SYNTHESIS OF SPIROOXINDOLE-PYRROLIZIDINE COMPOUNDS USING Fe₃O₄-GO CATALYST AND THEIR BIOACTIVITY ASSAYS

Mutiara Hapsari, Antonius Herry Cahyana*, Syntia H. Oktavia
and Agus Rimus Liandi

Department of Chemistry, FMIPA, Universitas Indonesia, 16424, Depok, Indonesia

*E-mail: herrykim@ui.ac.id

ABSTRACT

Spirooxindole-pyrrolizidine is an essential compound of concern for researchers, especially in its structure which shows some existing bioactivities in medicine. A novel series of spirooxindole-pyrrolizidine were prepared by a one-pot multicomponent reaction method. The reaction occurred between α , β -carbonyl unsaturated, isatin, and L-proline through 1,3-dipolar cycloaddition reaction and assisted by Fe₃O₄-graphene oxide (GO) as a catalyst. The spectral and analytical data have established the structures of new compounds and the catalyst used. The results of the reaction and data analysis showed that two spirooxindole-pyrrolizidine derivatives had been successfully synthesized. The newly synthesized compounds have been tested for their antioxidant and antimicrobial activity.

Keywords: Spiro-oxindole, Pyrrolizidine, 1,3-dipolar-cycloaddition, Fe₃O₄, Graphene Oxide.

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INTRODUCTION

Curcumin is the main compound found in turmeric plants (*Curcuma longa* Linn), and plants of other genus *Curcuma*.^{1,2} Curcumin is a compound that has several multifunctional groups, including β -dicarbonyl conjugated with alkene double bonds (bis- α,β -unsaturated β -diketones) and two phenolic ortho-methoxy groups.^{3,4} Curcumin has many benefits, has bioactivity as an antioxidant, anti-inflammatory, anticancer, antimicrobial, and anti-HIV.⁵⁻⁷ However, curcumin has less bioavailability and effectiveness compared to commercial drugs. During this time, various methods are used to increase the bioactivity of curcumin compounds, one of them by making curcumin derivatives.

Spiro compounds (also called spiran) are compounds with two or more rings connected by one chiral carbon atom. The spiro means a polycyclic compound in which one carbon is a member of two different rings.⁸ In recent times, the spirooxindole compound has become a center of attraction for scientists because of its unique structural core and potential application of these compounds in the pharmaceutical world.^{9,10}

Spirooxindole-pyrrolizidine is a group of spirooxindole compounds that in the last two decades have been widely studied by scientists because of their structure, which resembles cytostatic and chemotherapy agents such as vinblastine and vincristine.¹¹ These compounds are also present in active alkaloid compounds such as horsfilin and spirotriprostatin.^{12,13} The synthesis of the spirooxindole-pyrrolizidine compound can be synthesized through the 1,3-dipolar cycloaddition reaction with the formation of azomethine ylide. This reaction is an excellent reaction because it involves zwitterionic molecules, positively charged nitrogen atoms, and carbon atoms with sp² hybridization¹⁴. This reaction has a high level of regio-, diastereo-, and enantioselectivity, so it is interesting to study. Also, this reaction facilitates the formation of pyrrolizidine rings which are essential compounds in the pharmaceutical world.¹⁴⁻¹⁶

Recently scientists developed the synthesis of the spirooxindole-pyrrolizidine compound from the reaction between isatin, α -amino acids, and alkenes using Graphene Oxide (GO) catalyst.¹⁷ By using a GO catalyst, the reaction of the formation of the compound Spiro-oxindole-pyrrolizidine can give a higher result with a shorter reaction time. According to previous studies¹⁴, several types of α -amino acid show different reaction characteristics in the formation of spirooxindole-pyrrolizidine compounds which are very interesting to

study. Therefore, in this study, the synthesis of spirooxindole-pyrrolizidine compounds will be carried out through a multicomponent reaction between curcumin, isatin, and l-proline with the help of the Fe_3O_4 -GO catalyst. The addition of magnetic properties of Fe_3O_4 intended to simplify the separating process of GO from the reaction mixture.

