www.dergipark.gov.tr
ISSN:2148-3736
El-Cezerî Fen ve Mühendislik Dergisi
Cilt: 9, No: 3, 2022 (1147-1156)
El-Cezerî Journal of Science and Engineering
Vol: 9, No: 3, 2022 (1147-1156)
DOI : 10.31202/ecjse.1131913

ECJSE

Research Paper / Makale

Some Azo Compounds Containing Black Tea Processing Waste Catechins as Antioxidant and Urease Enzyme Inhibitory

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Received/Geliş: 20.06.2022 **Accepted/Kabul:** 07.09.2022

Abstract: Although a lot of azo compounds containing some natural origin catechins had been synthesized and determined their dyeing properties for various textile products, the azo compounds containing black tea processing waste catechins and their antioxidant capacity and urease enzyme inhibition were not investigated until this study. The urease enzyme is the most important enzyme that allows *Helicobacter pylori*, which is considered the main factor of stomach cancer, to live in the stomach. Inhibition of urease is very important for the treatment of *Helicobacter pylori* infection. It has been known that catechin extracts of natural origin inhibit the urease enzyme of *Helicobacter pylori* from literature. Black tea processing waste is a residue that is separated from the sieves during tea processing and has no economic value. The transformation of this residue into products that produce added value is very important because it contains many chemicals contained in the tea plant. In this study, some azo compounds containing black tea processing waste catechins were synthesized and investigated their antioxidant capacity, urease enzyme inhibition properties.

Keywords: Tea catechins, azo dyes, antioxidant, urease enzyme inhibition

Antioksidan ve Üreaz Enzim İnhibitörü Olarak Siyah Çay İşleme Atığındaki Kateşinleri İçeren Bazı Azo Bileşikleri

Öz: Bu çalışmaya kadar bazı doğal kaynaklı kateşinleri içeren bir çok azo bileşiği sentezlenmiş ve çeşitli tekstil ürünleri için boyama özellikleri incelenmiş olmasına rağmen, siyah çay işleme atığı kateşinlerini içeren azo bileşikleri ile bunların antioksidan kapasiteleri ve üreaz enzim inhibisyon etkileri araştırılmamıştır. Üreaz enzimi, mide kanserinin ana faktörü olarak kabul edilen *Helicobacter pylori* adlı bakterinin midede yaşamasını sağlayan en önemli enzimdir. Bu enzimin inhibisyonu *Helicobacter pylori* enfeksiyonu tedavisi için çok önemlidir. Doğal kaynaklı kateşin ekstraktlarının *Helicobacter pylori* üreazını inhibe ettiği literatürden bilinmektedir. Siyah çay işleme atığı, çayın işlenmesi sırasında eleklerden ayrılan ve ekonomik değeri olmayan bir kalıntıdır. Çay bitkisinin içerdiği birçok kimyasalı içeren bu tortunun katma değer üreten ürünlere dönüştürülmesi çok önemlidir. Bu çalışmada, siyah çay işleme atığındaki kateşinleri içeren bazı azo bileşikleri sentezlenmiş antioksidan kapasiteleri ve üreaz enzim inhibisyon etkileri araştırılmıştır.

Anahtar Kelimeler: Çay kateşinleri, azo boyaları, antioksidan, üreaz enzim inhibisyonu

1. Introduction

The tea plant is an evergreen perennial herb belonging to the *Camellia* genus (*Camellia sinensis*) of the Theaceae family [1]. The tea plant, which is one of the most widely consumed beverages in the world and also known to have medical effects, is grown in about 40 countries around the world [2]. In the production of black tea, the tea fiber separated by the electrostatic separators during the production process and the tea litter that is separated from the sieves of different grain sizes and

How to cite this article
Kantar C., Er Z., Baltaş N., Şaşmaz S., "Some Azo Compounds Containing Black Tea Processing Waste Catechins As Antioxidant and Urease Enzyme Inhibitory", El-Cezerî Journal of Science and Engineering, 2022, 9 (3); 1147-1156.

