Characterization and Antioxidant Activity of Gallic Acid Derivative

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Abstract. Peroxidase enzyme was used to catalyze the dimerization process of gallic acid. The structure of the dimerization product was characterized by ^{1}H NMR and LC-MS-MS. The mechanism of gallic acid dimerization was also discussed. It was proposed that ellagic acid was formed through an oxidative coupling mechanism that lead to the formation of a C-C bond and followed by an intramolecular Fischer esterification mechanism that lead to the formation of two C-O bonds. Moreover, the antioxidant activity of gallic acid and ellagic acid were also studied. Gallic acid and ellagic acid exhibited the DPPH radical scavenging activity with IC_{50} values of 13.2 μM and 15.9 μM, respectively.

INTRODUCTION

Ellagic acid is a condensed dimer of gallic acid, which is also the prevalent phenolic acid in plant tannins. With the molecular weight *m/z* 302.2, ellagic acid is classified as a polyphenolic compound present in some natural sources, including pomegranate, strawberries, blackberries, and raspberries [1]. Ellagic acid was found to be an effective antioxidant after subjecting it in different *in vitro* antioxidant activity and radical scavenging assays. Compared to standard antioxidant compounds such as BHA and BHT, results showed that ellagic acid has a higher antioxidant activity in DPPH free radical scavenging activity assay, superoxide radical scavenging assay, and Fe^{3+} reducing power assay [2].

Noticing this potential, there has been increasing attention to the use of gallic acid as a basis for drug development. However, since extraction method was considered to have a great dependency on nature and uncertain quantities of phytochemicals on each fruit [3], various ways were done to synthesize ellagic acid chemically, including through the dimerization process. Dimerization is the process of combining two smaller (identical) molecules into a larger molecule. To catalyze the reaction, it was claimed in the US Patent 5541091A that peroxidase enzyme can be used in the dimerization of an aromatic compound [4].

An earlier study on the dimerization of gallic acid indicated that peroxidase enzyme can be used to catalyze the dimerization process of gallic acid via an oxidative coupling mechanism. The Liquid Chromatography – Mass Spectrometry (LC-MS) analysis showed the presence of the product with a molecular weight which is similar to ellagic acid [5].

In this study, the dimerization process of gallic acid as well as product characterization were examined. In addition, the antioxidant activity of ellagic acid was also evaluated in comparison to gallic acid.

Proceedings of the 3rd International Symposium on Applied Chemistry 2017
AIP Conf. Proc. 1904, 020030-1–020030-7; https://doi.org/10.1063/1.5011887
Published by AIP Publishing. 978-0-7354-1594-2/$30.00

MATERIALS AND METHODS

Materials

Gallic acid monohydrate, Folin Ciocalteu's phenolic reagent, sodium carbonate, methanol, buffer pH 7, hydrogen peroxide 30%, and ethyl acetate were obtained from Merck, Germany. Peroxidase enzyme from horseradish with activity of ≥ 250 units/ mg and ellagic acid were obtained from Sigma-Aldrich, USA. Free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich, Germany.

Methods

Dimerization of Gallic Acid

In 2.5 ml of methanol, 100 mg of gallic acid was diluted (the initial concentration of gallic acid is equal to 0.235 M). As much as 15 mg of peroxidase enzyme in 20 ml buffer (pH 7) solution and 4 ml of 30% hydrogen peroxide were added. The mixture was stirred for 9 hours in 37°C [5].

The reaction product was separated using separatory funnel, and ethyl acetate was used as the solvent to captivate the desired product, which was the gallic acid dimer. After the addition of the solvent, two layers were formed. The upper (organic) layer was collected, while the bottom layer was rinsed repeatedly until two phases were no longer formed. The upper phase was brought to a rotary evaporator, where the solvent evaporates and leaves a concentrated product.

Structure Elucidation

The process of determining the chemical structure of the dimerization product was conducted by using ^{1}H Nuclear Magnetic Resonance Spectroscopy (Jeol JNM ECA-500, Japan). The confirmation of the molecular weight was done by using Liquid Chromatography – Tandem Mass Spectrometry (Waters Corporation UPLC Acquity 1, USA). The analysis results were compared with an appropriate reference, which is the ellagic acid obtained from Sigma-Aldrich, USA.

In vitro Antioxidant Activity.

