

Phenethyl *p*-coumarate and *N*-phenethyl-*p*-coumaramide: Synthesis, Characterization, Docking Studies and Anticancer Activity through P388 Cell (Fenetil *p*-kumarat dan *N*-fenetil-*p*-kumaramida: Sintesis, Pencirian, Kajian Mengedok dan Aktiviti Antikanser melalui Sel P388)

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ABSTRACT

Most *p*-coumaric acid derivatives and molecules containing phenethyl moiety have a potential in anticancer activity. Thus, combining a *p*-coumaroyl group and a phenethyl moiety in one compound will increase anticancer activity. The principal objective of this research was to incorporate *p*-coumaroyl and phenethyl moieties to form an ester, phenethyl *p*-coumarate (**5**), and an amide, *N*-phenethyl-*p*-coumaramide (**6**), then tested their anticancer activity using P388 leukemia murine cells. The characterization by FTIR method, compound **5** gave a strong absorption band of alkyl C-O bond that appears at 1165,00 cm⁻¹, and compound **6** gave a sharp and medium absorption band of N-H bond that appears at 3396.64 cm⁻¹. Docking studies of both compounds showed a hydrogen bond with Ile839 residue, and an additional hydrogen bond appeared between compound **6** and Ser991 residue. Based on their activity against P388 leukemia murine cells, these compounds are more active than their analog compounds of *N*-feruloylpiperidine and *N*-feruloylmorpholine, which have been synthesized previously. Compounds **5** and **6** have a high potential to be used as anticancer drugs.

Keywords: Anticancer; docking; *N*-phenethyl-*p*-coumaramide; *p*-coumaric acid; phenethyl *p*-coumarate

ABSTRAK

Sebilangan besar molekul dan terbitan asid *p*-kumarat yang mengandungi fenetil fenil mempunyai potensi aktiviti antikanser. Oleh itu, menggabungkan kumpulan *p*-koumaroyl dan gugus fenil dalam satu sebatian akan meningkatkan aktiviti antikanser. Objektif utama penyelidikan ini adalah untuk menggabungkan bahagian *p*-koumaroyl dan fenetil untuk membentuk ester, fenetil *p*-kumarat (**5**) dan amida, *N*-fenetil-*p*-kumaramida (**6**), kemudian menguji aktiviti antikanser menggunakan sel P388 leukemia murin. Pencirian dengan kaedah FTIR, sebatian **5** memberikan jalur penyerapan yang kuat di ikatan alkil C-O yang muncul pada 1165,00 cm⁻¹ dan sebatian **6** memberikan jalur penyerapan ikatan N-H yang tajam dan sederhana yang muncul pada 3396.64 cm⁻¹. Kajian mengedok untuk kedua-dua sebatian menunjukkan ikatan hidrogen dengan residu Ile839 dan ikatan hidrogen tambahan muncul antara sebatian **6** dan residu Ser991. Berdasarkan aktiviti mereka terhadap sel P388 leukemia murin, sebatian ini adalah lebih aktif daripada sebatian analog *N*-feruloylpiperidina dan *N*-feruloylmorfolina, yang telah disintesis sebelumnya. Sebatian **5** dan **6** berpotensi tinggi untuk digunakan sebagai ubat antikanser.

Kata kunci: Antikanser; asid *p*-kumarat; fenetil *p*-kumarat; kajian dok; *N*-fenetil-*p*-kumaramida

INTRODUCTION

Cancer is still the most severe medical problem globally, although many treatment methods have been developed for this disease, such as immunotherapy, surgical operation,

radiotherapy, and chemotherapy (El-Refaei & El-Naa 2010). Accordingly, new approaches for cancer therapy are hugely demanded, and among those wide varieties of cancer treatment, chemotherapy plays a significant

role. Therefore, the discovery and development of novel anticancer agents become one of the necessities (Ketabforoosh et al. 2013).

It has been reported that there was a significant correlation between the antioxidant activity of phenolic compounds and their anticancer activity (Cai et al. 2004). The vast majority of phenolic compounds, including phenolic acids, flavonoids, tannins, coumarins, lignans, quinones, stilbenes, and curcuminoids, exhibited strong antioxidant activity (Nakamura et al. 2014). Curcumin, one example of phenolic compounds, showed high activity against P388 murine leukemia cells (Eryanti et al. 2016). Furthermore, phenolic acids are another important group of secondary metabolites of a plant with potent antioxidant activity (Razzaghi-Asl et al. 2013), usually divided into two main classes: Benzoic acid and cinnamic acid.

Cinnamic acid, often represented by *p*-coumaric acid, ferulic acid, sinapic acid, and caffeic acid, consists of an aromatic ring and a carboxyl group. The structural features of cinnamic acid were found significantly crucial because particular modifications could be conducted in its two main sides (Razzaghi-Asl et al. 2013). The difference in the substituents at cinnamoyl moiety of cinnamide compounds will possess different bioactivities (Wang et al. 2015; Zhang et al. 2015). Amide derivative of cinnamic acid, methyl 2-cinnamido-3-hydroxy propanoate was actively inhibited P388 cancer cell with IC_{50} of 10.78 $\mu\text{g/mL}$ (Ernawati et al. 2014). The other two amide derivatives of cinnamic acid analogs, with a hydroxyl group on para positions, *N*-feruloylpiperidine and *N*-feruloylmorpholine, were also active against P-388 leukemia cells with an IC_{50} of 46.67 and 57.10 $\mu\text{g/mL}$, respectively (Firdaus et al. 2017). All the above findings indicated that amine moiety is also responsible for the bioactivity of cinnamide compounds.