Bu makaleye atıf yapmak için
Kantar C., Er Z., Baltaş N., Şaşmaz S., "Antioksidan ve Üreaz Enzim İnhibitörü Olarak Siyah Çay İşleme Atığındaki Kateşinleri İçeren Bazı Azo Bileşikleri", El-Cezerî Fen ve Mühendislik Dergisi 2022, 9 (3); 1147-1156.
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mostly originating from the petiole, thick veins, hard leaves, etc. cannot be turned into tea in the processed leaf. The resulting tea powder mixture is called black tea processing waste [3]. While this amount of waste is between 3-5% compared to the weight of fresh leaves, it rises to 17-18% due to the non-standard collection of fresh tea in the Eastern Black Sea Region [4]. According to the fresh tea purchase regulation, a maximum of 10% non-standard tea purchase is allowed, while in practice this rate goes up to 25-30%. Approximately 30,000 tons of factory black tea waste comes out of 600,000 tons of fresh leaves that only the General Directorate of Tea Businesses purchases every year [5].

The amount of chemicals that can be isolated from tea and tea waste is very important. Considering the 7-8% catechin content of green tea, a significant amount of catechin can be obtained [6]. No study was found about synthesis of azo compounds containing black tea processing waste catechins and the investigation of biological effects. There are only studies about synthesis of some azo compounds containing phenolic compounds in the skins of some plants and vegetables and their dyeing performance for various textile products. The synthesis of azo dyes containing phenolic compounds in red onion peel and the dyeing properties of various textile products have been investigated and it was observed that the results were promising and it was stated that agricultural wastes with similar content could also be evaluated in this direction, by Akaranta et al. [7]. Three different azo dyes containing phenolic compounds containing Areca nut extracts have been synthesized and their dyeing and UV protection properties of textile products such as nylon and polyester were investigated, and they stated that the poor fastness properties of natural dyes can be improved with this method In a study conducted by Pawar et al. [8]. Some azo compounds containing polyphenols obtained from a kind of acacia tree were synthesized and their dyeing properties were examined for silk fabric and it was emphasized that natural origin semi-synthetic dyes are environmentally friendly and have low toxic effects by Rabbi et al. [9].

Helicobacter pylori (H. pylori) is a gram-negative bacterium first isolated in 1982 by Warren and Marshall [10, 11]. Actually, *Helicobacter pylori* can only survive at stomach lumen for a few minutes due to has an acid pH [12]. However, the urease enzyme breaks down the urea to form ammonia and forms the pH that it can live on in the ammonia cloud [13]. Inhibition of the urease enzyme is very important for the treatment of *Helicobacter pylori*. Already, It has been known that the extract of *Camellia sinensis* and apple peel polyphenols inhibit the urease enzyme of H. pylori from literature [14, 15].

Azo compounds are of great interest due to having many application fields like dyeing textile products, biomedical studies, advanced applications of organic synthesis and advanced technology areas (laser, electro-optical devices and printers) [16]. Another interesting feature of azo compounds is their antimicrobial effects. 4-phenylazophenoxyacetic acids show antimicrobial activity against two gram-positive bacteria such as *Staphylococcus ureus* and *Streptococcus pyegenes*, which cause various diseases, and three gram-negative bacteria such as *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Esherichia coli* [17, 18]. It has been reported that some azo compounds with high antioxidant capacities act as Anti-*Helicobacter pylori* and Urease enzyme inhibitors by our research group [19, 20]. Some of the studies investigating different antibiotic effects of azo compounds are given as literature [21-24]. Azo compounds are the bioactive ingredients for many drugs currently in use, such as Azo, Azosilin, Uriseptin, Azogantricin, Azulfidin, Salazopyrin, Colazal, Ipsalazid and Dipentum.

In this study, we have determined the antioxidant capacities and urease inhibition properties of azo compounds containing catechins that extracted from black tea processing waste.

2. Experimental Methods

2.1. Materials

Black tea processing waste samples were obtained from the processing line at a black tea factory in the Black Sea region of Turkey during the second harvest season of 2021.

2.2. Solvent Extraction

Black tea processing waste catechins (TWC) were extracted by conventional hot water extraction method at literature [5]. Mp: 76-77 ˚C, Yield % 0,5. FTIRν_{max}/cm^{-1} 3232(OH), 2992, 2923 (CH aliphatic), 1689, 1605, 1515, 1449, 1229, 1309, 1190, 1140, 1090, 1029, 821, 763. UV/Vis λ_{max}(233, 271 nm).