Curcumin in this reaction acts as dipolarophils. As a comparison, isatin and l-proline will also be reacted with α , β -carbonyl unsaturated compounds from other functional groups that can act as dipolarophils such as chalcone derivatives, and the results are then characterized and analyzed to understand their bioactivity.

EXPERIMENTAL

Material and Methods

Tools and instruments used in this study were laboratory glassware, filter paper, magnetic stirrer, TLC plate, stirring hot plate IKA C-MAG HS 7 Julabo SW22 shaker, micropipettes, tweezers, spreaders, ose needles, incubators, and autoclave. The analysis instruments used are melting point measuring devices, FTIR Spectrophotometer (IRPrestige-21 Shimadzu), UV-Vis Spectrophotometers (UV-2450 Shimadzu), LC-MS/MS, TEM, EDS, and XRD. Materials used to make Fe_3O_4 -Graphene Oxide (GO) were graphite powder, NaNO_3 , H_2O_2 , KMnO_4 , H_2SO_4 , FeCl_3 , FeCl_2 . The chemicals used for the synthesis of target products are curcumin, acetophenone, vanillin, isatin, l-proline, ethanol, methanol, ethyl acetate, n-hexane, HCl, and aquabides. The compounds used for the antibacterial and antioxidant tests are antibiotic Gentamicin, DMSO, Pepton, Nutrient agar, yeast extract, 70% alcohol, and 1,1-diphenyl-2-picrylhydrazyl (DPPH•).

Synthesis of Graphene Oxide (GO)

Graphene Oxide (GO) synthesis is carried out by referring to the Hummers' method that has been carried out before.¹⁸ A total of 1.5 grams of graphite powder and 0.75 grams of NaNO_3 and 34.5 mL of concentrated H_2SO_4 were mixed in a three-neck flask and stirred with a magnetic stirrer for 20 minutes. Then, this mixture is allowed to stand and cool for 2 hours in an ice bath. After that, the addition of KMnO_4 (4.5 g) was carried out slowly by maintaining the mixture temperature below 20°C and then heated at 35°C under stirring conditions. Subsequently, 69 mL of distilled water was added while continuing to stir for 20 minutes, followed by the addition of H_2O_2 30% droplets (1.5 mL) and discoloration of the solution occurred to yellow. After this, the addition of distilled water (50 mL) was carried out, and the reaction mixture was sonicated for 2 hours. The final reaction solution is purified by centrifugation, filtering, and washed with bidistilled water and dried at 100°C for one day to produce GO.

Synthesis of Fe_3O_4 -GO

Synthesis of Fe_3O_4 -GO was carried out by the co-precipitation method.¹⁹ Graphene oxide (100 mg) was distributed in distilled water (100 mL) by sonication. In another flask, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (0.75 g), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2 g), and distilled water (25 mL) were added and stirred at room temperature for 30 minutes with a magnetic stirrer. The two mixes were transferred to the three-neck flask while continuing to stir for 30 minutes at 85°C . After that, ammonia solution (25%) was dripped slowly into the reaction and obtained pH 10, and stirring was continued for 45 minutes. The product was cooled and decanted with magnets from the outside. The solid phase was washed dried at 60°C for 12 hours.

Synthesis of 4-hydroxy-3-methoxychalcone

The method for 4-hydroxy-3-methoxychalcone synthesis was carried out by referring to the Claisen-Schmidt condensation reaction that previously reported.^{20,21} The reaction was conducted by mixing acetophenone (1.98 mL), NaOH 60% (10 mL), and 4-hydroxy-3-methoxy benzaldehyde (2.5 g) in ethanol solvents (15 mL). The mixture was refluxed for 3 hours at 70°C and monitored by TLC. After completion, the solvent was removed and acidification of the mixture to pH=1 was conducted by using 1M HCl. The organic phase obtained from liquid-liquid extraction (ethyl acetate) was purified by adding anhydrous Na_2SO_4 to remove the remained water. Furthermore, the solvent was evaporated, and the product was refined by column chromatography. The product was evaluated and analyzed its maximum wavelength,

functional group vibrations, and molecular weight using UV-Vis spectrophotometer, FTIR, and LCMS/MS, respectively. The reaction is shown in Fig.-1 below.

