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Antioxidant activity of 1.3-bis(*p*-hydroxyphenyl)urea by CUPRAC and FRAP methods

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ABSTRACT
An imbalance in the number of free radicals produced in the body can result in oxidative stress. Excessive oxidative stress can lead to chronic inflammation, which in turn can lead to most chronic diseases. Inflammation is related to oxidation through increased reactive oxidative stress, which can target modulators associated with inflammation, such as inflammatory cytokines. Antioxidants can inhibit or stop oxidation by protecting the body and neutralizing free radicals. 1.3-bis(*p*-hydroxyphenyl)urea is a modification of *p*-aminophenol and has hepatotoxic side effects such as those caused by acetaminophen. This compound can relieve pain, is anti-inflammatory, and has fewer side effects. This research was conducted to evaluate the antioxidant activity of 1.3-bis(*p*-hydroxyphenyl)urea using the CUPRAC (Cupric Ion Reducing Antioxidant Capacity) method and the FRAP (Ferric Reducing Antioxidant Power) method. The results of the CUPRAC method research show that the 1.3-bis(*p*-hydroxyphenyl)urea compound has an IC_{50} value of 4.40 ± 0.07 µg/mL. Meanwhile, the FRAP method was 29.36 ± 1.20 µg/mL. Apart from suppressing inflammation, this compound has the potential to be an antioxidant compound.
Keyword: Antioxidant, Free radicals, Inflammation, *p*-aminophenol, *1.3-bis(p-hydroxyphenyl)urea*

ABSTRAK
Ketidakseimbangan jumlah radikal bebas yang diproduksi dalam tubuh dapat mengakibatkan stres oksidatif. Stres oksidatif yang berlebihan dapat menyebabkan peradangan kronis, yang selanjutnya dapat menyebabkan sebagian besar penyakit kronis. Peradangan berhubungan dengan oksidasi melalui peningkatan stres oksidatif reaktif, yang dapat menargetkan modulator yang terkait dengan peradangan, seperti sitokin inflamasi. Antioksidan dapat menghambat atau menghentikan oksidasi dengan melindungi tubuh dan menetralisir radikal bebas. 1.3-bis(*p*-hidroksifenil)urea merupakan modifikasi dari *p*-aminofenol dan memiliki efek samping hepatotoksik seperti yang disebabkan oleh asetaminofen. Senyawa ini mampu meredakan nyeri, bersifat anti inflamasi, dan memiliki efek samping yang lebih sedikit. Penelitian ini dilakukan untuk mengevaluasi aktivitas antioksidan 1.3-bis(*p*-hidroksifenil)urea menggunakan metode CUPRAC (Cupric Ion Reducing Antioxidant Capacity) dan metode FRAP (Ferric Reducing Antioxidant Power). Hasil penelitian metode CUPRAC menunjukkan bahwa senyawa 1.3-bis(*p*-hidroksifenil)urea mempunyai nilai IC_{50} sebesar 4.40 ± 0.07 µg/mL. Sedangkan metode FRAP sebesar 29. 1.336 ± 1.20 µg/mL. Selain menekan peradangan, senyawa ini berpotensi menjadi senyawa antioksidan.
Keyword: Antiooksidan, Radical bebas, Inflamasi, *p*-aminophenol, 1.3-bis(*p*-hydroxyphenyl)urea

1. Introduction

Free radicals cause tissue and cell damage, as demonstrated by free radical reactions with proteins, fatty acids, and even DNA [1]. In the human body, free radicals are metabolic products of normal cells. Under normal circumstances, free radicals function as one of the body's defence systems. Free radicals can be Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), both of which can be obtained from within (endogenous) or from outside the body (exogenous) [2] [3]. An imbalance in the number of free radicals produced in the body can result in oxidative stress [4],[5]. Sustained oxidative stress can lead to chronic inflammation, which in turn can lead to most chronic diseases, including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases [6] [7]. Antioxidants can inhibit or stop the free radical oxidation process, which causes cell damage [8]. Antioxidants protect the body from reactive oxygen free radicals by providing extra electrons to free radicals. By neutralizing free radicals, antioxidants stop damage to the body. Free radicals are produced when other molecules undergo oxidation, which can be prevented or slowed by antioxidants [9], [10].