Moreover, the phenethyl group was found to be about the same interest compared to the *p*-coumaroyl group. This group was responsible for the potency of phenethyl ester carboxylate in the sensitivity of *Mycobacterium tuberculosis* to ethionamide (Weber et al. 2008). Several common dietary foods like cruciferous vegetables containing phenethyl isothiocyanate gave highly remarkable anticancer effects (Gupta et al. 2014). Further, propolis extract, which can be used in the treatment of inflammation and cancer, contains phenethyl polyphenolic ester (Murtaza et al. 2014). Due to these findings, there was a quantitative relationship between structures and bioactivities of compounds (Wang et al. 2015; Zhang et al. 2015).

This research aims to find the compound with potent bioactivity by synthesizing ester and amide compounds containing phenethyl and *p*-coumaroyl groups (compounds **5** and **6**) using the indirect method, characterize the structures, and study their potency as anticancer agents. The use of ester and amide as target compounds was justified based on several studies that have been reported previously. Phenethyl ester of caffeic acid was found to be a strong antioxidant (Razzaghi-Asl et al. 2013), and amide methyl cinnamate was active against P388 leukemia murine cells (Ernawati et al. 2014). Moreover, putrescine amides of *p*-coumaric, caffeic, and ferulic acid exhibited strong antioxidant activity (Velikova et al. 2007). In addition, anilide amide of caffeic acid was also active as an antioxidant against ABTS and DPPH (Hung et al. 2005).

In silico study through molecular docking was attempted to know the binding affinity of synthesized compounds against P-glycoprotein. This protein receptor was chosen among the others due to the finding that showed overexpression of P-glycoprotein when P388 leukemia cell was treated using small molecule inhibitor (Gatoillat et al. 2015; Kamath et al. 1992; Yamaoka et al. 1999). In order to determine the activity of compounds **5** and **6** as anticancer agents, both compounds were put on a bioassay test against P388 leukemia murine cells using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method. This method has been used in many pieces of research, for example, to test the stimulatory effects of ferulic acid derivatives on insulin secretion (Nomura et al. 2003), the anticancer activity of curcumin analogs against P388 leukemia murine cells (Ernawati et al. 2014), antituberculous of chalcones (Moodley et al. 2014), and the bioactivity of *N*-feruloyl morpholine and *N*-feruloyl piperidine compounds against P388 leukemia murine cells (Son & Lewis 2002).

MATERIALS AND METHODS

MATERIALS AND INSTRUMENTATIONS

FT-IR spectra were obtained on Shimadzu Prestige 21 FTIR spectrometer, and $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ were recorded in CDCl_3 solution using Agilent magnetic resonance spectrometer (operating at 500 MHz for ^1H and 125 MHz for ^{13}C nuclei). *p*-Coumaric acid was purchased from Sigma Aldrich, and it was used without further purification. Acetic anhydride, thionyl chloride, benzene, phenethylamine, phenethyl alcohol, pyrrolidine, methanol, dichloromethane (DCM), 4-(dimethylamino)

pyridine (DMAP), triethylamine (TEA), hydrochloride acid, ammonium chloride, sodium sulfate anhydrous, ethyl acetate, and *n*-hexane were pro synthesis and pro analysis grades which purchased from Merck and used without further purification.

SYNTHESIS OF (*E*)-3-(4-ACETOXYPHENYL)ACRYLIC ACID (1)

p-Coumaric acid (3.0 mmol) was mixed with pyridine (3.0 mL) and acetic anhydride (8.36 mmol) in a round bottom flask and stirred for 5 h at room temperature and then poured into cold water (20 mL) while stirring. The formed precipitate was filtered, washed with water, and dried. The solid was then crystallized and recrystallized with hot methanol to obtain compound 1 as a yellowish crystal with mp of 201-203 °C (63.93% yields). Pure compound 1 was analyzed subjected to an FTIR spectrometer analysis. IR (KBr): ν (cm⁻¹) = 2750-3200 (O-H), 1667.71 (C=O carboxylic), 1745.58 (C=O ester), 3047.53 (C-H unsat.), 1598.99 & 1506.41 (C=C Ar), 1625.99 (C=C olefin), 2981.95 & 2827.64 (C-H sat.), 1427.32 & 1371.39 (CH₃), 991.41 (*trans*-olefin), and 839.03 (*p*-subst. Ar).

SYNTHESIS OF (*E*)-3-(4-ACETOXYPHENYL)ACRYLOYL CHLORIDE (2)

Compound 1 (1.5 mmol) was refluxed with 0.42 mL thionyl chloride (6 mmol) in 20 mL of benzene under a nitrogen atmosphere for 4 h at 70 °C. After the reaction was finished, the mixture was evaporated to obtain a yellowish powder 2. Without any further purification, compound 2 was used for the next steps.

SYNTHESIS OF PHENETHYL (*E*)-3-(4-ACETOXYPHENYL)ACRYLATE (3)

Compound 2 (1.5 mmol) and phenethyl alcohol (1.36 mmol) was dissolved in 100 mL of DCM. The mixture was then mixed with DMAP (0.4 mmol) and TEA (1.2 mmol) and continuously stirred for two h. The precipitate was washed with 3% HCl and aqueous saturated NH₄Cl sequentially, dried with anhydrous Na₂SO₄, and evaporated to remove the solvent. The solid was purified by gravity column chromatography (eluent EtOAc/*n*-hexane 1:19), crystallized, and recrystallized from EtOAc/*n*-hexane to obtain a yellowish needle crystal with mp of 68-69 °C (47.20% yield). Pure compound 3 was analyzed by FTIR spectrometer. IR (KBr): ν (cm⁻¹) = 1703.14 (C=O phenetyl ester), 1761.01 (C=O acetyl ester), 3050.0 (C-H unsat.), 1598.99 & 1506.41 (C=C

Ar), 1631.78 (C=C olefin), 2931.80 & 2870.08 (C-H sat.), 1456.26 & 1369.46 (CH₃), 999.13 (*trans*-olefin), 840.96 (*p*-subst. Ar), and 756.10 & 702.09 (mono subst. Ar).

