Synthesis of 2-methyl-5-methoxy-1,4-benzoquinone and In-silico Activity Profiling Toward Cytochrome P450-3A4

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Abstract. The synthesis of 2-methyl-5-methoxy-1,4-benzoquinone (2) was carried out by reflux of 2-methyl-1,4-benzoquinone (1) with MeOH and ZnCl2 for 12 hours at ambient. The reaction resulted a yellow needle crystal in 6.92 % yield (m.p 173-175°C). The FT-IR analysis showed the presence of -OCH3 group at 1210 cm-1. The analysis using 1H-NMR showed chemical shift at δ = 3.81 ppm and the 13C-NMR gave 56.38 ppm for hydrogen and carbon from -OCH3 group. The bioavailability tests were determined by using in silico approach included lipophilicity (log P) and half maximum inhibitory concentration (IC50). It showed that the lipophilicity of 2 (log P = 0.92) is lower than 1 (log P = 1.79) which is calculated using Hyperchem software. The molecular docking of 2 towards cytochrome P450 3A4 showed a good result with IC50 13.68 ppm better than 1 (IC50 65.617 ppm). Further experimental is proposed for bromoalkylation of 2 using bromooctanoic acid to give 3-(7-bromoheptyl)-2-methyl-5-methoxy-1,4-benzoquinone (3). The in-silico calculation of 3 showed better lipophilicity (log P = 3.64) and IC50 (9.725 ppm) compared to 2. This result indicated that the addition of methoxyl and bromoalkyl group can improve the activity of the compound as a drug candidate.

1. Introduction
Quinone derivative is one of compounds that has shown biological and pharmacological properties. One of the quinone derivatives namely thymoquinone (TQ) or 2-isopropyl-5-methyl-1,4-benzoquinone is the main active compound in black cumin essential oil (Nigella sativa L.) which has biological activity as anti-inflammatory [1], antioxidant [2], antitumor [3], and anti-cancer [4]. However, the pharmacokinetic properties of thymoquinone have a high rate of elimination and a slow rate of absorption in the body which limits its bioavailability and lipophilicity [5].

The oral administration of drug is have a challenge since the modification of the structure should meet the requirements for solubility, stability in acid, and bioavailability [6]. Modification structure of thymoquinone by addition the alkyl chain proved to increase lipophilicity and antioxidant activity in the mitochondrial membrane [7]. Research on modification of thymoquinone and its derivatives by bromoalkylation gave better lipophilicity and anticancer activity [8][9]. The substitution of methoxyl and methyl groups in quinones also increasing the activity as antioxidants [10].

Cytochrome P450-3A4 (CYP3A4) isofrom is the most abundant and is mainly expressed in the liver and gastrointestinal tract which is able to accommodate and metabolize compounds in various chemical sizes and structures. This allows CYP3A4 to oxidize more than half of all administered drugs. However, through fast degradation, CYP3A4 can decrease bioavailability and therapeutic efficiency of pharmaceuticals and vice versa. CYP3A4 exerts genotoxic effects via activation of
procarcinogens. The participation of CYP3A4 in the carcinogen activation reaction is 10%, and participation in all metabolic reactions is 20% [11]. Cytochromes 450 3A4 is also an enzyme which responsible for carcinogen activation and cellular DNA damage [12].

The use of in vitro and in vivo technology has been carried out in the process of drug development. However, a simulation approach is needed to be carried out to accurately predict the activity by minimizing time and costs. A computational model is considerably interesting in order to predict drug safety in drug discovery and development [13]. To address these issues, we reported the synthesis of quinone derivative by addition of methoxyl group (-OMe) and predicted by in silico approach using molecular docking to assess its activity towards cytochrome P450-3A4 (CYP3A4) as macromolecule model. It is expected that the physicochemical properties of the synthesized compound inhibit CYP model by blocking the mutagenic and carcinogenic activity.

2. Materials and methods

2.1. Materials and instruments

The 2-methyl-1,4-benzoquinone (1) with 99% purity and zinc (II) chloride (ZnCl$_2$) were purchased from Sigma Aldrich (Singapore). The solvents used included methanol, ethyl acetate, and n-hexane were purchased in pro analysis grade from Merck (Singapore).

The instruments applied in this research were spectrophotometer UV-Vis Shimadzu 1600 type, spectrometer Fourier-Transform Infrared FTIR Shimadzu 8400S, $^1$H-NMR 600 MHz and $^{13}$C-NMR 150 MHz.