1.3-bis(*p*-hydroxyphenyl)urea, a modified version of the *p*-aminophenol derivative from Hari Purnomo (2016), is designed to mitigate hepatotoxic side effects such as those caused by acetaminophen. This compound not only alleviates pain but also exhibits fewer side effects compared to acetaminophen [11], [12]. Importantly, it has been shown to possess anti-inflammatory properties in both in vitro and in vivo tests, and it has low toxicity [13], [14], [15], [16]. In our previous research, this compound had anti-inflammatory activity by reducing the percentage of COX-2, TNF-α, IL-1β, and IL-6 in inflammatory rat paw tissue [17],[18].

Inflammation is related to oxidation through increased ROS. ROS can target the level, presence, and type of modulating factors associated with inflammation, such as inflammatory cytokines [19]. Inflammatory cells direct ROS generation through activation of oxidant-producing enzymes such as upregulation of lipoxygenase (LOX), myeloperoxidase (MPO), cyclooxygenase 2 (COX-2), xanthine oxidase (XO), NADPH oxidase, and inducible nitric oxide synthase (iNOS) [20],[21]. This study aims to evaluate the antioxidant activity of 1.3-bis(*p*-hydroxyphenyl)urea using the CUPRAC method and the FRAP method.

2. Method

1.1. Tools and materials

The tools used are a stir bar, beaker glass (Pyrex®Iwaki), Erlenmeyer (Pyrex®), FTIR spectrometer (Shimadzu), measuring cup (Pyrex®), cuvette, analytical balance (Sartorius), drop pipette, micropipette (Eppendorf), centrifuge (Eppendorf), spatula, UV-Vis spectrophotometer (Orion AquaMate 8000), vial. Meanwhile, the material used is 1.3-bis(*p*-hydroxyphenyl)urea obtained from Dr. apt. Hari Purnomo (Universitas Gadjah Mada), ammonium acetate (SMART-LAB), aquadest, oxalic acid, trichloroacetic acid (Merck), $CuCl_2.2H_2O$ (Merck), ethanol asbsolut (SMART-LAB), ferric chloride (SMART-LAB), potassium dihydrogen phosphate (Merck), potassium ferricyanide (SMART-LAB), quercetin (TCI), methanol absolute (Merck), sodium hydroxide (SMART-LAB), neocuproine (Sigma), *p*-aminofenol (Sigma).

1.2. Characterization by UV-Vis spectrophotometry

5 mg of each *p*-aminophenol and 1.3-bis(*p*-hydroxyphenyl)urea were dissolved in 5 mL of methanol. Both samples were filtered and then tested using UV-Vis spectrophotometry at a wavelength of 200-800 nm.

1.3. Characterization by FT-IR (Fourier Transform Infra-red)

1 mg of each p-aminophenol and 1.3-bis(*p*-hydroxyphenyl)urea compound, the samples were ground with 100 mg KBr homogeneously, then the infrared absorption at wave numbers 500-4500 cm^{-1} was measured.

1.4. Antioxidant activity test using the CUPRAC method

Weighed 10 mg of the sample and dissolved it in 10 mL of methanol. Created a concentration series of 1.87, 3.75, 7.5, 15, 30 µg/mL by pipetting 0.00935, 0.0187, 0.0375, 0.075, 0.15 mL of sample into the vial, then filling up to 5 mL with methanol. Pipette 1 mL each into vials. Added 1 mL of 0.2 M phosphorus buffer solution (pH 6.6) and 1 mL of 1% potassium ferricyanide. After that, it was incubated for 20 minutes at 50°C. 1 mL of TCA was added and centrifuged at 3000 rpm for 10 minutes. After centrifugation, pipette 1 mL of the supernatant each, then add 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The solution mixture was incubated for 10 minutes, and the absorbance was measured using a UV-Vis spectrophotometer with a wavelength of 700 nm.