SYNTHESIS OF (*E*)-3-(4-ACETOXYPHENYL)-N-PHENETHYLACRYLAMIDE (4)

Compound 2 (1.5 mmol) and phenethylamine (1.36 mmol) were dissolved in 100 mL of DCM. The mixture was mixed with DMAP (0.4 mmol) and TEA (1.2 mmol) and stirred for 2 h. The compound was washed with 3% HCl and aqueous saturated NH₄Cl sequentially, dried with anhydrous Na₂SO₄, and evaporated to remove the solvent. The solid was purified by gravity chromatography column (chloroform eluent), crystallized, and recrystallized from EtOAc/*n*-hexane to obtain a yellowish solid with m.p. of 143-144 °C (20.47% yield). Pure compound 4 was analyzed by FTIR spectrometer. IR (KBr): ν (cm⁻¹) = 3311.78 (N-H), 1656.65 (C=O amide), 1762.94 (C=O acetyl ester), 3068.75 (C-H unsat.), 1600.0 & 1502.55 (C=C Ar), 1624.06 (C=C olefin), 2931.80 & 2862.36 (C-H sat.), 1452.40 & 1371.39 (CH₃), 970.19 (*trans* olefin), 833.25 (*p*-subst. Ar), and 750.31 & 700.16 (mono subst. Ar).

SYNTHESIS OF PHENETHYL (*E*)-3-(4-HYDROXYPHENYL)ACRYLATE (5) AND (*E*)-3-(4-HYDROXYPHENYL)-N-PHENETHYL-ACRYLAMIDE (6)

Compounds 3 or 4 (2.58 mmol) were dissolved in 1 mL of pyrrolidine and diluted with 50 mL of EtOAc while stirring. The mixture was continuously stirred for 2 h at room temperature in a light-protected place. The mixture product was washed with 1 M H₂SO₄ (3 X 20 mL) and aqueous saturated NH₄Cl sequentially, drying over anhydrous Na₂SO₄, then evaporated to remove the solvent. The solid was crystallized from EtOAc/*n*-hexane to obtain a pure compound 5 as yellowish solid with mp of 68-69 °C (47.2% yield), and for compound 6 as yellowish solid with mp of 137-139 °C (76.02% yield). The products were analyzed using FTIR and NMR spectrometers.

Compound 5, IR (KBr): ν (cm⁻¹) = 3278.99 (O-H phenolic), 1680.0 (C=O phenetyl ester), 3026.31 & 3080.0 (C-H Ar), 1602.85, 1579.70, & 1512.19 (C=C Ar), 1631.78 (C=C olefin), 2953.02 (C-H sat.), 1448.54 (CH₂), 985.62 (*trans* olefin), 833.25 (*p*-subst. Ar), and 748.38 & 698.23 (mono subst. Ar); ¹H-NMR (CDCl₃): τ^m (ppm) = 3.03 (*t*, 2H, *J* = 14.05 Hz, CH₂), 4.45 (*t*, 2H, *J* = 14.05 Hz, CH₂), 6.29 (*d*, 1H, *J* = 15.95 Hz, =CH), 7.64 (*d*, 1H, *J* = 15.95 Hz, =CH), 6.87 (*d*, 2H, *J* = 8.45 Hz, Ar-H), 7.40 (*d*, 2H, *J* = 8.45 Hz, Ar-H), 7.24-7.33

(*m*, 5H, $J = 7.25$ - 8.05 Hz, Ar-H); ^{13}C -NMR (CDCl_3): δ (ppm) = 35.3, 65.3, 114.9, 116.1, 126.7, 126.8, 128.6, 129.0, 130.2, 137.9, 145.4, 158.4, and 168.2.

Compound **6**, IR (KBr): ν (cm^{-1}) = 3396.64 (N-H), 3153.61 (O-H phenolic), 1654.92 (C=O amide), 3020.53 (C-H unsat.), 1602.85 & 1512.19 (C=C Ar), 2929.87 (C-H sat.), 1442.75 (CH_2), 970.19 (*trans*-olefin), 833.25 (*p*-subst. Ar), and 750.31 & 700.16 (mono subst. Ar); ^1H -NMR (CDCl_3): δ (ppm) 2.89 (*t*, 2H, $J = 7.0$ Hz, $-\text{CH}_2-$), 3.64 (*t*, 2H, $J = 7.0$ Hz, $-\text{CH}_2-$), 5.59 (*s*, 1H, O-H), 6.17 (*d*, 1H, $J = 15.15$ Hz, =C-H), 7.56 (*d*, 1H, $J = 15.15$ Hz, =C-H), 6.84 (*d*, 2H, $J = 8.2$ Hz, Ar-H), 7.23 (*d*, 2H, $J = 8.2$ Hz, Ar-H), 7.32-7.41 (*m*, 5H, $J = 7.4$ - 8.2 Hz, Ar-H), 6.59 (*d*, 1H, $J = 15.5$ Hz, N-H), 7.65 (*d*, 1H, $J = 15.4$ Hz, N-H). ^{13}C -NMR (CDCl_3): δ (ppm) 35.8, 40.9, 115.9, 117.9, 126.7, 127.4, 128.8, 128.9, 129.7, 138.9, 141.1, 157.7, and 166.6.