2.3. General Synthesis of New Azo Dyes Containing Black Tea Processing Waste Catechins

To synthesize all azo compounds, 5-amino salicylic acid and sulphanilic acid (0,26 g) were converted to diazonium salts by addition HCl/$NaNO_2$ and were coupled to tea waste extract (0,5 g) in % of 10 NaOH solution. After the mixture became neutral by adding dilute HCl solution formed precipitate was filtered off and washed with water. Pure compounds were obtained by recrystallization from ethanol. All novel azo compounds were soluble in DMSO, ethanol, methanol. Spectral analysis data were as follow. The new azo dyes were named as 5ASATWC and SATWC synthesized from 5-amino salicylic acid and sulphanilic acid, respectively.

Physical properties and spectroscopic characterization of novel azo compounds

5ASATWC (1) Mp:>250 ˚C **,** Yield % 50, 0,15 g. FTIRν_{max}/cm^{-1} 3213-2500 (COOH), 2922 (CH aliphatic), 1614, 1508, 1490 (N=N), 1447, 1372, 1203, 1033, 830. UV/Vis λ_{max}(213, 271, 438 nm).

SATWC (2) Mp: >250 ˚C, Yield % 50, 0,15 g. FTIRν_{max}/cm^{-1} 3274(OH), 2920 (CH aliphatic), 2851, 1707, 1621, 1516, 1462 (N=N),1363, 1182, 1119, 1079, 1031, 1006, 966, 719. UV/Vis λ_{max}(211, 272, 420 nm).

2.4. Antioxidant Activity

Antioxidant activities and radical scavenging properties of the synthesized azo compounds were studied using various in vitro methods, including CUPric Reducing Antioxidant Capacity (CUPRAC), DPPH (1,1- diphenyl-2-picrylhydrazyl) and ABTS cationic radical scavenging assays. Catechin (Sigma Chemical Co, USA) was used as standard compound.

2.4.1. CUPric Reducing Antioxidant Capacity Assay (CUPRAC)

To determine the cupric ions (Cu^{2+}) reducing the ability of the newly synthesized azo compounds was determined according to the literature [25-27]. Trolox® was used standard, linear between 8.0 and 0.03125 mM (r^2= 0.999). Cuprac values were expressed as mM Trolox® equivalent 1 mg newly synthesized azo compound.

2.4.2. DPPH-Free Radical Scavenging Assay

The DPPH˙ (2,2-diphenyl 1-picrylhydrazyl) radical scavenging activity of the newly synthesized azo compounds was measured using the method of Blois [28]. Radical scavenging activity values are

expressed as SC_{50} (µg/mL), the concentration of the samples that causes 50% scavenging of DPPH$^{\bullet}$ radical. All determinations were carried out three times.

2.4.3. ABTS$^{\bullet+}$ Radical Cation Decolorization Assay

The ability of the synthesized compound to scavenge ABTS$^{\bullet+}$ radical was determined according to the literature [29]. All determinations were carried out three times.

2.5. In vitro Urease Inhibition Assay

The preparation of the *Helicobacter pylori* urease enzyme was the same as the previously published research article [30].

Urease catalyzes the hydrolysis of urea to carbondioxide and ammonia. The production of the ammonia was measured using the indophenol method [31]. *Helicobacter pylori* urease inhibitory activity and kinetic studies steps of the newly synthesized azo compounds and thiourea were examined to the published studies [30, 32]. Thiourea was used as the standard inhibitor drug. The urease inhibition percentage was calculated as follows:

$$Urease\ inhibition\ (\%) = [(Acontrol - Acompound)\ /\ Acontrol]\ x100 \qquad (1)$$

where A control is the activity of enzyme without compound/standard and A compound is the activity of the enzyme with compound/standard at different concentrations. The concentration of the inhibitor required for inhibiting 50% of the enzyme activity under the assay conditions was defined as the IC_{50}. The enzyme activity was determined by using urea as a substrate in the buffer (pH 8.2). Increasing concentrations of substrate (0.50–8.0 mg/mL) and 200 µL of urease enzyme solution were added in a test tube and the mixture was incubated at 25 °C for 50 min. The absorbance was measured at 625 nm. The obtained data were plotted as 1/activity (1/V) against 1/substrate concentration (1/[S]), according to the Lineweaver-Burke graph [33].