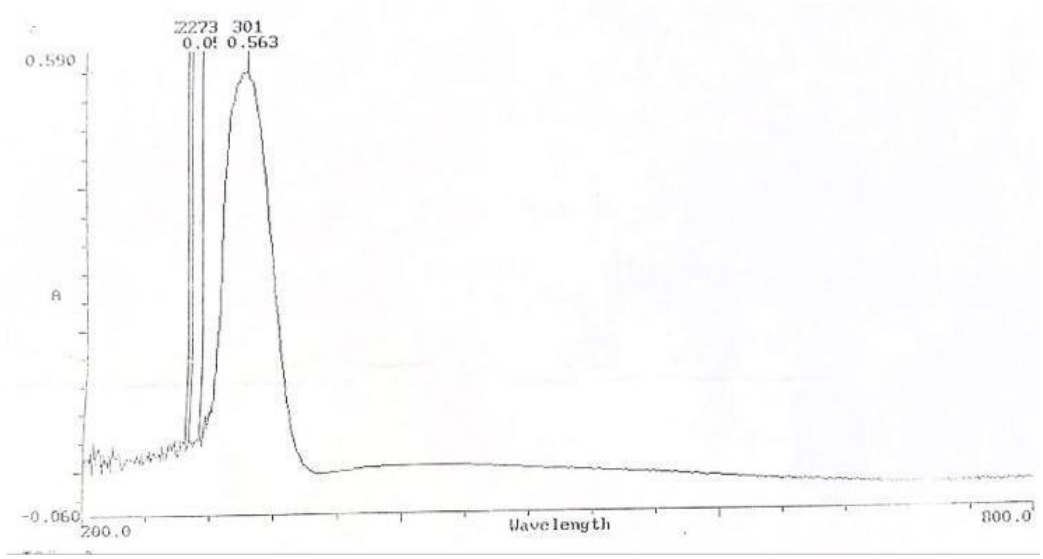
1.5. Antioxidant activity test using the FRAP method

5 mg of the sample was dissolved in 5 mL of 96% ethanol. Then, 1 mL of the solution was pipetted and mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% $K_3[Fe(CN)_6]$. The mixture was then incubated at 50°C for 20 minutes. After incubation, 1 mL of TCA (trichloroacetic acid) was added, and the mixture was centrifuged at 3000 rpm for 10 min. After centrifugation, 1 mL of the top layer was transferred into a test tube, and 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ were added. The solution was left for 10 minutes, after which the absorbance was measured at a wavelength of 720 nm.