DOCKING STUDY

Docking analysis was done using AutoDock 4.2 software and AutoDockTools program (Morris & Huey 2009). It started with redocking analysis to know the suitable procedure such as grid size and spacing. The protein that was used in this docking was P-glycoprotein since its protein receptor found overexpression in P388 leukemia cells (Gatoillat et al. 2015). This protein was downloaded in a protein data bank with ID 6FN4. Redocking analysis was built by box size $40 \times 44 \times 64 \text{ \AA}^3$ and spacing 0.375 \AA . Successful redocking analysis was achieved if RMSD value was lower than 2 \AA due to the great conformation results (Huey et al. 2007). After the redocking step was done, the procedure was adopted in docking analysis of the new ligands (compounds **5** and **6**). The parameter file was saved as a .gpf format file containing grid box size and spacing as in the redocking stage. The docking procedure was set to produce ten conformations and run for maximum energy evaluation of 2500000. The Lamarckian Genetic Algorithm was used to obtain data in the form of binding energy and inhibition constant. Discovery Studio Visualizer was performed to give the 2D visualization of ligand and protein interaction (Dassault Systemes 2019).

BIOACTIVITY ASSAY

The activity of compounds **5** and **6** against P388 leukemia murine cells was carried out using references from Kuncoro et al. (2017) and Salahuddin et al. (2013). By this method, compounds **5** and **6** each gave IC_{50} values of 1.0 and $5.89 \mu\text{g/mL}$, respectively.

RESULTS AND DISCUSSION

SYNTHESIS AND CHARACTERIZATION

Several direct methods have been reported for converting the carboxylic acid to amide and ester, e.g. esterification using boron trichloride as the catalyst (Dyke & Bryson 2001), and amidation using a boric acid catalyst (Tang 2005). However, these methods failed to convert *p*-coumaric acid to its ester and amide due to the low nucleophilicity of the carbonyl group of *p*-coumaric acid caused by the resonance of the phenolic hydroxyl moiety to the carbonyl group, which increases the electron density at the carbonyl group. Therefore, to convert the *p*-coumaric acid into its amide and ester derivatives, the activity of the carbonyl group should be increased by converting it to its acid halide. However, when *p*-coumaric acid is converted to its acid halide, the dimerization reaction between acid halide and hydroxyl group on the phenolic moiety will occur. Therefore, the acid halide formation must be preceded by protecting the hydroxyl group of the phenolic moiety. One method to protect the hydroxyl group is by acetylation. The advantages of using this reaction are not only that it can protect the hydroxyl group from the dimerization reaction, but it is also easy to release by using a deprotecting agent, such as pyrrolidine. Thus, after esterification or amidation of acid halide, a deprotection reaction should take place to produce the desired ester and amide derivatives.

Under the above discussion, to get the successful conversion of *p*-coumaric acid to compounds **5** and **6**, the synthesis reaction should involve four steps of reaction, i.e. (i) acetylation to protect the hydroxyl from phenolic moiety, (ii) chlorination to activate the carboxylic group (Helm et al. 1992), followed by (iii) esterification using phenethyl alcohol or amidation using phenethylamine, and (iv) deacetylation to remove protecting group (Lu & Ralph 1998) (Figure 1). In the last step (iv), compounds **3** and **4** were treated with the same procedure to obtain compounds **5** and **6** with a yield of 40.20 and 76.02%, respectively. Except for the product of step 2, all the structures of the compound on each reaction step have been confirmed by the FTIR data and followed by NMR data for the final step.

The difference in the yields was due to the differences in the stability of ester and amide toward pyrrolidine as a deprotecting agent. In compound **3**, there were two ester groups, acetyl ester as a protecting group and phenethyl ester, which were both ready to convert to amides. When compound **3** was deprotected to give compound **5**, some compound **5** was converted to its pyrrolidine amide as

a side product. However, in compound **4**, there was an amide group which was resistant to pyrrolidine attack. When this compound was deprotected to give compound **6**, compound **6** was not converted. This condition also explained why pyrrolidine as a deprotecting agent should be used in a limited amount in the reaction. Pyrrolidine, which is an amine, can also react to the carbonyl group to produce undesired pyrrolidine amide. Thus, if the use of pyrrolidine was excessive, more pyrrolidine amide would be produced as a side product.

All products in this reaction, including intermediate products (except the chlorination product), have been characterized by FTIR. In particular, the characterization of the final products was completed by ^1H NMR and ^{13}C NMR data.

CHARACTERIZATION OF COMPOUNDS **1**, **3**, AND **4**

As an intermediate product, except for compound **2**, the

existence of compounds **1**, **3**, and **4** has been confirmed by FTIR data. Compound **1** gave an absorption band of the carbonyl group at 1747.51 cm^{-1} and methyl group at 1427.32 and 1373.32 cm^{-1} . Compounds **3** and **4**, obtained via chlorination followed by esterification and amidation *in situ*, gave absorption bands for the carbonyl group at 1703.14 and 1656.65 cm^{-1} , respectively. Except for the N-H absorption of the amidation product (compound **4**), the other absorption bands from both products showed no significant difference.