3. Results and Discussion

3.1. Synthesis and Characterization

Extraction of black tea processing waste catechins were accomplished by conventional hot water extraction method at literature [5]. Catechin extraction yield from black tea processing waste was % 0.5 about. The phenolic compound content of black tea waste extract was not determined.

FT-IR spectrum of black tea processing waste catechins show the broad OH band at 3232 cm^{-1} due to weak intermolecular hydrogen bonding interaction between corresponding compound molecules, aliphatic CH peaks at 2923 cm^{-1}, aromatic CH peaks at 1689 ve 1605 cm^{-1}, cyclic alcohol OH peaks at 1029 cm^{-1} [34, 35]. Black tea processing waste catechins exhibit typicaly electronic spectra with two absorption peaks in the UV region, one of them at about 233 nm and the other one at 271 nm, compatible with literature values [36].

Novel azo compounds containing black tea processing waste catechins were characterized using FT-IR and UV/Vis spectroscopy techniques. The synthetic route azo compounds containing black tea processing waste catechins can be seen in Scheme 1. More than one type of azo compounds were obtained due to there is more than one type of catechin in black tea procesing waste extracts [7, 8].

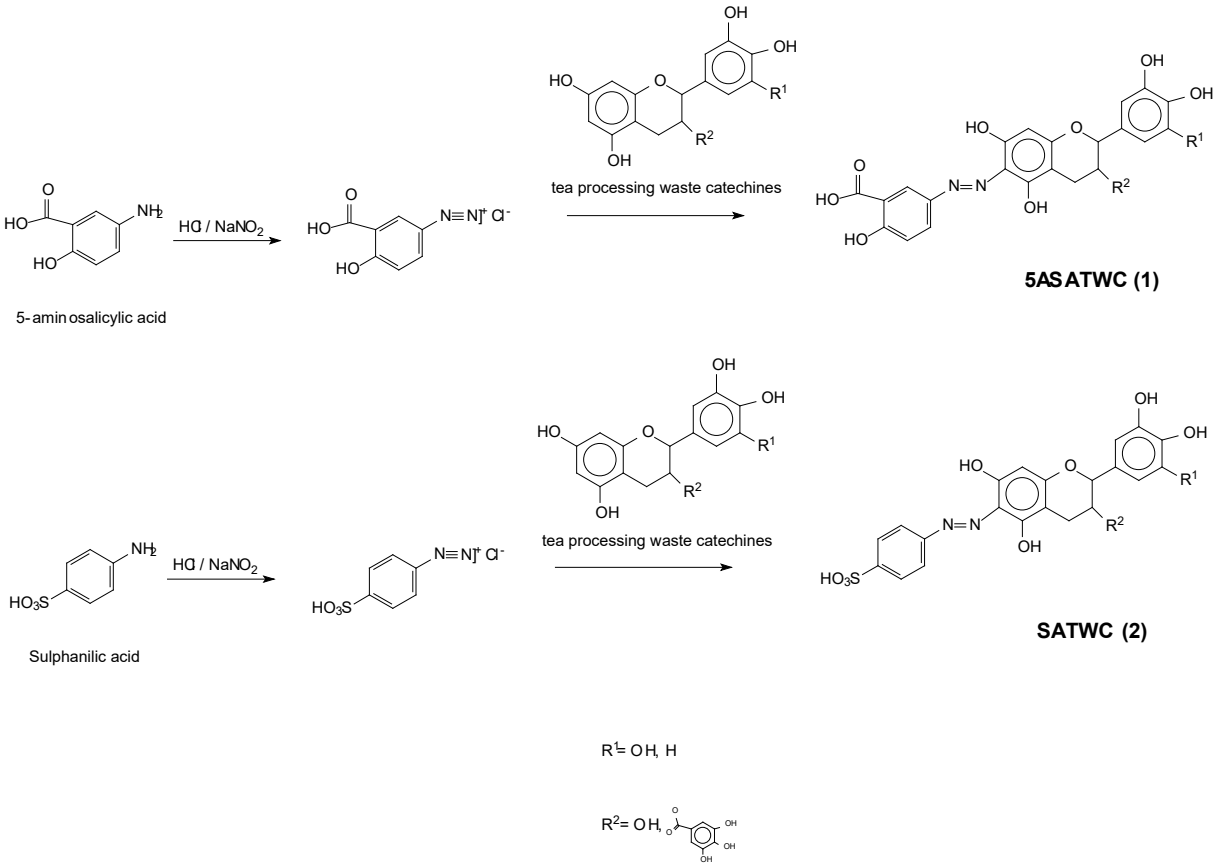

Figure 1a. Synthetic route of azo compound containing black tea processing waste catechins (hypothetical)