The DPPH (1.1-diphenyl-2-picryl-hydrazyl) scavenging assay was performed based on the method used by Mishra, et al., (2012) with slight modification [6]. The objective of this method was to investigate the scavenging activity of the sample (gallic acid and ellagic acid) towards free radical DPPH.

The DPPH solution was prepared. To make a 2536 μM of DPPH (Mr = 394.32 g/mol) grandparent solution, 10 mg DPPH powder was diluted in 10 ml of methanol. From the grandparent solution, DPPH parent solution of 253.6 μM was made. Both grandparent and parent solution were stored in an amber glass bottle covered in aluminum foil at 4°C. As a note, DPPH solution that was going to be used in the procedure described below was the one that has a concentration of 253.6 μM (the one that was referred as the parent solution).

In brief, the sample with various concentrations was prepared in methanol solution. In the test tubes, 1.5 ml of the sample with various concentrations was mixed with 1.5 ml of 253.6 µM DPPH solution in methanol. Control solution was made by replacing the proportion of the sample with 1.5 ml of methanol. The test tube for blank solution was filled with 3 ml of methanol. All test tubes were brought to vortex shaker and incubated at room temperature and in a dark chamber for 30 minutes. The absorbance of each sample was analyzed using UV-Vis spectrophotometer (λ = 517 nm). The percentage scavenging of DPPH radical was calculated using the equation: [1 - (B/A)] x 100%; whereas A is absorbance of the control solution and B is absorbance of the sample solutions.

For each sample, a graph of DPPH radical scavenging activity (in %) versus sample concentration (in µM) was made in order to calculate the IC50 (the half maximal inhibitory concentration) of each sample. In this study, the parameter IC50 was used to estimate the amount of antioxidant necessary to decrease the initial DPPH by 50%.

RESULTS AND DISCUSSIONS

Dimerization of Gallic Acid

The mixture of gallic acid solution and peroxidase enzyme solution produced a light brown color. Upon the addition of H_2O_2, the color of the solution changed rapidly to dark brown. After 9 hours of incubation, the color of the solution turned into yellow. The color changes indicated that gallic acid has been transformed into a new substance.

The reaction product was brought into a separatory funnel to be separated by using ethyl acetate. Ethyl acetate was used as the solvent due to its efficiency in obtaining extracts enriched in ellagic acid [7]. Moreover, gallic acid has a low solubility in ethyl acetate [8]. This fact supports the use of ethyl acetate as a solvent to separate the dimerization product [Compound **III**] from gallic acid.

Compound **III** ^{1}H NMR (500 MHz in CD_3OD): δ 7.4868 (s, 2H, H-3/3'). ESI-MS: $[M+H]^+$ *m/z* 303.01088.

During the dimerization process of gallic acid, the catalytic cycle of horseradish peroxidase (HRP) enzyme was started upon the addition of hydrogen peroxide to the solution of gallic acid and peroxidase enzyme. Figure 1 shows the catalytic cycle of horseradish peroxidase enzyme with gallic acid.