2.2. Synthesis of 2-methyl-5-methoxy-1,4-benzoquinone (2).

Synthesis of 2-methyl-5-methoxy-1,4-benzoquinone (2) was carried by reflux. ZnCl$_2$ (6.815 g, 50 mmol) was placed in 50 mL methanol, then heated to 60°C followed by the slowly addition of 2-methyl-1,4-benzoquinone (1) (6.106g, 50 mmol). Continued to stir and reflux for 12 h. The solution was cooled and stirred for additional 5 h at 0-10°C. The yellow needle crystal was precipitated, collected and washed with cold methanol [14]. The product was analyzed using TLC, UV-Vis, IR, $^1$H and $^{13}$C NMR.

2.3. Activity test of 2-methyl-5-methoxy-1,4-benzoquinone (2).

The activity was calculated by using Auto Dock 4 for molecular docking. The ligand structure (TQ, (1), (2), and (3)) was optimized by HyperChem and saved in format .pdb. The cytochrome P450 3A4 protein (CYP3A4) as macromolecule was downloaded by www.pdb.org and saved in format .pdb. The structure of macromolecule must be free of water and external ligand. The docking process consists of two main steps, running Autogrid (modified format changed to .gpf) and running Autodock (format changed to .dpf). AutoDock parameter was used in the calculation of the van der Waals and electrostatic terms. Docking simulations were performed using the Lamarckian genetic algorithm. The grid box for CYP3A4 in this research was determined using Autogrid4 with box dimension is $x=64.34$; $y=78.191$, and $z=8.528$. Then, run Autodock4 to get the minimum energy for each bind. Discovery studio was used to show the results of analysis of activities included binding interaction (Ki) with minimum energy, interaction of amino acid and ligand.

3. Result and Discussion

3.1. Synthesis of 2-methyl-5-methoxy-1,4-benzoquinone (2)

The synthesis of 2-methyl-5-methoxy-1,4-benzoquinone (2) was performed by nucleophilic addition of methoxyl group at the quinone ring of 2-methyl-1,4-benzoquinone (1). The addition of methoxyl group is expected could increase hydrophilicity of the compound quinone ring. The synthesis is described in Scheme 1.
Scheme 1. The synthesis of 2-methyl-5-methoxy-1,4-benzoquinone (2)

The obtained product is yellow needle crystal with melting point value 173-175°C (6.92 % yield). Analysis using TLC showed that compound 2 has a higher polarity than 1 may be the insertion of -OMe group. Analysis using UV-Vis gave maximum wavelength ($\lambda_{\text{max}}$) 261 nm. The FTIR analysis revealed the appearance of C-O ether group at 1210 cm$^{-1}$ that indicated the methoxy moiety.

Analysis using $^1$H-NMR (Figure 1) and $^{13}$C-NMR (Figure 2) was carried. From the $^1$H-NMR, the emergence of a singlet peak with high intensity in $\delta$ 3.81 ppm (3H, s) is indicated as proton attached in a methoxy group. The methoxyl group is very easy to identify because it appears as a high and sharp singlet peak in the region of 3.2-3.8 ppm due to electronegativity effect. The other proton is identified in $\delta$ 6.54 (1H, s); 5.92 (1H, s); 2.05 (3H, s). Analysis using $^{13}$C-NMR gave the decoupled of eight carbons as expected for compound 2. The CH$_3$O peak appears at 56.3 ppm which is indicated as carbon connected to ether group. This result showed that the methoxyl already attached to the quinone ring. The other carbon peak is detected in $\delta$ 107.7, 131.40, 147.03, 158.8 ppm for C=C in quinone ring, C=O at $\delta$ 182.3 and 187.8 ppm, and -CH$_3$ at $\delta$ 15.9. All the data is in accordance with 2.

Figure 1. $^1$H NMR of compound 2 (CDCl$_3$, 600 MHz)
3.2. Activity test of 2-methyl-5-methoxy-1,4-benzoquinone (2).

The activity test was represented by coefficient partition (Log P) which is widely used to measure molecular lipophilicity [15]. Log P value were calculated by using Hyperchem software. The log P value of 2 showed the smallest result, that is, log P = 0.92. This small log P value can be due to the high polarity of the compound, which affects its solubility in water. A large log P value indicates large lipophilicity, so the compound will easily penetrate the biological membrane [16].

In order to increase the lipophilicity of the compounds, the structural modification was designed with the substitution of bromoalkyl group (C7), proposing the formation of 3-(7-bromoheptyl)-2-methyl-5-methoxy-1,4-benzoquinone (3). The result showed that 3 has the best lipophilicity, with log P = 3.64, compared to the thymoquinone (TQ) (log P = 2.80), compound 1 (log P = 1.79), and compound 2 (log P = 0.92). The high value of the partition coefficient should increase the solubility of a compound in the fat phase thereby reducing its polarity [17]. The result showed that the modification of the structure by the addition of bromoalkyl is promising strategy to increase the lipophilicity.