CHARACTERIZATION OF COMPOUND **5**

Compound **5** was obtained by deprotection of the phenolic group in compound **3**. The deprotection was confirmed by FTIR data in which the carbonyl group appeared at absorption bands of 1703.14 cm^{-1} , while the methyl group peaks at 1427.32 and 1371.39 cm^{-1} disappeared. All other absorption peaks were consistent with the structure of compound **5**, including the bands

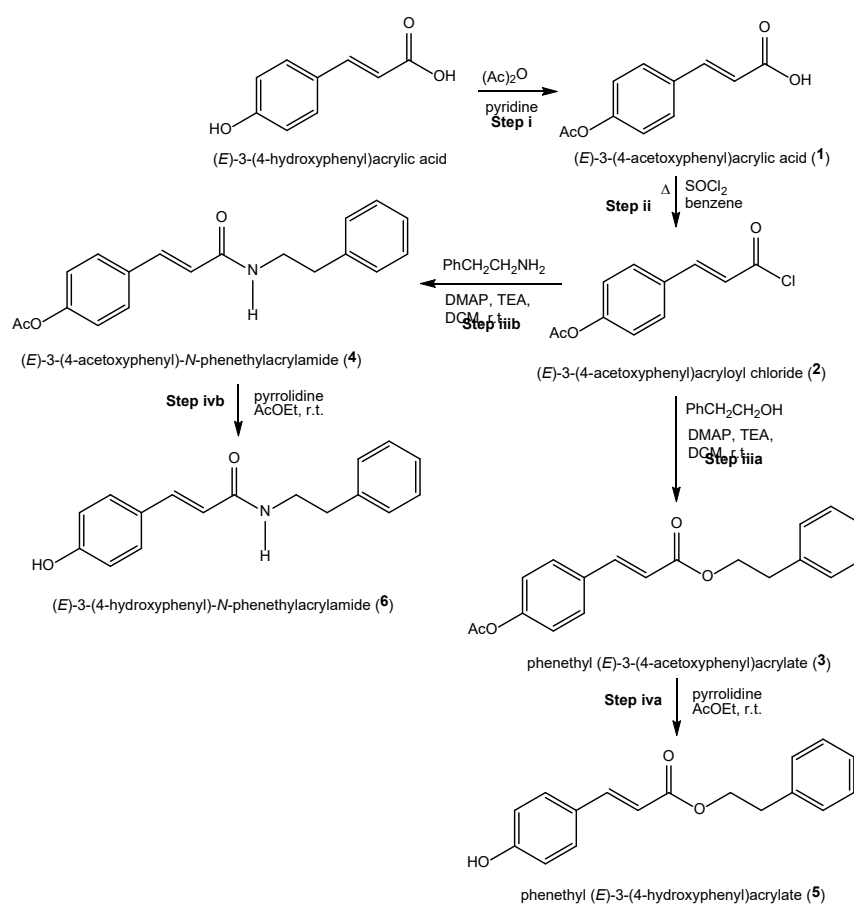


FIGURE 1. Synthesis pathway of target compounds

that indicate the presence of phenethyl groups, which are the bands at 1448.54 cm^{-1} from methylene, and at 748.38 and 698.23 cm^{-1} from the mono-substituted aromatic group.

Those FTIR data are also in accordance with the ^{13}C -NMR data, which indicates seventeen carbons within thirteen different electronic environments. Peaks at 35.3 and 65.3 ppm are related to the methylene of phenethyl moiety, and the peak at 168.2 ppm corresponds to the carbonyl group. The other remaining peaks were consistent with the carbon of both aromatic groups in

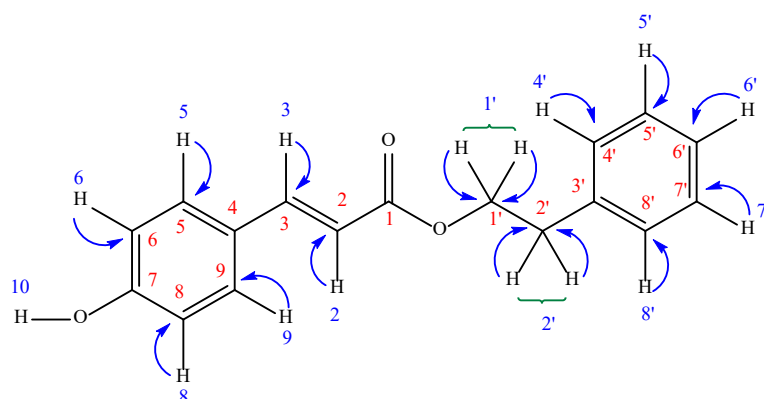
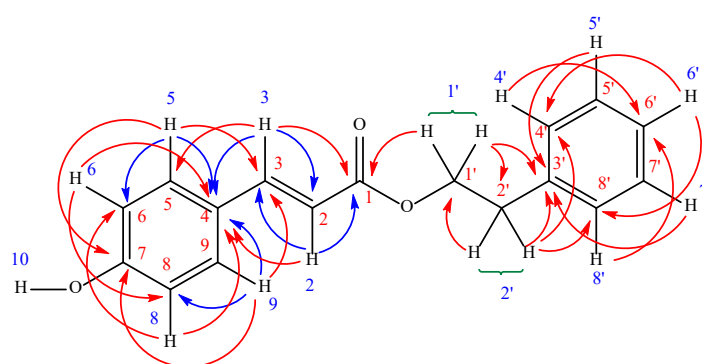
compound **5**. Further, a triplet splitting peaks at 3.03 and 4.45 ppm with coupling constant $J = 14.05\text{ Hz}$ corresponds to the methylene protons of phenethyl moiety, and a doublet peak at 6.29 and 7.64 ppm with $J = 15.95\text{ Hz}$ corresponds to the presence of olefin protons of the *p*-coumaroyl moiety. The peaks of aromatic protons in phenethyl and *p*-coumaroyl moieties appear at $6.87 - 7.40\text{ ppm}$ with $J = 7.25 - 8.45\text{ Hz}$. The structure of compound **5** has also been confirmed by HSQC and HMBC NMR, shown in Tables 1 and 2. The correlation between carbon and hydrogen atoms in compound **5** is shown in Figures 2 and 3.

