was measured using the method of Blois.[40] All determinations were carried out three times. Radical scavenging activity was measured by using Ascorbic Acid, Trolox® and Catechin (Sigma Chemical Co, USA) as standards and all values are expressed as % scavenging of DPPH radical.

$ABTS^{\bullet+}$ Radical cation decolorization assay

The ability of the synthesized compound to scavenge $ABTS^{\bullet+}$ radical was determined according to the literature.[41, 42] All determinations were carried out three times. ABTS radical scavenging activity was measured by using Trolox®, Ascorbic Acid and Catechin (Sigma Chemical Co, USA) as standards.

In vitro Urease inhibition assay

Urease catalyzes the hydrolysis of urea into carbon dioxide and ammonia. The production of the ammonia was measured using the indophenol method.[43]

Anti-Helicobacter pylori activity assessment

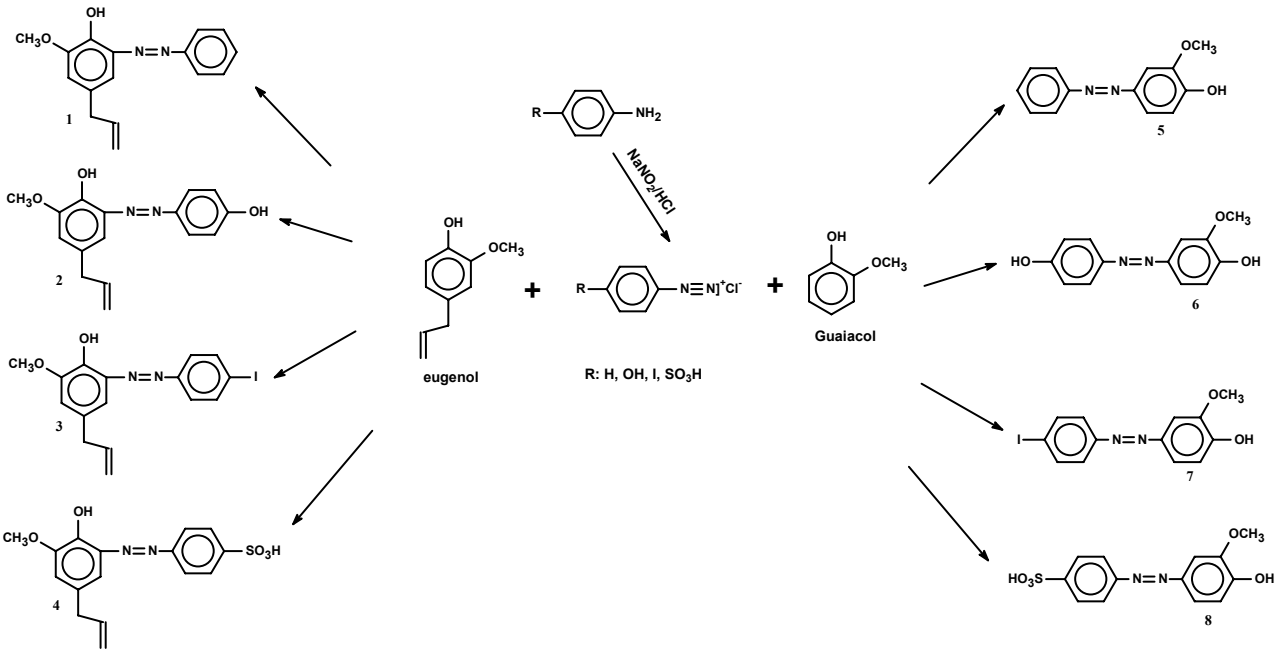
Test microorganism (*Helicobacter pylori* J99) was obtained from the Hifzissihha Institute of Refik Saydam (Ankara, Turkey).