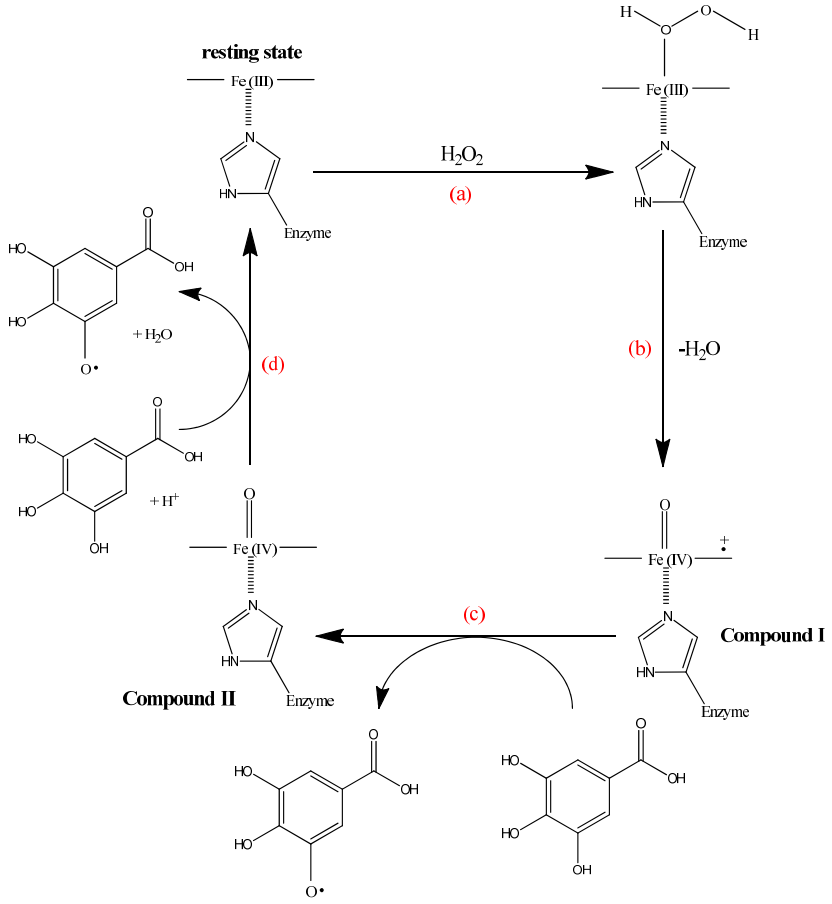

FIGURE 1. Catalytic Cycle of Horseradish Peroxidase Enzyme with Gallic Acid as Reduction Substrate; (a) the reaction between hydrogen peroxide and HRP-Fe(III) at its resting state; (b) The reaction lead to the formation of water and compound I which was known as an Fe(IV) oxoferryl centre; (c) the reaction of gallic acid, as a reducing substrate, with compound I. The reaction resulted in the formation of gallic acid radical and compound II; (d) the reaction of compound II with other gallic acid resulted in the recovery of HRP-Fe(III) and another gallic acid radical

The catalytic cycle of horseradish peroxidase enzyme marked the first and second step of the radical dimerization reaction, which was chain initiation and chain propagation. At the end of the propagation step, gallic acid radicals were formed. As free radicals were not stable, they were undergoing delocalization to stabilize themselves.

Moreover, during the catalytic cycle, there were two different possible hydrogen positions that could be attacked by compound I and II; namely the hydroxyl group in the *meta* and *para* position. Figure 2 and 3 presents the radical stabilization of gallic acid radical against attacks on hydrogen at *meta* and *para* substituted – OH Group, respectively.

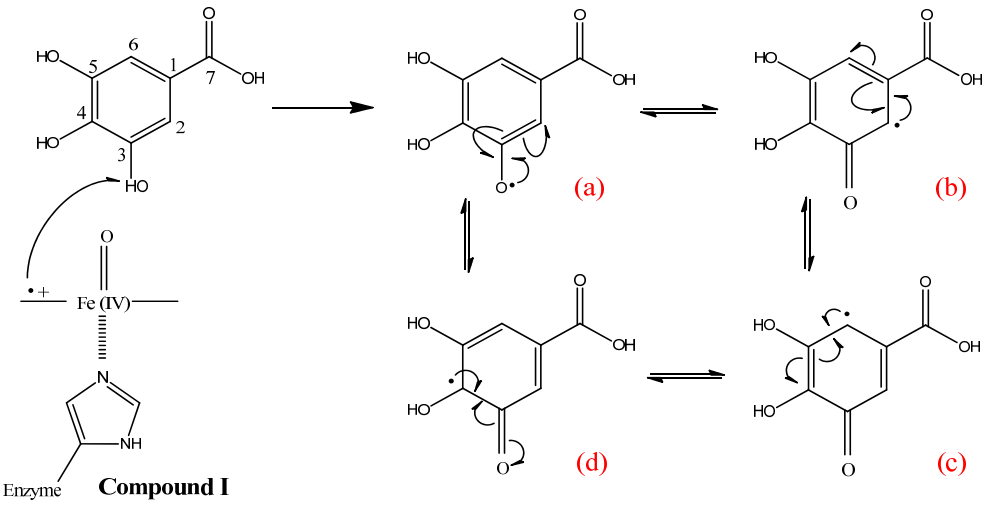

FIGURE 2. Resonance Stabilization of Gallic Acid Radical Against Attacks at *Meta* Substituted – OH Group