Fig.-1: Reaction of 4-hydroxy-3-methoxychalcone

Synthesis of Spirooxindole-pyrrolizidine Derivatives

The synthesis of spirooxindole-pyrrolizidine derivatives was carried out based on the development of the procedure previously reported.^{14,17} Some Fe₃O₄-GO catalysts in various mediums were mixed to a mixture of isatin, curcumin, and l-proline (2: 1: 2 mole ratio). The reaction is carried out at 65°C and monitored using TLC. The catalyst is separated from the external magnetic source, and the solution is evaporated until solids remain. Recrystallization of solids is carried out by washing solids formed using warm ethanol to obtain the derivative of the bis-spirooxindole-pyrrolizidine-curcumin compounds. Under the same procedure, isatin and l-proline were reacted with 4-hydroxy-3-methoxychalcone in 1:1:1 mole ratio to obtain Spirooxindole-pyrrolizidine-4-hydroxy-3-methoxychalcone. The reaction can be seen in Fig.-2 and Fig.-3.

Fig.-2: Reaction of bis-spirooxindole-pyrrolizidine-curcumin

Fig.-3: Reaction of Spirooxindole-pyrrolizidine-4-hydroxy-3-methoxychalcone

Antioxidant Activity Test

Free radical scavenging or antioxidant activity test of the synthesized products was calculated by using DPPH• method.^{20,22} Five different concentrations of synthesized products and curcumin as its standard were made using ethanol as its solvent (200, 100, 50, 25, and 12.5 µg/mL). Then, each 2 mL of DPPH• 0.1 mM solution, and the test sample was mixed and incubated for 30 minutes in a dark room. As a negative control, 2 mL ethanol was mixed with 2 mL of 0.1 mM DPPH• solution. The absorbance was measured and evaluated by a UV-Vis spectrophotometer at 517 nm. The IC₅₀ of all test samples determined by plotting its concentration and % DPPH scavenged in a linear graphic. Determination of radical scavenging activity was calculated using the formula:

$$\% \text{ DPPH scavenged} = [A_0 - A_1 / A_0] \times 100$$

Where A_0 = DPPH solution absorption, and A_1 = sample solution absorption.

Antimicrobial Activity Test

The determination of the antimicrobial activity of the synthesized products was conducted by using the disc diffusion method.²¹ A total of 15 mL of nutrient agar media was put into a petri dish. After solidification, the process of bacterial suspension inoculation (0.1 mL) was carried out on the media. Then, sterilized disc paper was dipped for one minute in a product that has been diluted with varying concentrations. In the final step, the disc paper was distributed over the surface of the agar medium and incubated for one day at 37°C. Clear zone diameters were observed and measured.

RESULTS AND DISCUSSION

Synthesis and Characterization of Fe₃O₄-Graphene Oxide

The synthesis of Fe₃O₄-GO was prepared by oxidized graphite powder to graphene oxide (GO) using Hummer's method and Fe₃O₄ insertion into GO layers by co-precipitation method using Fe²⁺ and Fe³⁺ ions which are assisted by ammonia bases. The product Fe₃O₄-GO synthesized was obtained in the form of black powder. The scientific evidence that Fe₃O₄-GO has been successfully synthesized was carried out by analysis using FTIR, EDS, XRD, and TEM.

The analysis using FTIR was conducted to evaluate the functional groups of GO and Fe₃O₄ and analyze the interactions that occur in both. The comparison FTIR spectra of Fe₃O₄-GO composite is shown in Fig.-4. The GO spectrum showed there were four peaks gained at 3610.89 cm⁻¹ (O-H), 3138.31 cm⁻¹ (C-H sp²), 1731.18 cm⁻¹ (C=O Carboxylate), 1258.60 cm⁻¹ (C-O-C Epoxide group). Then, in the Fe₃O₄ spectrum, there is a unique peak which shows that Fe₃O₄ formation has occurred at the wave number 628.82 cm⁻¹, which indicates the existence of Fe-O stretching vibrations. The process of inserting Fe₃O₄ into GO layers can be seen from the spectrum of Fe₃O₄-GO formed. In this spectrum, there are no new peaks formed but only a combination of peaks that exist on GO and Fe₃O₄.