Agar well diffusion method. Simple susceptibility screening test using agar-well diffusion method was used. *Helicobacter pylori* were suspended in Mueller Hinton (MH) (Difco, Detroit, MI) broth. Brain Heart Infusion Agar (BHI) with 7 % human blood and supplement (DENT) was used for *H. pylori* J99. *H. pylori*-selective supplement (Dent) containing vancomycin (10 mg/liter), trimethroprim lactate (5 mg/liter), cefsulodin (5 mg/liter), and amphotericin B (5 mg/liter), purchased from Oxoid (Oxoid, Hampshire, England). They were "flood-inoculated" onto the surface of MH and SD agars and then dried. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer, and 80 μL of the compounds were delivered into the wells. The plates were incubated for 18 h at 35°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Amoxicillin (10 μg) was standard drug. Hexane was used as solvent control.

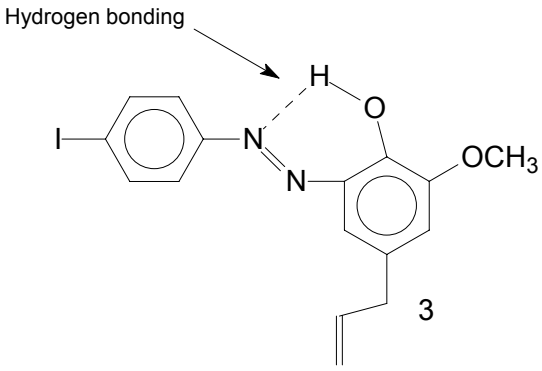
RESULTS AND DISCUSSION

Synthesis and Characterization

The synthetic route of azo compounds can be seen in Scheme 1. All compounds were synthesized by azo coupling reaction of different amines with eugenol and guaiacol, respectively. All spectroscopic data of compounds **1**, **2**, **4**, **5** and **8** show good agreement with the literature values.[34-37] Newly synthesized azo compounds were characterized by FT-IR, UV-Vis, ^{1}H NMR, ^{13}C NMR and Mass spectroscopy techniques.



Scheme 1 – Synthetic route of azo compounds (**1**–**8**).



Scheme 2 – Hydrogen bonding at compound 3.

The compound **3** exhibited the broad OH band at 3357 cm^{-1} in the FT-IR spectrum. The formation of compound **3** was clearly indicated by the appearance of OH peak at 10.54 ppm and aromatic peaks at 7.92-7.90 and 7.76-7.74 ppm as two doublets, at 7.11 and 6.95 ppm as two singlets, aliphatic protons at 5.97-5.92 and 5.11-5.03 as two multiplets, at 3.82 ppm as a singlet and 3.33-3.31 as a doublet in its ^{1}H NMR spectrum. Singlet peak at 10.54 ppm can be assigned to proton of OH group which is intramolecular hydrogen bonding with the nitrogen atom in neighboring azo group (Scheme 2). The ^{13}C NMR spectrum of compound **3** compatible to suggested structure.

The compound **6** signified the broad OH band at 3363 cm^{-1} in the FT-IR spectrum. The formation of compound **6** was clearly indicated by the appearance of two OH peaks at 10.09 and 9.73 ppm and aromatic peaks at 7.71-6.87 ppm in its ^{1}H NMR spectrum. The ^{13}C NMR spectrum of compound **6** showed the presence of expected carbon atoms.

The compound **7** exhibited the broad OH band at 3488 cm^{-1} in the FT-IR spectrum. The formation of compound **7** was clearly indicated by the appearance of OH peaks at 10.02 ppm and aromatic peaks at 7.90-6.94 ppm in its ^{1}H NMR spectrum. The ^{13}C NMR spectrum of compound **7** showed the presence of suggested carbon atoms.

Azo dyes are capable of exhibiting two geometrical isomerism as *cis-trans* because of rotation about the –N=N– double band. All of our azo compounds exist as *trans* isomer. Compounds **1-4** contain a hydroxy group *ortho* the azo group. As illustrated in scheme 2, this gives rise to intramolecular hydrogen-bonding which further stabilises the *trans* isomer and effectively prevents its conversion into the *cis* form. As can be understood from the crystal structure of compound **8** (given in the supporting figure S11), compounds **5-8** is in trans form.

