Synthesis of Piperine Amide Derivatives and Evaluation of Their Anti-inflammatory Activity

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Abstract

Chronic inflammation has been recognized as a significant leading cause of disability and mortality worldwide. More than 50% of deaths being attributed to inflammation-linked diseases such as stroke, cancers, kidney, and cardiovascular diseases. In searching for new potential anti-inflammatory agents, a novel piperine dimer and an amide series of piperine derivatives were designed, synthesized, and screened for their anti-inflammatory activity. Piperine was isolated from fruits of *Piper longum L.* and hydrolyzed under reflux conditions to obtain piperic acid as a starting material for the synthesis of the piperine derivatives. The conversion of piperic acid to an amide series was involving the *N*,*N*'-dicyclohexylcarbodiimide (DCC) coupling method in the presence of 4-dimethylamino pyridine (DMAP) as a catalyst. Meanwhile, the piperine dimer (**7**) was obtained by SiO_2-mediated Diels-Alder reaction of piperine. The isolated and synthesized compounds were elucidated using ^1H and ^{13}C NMR spectroscopy. The anti-inflammatory activity of the compounds was determined by nitric oxide (NO) inhibition assay of LPS-activated macrophage J774.A1 cells and reported as IC_{50} values. Among the compounds tested, compound **3** showed the most potent activity at an IC_{50} value of 19.5 μM. Piperine (**1**) and its amide derivatives **4-6** showed moderate anti-inflammatory activity with IC_{50} values in the range of 26.7-44.4 μM, whereas piperic acid (**2**) did not show any significant activity at 50 μM. These findings indicated that the amide moiety of the piperine derivatives might play an important role in their anti-inflammatory activity. Moreover, the cytotoxicity of all compounds against J774.A1 cells was evaluated using an MTT assay. None of the compounds showed cytotoxic to the cells, except compound **7** with the cell viability less than 80% at a dose of 50 μM.

Keywords**:** *Amide, Anti-inflammatory activity, Cytotoxicity, Piperine derivatives*

1. Introduction

Inflammation is a response of the body's immune system to cellular damage. An immune system such as macrophages will release inflammatory mediators like cytokines, nitric oxide (NO), and others (Sharma, Kumar, Singh, Monga, & Kumar, 2020). Acute inflammation aims to localize and eliminate damaged tissue components so that the body can begin to heal. However, prolonged acute inflammation will evolve into chronic inflammatory, leading to severe diseases including asthma, cardiovascular diseases, neurodegenerative disorders, and cancers (Dvorakova & Landa, 2017). Non-steroidal anti-inflammatory drugs (NSAID) are commonly used to treat inflammation. Still, the current drugs are associated with gastrointestinal, cardiovascular, and kidney disease side effects (Bai et al., 2021). Therefore, more research is necessary for the field of discovery and development of anti-inflammatory agents.

Piperine is a natural alkaloid that is abundantly found in the fruits of *Piper nigrum L.* and *Piper longum L.* species of the Piperaceae family with constituent 2.0-7.4% (Chavarria, Silva, Magalhaes e Silva, Remiao, & Borges, 2016). Piperine was reported to display a wide spectrum of biological activities such as anti-oxidant, anti-inflammatory, anti-microbial, and anti-cancer (Greenshields et al., 2015; Ying et al., 2013; Zarai, Boujelbene, Ben Salem, Gargouri, & Sayari, 2013). It was reported that piperine significantly decreased the level of LPS-stimulated inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), inducible NO synthase (iNOS), and cyclooxygenase (COX)-2 in RAW264.7 macrophage cells (Ying et al., 2013). Besides, piperine was found to inhibit pro-inflammatory cytokines including TNF-α, interleukin (IL)-1β, and IL-6 highly expressed in the rheumatoid joint, which play a significant role in the pathogenesis of rheumatoid arthritis (Umar et al., 2013).

Owing to its numerous pharmacological effects, piperine has been used for therapeutic purposes and is expected to remain so in the future. The piperine structure contained three subunits; methylenedioxy, butadiene chain, and an amide linkage with piperidine moiety that contributed to binding with some protein

and showed promising activity as an anti-inflammatory. Nevertheless, to the best of our knowledge, even though publications were reporting the activity of piperine as mentioned above, none of them revealed the potency of piperine-amide derivatives as anti-inflammatory agents. Therefore, this present study deals with the preparation of piperine derivatives by modifying the piperidine moiety with a series of the amide to increase the natural derived piperine's potentiality as an anti-inflammatory. All compounds were characterized by NMR spectral data, and the anti-inflammatory activity was evaluated by nitric oxide (NO) inhibition assay of LPS-activated macrophage J774.A1 cells in comparison with indomethacin, as the commercial anti-inflammatory drug, as well as compared with the parent compound piperine.