The XRD pattern of Fe₃O₄-GO composite is shown in Fig.-5. These diffraction peaks showed at Bragg angle $2\theta = 30.30^\circ$, 35.69° , 43.41° , 57.54° , and 62.81° indicated the characteristics of Fe₃O₄ (JCPDS 19-0629)²³. These diffraction peaks implied that the Fe₃O₄ core crystal structure was still well-maintained after functionalization. Unfortunately, there were no diffraction peaks indicated as GO, due to its low concentration. However, determination by EDS (Fig.-6) to analyze the elemental composition confirmed that there was a carbon atom in the synthesized Fe₃O₄-GO. In the synthesized Fe₃O₄-GO, there were 04.49 wt% of C atom, 31.76 wt% of O atom, and 63.74 wt% of Fe atom. The calculation with the formula of Debye-Scherrer's XRD pattern showed that the average size of the particle of Fe₃O₄-GO had an estimated approximately 19,9 nm. Typically TEM image of Fe₃O₄-GO composite (Fig.-7) showed that most particles of Fe₃O₄ have a spherical shape, and they distributed well in the graphene oxide layer.

Fig.-4: FT-IR Spectra of Fe₃O₄, Fe₃O₄-GO, GO

Fig.-5: XRD Pattern of Fe₃O₄-GO

Fig.-6: EDS Spectrum Fe₃O₄-GO Fig.-7: TEM Image of Fe₃O₄-GO

Catalytic Ability of Fe₃O₄-GO in the Synthesis of Spirooxindole-pyrrolizidine Compounds

In this research, the optimization of reaction condition was carried out using the model of reaction between isatin, l-proline, and 4-hydroxy-3-methoxychalcone. All reaction was conducted using methanol solvent at 65°C for approximately 3 hours. The yield of all the reactions is shown in Table-1.

Table-1: Variation Catalyst Concentration of Optimization Reaction Condition

Catalyst	Quantity (wt%)	Yield (%)
Fe ₃ O ₄ -GO	5	95.82
Fe ₃ O ₄ -GO	10	81.50
GO	5	94.24
GO	10	90.15
Fe ₃ O ₄ -GO	5	93.03 ^a
GO	5	79.22 ^a
No Catalyst	-	78.16 ^a

^a reaction of isatin, curcumin, and l-proline (1:2:2 mole ratio) after 6 hours of stirring

The results of variations in reaction conditions in Table-1, the use of Fe₃O₄-GO as much as 5 wt% generated the highest result of the desired product. The reaction with the highest yield was carried out for 3 hours and obtained a yield of 95.82 %. However, when the condition was applied to the reaction of isatin, curcumin, and l-proline, the desired product was not obtained at 3 hours reaction. The reaction with that reactants took two times longer (6 hours) to obtain a reaction yield of 93.03%. This condition is probably influenced by the presence of two symmetrical α,β -unsaturated carbonyl group at curcumin, so the reaction occurs in its two sides of alkenes produced bis-spirooxindole-pyrrolizidine-curcumin.

Table-2: Spectral Data of the Synthesized Products

No.	Compound	Spectral Data	Yield (%)
1.	Bis-spirooxindole-pyrrolizidine-curcumin	LC-MS/MS [M+H] ⁺ : 769.3181 FTIR (cm ⁻¹): 3517.34 (N-H amide), 3364.96 (O-H), 3107.45 (C-H sp ²), 2980.14 (C-H sp ³), 1732.15 (C=O), 1622.20 (C=C aromatic), 1474.64 (-CH ₂ - methylene), 1347.33 (-CH methyne), 1288.50 (C-O-C from methoxy group), 762.87 (ortho substituted benzene).	93.03