High resolution MS spectra APCI (Atmospheric Pressure Chemical Ionization) of compounds **3, 6** and **7** provided a definitive proof for their characterization. Molecular ion peaks of compounds **3, 6** and **7** were detected as expected. MS measurements confirmed unambiguously the molecular mass of compounds **3** (m/z =395.4 M+1), **6** (m/z = 245.3 M+1), **7** (m/z = 355.3 M+1).

CUPRAC Antioxidant capacity

The CUPRAC method is based on the absorbance measurement of the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine)copper(II) cation [Cu(II)-Nc], where absorbance is recorded at the maximal light absorption wavelength of 450 nm.[38] The antioxidant effects were classified by three groups; compound **6**, the most effective , was the first. Compounds **1, 2, 3, 5, 7** and **8**, evaluated by the range of moderately, were the second (**Figure 1**). The others were determined as the third group, the compound **4**, weak antioxidant activity.

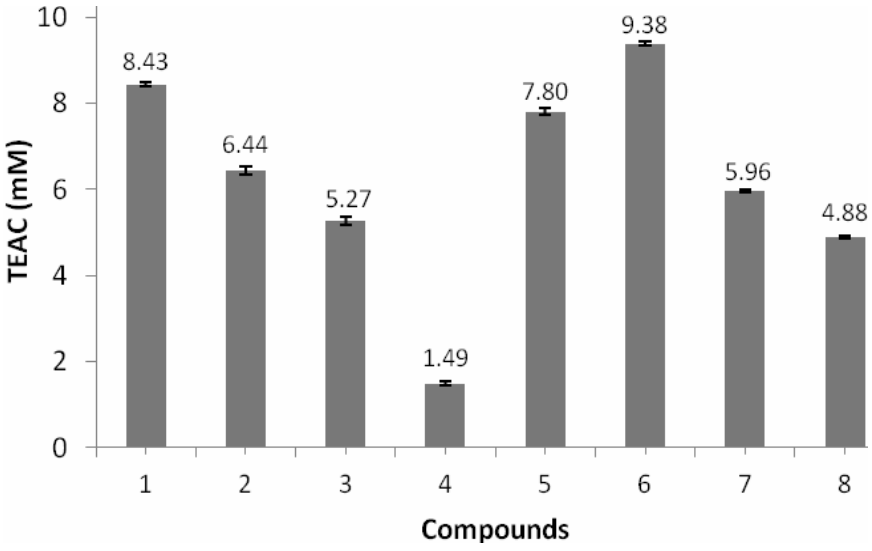


Fig. 1 – CUPRAC test results of all the synthesized compounds as mM TEAC (Trolox equivalent antioxidant capacity) values obtained from [Trolox]- absorbance calibration graph. TEAC values of compounds are expressed as the mean ±S.D. in triplicate.