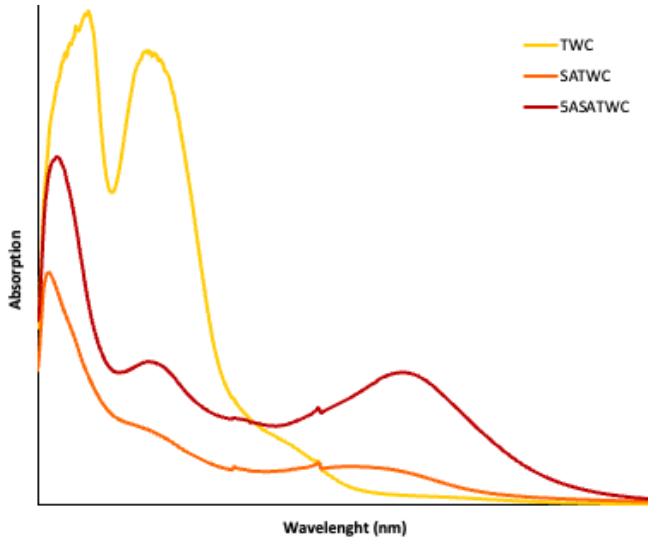

Figure 1b. UV/Vis spectra of black tea processing waste catechins (TWC) and azo compounds (5ASATWC, SATWC)

In the FT-IR spectrum of azo compounds containing black tea processing waste catechins (5ASATWC) exhibited the broad OH band at 2500-3213 cm^{-1}. The formation of compounds (5ASATWC) was clearly indicated by the appearance of azo (N=N) peaks at 1490 cm^{-1} in its FT-IR spectrum [34, 36]. In the UV/Vis spectrum of azo compounds containing black tea processing waste catechins (5ASATWC) the absorption peaks were observed at 213, 233 and 438 nm.

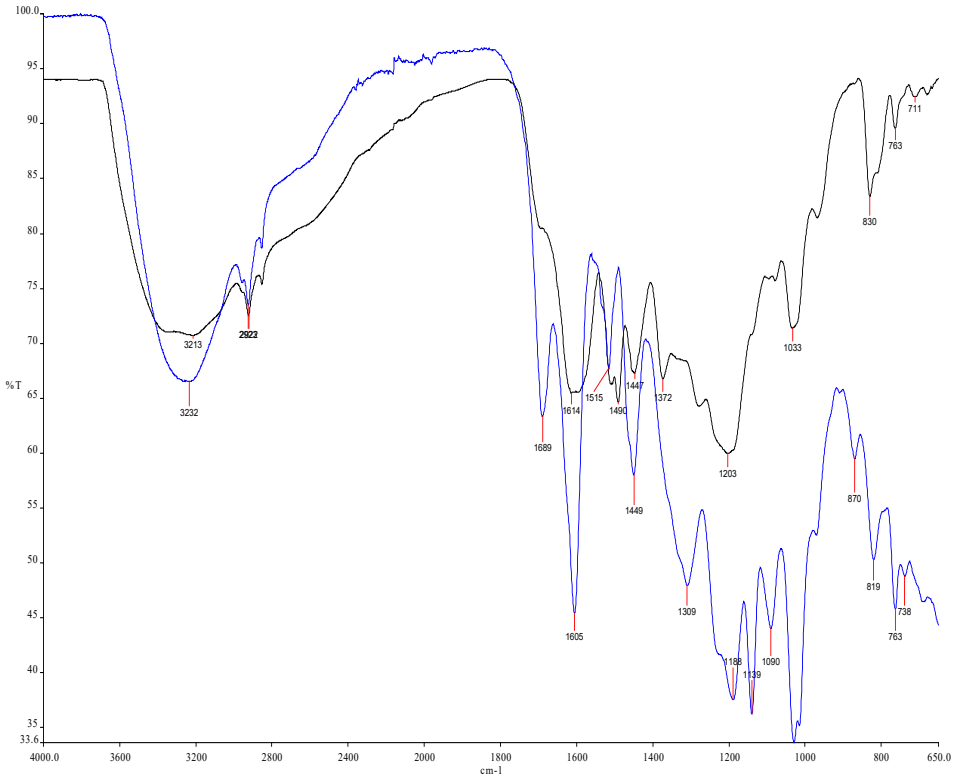

Figure 2. FT-IR spectra of black tea processing waste catechins (Blue line) and azo compounds 5ASATWC (Black line)