TABLE 1. HSQC correlation of compound **5**

^1H NMR δ (ppm)	Profile of peaks (Coupling constant, Hz)	^{13}C NMR δ (ppm)	Correlation to carbon
3.036	<i>t</i> ($J = 7.00 - 7.05$)	35.291	H-2' to C-2'
4.448	<i>t</i> ($J = 7.00 - 7.05$)	65.347	H-1' to C-1'
6.294	<i>d</i> ($J = 15.95$)	114.995	H-2 to C-2
6.729	<i>s</i>		H-O (no correlation)
6.876	<i>d</i> ($J = 8.45$)	126.863	H-6 to C-6 and H-8 to C-8
7.243 – 7.262		116.727	H-6' to C-6'
		126.727	C-4 (no correlation to H)
7.270	<i>d</i> ($J = 8.05$)	128.657	H-4' to C-4' and H-8' to C-8'
7.333	<i>t</i> ($J = 7.25 - 7.70$)	129.052	H-5' to C-5' and H-7' to C-7'
7.406	<i>d</i> ($J = 8.45$)	130.221	H-5 to C-5 and H-9 to C-9
		137.901	C-3' (no correlation to H)
7.637	<i>d</i> ($J = 15.95$)	145.365	H-3 to C-3
		158.420	C-7 (no correlation)
		168.213	C-1 (no correlation)

TABLE 2. HMBC correlation of compound **5**

Proton Number	Long-range correlation to carbon	Short-range correlation to carbon
H-2	C-4	C-5 & C-9
H-3	C-1, C-5 & C-9	C-2 & C-4
H-5 & H-9	C-3 & C-7	C-4, C-6 & C-8
H-6 & H-8	C-4	C-7, C-5 & C-9
H-1'	C-1, C-3' & C-2'	C-2
H-2'	C-1', C-3', C-4' & C-8'	C-5' & C-7'
H-4' & H-8'	C-2' & C-6'	C-5' & C-7'
H-5' & H-7'	C-3'	C-4' & C-8'
H-6'	C-4' & C-8'	C-5' & C-7'

FIGURE 2. HSQC correlation of the structure of compound **5**FIGURE 3. HMBC correlation of the structure of compound **5** (red = long-range correlation, blue = short-range correlation)

CHARACTERIZATION OF COMPOUND **6**

Structurally, the compounds **5** and **6** only differ in the oxygen and nitrogen atoms attached to the carbonyl group. Thus, the FTIR spectrum of both compounds only shows significant differences at carbonyl group bands. The carbonyl absorption band of compound **5** appeared at 1703.14 cm^{-1} , while the carbonyl absorption band of compound **6** appeared at 1656.85 cm^{-1} . Compound **6** also gave an absorption band at 3398.57 cm^{-1} which correlated with N-H group. This peak did not appear in the FTIR spectrum of compound **5**. The other peaks appeared mostly at a similar wavenumber of compound **5**.

In addition, the ^{13}C NMR spectrum of compound **6** showed thirteen peaks representing seventeen carbons. The carbon belongs to the carbonyl functional group appears at 166.60 ppm , while peaks at 2.89 and 3.64 ppm indicated the presence of phenethyl groups in the

compound. Two peaks at 6.17 and 7.56 emerge from olefin carbons. The other remaining bands were corresponding to the carbons of aromatic groups.

The ^1H -NMR spectrum also supported the presence of the amide group in compound **6**. The signal of proton belong to a secondary amide group appears at a chemical shift of 6.59 ppm , methylene protons of phenethyl group appear at 2.89 , and 3.64 ppm as triplet peaks with $J = 7.0\text{ Hz}$, and olefin protons peaks appear at 6.17 and 7.56 as doublet peak with $J = 15.15\text{ Hz}$. The other bands correspond to aromatic protons.

The structure of compound **6** also has been confirmed with HSQC and HMBC NMR methods. HSQC and HMBC data spectrums were shown in Tables 3 and 4, respectively. In addition, the correlation of carbon and hydrogen atoms in compound **6** was displayed in Figures 4 and 5.

TABLE 3. HSQC data of compound 6

¹ H NMR ™ (ppm)	Profile of peaks (Coupling constant, Hz)	¹³ C NMR ™ (ppm)	Correlation to carbon
2.888	<i>t</i> (<i>J</i> = 6.90 – 6.95)	35.830	H-2' to C-2'
3.667	<i>t</i> (<i>J</i> = 6.65)	40.968	H-1' to C-1'
6.417	<i>d</i> (<i>J</i> = 15.5)	117.924	H-2 to C-2
6.839	<i>t</i> (<i>J</i> = 8.15)	115.983	H-6 to C-6 and H-8 to C-8
7.230	<i>t</i> (<i>J</i> = 7.45 – 8.35)	126.723	H-6' to C-6'
		127.426	C-4 (no correlation to H)
7.326	<i>t</i> (<i>J</i> = 7.35 – 7.40)	128.972	H-5' to C-5' and H-7' to C-7'
7.365	<i>d</i> (<i>J</i> = 8.10)	128.853	H-4' to C-4' and H-8' to C-8'
7.413	<i>d</i> (<i>J</i> = 8.15)	129.819	H-5 to C-5 and H-9 to C-9
		138.996	C-3' (no correlation to H)
7.556	<i>d</i> (<i>J</i> = 15.5)	141.161	H-3 to C-3
		157.694	C-7 (no correlation to H)
		166.581	C-1 (no correlation to H)