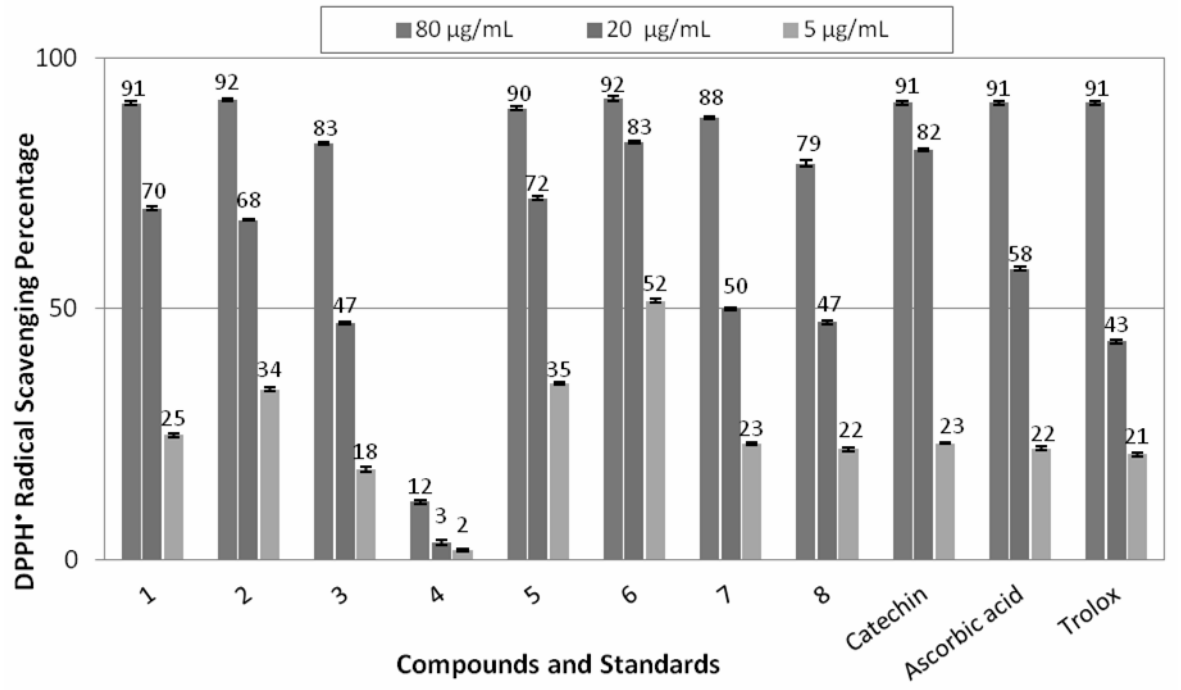


Fig. 2 – % DPPH radical scavenging capacity of all the compounds and standards. %DPPH• radical scavenging values are defined as the mean ±S.D. in triplicate.

DPPH Scavenging capacity

The total radical scavenging capacity of the compounds was determined and compared to that of Trolox®, ascorbic acid and catechin by using the DPPH• and $ABTS^{\bullet+}$ radical scavenging methods. The compounds **1**, **2**, **5**, **6** and **7** showed fairly well DPPH radical scavenging activity, at the 20 µg/mL final concentration (Figure 2). Beside this reality, it has been found that these compounds have greater scavenging activity than Trolox® at 20.0 µg/mL final concentration. On the other hand, the compounds **3, 7** and **8** showed good activity at the same concentration. In addition to, at 5.0 µg/mL final concentration, it was found that the compound **6** had greater scavenging activity than the other compounds and all standard antioxidant molecules.

$ABTS^{\bullet+}$ Scavenging capacity

The pre-formed radical monocation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity.[41] The compounds **1**, **2**, **5**, **6** and **7** showed more scavenging activity than Trolox®, standard at the 2.0 µg/mL final concentration (Figure 3).

Basic correlation analysis was performed to investigate the relationship between the $ABTS^{\bullet+}$ radical scavenging and cupric reducing activities of the compounds, as shown in Figure 4. It was expected that the antioxidant activities of the compounds in both assays would show similar trends and that the values of both could, therefore, be correlated. The results of the correlation of the two antioxidant tests are plotted at **8.0, 2.0 and 0.50 µg/mL** final concentration (Figure 4), and a relatively good correlation was determined (R^2 = 0.9284-0.9615). All of the compounds active in the CUPRAC antioxidant assay exhibit highly radical scavenging activities in the $ABTS^{\bullet+}$ method. Some differences seen between the results of the two antioxidant methods results are probably due to the differences between the reaction mechanisms and in dependence on the reaction conditions and sterical issues in the case of $ABTS^{\bullet+}$ test.[44]

Anti-urease activities

All compounds were assayed for their *in vitro* inhibitory activity against Jack Bean urease. Thiourea and acetohydroxamic acid were used as standard

inhibitors. Initially, all the synthesized compounds were screened at the 80 µg/mL final concentration. Among these compounds, **6** exhibited the best inhibitory effect against urease (Figure 5).