2. Objectives

1) To synthesize and characterize a piperine dimer and an amide series of the piperine derivatives
2) To evaluate the anti-inflammatory activity of the piperine derivatives

3. Materials and Methods

3.1 Materials and instruments

All reagents and solvents were of reagent grade or purified according to standard methods. Pyrrolidine, *n*-butylamine, di-*n*-butylamine, *N*-methylaniline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Hydrochloric acid (HCl), anhydrous sodium sulfate (Na_2SO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), and potassium hydroxide (KOH) were obtained from Merck KGaA (Germany). *N,N'*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were bought from Fluka Chemical Company Ltd. (Switzerland). The macrophage J774.A1 cell was purchased from American Type Culture Collection (Manassas, Virginia, United States). Lipopolysaccharides (LPS), fetal bovine serum (FBS), and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Sigma-Aldrich (St. Louis, Missouri, United States).

^{1}H and ^{13}C NMR spectra were recorded on JEOL JNM-ECZ500R/S1 spectrometer at 500 MHz for ^{1}H and 126 MHz for ^{13}C, respectively, using tetramethylsilane (TMS) as the internal standard. Silica gel 60 (60–200 μm, Silicycle, Quebec, Canada) was used for column chromatography (CC).

*3.2 Isolation and purification of piperine (**1**) from Piper longum L.*

The fruits of *Piper longum L.* (0.50 kg) were powdered and extracted with methanol at room temperature, then partitioned with ethyl acetate. The piperine was isolated from ethyl acetate crude extract and the purification was performed by column chromatography over silica gel 60 eluted with solvent system hexane: ethyl acetate (7:3). The structure of the isolated compound was then confirmed by NMR spectroscopy.

3.3 *Synthesis of piperic acid (**2**)*

Piperic acid was prepared by alkaline hydrolysis (Scheme 1). 500 mg of piperine was refluxed with 20% methanolic KOH for 26 hours. The reaction was monitored by thin-layer chromatography (TLC). After the completion of the hydrolysis process, methanol was evaporated under reduced pressure. The result was suspended in water and gradually acidified with HCl to pH <1, then extracted with ethyl acetate. The organic layer was separated and washed with saturated NaCl (brine), followed by the addition of anhydrous sodium sulfate (Na_2SO_4). The solution was then filtered and evaporated to obtain the yellow solid piperic acid.

*3.4 Synthesis of piperic-amide (**3-6**)*

Piperic acid (50 mg, 0.02 mmol), DCC (47 mg, 0.02 mmol), and 5% of DMAP were dissolved in 8 mL of dry dichloromethane. After 2 hours of vigorous stirring, various amine (0.02 mmol) was added to the solution and continued stirring under the inert condition overnight (Table 1). The reaction was monitored by TLC. After the completion of the reaction, the result was diluted in the ethyl acetate then washed using sodium bicarbonate ($NaHCO_3$), followed by brine. The organic layer was separated from the aqueous phase, and anhydrous sodium sulfate (Na_2SO_4) was added to trap the rest of the water contained in the organic phase.

The solution was then evaporated under pressure, followed by the purification of the product using column chromatography over silica gel and eluted with a gradient mixture of hexane-ethyl acetate to yield the series of amide piperine derivatives (**3-6**)

*3.5 Synthesis of piperine dimer **(7)***

A novel piperine dimer was prepared by lewis acid-mediated Diels-Alder reaction of piperine under solventless conditions (Scheme 2) following the previous report (Wei et al., 2013). 50 mg of piperine was heated at 160°C in the presence of silica gel (10 mg) as the lewis acid for 6 hours. After the reaction was completed, the product was then purified by silica gel column chromatography and eluted with hexane: ethyl acetate (7:3).

3.6 Anti-inflammatory assay

The anti-inflammatory activity of the synthesized compounds was evaluated by monitoring the inhibition of nitric oxide (NO) production in LPS-activated macrophage J774.A1 cells, with indomethacin as the positive control (Sarigaputi, Sommit, Teerawatananond, & Pudhom, 2014). The cells were cultured and seeded in the 96-wells plate in Dulbecco's Modified Eagle's Medium (DMEM) at a density of 1×10^5 cells/well and incubated at 37°C of 5% CO_2. After 24 hours of incubation, the cells were pretreated with various concentrations of every compound following the incubation for 2 hours. The cells were then activated by LPS with a concentration of 1μg/mL and incubated for the next 20 hours. The nitrite concentration produced was determined by the Griess reagent, and the absorbance was measured at 540 nm to obtain the % inhibition of NO production. The result was then reported as IC_{50} value.