TABLE 4. HMBC data of compound 6

Proton number	Long-range correlation to carbon	Short-range correlation to carbon
H-2	C-4	C-1
H-3	C-1, C-5 & C-9	C-2
H-5 & H-9	C-3 & C-7	
H-6	C-4 & C-8	C-7
H-8	C-4 & C-6	C-7
H-1'	C-1 & C-3'	C-2'
H-2'	C-4' & C-8'	C-1' & C-3'
H-4' & H-8'	C-2' & C-6'	
H-5'	C-3'	C-4'
H-7'	C-3'	C-8'
H-6'	C-4' & C-8'	

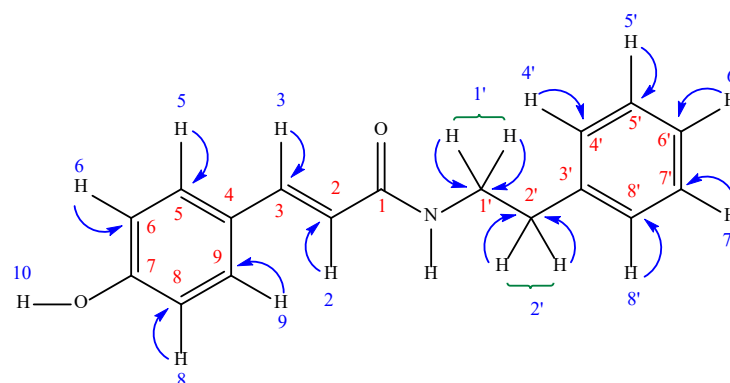


FIGURE 4. HSQC correlation of the structure of compound 6

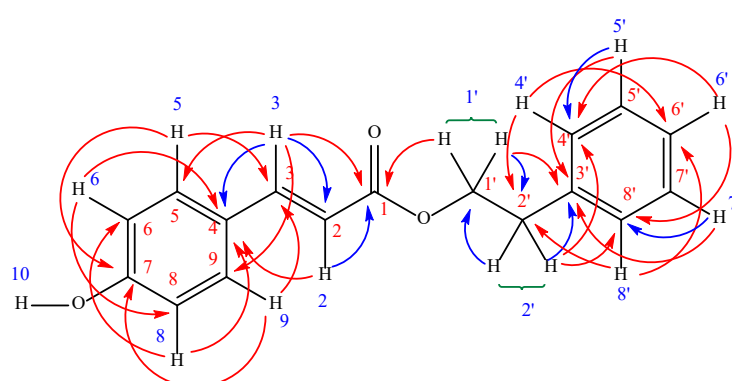


FIGURE 5. HMBC correlation of the structure of compound 6 (red = long-range correlation, blue = short-range correlation)

DOCKING STUDIES OF COMPOUNDS 5 AND 6 AGAINST P-GLYCOPROTEIN RECEPTOR

Human P-glycoprotein (P-gp) is a member of ATP binding cassette (ABC) that belongs to transporter protein. This protein was chosen among the others because the previous result by Gatoillat et al. (2015) showed this protein,

P-glycoprotein, express in P388 leukemia cells when that cell was treated by two flavonoids (Medicarpin and Millepurpan). The overexpression of P-gp was responsible for the inhibition mechanism of Medicarpin and Millepurpan in P388 leukemia cell. This fact makes us choose P-gp protein in docking analysis.

TABLE 5. Docking analysis result of compounds 5 and 6 against P-gp

Ligand	Binding free energy (kcal/mol)	Inhibition constants (μM)
5	-4.27	744.97
6	-4.62	411.07

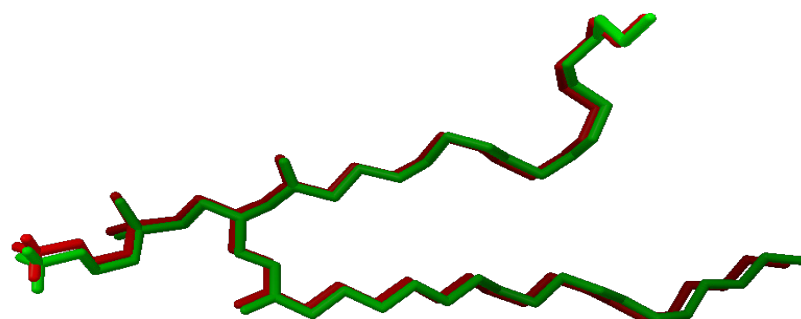


FIGURE 6. Overlapping of standard ligand and conformation 1 after redocking proses (green: standard ligand; red: conf.1)

The redocking analysis resulted in a lower RMSD value of about 0.8 Å. Figure 6 shows the overlap conformation before and after redocking the standard ligand (3PE), which complexed with P-gp (PDB ID 6FN4). The good overlapping conformation was corresponding to the lower RMSD value. After reaching the successful redocking analysis, we adopted that procedure in docking analysis of the synthesized compounds **5** and **6**. Table 5 performed the resulted docking analysis of both compounds. The binding energy levels of both compounds have a similar value due to the similarity interactions formed against P-gp protein receptor. Binding energy is a sum of intermolecular interaction energy formed between ligand and protein, which resulted from hydrogen bonding energy, van der Waals, - stacking. The more interactions give the lower binding energy. Visualization of interaction compounds **5** and **6** against P-gp protein was showed in Figure 7. It was seen that compound **6** has a more hydrogen bond due to the presence of -NH groups as a donor, while Ser991 acts as a donor of the hydrogen bond. Furthermore, a similar hydrogen bond was found between Leu839 and -OH groups of both compounds, indicating that -OH groups should be present as the structure-activity relationship in order to give good inhibition of bioactivity.