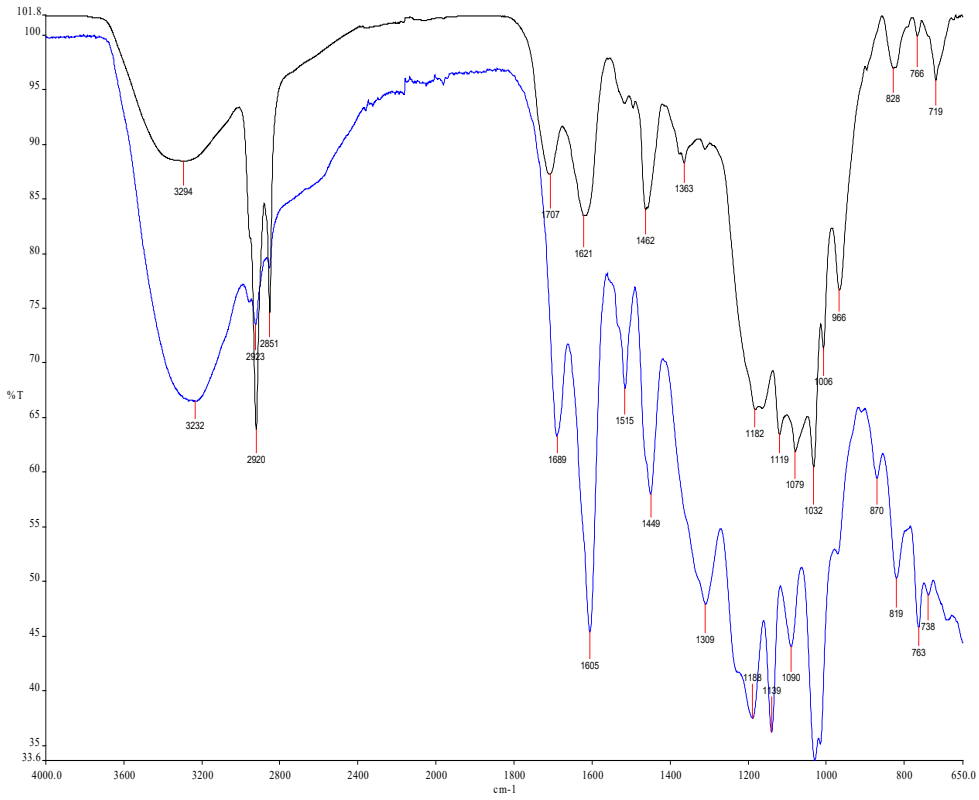

Figure 3. FT-IR spectra of black tea processing waste catechins (Blue line) and azo compounds SATWC (Black line)

New peaks at approximately 420-433 nm in the UV/Vis spectra of azo compounds clearly show that azo compounds were synthesized successfully. Comparative FT-IR and UV/Vis spectra of black tea processing waste catechins and new azo compounds containing them are shown in Figure 1-3.

In the FT-IR spectrum of azo compounds containing black tea processing waste catechins (SATWC) exhibited the broad OH band at 3274 cm-1. The formation of compounds (SATWC) was clearly indicated by the appearance of azo (N=N) peaks at 1462 cm^{-1} in its FT-IR spectrum [34, 36]. In the UV/Vis spectrum of azo compounds containing black tea processing waste catechins (SATWC) the absorption peaks were observed at 211, 272 and 420 nm.

3.2. 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. The orange-yellow color is due to the Cu(I)-Nc charge-transfer complex formed. Increased absorbance of the reaction mixture indicates increased reduction capability [25]. The CUPRAC results of all compounds were within the range of 5.943-12.175 mM Trolox/mg compound, (Table 1). Compound TWC was found to be the most effective compound in cuprac antioxidant assay. The other compounds, evaluated by the range of highly to moderately (Table 1).