Until now, only one compound, acetohydroxamic acid (AA), has been clinically used for the treatment of urinary tract infections by urease inhibition. Unfortunately, it exhibits severe side effects. Thus, it seems that the full potential of urease inhibition has not yet been fully explored. In this study, all compounds, except for compound **4**, had higher inhibitory effect on urease and exhibited lower IC_{50} inhibition values than acetohydroxamic acid (Figure 5). Dose-dependent inhibitory effect of the some compounds and standards were depicted in Figure 6.

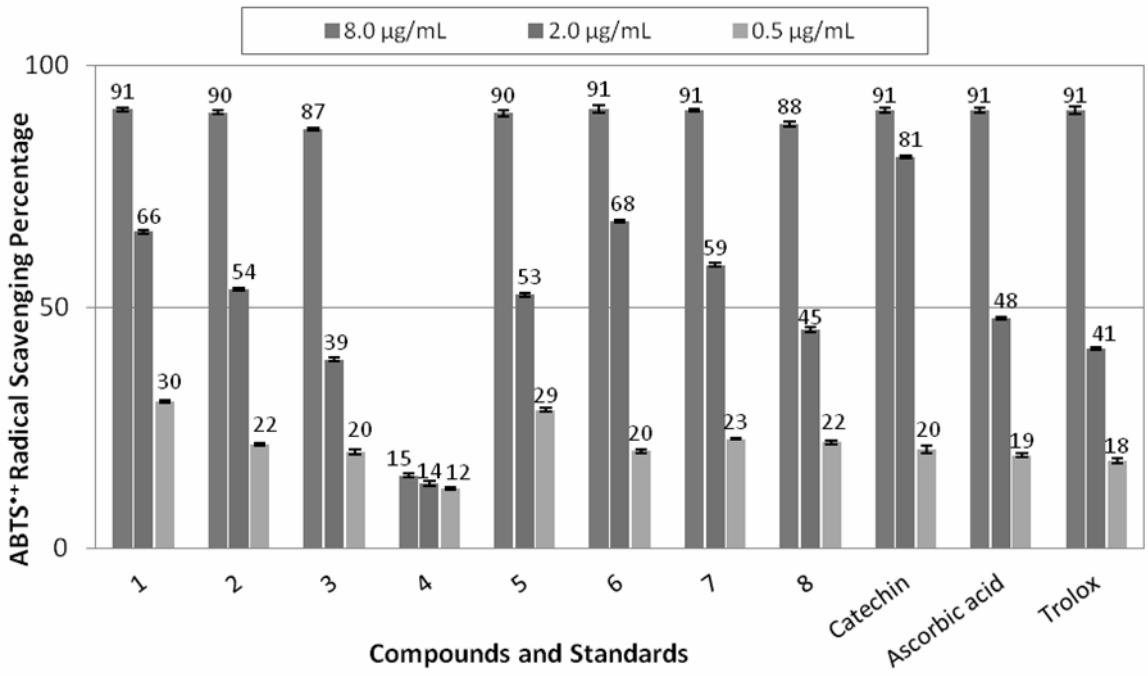


Fig. 3 – % $ABTS^{\bullet+}$ scavenging of the compounds and standards. $ABTS^{\bullet+}$ scavenging values of compounds are expressed as the mean ±S.D. in triplicate.

