Synthesis, in vitro Antioxidant Activity, and Toxicity Evaluation of Hydrazone Derivatives Naphthalene-1-ylmethylene hydrazine

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Abstract. Hydrazone is a versatile organic compound that has a basic structure (-NHN=CH-) called the azomethine group. This structure is responsible for the physical and chemical of hydrazone, which makes this compound has variety bioactivities such as antioxidant, antitumor, and anticancer. In this work, two hydrazone derivatives from 1-naphthaldehyde and hydrazine (phenylhydrazine/hydrazine hydrate) have been synthesized under microwave irradiation. Their antioxidant activity and toxicity were evaluated by DPPH and BSLT method, respectively. Structures of the synthesized compounds were confirmed based on spectroscopic data included UV, FTIR, HRMS, and 1H-NMR. Based on the DPPH assay, hydrazone from phenylhydrazine has strong antioxidant (IC$_{50}$ 28.90 μg/mL) but inactive antioxidant for hydrazine hydrate (IC$_{50}$ >1000 μg/mL). However, both compounds have a high toxicity effect on Artemia Salina Leach with each LC$_{50}$ 1.45 and 47.20 μg/mL, hence they have the potential to be developed into anticancer drugs.

1. Introduction
Oxidative stress is the cause of different pathological states [1]. Cancer, heart, cataract, premature aging, and other degenerative diseases are the result of free radical reactions [2]. It is known that the use of antioxidants could be beneficial in the prevention or delay of numerous diseases associated with oxidative stress [3]. Hydrazone is one of the potential compounds as an antioxidant with azomethine group (-NHN=CH-) that can donate an electron or proton to free radicals [4]. In our previous work, hydrazone with chloro substituent in ortho, meta [5], and para [6] position give active to highly active antioxidant activity with IC$_{50}$ 57, 25.8, and 41.33 μg/mL, respectively by the DPPH method. Furthermore, 4-methoxy hydrazone compound exhibited very strong antioxidant activity with an IC$_{50}$ value of 9.5 ± 1.1 μg/mL, it is close to the IC$_{50}$ value of ascorbic acid which is used as an antioxidant standard [7].

Besides having antioxidant activity, hydrazone also has a high toxicity effect on Artemia salina Leach larvae in the BSLT (Brine Shrimp Lethality Test) method. BSLT is a method used for pre-screening of potential anticancer compounds [8]. Afriana et al. have synthesized p-chlorohydrazone from p-chlorobenzaldehyde and phenylhydrazine and possessed highly toxic with LC$_{50}$ of 0.018 μg/mL [6]. Naphthalenylmethylen hydrazine derivatives are hydrazone compounds containing...
naphthalene ring, they have been synthesized by Shirindazeh et al. and most of the synthesized compounds showed significant antioxidant activity against DPPH radicals [9]. Therefore, in this study, instead of using substituted benzene rings as a side chain, we designed hydrazones with naphthalene rings especially from 1-naphthaldehyde to the formation of hydrazones. Then we evaluated naphthalene-1-ylmethylene hydrazine as antioxidant and their toxicity using DPPH and BSLT method.

2. Methodology

2.1. Materials and Animal

The materials were used for synthesis, antioxidant, and toxicity evaluation are 1-naphthaldehyde (Merck), phenylhydrazine (Merck), hydrazine hydrate (Merck), glacial acetic acid (AcOH) (Merck), potassium hydroxide (KOH) (Merck), 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution (100 μg/mL), aqua DM, and some organic solvents such as absolute ethanol (Merck), dimethyl sulfoxide (DMSO) (Merck), n-hexane, ethyl acetate (EtOAc), and chloroform (CHCl₃). The animal that was used is Artemia salina Leach larvae. Eggs of Artemia salina Leach were obtained from the collection of Chemical Oceanography Laboratory of Faculty of Fisheries and Marine Science, Riau University.