BIOACTIVITY OF COMPOUNDS **5** AND **6**

The bioactivities of the compounds quantitatively correspond with the groups that are present in them. Combining several groups with a certain degree of individual activity may produce a more active compound. In particular, most *p*-coumaric acid

derivatives and other molecules containing phenethyl moiety have potential activity as anticancer agents. Thus, combining *p*-coumaroyl groups and phenethyl groups in a compound will increase anticancer activity. This is in accordance with the facts obtained in this study. Both compounds **5** and **6** are classified as cytotoxic (Cao et al. 1998) because bio-assay against P388 leukemia murine cells gave a quite good IC_{50} of 1.0 and 5.89 $\mu\text{g}/\text{mL}$, respectively. Compared with its analog compounds like methyl 2-cinnamamido-3-hydroxy propanoate with IC_{50} 10.78 $\mu\text{g}/\text{mL}$, *N*-feruloylmorpholine, and *N*-feruloylpyperidine with IC_{50} value of 10.78, 46.67, and 57.10 $\mu\text{g}/\text{mL}$, the synthesized compounds **5** and **6**, are much more active.

Nevertheless, the bioactivity data seems a bit inconsistent with the data of phenethyl derivatives of caffeic acid in which the amide derivatives were more active as an antioxidant than the esters (Son & Lewis 2002). This dispute could be clearly understood because bioactivity not only depends on the main functional group but also on the type of the cinnamic compound (Venkateswarlu et al. 2006). The level of bioactivity of the *p*-coumaric derivative corresponds with the conjugation system in its structure (Zhang et al. 2014). Releasing of hydrogen radical from the hydroxyl of the phenolic group was facilitated by the electron de-localization alongside the conjugation system. However, the presence of a methoxy group in the *meta*-position of ferulic compounds causes difficulty for the hydrogen atom to be released due to the formation of hydrogen bonding with oxygen from the methoxy group. These explain the less activity of *N*-feruloylmorpholine and *N*-feruloylpyperidine compared to compounds **5** and **6**.

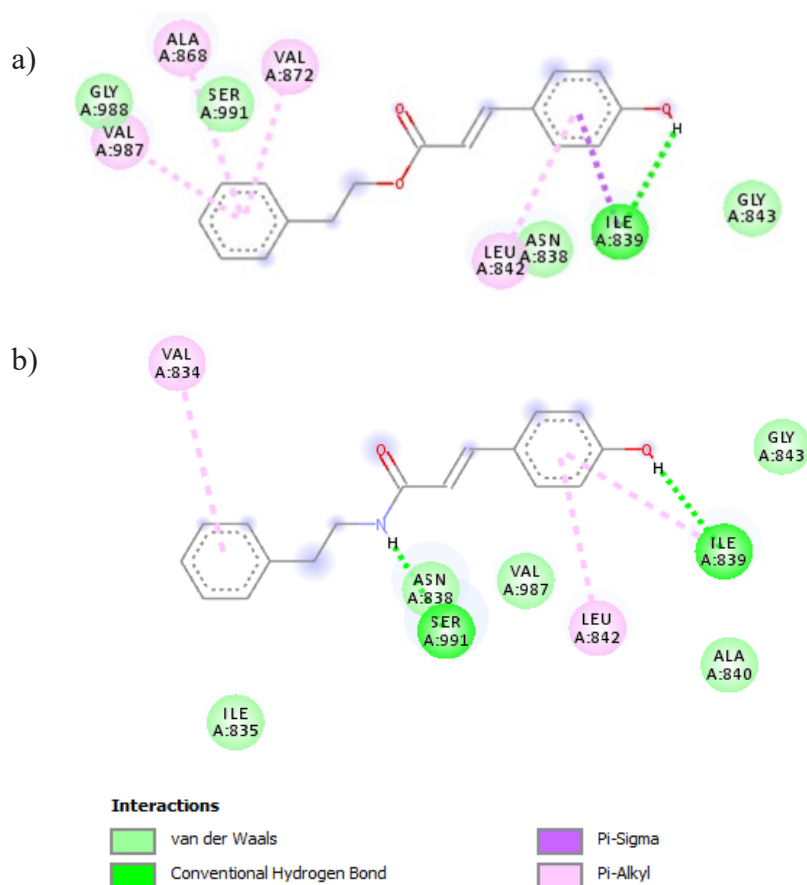


FIGURE 7. Interactions of compounds **5** and **6** against P-gp

The difference of IC_{50} value between compounds **5** and **6** relates to the differences in the stability of ester and amide. As carboxylic acid derivatives, the ester is much more active than amide. This fact is consistent with some of the previous studies. Bioactivity *N*-(2-serinyl)-3-hydroxypicolinamide against P388 leukemia murine cells increased after esterification (Anita et al. 2007), and ester derivatives of naphthoquinone aliphatic were more active against human epidermoid carcinoma cells than their amide (Kongkathip et al. 2013)

CONCLUSION

Two new compounds, namely phenethyl *p*-coumarate (**5**) and *N*-phenethyl-*p*-coumaramide (**6**), have been successfully synthesized through acetylation, chlorination, oxidation, and deacetylation reactions in a yellowish solid with m.p. of 68-69 °C and 137-139 °C, respectively. Both compounds were active as anticancer agents against P388 leukemia cells with an IC_{50} of 1.00 and 5.89 $\mu\text{g/mL}$.

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