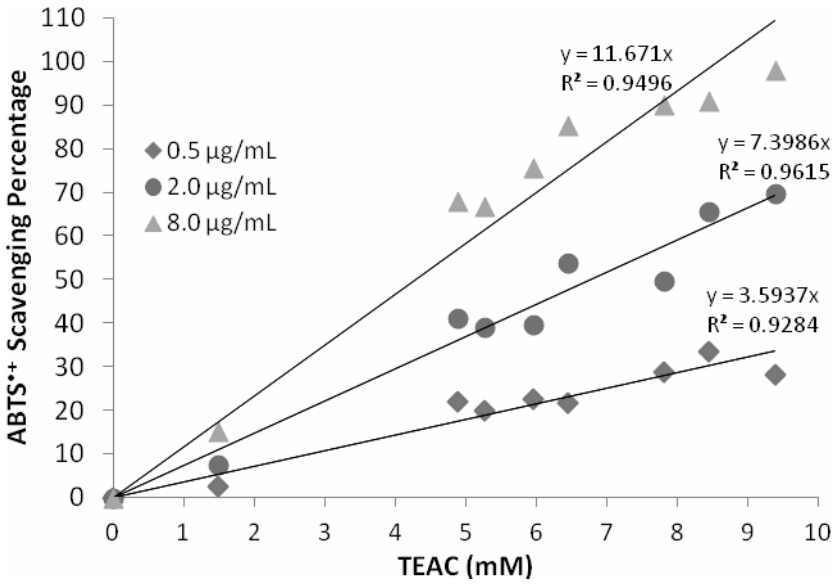


Fig. 4 – Basic correlation analysis graph between the results of CUPRAC antioxidant activities test (TEAC values) and $ABTS^{\bullet+}$ radical scavenging activities of the synthesized compounds at 0. 5, 2.0 and 8.0 µg/mL concentrations (percentage scavenging of $ABTS^{\bullet+}$ at 5 min incubation time).

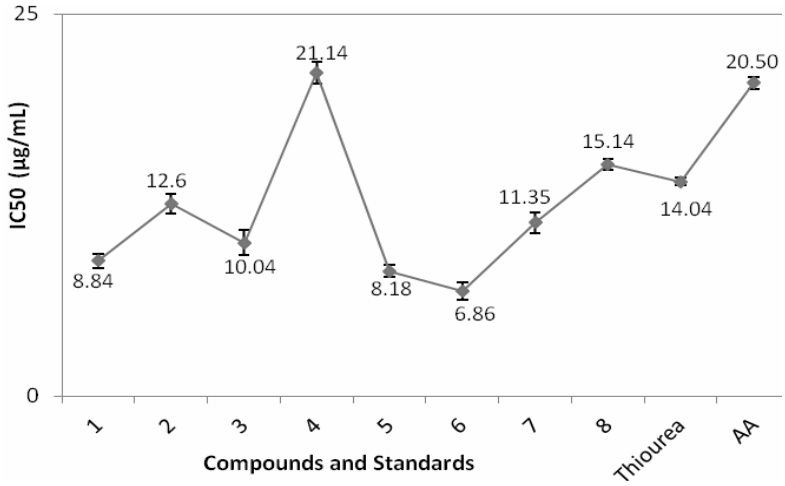


Fig. 5 – IC_{50} values of all compounds against Jack Bean urease. Thiourea and acetohydroxamic acid were used as standard inhibitors.