2.2. Instrumentation

The synthesis of hydrazone compounds were performed in a domestic microwave, Samsung (ME109F). The progress of the 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 (CamagTM). The HPLC analysis was performed in a Shim-pack VP-ODS column (250 x 4.6 mm), with methanol/acetonitrile and water as mobile phases in gradient system for 20-30 min and flow rate of 0.75 mL/min (UFLC Prominence-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 mass spectra were measured by High-Resolution Electrospray Ionization-Time-of-Flight Mass Spectrometry (HR-ESI-TOFMS). The ultraviolet (UV) spectra were measured by UV-Vis spectrophotometer (Genesys 10S UV-Vis v4.002 2L9N175013). The Fourier Transform-Infra Red (FT-IR) spectra were measured by FT-IR Shimadzu, IR Prestige-21. The 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-(naphthalen-1-ylmethylene)-2-phenylhydrazine (4). 1-naphthaldehyde (1) (0.156 g; 1 mmol) in 2.5 mL of absolute ethanol was put into the Erlenmeyer flask. Then, 1.5 mL of glacial acetic acid and phenylhydrazide (2) (0.108 g; 1 mmol) were added into the solution, then the mixture was homogenized. The compound mixture was irradiated by microwave at 180 Watt for 2 minutes. Progress of the reaction was observed every 60 seconds using TLC. After the reaction was finished, then the mixture was added 1 mL of cold aqua DM and neutralized by KOH. The mixture was cooled in the refrigerator for 24 hours. The obtained solid compound was then filtered by Buchner funnel and washed with cold aqua DM and n-hexane, and then dried at room temperature [5].

2.3.2. Synthesis of 1,2-bis(naphthalen-1-ylmethylene)hydrazine (5). 1-naphthaldehyde (1) (0.312 g; 2 mmol) in 5 mL of absolute ethanol was put into the Erlenmeyer flask. Then, 2 mL of glacial acetic acid and hydrazine hydrate (3) (0.050 g; 1 mmol) were added into the solution, then the mixture was homogenized. The compound mixture was irradiated by microwave at 180 Watt for 9 minutes. Progress of the reaction was observed every 60 seconds using TLC. After the reaction was finished, the mixture was cooled in the refrigerator for 24 hours. The obtained solid compound was then filtered by Buchner funnel and washed with cold aqua DM and n-hexane, and then dried at room temperature [5]. The synthetic pathway of compounds (4) and (5) are depicted in figure 1.
Figure 1. The synthetic pathway of compounds (4) and (5).

1-(naphthalen-1-ylmethylene)-2-phenylhydrazine (4). The product was obtained as brown solid. Yield: 72.35%, m.p. 50-51°C, UV (MeOH): $\lambda_{\text{max}} = 242$ nm. FTIR (KBr, cm$^{-1}$): 3312 (N-H str.), 3053 (Ar C-H str.), 1594 (C=N str.), 1516 (Ar C=C str.). $^1$H-NMR (500 MHz, DMSO-d$_6$) $\delta$ (ppm): 10.49 (s, 1H, NH); 8.77 (d, 1H, $J$ = 8.6 Hz, Ar-H); 8.53 (s, 1H, azomethine H); 7.97 (d, 1H, $J$ = 8.1 Hz, Ar-H); 7.88 (t, 2H, $J$ = 7.6 Hz, Ar-H); 7.63 (t, 1H, $J$ = 8.7 Hz, Ar-H); 7.56 (q, 2H, $J$ = 8.1 Hz, Ar-H); 7.26 (t, 2H, $J$ = 7.9 Hz, Ar-H); 6.78 (t, 1H, $J$ = 7.3 Hz, Ar-H). The molecular ion peak in HRMS spectra [M + H]$^+$ found at m/z 247.1235, calculated as m/z 247.1244.

1,2-bis(naphthalen-1-ylmethylene)hydrazine (5). The product was obtained as yellow crystal. Yield: 54.87%, m.p. 150-151°C, UV (EtOAc): $\lambda_{\text{max}} = 355$ nm. FTIR (KBr, cm$^{-1}$): 3056 (Ar C-H str.), 1609 (C=N str.), 1507 (Ar C=C str.). $^1$H-NMR (500 MHz, CDCl$_3$) $\delta$ (ppm): 9.49 (s, 1H, azomethine H); 8.99 (d, $J$ = 10 Hz, 1H, Ar-H); 8.15 (d, $J$ = 10 Hz, 1H, Ar-H); 7.99 (d, $J$ = 10 Hz, Ar-H); 7.68 (t, $J$ = 10 Hz, 1H, Ar-H); 7.60 (m, 2H, Ar-H). The molecular ion peak in HRMS spectra [M + H]$^+$ found at m/z 309.1392, calculated as m/z 392.1440.

