Microwave-Assisted Synthesis, Antioxidant and Toxicological Evaluation of a Hydrazone, 1-(4-chlorobenzylidene)-2-phenylhydrazine

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Abstract. Hydrazone is an imine-derived compound that has various biological activities such as anticancer, antitumor, antioxidant, antimicrobial, and antidepressant. Antioxidants play an important role in removing oxidants and preventing cell damage and the toxicity of a compound is one of the parameters in drug discovery, especially anticancer. In this work, a hydrazone, 1-(4-chlorobenzylidene)-2-phenylhydrazine has been synthesized under microwave irradiation and evaluated for its antioxidant activity by DPPH assay. The toxicity of this compound was evaluated by Brine Shrimp Lethality Test (BSLT) for pre-screening of anticancer drugs. The compound was elucidated for its structure using UV-Vis, FTIR, GC-MS, 1D, and 2D NMR spectrometers. Based on the toxicological evaluation using BSLT, the synthesized compound possessed a highly toxic with LC₅₀ value of 0.018 µg/mL and has strong antioxidant activity with IC₅₀ value of 41.33 µg/mL. These suggest that the synthesized hydrazone compound is a potential compound in the development of antioxidant and anticancer drugs.

1. Introduction

The development of new compound structures that have potential and biological activities such as anti-inflammatory, anticancer, antimicrobial, antihypertensive, and others remain to be the main objective by many researchers, including imine-derived compounds, hydrazone which has an azomethine group (-NHN=CH). This azomethine group has received great attention because of its diverse bioactivities [1]. Formation of hydrazone can be catalyzed either with acids or bases, but generally with acids such as acetic acid [2]. Some synthesis methods have been used to synthesize hydrazone such as reflux and stirring but this conventional method required more time and low yield compared to microwave-assisted [3].

Many studies have been conducted on hydrazone derivatives that show these compounds have very diverse biological activities. Dilek et al. [4] have synthesized hydrazone derivative compounds through nucleophilic addition-elimination reactions between 2-[(1-methyl-1H-tetrazole-5yl)thio]acetohydrazide with aromatic aldehydes/ketones with promising anticancer properties, because it has an inhibitory effect on cancer cells A549 (lung) and has low toxicity to NIH3T3 (mouse embryonic fibroblast) cells. Cui et al. [5] have synthesized N-acylhydrazone compounds through reactions with high diastereoselectivity with good antitumor activity. Abdu et al. [6] have synthesized hydrazone derivatives that have radical scavenging activity which shows the antioxidant properties of these compounds. Hydrazone also has other bioactivities such as anticonvulsants [7], antidepressants...
[8], analgesics, and anti-inflammatory [9]. Besides, the combinations of hydrazones with other functional groups form the other compounds with interesting physical and chemical properties. Therefore, the hydrazones are also widely used in organic synthesis as intermediates [10] and also as target structure in drug discovery and drug design works.

The toxicity of a compound is one of the parameters in drug discovery. BSLT is widely used as one of the methods for testing the toxicity of compounds, using Artemia salina Leach larvae which is 48 hours (2 days) old as a test animal. The advantages of using this method are a short time test (24 hours), low cost, and only use a small amount of tested compound (2-20 mg). The results of the toxicity test with the BSLT method can be known from the number of Artemia salina Leach larvae deaths due to the influence of certain extracts or compounds from a predetermined dose [11].

Antioxidants play an important role in removing oxidants and preventing cell damage. Oxidative damage is caused by the presence of free radicals or radical-generating agents in concentrations that overwhelm natural radical-blocking or radical-scavenging mechanisms [12]. Antioxidants have been identified as a major antidote to oxidative damage. In this study, we are interested to synthesize a hydrazone, 1-(4-chlorobenzylidene)-2-phenylhydrazine and evaluate its toxicity using BSLT and its antioxidant using DPPH assay.

2. Methodology

2.1. Materials and Animal

The materials were used in this study are 4-chlorobenzaldehyde (Merck), phenylhydrazine (Merck), glacial acetic acid (Merck), sodium hydroxide (NaOH) (Merck), 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (100 μg/mL), distilled water, and some organic solvents such as absolute ethanol (Merck), n-hexane, ethyl acetate (EtOAc), chloroform (CHCl₃), and dimethyl sulfoxide (Merck). The animal that was used is Artemia salina Leach larvae. Eggs of Artemia salina Leach obtained from the collection of Marine Chemical Laboratory of Marine Science Department, Faculty of Fisheries and Marine, Universitas Riau.