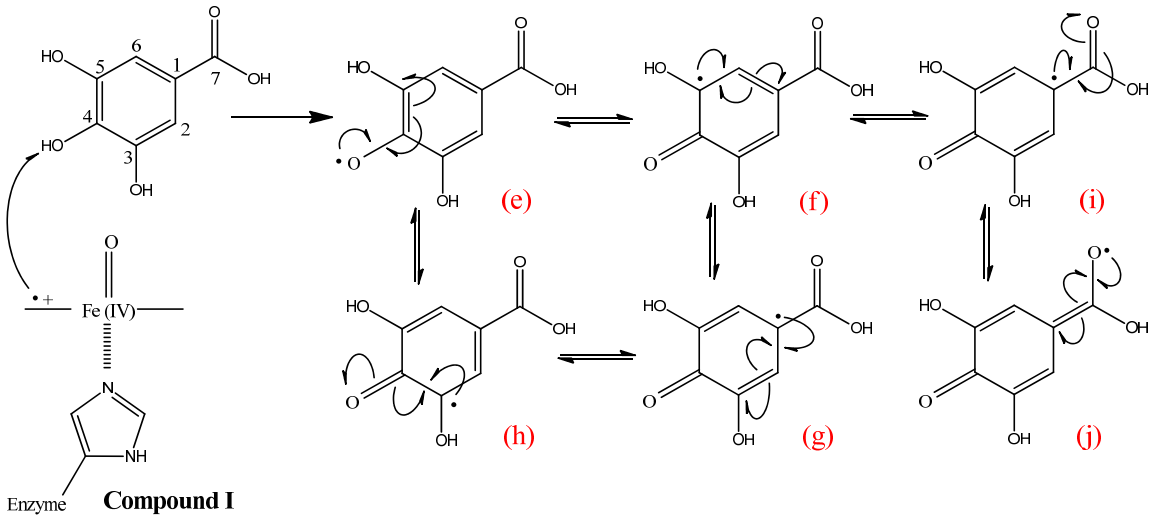

FIGURE 3. Resonance Stabilization of Gallic Acid Radical Against Attacks at *Para* Substituted – OH Group

The final step of the radical dimerization reaction was termination. From many possible gallic acid radicals that may form (as shown in Figure 2 and 3), not all of them would be so easily form connections with each other due to the stability of the radicals formed and the steric factors.

An interesting phenomenon was observed when the gallic acid radical (b) initiated a chain termination with the same gallic acid radical (b), as shown in Figure 4. The compound shows an equilibrium of keto and enol form. James (2010) stated that "aldehydes and ketones are somewhat lycanthropic (having an altered behavior) chemical

species". It means that they can form equilibrium between isomers, but not resonance [9]. For aldehydes and ketones, the keto form is mostly considered as the most stable. However, due to aromatic stabilization, the enol form is greatly favored in phenols. The chain termination mechanism also marked the formation of a new C-C bond.

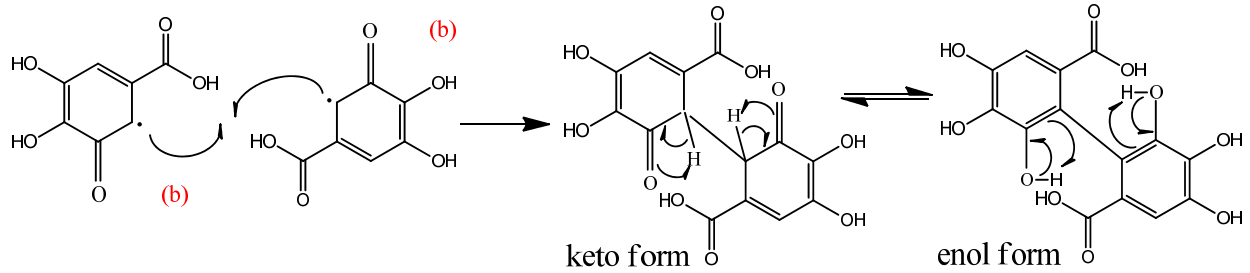

FIGURE 4. Chain Termination of Gallic Acid Radicals

Apparently, there was a possibility for the compound (in enol form) to undergo an intramolecular Fischer esterification mechanism as displayed in Figure 5.