2.3.3. DPPH Assay. Compounds (4) and (5) were prepared with a certain concentration in methanol. Approximately 100 μL of the samples were put into row A of the microplate (plate consist of A-H rows, each row consists of 12 wells). Two-fold dilutions of the compounds were added to the next row so that the concentrations of each A-F line were 100, 50, 25, 12.5, 6.25, and 3.125 μg/mL, respectively for compound (4). Then 1000, 500, 250, 125, 62.5, and 31.25 μg/mL, respectively for compound (5). 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 a dark room [10]. Then, the absorbance was measured in triplicate and the total percentage of radical scavenging activity was calculated based on equation (1).

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\text{Inhibition (\%)} = \left( \frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance control}} \right) \times 100\%
\] (1)

Then, the inhibition concentration 50 (IC$_{50}$) values were calculated based on the linear regression equation ($y = ax + b$) from the curve by plotting the Ln concentration on the x-axis and percentage of inhibition on the y-axis.

2.3.4. Toxicity Evaluation using Brine Shrimp Lethality Test. The test vial was calibrated using 5 mL of distilled water. 2 mg of synthesized compounds were dissolved in 2 mL of ethyl acetate (concentration: 1000 μg/mL), then diluted to make different concentrations of 100, 10, 1, 0.1, and 0.01 μg/mL. The sample solution was pipetted into each of the calibrated vials of 0.5 mL, after that the
solvent was evaporated. 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 synthesized compound as a control. Next, the test and control vials were incubated for 24 hours. After 24 hours, dead shrimp larvae were calculated and recorded. The level of toxicity was measured by looking at the mortality percentage. The test was performed three times of repetition with the same treatment for each concentration. The data obtained were analyzed to determine the LC$_{50}$ value by curve method using a probit analysis table [6].

3. Results and Discussion

3.1. *Synthesis of naphthalene-1-ylmethylene hydrazine*

Naphthalene-1-ylmethylene hydrazine derivatives were synthesized from a condensation reaction between 1-naphthaldehyde with phenylhydrazine (4) or hydrazine hydrate (5). This reaction was assisted by applying microwave irradiation at 180 Watt under the acid condition in just 2 and 9 minutes, respectively. This microwave-assisted organic synthesis (MAOS) is a more favorable method for hydrazone because produces high yields of products, short-time reaction, facile, and mild reaction [6]. In our previous work, we have synthesized chloro-substituted hydrazone with a high yield of up to 80% [5]. In this study, we designed the structure of hydrazone compound with naphthalene ring especially from 1-naphthaldehyde with moderate to high yield 54.87 and 72.35% for each compound (4) and (5) (figure 2).

![Figure 2. Product of synthesized compounds; a. Brown solid of compound (4) b. Yellow crystals of compound (5)](image)

The structures of synthesized compounds were confirmed by spectroscopy analysis (UV, FTIR, MS, and $^1$H-NMR). UV spectra showed absorption at maximum wavelength $\lambda_{max}$ 242 and 355 nm for compounds (4) and (5), respectively. This absorption indicated the presence of conjugated double bond. FTIR spectra showed absorption bands for the functional group of azomethine at wavenumber 1600 cm$^{-1}$ (C=N str.) for both synthesized compounds and 3312 cm$^{-1}$ (N-H str.) just for compound (4). The presence of aromatic rings can be seen at absorption bands 3050 and 1500 cm$^{-1}$. Mass spectra also confirmed the molecular mass of compounds (4) and (5) with molecular ion peak [M + H]$^+$ m/z 247.1244 and 309.1392, respectively. $^1$H-NMR spectra were performed to observe the total protons, positions, and chemical environment of protons. Methine proton in azomethine group (N=C-H) of compounds (4) and (5) was observed each at $\delta$ 8.53 and 9.49 ppm with singlet signal. In addition, the singlet signal at $\delta$ 10.49 ppm is due to NH azomethine was only observed in compound (4). All the spectroscopic data showed that the obtained compounds corresponded to the expected molecule targets.
3.2. *In vitro antioxidant Assay*