2.2. Instrumentation

The synthesis of compound 3 was performed in a domestic microwave, Samsung (ME109F). The progress of reaction was observed by TLC analysis using silica gel plate, GF₂₅₄ (Merck). The spots on the TLC plate were observed under UV lamp 254 nm (Camag™). The HPLC analysis was performed in a Shim-pack VP-ODS column (250 x 4.6 mm), with methanol and water as mobile phases in gradient system for 25 min and flow rate of 0.75 mL/min (UFLC Prominance-Shimadzu LC solution, UV detector SPD 20AD). The melting point of the synthesized compound was measured in a Fisher John melting point apparatus (Uncorr.). The molecular mass was determined in a Gas Chromatography-Mass Spectrometer (GC-MS), Agilent 6890 GC. The ultraviolet (UV) spectrum was measured by UV-Vis spectrophotometer (Genesys 10S UV-Vis v4.002 2L9N175013). The Fourier Transform-Infra Red (FT-IR) spectrum was measured by FT-IR Shimadzu, IR Prestige-21. The 1D and 2D-Nuclear Magnetic Resonance (NMR) spectra were measured by NMR spectrometer (Agilent). The antioxidant activity assay was performed by 96-wells microplate reader (Berthold).

2.3. Procedure

2.3.1. Synthesis of 1-(4-chlorobenzylidene)-2-phenylhydrazine (3). 4-chlorobenzaldehyde (1) (0.1405 g; 1 mmol) in 5 mL of absolute ethanol was put into Erlenmeyer flask. Next, 2 mL of glacial acetic acid and phenylhydrazine (2) (0.1081 g; 1 mmol) was added into the solution, then the mixture was homogenized. The compound mixture was irradiated by microwaves at 180 Watt for 2 minutes. The progress of reaction was observed every 60 seconds using TLC. If the reaction was finished, the mixture was then neutralized by NaOH 3N and then added 3 mL of cold aqua DM. The mixture was cooled in the refrigerator for 24 hours. The obtained yellowish-white solid was then filtered by Buchner funnel and washed with cold aqua DM and n-hexane, and then dried at room temperature. The synthetic pathway of compound 3 is depicted in Figure 1.
1-(4-chlorobenzylidene)-2-phenylhydrazine (3). The product was obtained as yellowish-white solid. Yield: 85%, m.p. 126-127°C, UV (EtOH): λ_{max} = 206, 242, 299 and 353 nm. FTIR (KBr, cm⁻¹): 3312 (N-H str.), 3053 (Ar C-H str.), 1601 (C=N str.), 1518 and 1445 (Ar C=C str.), 1267 (C-N str.), 748 (C-Cl str.). \(^1\)H-NMR (500 MHz, CDCl₃) δ (ppm): 7.65 (br-s, 1H, NH); 7.63 (s, 1H, azomethine H); 7.59 (d, 2H, J = 8.5 Hz, Ar-H-2', Ar-H-6'); 7.35 (d, 2H, J = 8.5 Hz, Ar-H-3', Ar-H-5'); 7.30 (t, 2H, J = 8.5 Hz, Ar-H-3", Ar-H-5"); 7.12 (d, 2H, J = 8 Hz, Ar-H-2", Ar-H-6"); 6.91 (t, 1H, J = 7.5 Hz, Ar-H-4"). \(^{13}\)C-NMR (125 MHz, CDCl₃) δ (ppm): 144.40 (C-1"); 135.80 (C-1); 133.98 (C-4"); 133.87 (C-1); 129.34 (2C, C-3", C-5"); 128.83 (2C, C-3', C-5'); 127.27 (2C, C-2', C-6'); 120.37 (2C, C-2", C-6"). GC-MS: tₓ = 8.042 minutes, m/z [M]+ = 230.1 (100%), 232.1 (59%).