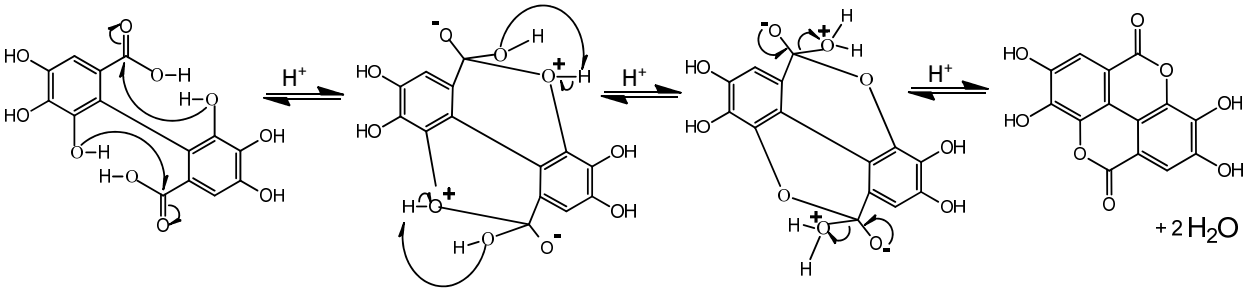

FIGURE 5. Gallic Acid Intramolecular Fischer Esterification Mechanism

During the mechanism, a nucleophilic (a molecule that has a tendency of donating electrons to create a new bond) oxygen atom from the hydroxyl group attacked the carbonyl carbon atom, forming an oxonium (oxygen cation with three bonds) ion. Proton transfer from the oxonium ion to the adjacent hydroxyl group gave a tetrahedral intermediate and a new oxonium ion. The loss of water from this oxonium ion gave the ellagic acid and water [10]. Therefore, during the dimerization process of gallic acid, the formation of ellagic acid was associated with the formation of three bonds. One C-C bond was formed during the chain termination of two gallic acid radicals. Meanwhile, two C-O bonds were formed during the intramolecular Fischer esterification mechanism, in which water was removed. Figure 6 shows the molecular structure of ellagic acid.

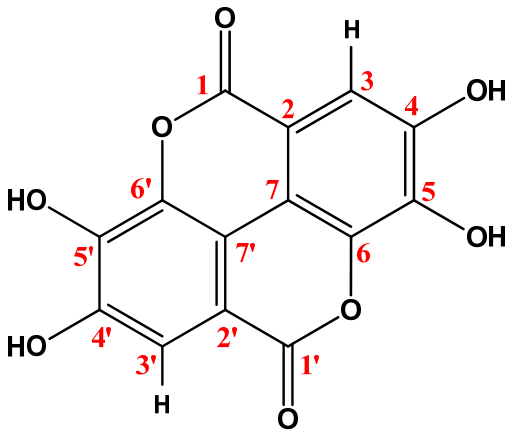

FIGURE 6. Molecular Structure of Ellagic Acid with C Atoms Numbering

Structure Elucidation

To characterize Compound **III**, the number and types of hydrogen was analyzed by ^{1}H NMR spectroscopy. As a reference, the standard ellagic acid were also subjected to ^{1}H NMR spectroscopy.

Compound **III** ^{1}H NMR (CD_3OD, 500MHz): δ 7.4868 (s, 2H, H-3/3'). The ^{1}H NMR spectrum of Compound **III** shows a singlet signal at δ 7.4868 for two hydrogens of the aromatic ring, since it is recognized that hydrogens bonded to a substituted benzene ring have signals that appear in the region δ 6.5-8.5 [11].

Due to the spectrum similarity with the standard ellagic acid that was also examined, the interpretation of Compound III by ^{1}H NMR spectroscopy indicates the presence of ellagic acid. The ^{1}H NMR spectrum of the standard ellagic acid shows a singlet signal at δ 7.4583 for two hydrogens of the aromatic ring [^{1}H NMR (DMSO, 500MHz): δ 7.4583 (s, 2H, H-3/3')]. Moreover, a literature review also reported that the characterization of ellagic acid was marked by the presence of a singlet signal at δ 7.45 for two hydrogens of the aromatic ring [^{1}H NMR (DMSO-d6) ppm: δ 7.45 (s, 2H, ArH) [12].

In order to identify the molecular mass of the components present in the sample, the Compound **III** was subjected to LC-MS-MS. As a reference, a standard ellagic acid was also observed. The solvent used was a gradient of acetonitrile and water.