In this work, the antioxidant activities of synthesized compounds were tested *in vitro* by the DPPH method. The result of the antioxidant activity evaluation is presented in Table 1. Compound (4) possesses strong antioxidant with IC$_{50}$ value of 28.90 μg/mL and compound (5) is inactive with IC$_{50}$ >1000 μg/mL [2].

| Table 1. The result of antioxidant activity of compounds (4) and (5) |
|---------------------------------------------------------------|
| **Compound (4)** | **Compound (5)** |
| Concentration (μg/mL) | %inhibition | Concentration (μg/mL) | %inhibition |
| 100 | 89.89 | 1000 | 9.99 |
| 50 | 66.90 | 500 | 1.97 |
| 25 | 43.92 | 250 | 1.50 |
| 12.5 | 26.47 | 125 | 1.24 |
| 6.25 | 17.81 | 62.5 | 0.98 |
| 3.12 | 13.23 | 31.25 | 0.47 |

IC$_{50}$ = 28.90 μg/mL  IC$_{50}$ > 1000 μg/mL

The active antioxidant compound has the ability to donate proton or an electron to DPPH radicals (purple color) to reduced DPPHH (yellow color). Then, the antioxidant compound can stabilize the radical in its structure because of the resonance in the conjugation system of the compound. Thus compound (4) is an active antioxidant because hydrazine proton is responsible for donor proton to the DPPH radicals [7]. On another hand, compound (5) does not have hydrazine proton so it cannot transfer a proton to DPPH radicals, thus inactive antioxidant activity (figure 3). Changes in the structure of the benzene ring to a naphthalene ring may increase antioxidant activity because it can help delocalize electrons in stabilizing free radicals. But this condition only applies if the hydrazone structure contains a proton donor group in the form of NH azomethine.

![Figure 3. The suggested antioxidant mechanism for the synthesized compounds](image)

3.3. Toxicological Evaluation

The synthesized compounds were tested for toxicity effect to *Artemia salina* Leach with the BSLT method. This method is used for pre-screening of potential anticancer compounds [8]. Compound (4) is highly toxic with LC$_{50}$ 1.45 μg/mL and compound (5) is toxic with LC$_{50}$ 47.20 μg/mL. According to Andini *et al.*, a compound is considered very toxic if it has LC$_{50}$ value < 30 μg/mL, and toxic if it has
LC₅₀ 30-1000 μg/mL [10]. Toxicity effect to *Artemia salina* Leach indicate the naphthalene-1-ylmethylene hydrazine derivatives have potential to be developed as anticancer agent and can be forwarded to the cytotoxic test on certain cancer cells. The toxicological evaluation of compounds (4) and (5) can be seen in table 2.

| Concentration (μg/mL) | % Mortality | Compound (4) | Compound (5) |
|-----------------------|-------------|--------------|--------------|
| 1000                  | 97          | 76           |
| 100                   | 77          | 53           |
| 10                    | 63          | 36           |
| 1                     | 50          | 20           |
| 0.1                   | 27          | 13           |
| LC₅₀ (μg/mL)          | 1.45        | 47.20        |

4. Conclusion

Naphthalenylmethylene hydrazine derivatives have been successfully synthesized under microwave irradiation with moderate to high yield. The spectroscopic data confirmed the structure expected. Antioxidant activity of hydrazone from phenylhydrazine (4) has strong antioxidant (IC₅₀, 28.90 μg/mL) but inactive antioxidant for hydrazine hydrate (5) (IC₅₀ >1000 μg/mL). Based on these results, the azomethine group determines the activity of the synthesized compounds, and the naphthalene ring as a side chain does not have much impact. Nevertheless, both compounds have a high toxicity effect on *Artemia Salina* Leach with each LC₅₀ 1.45 and 47.20 μg/mL, hence they have the potential to be developed to anticancer drugs.

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