Furthermore, a computational modeling is performed by using molecular docking to predict activity relationship between ligands and target macromolecule [18]. The optimized ligand is compound 1, 2, and 3, compared with thymoquinone (TQ). The macromolecules used are cytochrome CYP3A4. Among the four ligands used, compound 3 has the strongest interaction with the Ki value is 14.77 µm, compared to TQ (Ki = 72.23 µm) and compound 2 (Ki = 45.55 µm). It is reported that the smaller Ki value gave the strong and stable bonding between ligand and target macromolecule. The inhibition constant (Ki) is related to the inhibition concentration value (IC50) which indicates the quantity of drug needed to inhibit biological processes in the body [19].

Half maximum inhibitory concentration (IC50) showed the ability of the ligand to inhibit a specific disease-causing receptor by 50%. The smaller the IC50 gave the better inhibition [20]. Compounds 2 and 3 showed the best activity in inhibiting CYP3A4 with IC50 = 13.861 ppm and 9.725 ppm, respectively. All the result showed a good result for the possibility of the synthesis 3 as a procarcinogen activation inhibitor.

The interaction between amino acid from macromolecules and ligand from this model is shown in Table 1. These observations indicate the difference in the number of amino acid residues that are bound to each compound. Compound 3 have a specific interaction with 18 types of amino acid
residues. However, compound 2 only interact with 10 types of amino acid. This result indicated that the addition of alkyl substituents to the ligand can increase the bonding of ligand with macromolecule target in CYP3A4. The interaction then can affect its the affinity of ligand toward macromolecule [21].

| Table 1. Molecular docking of ligand with macromolecule CYP3A4 |
|-----------------|-----------------|-----------------|-----------------|
| Ligand | Ki (µm) | ΔG° (kcal/mol) | IC₅₀ (ppm) | Amino acid |
|--------|---------|----------------|-------------|------------|
| TQ | 72.23 | -5.69 | 23.720 | THR309, LIG1, ILE369, PRO434, ALA370, ALA448, PRO368, LEU364, VAL313, MET452, THR310 |
| 1 | 268.61 | -4.87 | 65.617 | LIG1, PRO434, THR309, ALA370, VAL313, PHE108, LEU364, ILE369, PRO368, PHE367, PRO434 |
| 2 | 45.55 | -5.92 | 13.861 | PHE108, ARG105, LIG1, PRO107, ASN104, ALA121, ILE120, SER119, ARG106 |
| 3 | 14.77 | -6.59 | 9.725 | LIG1, ARG10, ARG440, ARG130, PHE447, CYS442, PRO368, PHE435, LEU364, VAL313, MET452, THR310, GLU122, PHE137, SER119, THR310, TRP126 |

The ligand interaction of compound 2 and 3 with CYP3A4 is illustrated in Figure 3. It was found that the ILE120 and ALA448 interact with these two molecules by establishing pi-alkyl interaction with the quinone rings. In addition, the carbonyl from 2 in quinone ring formed H-bond with the acceptor oxygen atom of ARG105, PHE108 and ARG440. Meanwhile, compound 3 formed hydrophobic interactions with ALA448, CYS442, and ILE118. Compound 2 also showed the interaction with ARG105, PRO107, and ILE120. However, compound 3 showed the interaction with ALA448 by pi-pi stacking with the alkyl chain side of each compound, thereby contribute an increasing in binding affinity [12].

Figure 4 showed the visualization of the interaction between ligand and macromolecule. The relationship between structure and ligand activity was compared with TQ and showed that compound 2 and 3 provide a good activity with CYP3A4 from the evaluation of Ki, ΔG° and IC₅₀ values. All results showed that by adding methoxyl and bromoalkyl substituent into quinone can improve the bioavailability of drug candidate. The activities using in vitro and in vivo will be evaluated.
Figure 3. Interaction of ligand with amino acid residue. (a) compound 2 and (b) compound 3

Figure 4. Molecular visualization of ligand and macromolecule cytochrome CYP3A4. (a) TQ, (b) compound 1, (c) compound 2, and (d) compound 3

4. Conclusion
The synthesis of 2-methyl-5-methoxy-1,4-benzoquinone (2) was performed by nucleophilic addition of methoxyl group at the quinone ring in mild condition. The bioavailability tests using in silico approach by molecular docking with cytochrome P450 3A4 (CYP3A4) as the macromolecule showed that the synthesized compound 2 and target compound 3 had a better IC_{50} towards CYP3A4 compared to thymoquinone (TQ). The addition of methoxy group and bromoalkyl promote the activity of the compound as drug candidate.

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