2.3.2. Toxicological Evaluation using Brine Shrimp Lethality Test. The test vial was calibrated using 5 mL of distilled water. 2 mg of compound 3 was dissolved in 2 mL of ethyl acetate (concentration: 1000 μg/mL), then from this concentration were diluted to make different concentrations of 10, 1, 0.1, and 0.01 μg/mL. The sample solution was piped into each of the calibrated vials of 0.5 mL, then the solvent was evaporated. The concentrations used for the compound 3 were 10, 1, 0.1, and 0.01 μg/mL. Furthermore, each vial was added 50 μL of DMSO and added a little amount of seawater, then each added 10 shrimp larvae Artemia salina Leach and added seawater until the calibration limit. In another vial was filled with 50 μL of DMSO and added a little amount of seawater before the calibration limit, then added 10 shrimp Artemia salina Leach larvae without the compound 3 as a control. Next, the test and control vials were left for 24 hours. After 24 hours, dead shrimp larvae were calculated and recorded. The level of toxicity was measured by looking at the percentage of dead larvae. The test was performed three times of repetition with the same treatment for each concentration. The data obtained were analyzed to determine the LC₅₀ value by curve method using a probit analysis table.

2.3.3. DPPH Assay. Compound 3 was prepared with a certain concentration in methanol. Approximately 100 μL of the sample was put into row A of microplate (plate consist of A-H rows, each row consists of 12 wells). Two-fold dilutions of the compound were added to the next row so that the concentration of each A-F line were 1000 μg/mL, 500 μg/mL, 250 μg/mL, 125 μg/mL, 62.5 μg/mL, and 31.25 μg/mL respectively. A-G rows were added with 80 μL of DPPH with a concentration of 100 μg/mL. After that, it was incubated for 30 minutes in the dark room [13]. Radical scavenging activity was taken as a decrease in absorbance of DPPH with a microplate reader and spectrometer seen at a maximum absorption wavelength of 520 nm. Antioxidant activity was calculated based on the equation (1).

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\text{Inhibition (\%) =} \frac{(\text{Absorbance Control - Absorbance Sample})}{\text{Absorbance Control}} \times 100\%
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Then, the inhibition concentration 50 (IC₅₀) value was calculated based on the linear regression equation (y = ax + b) from the curve by plotting the Ln concentration on x-axis and percentage of inhibition on y-axis.

3. Results and Discussion

3.1. Synthesis of 1-(4-chlorobenzylidene)-2-phenylhydrazine (3)

Compound 3 was obtained from the condensation reaction between 4-chlorobenzaldehyde (2) and phenylhydrazine (1) through nucleophilic addition under acid condition. The synthesis reaction was

![Figure 1. The synthetic pathway of compound (3).](attachment:image)
assisted by applying microwave irradiation at 180 Watt for 2 minutes. This microwave-assisted organic synthesis (MAOS) has advantages such as short time reaction, energy-saving, and high yield of a product than the conventional method (reflux and stirring) [3]. Compound 3 was obtained as a yellowish-white solid with 85% of yield. The melting point is reached on 126-127 °C, with this sharp melting point (≤ 2 °C) showed that the obtained solid product was a pure solid. The TLC chromatogram showed a single spot with different eluent comparison systems and the HPLC chromatogram showed a single peak at tR of 18.24 min. These results indicated that the obtained solid product was a pure compound.

In the UV spectrum, maximum absorption at wavelength (λmax) 206, 242, 299, and 353 nm indicated the presence of conjugated double bond in compound 3. In the IR spectrum, stretching absorptions at 3111 cm−1 indicated the presence of N-H bonds of the hydrazone groups; 3053 cm−1 indicated the aromatic C-H bond vibrations of the two aryl rings, A and B; 2968 cm−1 indicated the existence of aliphatic C-H bond vibrations of carbon which were directly bound to the B ring; 1601 cm−1 indicated the bending vibration of the N-H bond; 1518 cm−1 indicated the presence of vibrations of the C=N bond which is typical of imine-derived compounds; 1444 cm−1 indicated the presence of aromatic C=C bond vibrations; and 748 cm−1 indicated the presence of C-Cl bond vibrations. For more details, the structure can be seen in Figure 2.

In the 1D and 2D NMR were performed to observe proton and carbon positions also the correlation between them (HSQC and HMBC) to confirm the structure of compound 3. The 1H-NMR spectrum of compound 3 confirmed the formation of azomethine group by the appearance of a broad singlet signal (N-H proton) at δ 7.65 ppm and singlet signal (methine proton) at δ 7.63 ppm. Besides, the roof effect of the doublet signal at δ 7.35 and δ 7.59 ppm indicated a para-substituted aromatic ring (see Figure 3). The 13C-NMR spectrum also showed characteristics of the carbon signal of hydrazone, which is the resonance of an azomethine carbon (C=N) that appeared at δ 135.80 ppm. Overall, the 1H and 13C-NMR spectra of compound 3 showed the number of protons and carbons corresponded to be expected molecule targets.