3.3. DPPH Scavenging Capacity

DPPH method is based on the fact that the free radical is purple, and that the purple color of DPPH decays in the presence of an antioxidant. The color changed from purple to yellow after reduction, which can be quantified by its decrease of absorbance at wavelength 517 nm. The results were expressed as SC_{50} (µg/mL), which was calculated from the curves by plotting absorbance values, the SC_{50} values representing the concentration of the compound (µg/mL) required to scavenge 50% of the radicals. DPPH radical is reduced in the presence of such hydrogen-donating antioxidants. The influences of DPPH the concentration of antioxidant and duration of reaction on the inhibition of the radical absorption are taken into account when determining the antioxidant activity. All compounds showed fairly well DPPH radical scavenging activity, at all final concentration (Table 1). Also, compound TWC showed more effective DPPH radical scavenging activity than any other compounds (Table 1).

3.4. $ABTS^{\bullet+}$ Radical Cation Decolorization Assay

$ABTS^{\bullet+}$ radical is reduced in the presence of such hydrogen-donating antioxidants. The influences of $ABTS^{\bullet+}$ the concentration of antioxidant and duration of reaction on the inhibition of the radical absorption are taken into account when determining the antioxidant activity. All compounds showed fairly well $ABTS^{\bullet+}$ radical scavenging activity, at all final concentration (Table 1). The compound TWC showed more effective $ABTS^{\bullet+}$ radical scavenging activity than any other compounds (Table 1).

3.5. Inhibition of *Helicobacter pylori* Urease

The newly synthesized compounds were examined in terms of their urease inhibition potential. The percentage relative activities versus inhibitor concentrations were plotted separately for each molecule. IC_{50} values were determined urease inhibitory activity of the synthesized compounds and thiourea were shown in Table 1. Among the tested compounds, TWC showed the most significant urease inhibitory activity, with IC_{50} value 0.20±0.06 µg/mL (Table 1). The urease inhibition

effectiveness of the compounds, which are more active than thiourea, are listed from the most active molecules to the least active molecules as follows, TWC > 5ASATWC > SATWC > thiourea (Table 1).

These compounds have a considerable inhibition potential against the urease enzyme (Table 1). Urease inhibition effects of azo compounds containing black tea processing waste catechins were found to be higher than thiourea used as a standard. The compound 5ASATWC was determined as the highest urease enzym inhibitory azo compound at the study.

Table 1. CUPRAC results, SC_{50} values of DPPH and ABTS methods and urease inhibition results of all the compounds. The results of all studies of the compounds are expressed as the mean ±SD in triplicate.

Compounds	CUPRAC Value mM Trolox/mg compound	DPPH Method SC_{50} Value, µg/mL	$ABTS^{\bullet+}$ Method SC_{50} Value, µg/mL	Urease (IC_{50} value, µg/mL)
TWC	12.175±0.009	3.42±0.09	2.78±0.05	0.20±0.06
SATWC	5.943±0.015	14.30±0.17	11.84±0.06	8.80±0.13
5ASATWC	7.096±0.010	7.15±0.07	5.62±0.09	3.05±0.09
Catechin	13.790±0.011	3.35±0.09	2.62±0.04	0.16±0.07
Thiourea	---	---	---	14.87±0.16

4. Conclusions

When the obtained data were evaluated, it was observed that the antioxidant capacity and urease enzyme inhibitory effect of black tea processing waste catechins were very close to pure standard catechin. Compound T**WC** has both the most antioxidant activity, radical scavenging capacity and most urease inhibitory. It has been determined that the antioxidant capacity of azo compounds containing black tea processing waste catechins is lower than the catechin used as a standard. A significant positive correlation was determined between the antioxidant capacity and urease inhibition. Urease inhibitory effects of all newly synthesized azo compounds containing black tea processing waste catechins were found to be higher than thiourea used as a standard.

Acknowledgments

This study was supported by the Scientific and Technological Research Council of Turkey (TUBİTAK, Project no: 1919B012100497)

Authors' Contributions

Each author's contribution to the study is 25%.

Both authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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