3.7 Cytotoxicity assay

The cytotoxicity of the compounds towards macrophage J774.A1 cells was evaluated using the MTT assay method. Briefly, J774.A1 cells were placed in a 96-wells plate (1×10^5 cells/well) in Dulbecco's Modified Eagle's Medium. After 24 hours of pre-incubation at 37°C in a humidified atmosphere of 5% CO_2, the various concentrations of all compounds were added and incubated for 24 hours under the above conditions. At the end of the incubation, tetrazolium reagent was added into each well, followed by further incubation for 4 hours, and then the absorbance was measured at 570 nm. The compound was categorized as toxic if the cell viability was less than 80% at a dose of 50 μM.

4. Results and Discussion

4.1 Isolation of piperine

Piperine (**1**) was isolated from the ethyl acetate crude extract of the fruits of *Piper longum L.* by silica gel column chromatography as yellow crystals. The structure of this compound was shown in Figure 1. The ^1H and ^{13}C NMR spectroscopic data of this compound indicated the presence of piperidine moiety showed by in the total of 10 proton and 5 carbon signals at [δ_H 3.59 (2H-1', t, J= 6 Hz), 3.47 (2H-5', t, J= 5.5 Hz), 1.61 (2H-3', m), 1.53 (2H-2'and 2H-4', m); δ_C 46.9, 43.2, 26.8, 25.7, 24.7]. The methylenedioxy group was proven by 2 protons as singlet peak and 1 carbon at [δ_H 5.92 (2H, s); δ_C 101.3], and the carbonyl group (C=O) appeared at (δ_C 165.4) as shown in Tables 2 and 3.

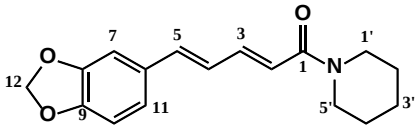
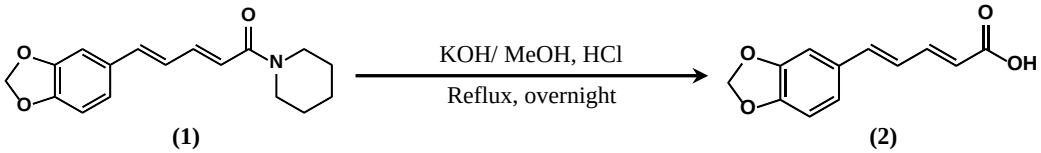


Figure 1 Chemical structure of piperine (**1**)

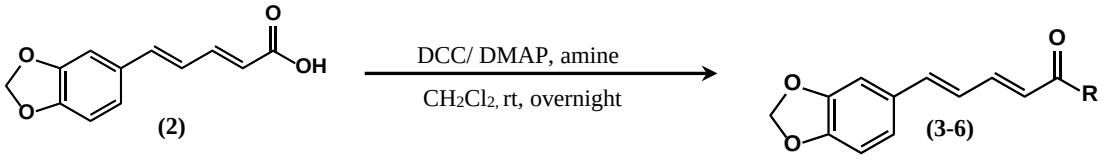
4.2 Synthesis of an amide series of piperine



Scheme 1 Hydrolysis of piperine

The synthesis of the piperine amide derivatives was started by converting piperine (**1**) to piperic acid (**2**) by alkaline hydrolysis. Compound **2** was achieved as a yellow solid with a 96% yield. The changing of piperine to piperic acid was proven by the absence of piperidine moiety signals in the ^{1}H and ^{13}C NMR spectroscopic data, compared with the NMR spectra of compound **1**. ^{1}H NMR spectra (Table 2) shown only in the total of 9 protons at δ_H 5.95-7.35 ppm and ^{13}C NMR spectra (Table 3) shown in the total of 12 carbons at δ_C 101.6-167.1 ppm indicated the loss of the piperidine ring.