The HSQC spectrum of compound 3 showed a direct correlation between the H-1 proton (δ 7.63 ppm) and the C-1 carbon atom (δ 135.8 ppm). Proton NH was no direct correlation to any carbon signal that showed NH was not bound to carbon, but it was bound to nitrogen. In the HMBC spectrum showed the correlation between proton and carbon within 2-3 bonds. Compound 3 showed correlations of proton H-1 to carbon C-1' (2 bonds), C-2' and C-6' (3 bonds), C-3' and C-5' (4 bonds). Meanwhile, the NH proton correlated with carbon C-1" (2 bonds), C-1 (3 bonds), C-3" and C-5" (4 bonds). Then, we performed a GC-MS analysis to confirm the molecular mass of the compound 3. The GC chromatogram showed that the synthesized compounds possess high purity and the MS spectra showed the molecular ion peak [M]+ at m/z 230.1 with a relative abundance of 100% which is the molecular weight of the compound 3 according to the molecular formula C13H11N2Cl.

![Figure 2](image-url)
Figure 3. $^1$H-NMR spectrum of compound 3.

3.2. Toxicological Evaluation using Brine Shrimp Lethality Test

In the BSLT method, a pure compound is considered toxic if it has an LC$_{50}$ value of $\leq$ 200 µg/mL [14]. BSLT results showed that compound 3 is highly toxic with LC$_{50}$ value of 0.018 µg/mL. Compound 3 at concentration of 0.01, 0.1, 1, 10 µg/mL was able to kill shrimp larvae *Artemia salina* Leach with percentage 50, 63, 76, 96% respectively. Therefore, it can be concluded that the higher concentration of compound 3 showed better toxicity. The toxicological evaluation of compound 3 is presented in Table 1. The toxicity can be measured by using the percentage of dead larvae to probit table then we got probit value. By plotting the log concentration on the x-axis and probit value on the y-axis, as depicted in Figure 4, we can get a linear regression equation. Then, this equation is used to calculate the LC$_{50}$ value.

| Concentration (µg/mL) | Death Percentage (%) | Probit Value | Log Concentration |
|-----------------------|-----------------------|--------------|-------------------|
| 0.01                  | 50                    | 5.000        | -2                |
| 0.1                   | 63                    | 5.332        | -1                |
| 1                     | 76                    | 5.706        | 0                 |
| 10                    | 96                    | 6.751        | 1                 |

LC$_{50}$ = 0.018 µg/mL
3.3. **DPPH Assay**

Compound 3 was evaluated for its antioxidant activity using DPPH assay. The method can be characterized by the formation of stable radicals with electrons that can be delocalized and give a deep purple colour, and the density of this colour can be reduced if reacted with a compound that can donate hydrogen atoms [15]. In this study, the result of the antioxidant activity assay for compound 3 was 41.33 µg/mL (Table 2) and was able to donate its hydrogen atom to DPPH radical compounds as evidenced by the colour of DPPH was reduced from purple to yellow (see Figure 5). Based on this category, it is concluded that the synthesized compound has strong antioxidant activity, but it was less active than ascorbic acid as standard with IC₅₀ value of 6.66 µg/mL [16].

| Concentration (µg/mL) | % Inhibition |
|-----------------------|-------------|
| 250                   | 90.2        |
| 125                   | 85.7        |
| 62.5                  | 65.0        |
| 31.25                 | 37.3        |

**Table 2.** The percentage of inhibition of compound 3 in various concentration.

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\text{IC}_{50} = 41.33 \mu g/mL
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4. Conclusion
A hydrazone compound, 1-(4-chlorobenzylidene)-2-phenylhydrazine has been successfully synthesized under microwave irradiation with high yield. Then, the synthesized compound was evaluated for its toxicity using Brine Shrimp Lethality Test (BSLT) and its antioxidant using DPPH assay. The synthesized compound possessed a highly toxic with LC₅₀ value of 0.018 μg/mL and has strong antioxidant activity with IC₅₀ value of 41.33 μg/mL. These suggest that the synthesized hydrazone compound is a potential compound in the development of antioxidant and anticancer drugs.

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