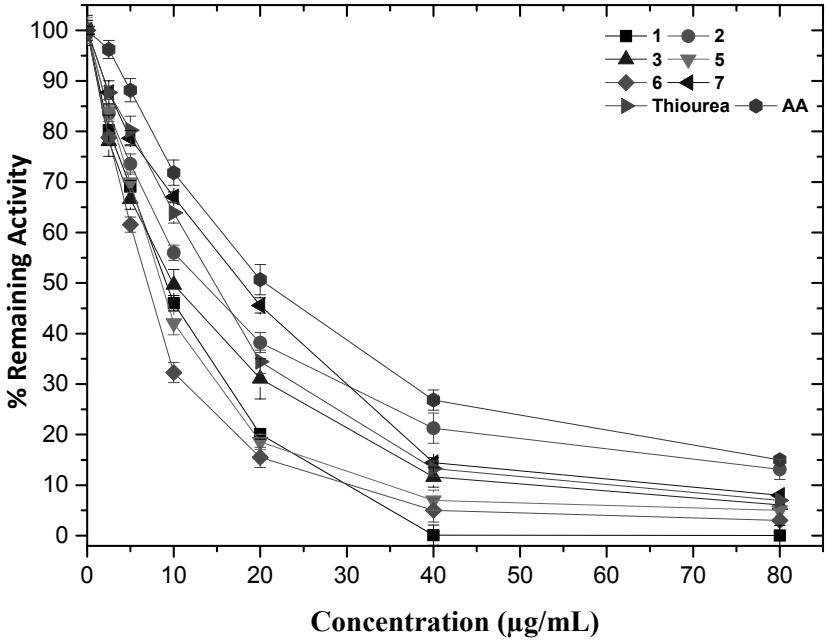


Fig. 6 – Dose-dependent inhibitory effect of some compounds (**1**, **2**, **3**, **5**, **6** and **7**) and standards. Thiourea and acetohydroxamic acid (AA) were used as standard inhibitors. Inhibitory effects of compounds and standards were measured in the range of 80 to 2.50 µg/mL concentrations.

A significant positive correlation was determined between the antioxidant capacity and Anti-Urease activity. Compounds **1**, **5** and **6** have both the most antioxidant capacity and most anti-urease activity.

Urease was the first enzyme crystallized and known to contain nickel ions.[45] The dependence on nickel ions for catalytic activity is a unique feature of urease among hydrolytic enzymes.[1] Inhibitors of urease can be broadly classified into two categories: (1) active site directed (substrate-like), (2) mechanism-based directed [46]. We believe that our compounds at the first group and directed binding to nickel ions at active site of enzyme. However, in order to determine the exact inhibition mechanism need more comphrensive studies like docking, kinetics.

Anti-*Helicobacter pylori* activities

Helicobacter pylori is the major microorganism for bacterial gastrointestinal infections, peptic ulcer and gastric cancer. Inhibition of this enzyme is very important for the treatment of *H.pylori* related diseases.

Table 1

Anti-*Helicobacter pylori* activity of all the compounds (80 µg), (Amoxicillin 10 µg)

Compounds	*Helicobacter pylori* inhibition zone (mm)
P1	9
P2	8
P3	8
P4	-
P5	8
P6	8
P7	10
P8	8
Control	-
Amoxicillin	40

Anti-*Helicobacter pylori* activity was obtained from all compounds except for compound **4**. Inhibition zones which were determined almost the same (8–10 mm) for all compounds at 80 µg, can be seen in Table 1.

It is determined that compound **7** has the highest anti-microbial activity against *Helicobacter pylori*. A positive relationship was found between Anti-*Helicobacter pylori* activities and anti-urease activities. Almost all compounds have both high antioxidant capacity and high Anti-*Helicobacter pylori* activity. Although antimicrobial properties of some azo dyes were known on bacterium that can cause several serious illnesses [27-31], only one compound (calvatic acid) has been confirmed as Anti-*Helicobacter pylori* agent [32, 33] so far. Our synthesized natural phenol based azo dyes could be considered as a new Anti-*Helicobacter pylori* agent at literature.

CONCLUSION

Natural phenol (eugenol and guaiacol) based some azo dyes were synthesized and their antioxidant capacity, urease inhibitory and Anti-*Helicobacter pylori* activity were investigated. Finally, we can suggest that the natural phenol based azo compounds (**1**, **5**, **6** and **7**) with high antioxidant capacity and effective urease inhibitor have Anti-*Helicobacter pylori* agent potential. However, more extensive studies (such as cytotoxicity, genotoxicity) are needed to evaluate these compounds as drugs.

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