The mass spectrum of peaks at retention time of 2.76 min indicates the presence of Compound **III** at high intensity by molecular ion $[M+H]^+$ *m/z* 303.01088. This compound was predicted to be ellagic acid. The identification of the compound as ellagic acid was made according to the suitability of the retention time and the mass spectrum of the compound to the standard ellagic acid that was also examined.

The chromatogram of the standard ellagic acid was observed in negative ion mode. There were two observed peaks with the retention time of 2.49 and 2.79 min. From the mass spectrum of peaks at retention time of 2.49 min, ellagic acid was detected at high intensity by molecular ion $[M–H]^–$ *m/z* 301.00125. Identification of peaks at retention time of 2.79 min also shows the presence of ellagic acid by molecular ion $[M–H]^–$ *m/z* 300.99770. The result indicated that the two ions were considered to originate from the same compound, which is ellagic acid.

Therefore, based on NMR and ESI-MS measurements, it can be confirmed that Compound **III** is ellagic acid.

In vitro Antioxidant Activity

The antioxidant activity of the samples (gallic acid and ellagic acid) were determined using the DPPH radical scavenging assay. The assay determines the antioxidant activity based on the ability of the sample to neutralize or inhibit the DPPH free radical. Gallic acid and ellagic acid possessed an antioxidant activity with IC_{50} values of 13.2 μM and 15.9 μM, respectively. The result of this experiment showed that based on DPPH radical scavenging assay, ellagic acid possessed a lower antioxidant activity compared to gallic acid.

By means of Density Functional Theory (DFT), Saqib, et al., (2015) mentioned that, even though the numbers of hydroxyl substituents in a compound are crucial, the antioxidant activity is mainly depending on the bond dissociation enthalpy (BDE) value of each substituent.

Among some possible reaction pathways, the mechanism of DPPH free radical scavenging assay is based on the hydrogen atom transfer (HAT) from an antioxidant to free radical DPPH. During the reaction, DPPH free radical is reduced by receiving a hydrogen atom from antioxidants. The HAT mechanism is governed by the O-H bond dissociation enthalpy (BDE). Low value of BDE indicates that the hydrogen atoms are more easily separated and act as a hydrogen donor during the reaction with DPPH. Therefore, from a theoretical perspective, low BDE values are identical with high antioxidant capacity [14]. By using DFT theory, the BDE values in gallic acid free radicals are in the range of 72.79 kcal/mol to 99.46 kcal/mol. Otherwise, the BDE values in ellagic acid free radicals are in the range of 77.08 kcal/ mol to 85.01 kcal/ mol [13, 14].

Additionally, Badhani, et al., (2015) stated that gallic acid possesses strong antioxidant activities among various polyphenols. The research declared that the defining factor of the antioxidant activity of gallic acid is the three hydroxyl groups that are bonded to its aromatic ring. Arranged in the *ortho* position with each other, these hydroxyl groups form an intramolecular hydrogen bond that not only affects the antioxidant activity of gallic acid, but also stabilizes the antioxidant radicals that are formed. The carboxylic group bonded to carbon number 1 is also believed to have a valuable impact to the antioxidant abilities of gallic acid. Compared to pyrogallol, whose molecular structure differs only in carboxylic group, gallic acid showed a higher antioxidant activity [15].

CONCLUSIONS

The dimerization process of gallic acid using peroxidase enzyme as the catalyst was successfully performed. ESI-MS analysis showed the presence of Compound **III** by molecular ion $[M+H]^+$ *m/z* 303.01088 at the retention time of 2.76 min, which is similar to ellagic acid. ^{1}H NMR spectroscopy result also indicated the presence of ellagic acid [^{1}H NMR (CD_3OD, 500MHz): δ 7.4868 (s, 2H, H-3/3')]. These results proved that Compound **III** is ellagic acid

Gallic acid and ellagic acid showed their IC_{50} value of 13.2 μM and 15.9 μM, respectively. Therefore, it can be concluded that based on the DPPH radical scavenging assay, gallic acid is a better antioxidant than ellagic acid.

ACKNOWLEDGEMENTS

The authors would like to express gratitude to the Research Center for Chemistry, Indonesian Institute of Sciences for providing the materials, equipment, and all of the analytical needs for this research. We also acknowledge Swiss German University, along with the lecturers and colleagues for the support, both financially and emotionally.

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