2.	Spirooxindole-pyrrolizidine-4-hydroxy-3-methoxychalcone	LC-MS/MS $[M+H]^+$: 455. 2042 FTIR (cm^{-1}) : 3549.173 (N-H amide), 3309.99 (O-H), 3089.13 (C-H sp^2), 2969.53 (C-H sp^3), 1727.32 (C=O), 1621.23 (C=C aromatic), 1472.71 (-CH ₂ - methylene), 1385.91 (-CH methyne), 1273.07 (C-O-C from methoxy group), 759.02 (ortho substituted benzene).	95.82
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Graphene oxide was also taken part in the optimization because it has functional groups such as O-H and -COOH that plays an essential role in this reaction.¹⁷ GO facilitated the reaction through hydrogen bonding and π -stacking between the catalyst and the substrates. Furthermore, the addition of Fe_3O_4 was aimed to facilitate its separation from the reaction mixture due to its magnetic properties. Also, Fe_3O_4 acts as a Lewis acid which will affect oxygen in the carbonyl group so that the C carbonyl atom is more positive²⁴. Graphene oxide is also known to have acidic properties and can facilitate the formation of reactive azomethine ylide. This reactive azomethine ylide leads to the formation of 5 membered heterocyclic compounds and the reaction is well known as 1,3-dipolar cycloaddition or Huisgen Cycloaddition. The plausible mechanism of synthesis spirooxindole-pyrrolizidine derivatives using the Fe_3O_4 -GO catalyst is shown in Fig.-8.

Fig.-8: Plausible Mechanism synthesis spirooxindole-pyrrolizidine derivatives using Fe_3O_4 -GO catalyst

Bioactivity Assay

After the synthesized product was well characterized (showed in Table-2), their antioxidant and antibacterial activity were determined. The IC_{50} of the synthesized product is demonstrated in Fig.-9. From the result, bis-spirooxindole-pyrrolizidine-curcumin possess the best free radical scavenging activity (IC_{50} 18.71 ppm) compared to spirooxindole-pyrrolizidine-4-hydroxy-3-methoxychalcone and curcumin. In comparison, the spirooxindole-pyrrolizidine-4-hydroxy-3-methoxychalcone possesses the lowest free

radical scavenging activity (IC_{50} 38.71 ppm) compared to the others. It is because 4-hydroxy-3-methoxychalcone has a hydroxyl group (-OH) while curcumin has two. As is known, the radical scavenging activity of a compound is influenced by the number of hydroxyl groups and conjugation of the double bonds that resonate in the structure of the compound. In carrying out its role, free radicals will release hydrogen atoms with a radical reaction system of hydroxyl groups so that these compounds become radicals.²⁵ Unfortunately, in the antibacterial test conducted on bacteria of *S. aureus*, *P. aeruginosa*, *E. coli*, and *B. subtilis*, there was no significant activity. This result was a bit surprising because many kinds of literature said that most pyrrolizidine alkaloids possess a good antibacterial activity.²⁶ Also, the presence of oxindole and spiro rings should increase its antibacterial activity. There are many possibilities, including the micro bacterial species which is less varied and the configuration of spirooxindole-pyrrolizidine since the configuration of a compound can affect its bioactivity. According to the previous report²⁷, the R-enantiomer of a compound will not necessarily act the same way as the S-enantiomer of the same compound when given by an organism. One enantiomer probably has a good activity while the other is inactive because it cannot bind the enzymes, receptors, or transporters no matter how it is rotated in space. Meanwhile, both synthesized products have some chiral carbon which has not been determined well.

Fig.-9: Free Radical Scavenging Activity of spirooxindole-pyrrolizidine derivatives: bis-spirooxindole-pyrrolizidine (1), spirooxindole-pyrrolizidine-4-hydroxy-3-methoxychalcone

CONCLUSION

In summary, the magnetic-graphene oxide (Fe_3O_4 -GO) catalyst was successfully synthesized. The catalytic ability of Fe_3O_4 -GO generated a high yield in reaction spirooxindole pyrrolizidine synthesis using a one-pot three-component reaction, by reacting of isatins, l-proline and two kinds of α,β -unsaturated carbonyl compounds such as curcumin and chalcone derivatives. The synthesized compound possesses a good antioxidant activity which is indicated by its IC_{50} values (< 50 ppm).

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