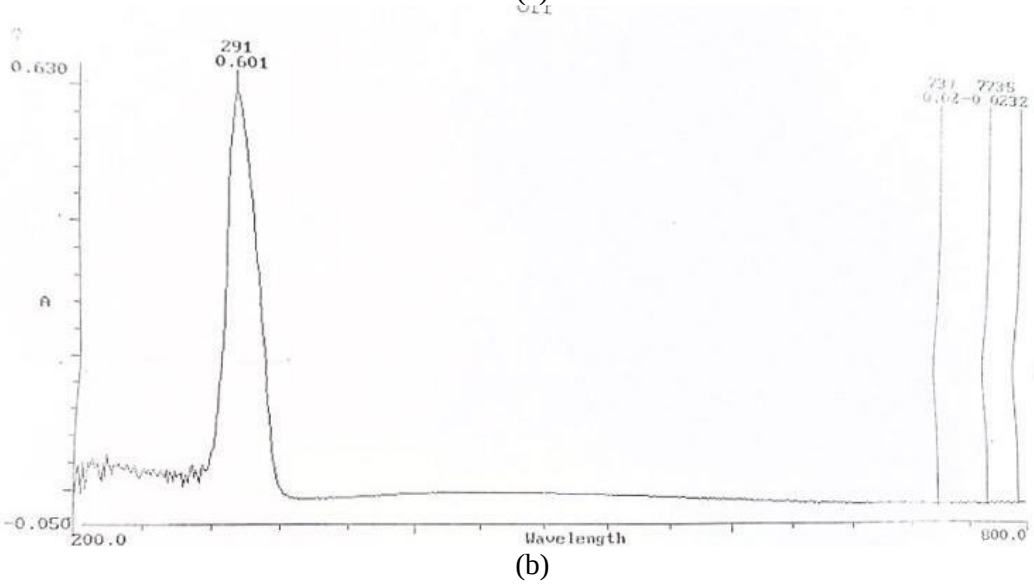
3. Results and Discussion

1.6. Characterization results by UV-Vis spectrophotometry

In testing the maximum wavelength of the *p*-aminophenol compound, the maximum wavelength was 301, with an absorbance of 0.563. Meanwhile, the maximum wavelength for the 1.3-bis(*p*-hydroxyphenyl)urea compound was 291 nm, with an absorbance of 0.601, see Fig. 1. There was a shift in the wavelength of the parent compound p-aminophenol with the compound 1.3-bis(*p*-hydroxyphenyl)urea to a shorter wavelength; this is called the blue shift or hypsochromic effect. The hypsochromic effect is caused by changes in the medium and the occurrence of several phenomena, such as the removal of conjugation [22].



(a)



(b)

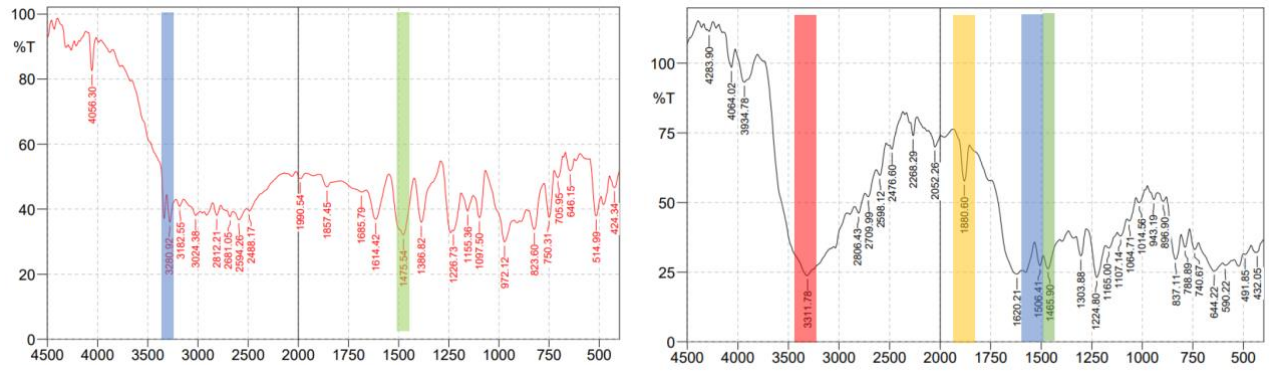
Figure 1. Maximum Wavelength, (a) *p*-aminophenol; (b) 1.3-bis(*p*-hydroxyphenyl)urea

1.7. Characterization results by FT-IR

Fig. 2 shows the functional group testing using an FT-IR instrument on the test compound. In *p*-aminophenol, the -NH_2 group was found at the peak of 3280.92 cm^{-1}, and the aromatic C=C group at 1475.54 cm^{-1}. The NH_2 group consists of two N-H group bonds between wave numbers 3100 to 3500 cm^{-1} [23]. Meanwhile, 1.3-bis(*p*-

hydroxyphenyl)urea and O-H groups were found at 3311.78 cm^{-1}. The O-H group appears between wave numbers 3000 to 3750 cm^{-1}, the C=O group between wave numbers 1650 to 1900 cm^{-1}, and the C=O group at 1880.60 cm^{-1} (Masfria et al., 2018). At wave number 1506.41 cm^{-1}, an NH group is experiencing bending (bending vibration). Meanwhile, the wave number 1465.90 cm^{-1} is the aromatic C=C group [11]. Bending is vibration caused by the bond angle, resulting in an increase or decrease in the bond angle [24].

1.3-bis(*p*-hydroxyphenyl)urea is a compound resulting from synthesising *p*-aminophenol with urea in an acidic environment [11]. The -NH_2 group found in *p*-aminophenol is not found in the 1.3-bis(*p*-hydroxyphenyl)urea compound (See Fig. 3) because the synthesis reaction carried out can change the functional group of a compound.



(a) (b)

Figure 2. FT-IR spectrum, (a) *p*-aminophenol; (b) 1.3-bis(*p*-hydroxyphenyl)urea

(a) (b)

Figure 3. Chemical structure, (a) *p*-aminophenol; (b) 1.3-bis(*p*-hydroxyphenyl)urea

1.8. Antioxidant activity test results using the CUPRAC method

The CUPRAC reagent is a selective reagent with a low reduction potential value and relatively fast oxidizing antioxidant thiols. It is also a selective reagent because it is redox. The reagent is stable and easily accessible compared to other chromogenic reagents [25]. The IC_{50} (Inhibitory Concentration) value describes the test compound's concentration, which can trap free radicals by 50% [26]. Based on Table 1, the average IC_{50} value of 1.3-bis(*p*-hydroxyphenyl)urea from three tests was found to be 4.40 ± 0.07 µg/mL, and quercetin was 1.43 ± 0.08 µg/mL. Quercetin is used for comparison because it is a pure compound with high antioxidant activity. Quercetin is a flavonol from polyphenolic flavonoid compounds found in almost every type of plant, and standard quercetin is a natural antioxidant with vigorous antioxidant activity [27].

According to the Blois classification, the level of the antioxidant power of a compound is said to have powerful antioxidant activity if the IC_{50} value is <50 µg/mL, strong antioxidant (50-100 µg/mL), moderate (101-150 µg/mL), and weak (150- 200 µg/mL). The smaller the IC_{50} value of a compound, the better the antioxidant activity of the compound [28], [29]. From these results, it can be concluded that the 1.3-bis(*p*-hydroxyphenyl)urea compound has powerful antioxidant activity.

Table 1. Results of testing the antioxidant activity of 1.3-bis(*p*-hydroxyphenyl)urea using the CUPRAC method

Sample	Concentration (µg/mL)	Absorbance	% Radical scavenging	IC_{50} value (µg/mL) ± SEM
1.3-bis(*p*-hydroxyphenyl)urea	Blank	0.107	0	4.40 ± 0.07
	1.25	0.142	24.02	
	2.5	0.163	34.32	
	5	0.232	53.96	
	10	0.355	67.77	
	20	0.606	82.35	
Quercetin	Blank	0.107	0	1.43 ± 0.08
	0.625	0.158	32.29	
	1.25	0.197	45.88	
	2.5	0.283	62.20	
	5	0.533	79.95	
	10	0.790	86.47	

1.9. Antioxidant activity test results using the FRAP method

The FRAP is a method for determining the total antioxidant content of a material based on the ability of antioxidant compounds to reduce Fe^{3+} ions to Fe^{2+} ions [30]. The advantages of the FRAP method are that it is cheap and fast, and the reagents used are simple and do not use special equipment to calculate total antioxidants [31]. However, it also has a weakness: the reagent is less stable, so a new reagent must be made when testing is carried out and used immediately [32]. In the FRAP method provided in Table 2, the average IC_{50} value for the compound 1.3-bis(*p*-hydroxyphenyl)urea was 29.36 ± 1.20 µg/mL, and quercetin was 13.23 ± 1.11 µg/mL. The 1.3-bis(*p*-hydroxyphenyl)urea can potentially be a potent antioxidant.

Table 2. Results of testing the antioxidant activity of 1.3-bis(*p*-hydroxyphenyl)urea using the FRAP method

Sample	Concentration (µg/mL)	Absorbance	% Radical scavenging	IC_{50} value (µg/mL) ± SEM
1.3-bis(*p*-hydroxyphenyl)urea	Blank	0.336	0	4.40 ± 0.07
	1.87	0.438	23.16	
	3.75	0.467	28.08	
	7.5	0.527	36.25	
	15	0.576	41.60	
	30	0.695	51.63	
Quercetin	Blank	0.336	0	1.43 ± 0.08
	0.935	0.391	13.91	
	1.87	0.415	19.02	
	3.75	0.466	27.87	
	7.5	0.549	38.80	
	15	0.769	56.08	

3. Conclusion

Based on tests using the CUPRAC and FRAP methods, the 1.3-bis(*p*-hydroxyphenyl)urea compound has the potential to be an antioxidant. The IC_{50} value was no more than 50 µg/mL in both methods, indicating antioxidant activity in the very high category.

4. Acknowledgements

Acknowledgements and Reference heading should be left justified, bold, with the first letter capitalized but have no numbers. Text below continues as normal.

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