Table 1 Synthesis of an amide series of piperine by DCC coupling method



Amine	Yield (%)
Pyrrolidine	45.6
n-Butylamine	58.1
Di-*n*-butylamine	28.2
N-Methylaniline	32.5

The conversion of an acid to an amide usually involves acyl chloride formation as the most active carboxylic acid derivatives, followed by immediate treatment with the amine. Thionyl chloride ($SOCl_2$) and oxalyl chloride $(COCl)_2$ are commonly used to generate acyl chlorides from their corresponding acids. Nevertheless, acyl chlorides are very reactive, leading to a danger of hydrolysis, racemization, and other side reactions. Moreover, the use of oxalyl chloride is accompanied by the production of chemical hazards such as CO_2, CO, and HCl (Dunetz, Magano, & Weisenburger, 2016; Montalbetti & Falque, 2005). Hence, the preparation of amide series in this experiment used a milder method that involved DCC to activate the piperic acid along with the addition of DMAP as a catalyst to yield compounds (**3-6**), as shown in Figure 2.

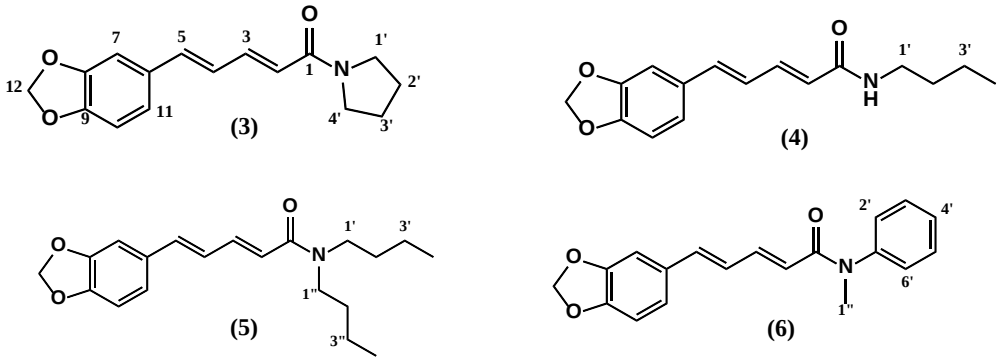


Figure 2 Chemical structures of synthetic piperine amide derivatives (**3-6**)

Table 2 ^{1}H NMR spectral data for compounds **1, 3-6** in $CDCl_3$ and **2** in acetone-d_6 (δ_H in ppm, mult., *J* in Hz)

Position	1	2	3	4	5	6
2	6.39 d (14.5.0)	5.95 d (15.0)	6.24 d (14.5)	5.89 d (15.0)	6.34 d (14.5)	5.87 d (15.0)
3	7.35 dd (14.5, 8.0)	7.35 dd (15.0, 6.0)	7.42 dd (15.0, 10.0)	7.35 dd (15.0, 10.0)	7.43 dd (15.0, 7.0)	7.42 dd (14.0, 2.0)
4	6.70 d (16.0)	6.83 d (8.0)	6.76 d (7.5)	6.76 d (11.0)	6.76 d (11.0)	6.51 dd (15.0, 11.0)
5	6.71 d (16.0)	7.01 dd (8.0, 2.0)	6.78 d (15.5)	6.75 d (7.0)	6.75 d (7.0)	6.72 d (12.5)
7	6.92 d (2.0)	7.14 d (2.0)	6.97 d (2.0)	6.97 d (2.0)	6.99 d (1.5)	6.89 d (1.5)
10	6.69 d (8.0)	6.94 d (6.0)	6.73 (10.0)	6.74 d (8.0)	6.74 d (7.5)	6.73 d (11.5)
11	6.83 dd (8.0, 2.0)	6.94 d (4.0)	6.89 dd (8.0, 2.0)	6.88 dd (8.5, 2.0)	6.89 dd (8.0, 1.5)	6.83 dd (8.0, 2.0)
12	5.92 s	6.01 s	5.96 s	5.97 s	5.96 s	5.92 s
1’	3.59 t (6.0)	-	3.55 t (7.0)	3.35 t (7.0)	3.39 t (8.0)	
2’	1.53 m	-	1.97 m	1.53 m	1.56 m	7.41 d (8.0)
3’	1.61 m	-	1.87 m	1.37 m	1.34 m	7.20 d (7.5)
4’	1.53 m	-	3.54 t (7.0)	0.93 t (7.5)	0.96 t (7.5)	7.34 t (7.0)
5’	3.47 t (5.5)	-	-	-	-	7.20 d (7.5)
6’	-	-	-	-	-	7.41 d (8.0)
1”	-	-	-	-	3.31 t (8.0)	3.36 s
2”	-	-	-	-	1.56 m	-
3”	-	-	-	-	1.34 m	-
4”	-	-	-	-	0.93 t (7.5)	-

Table 3 ^{13}C NMR spectral data for compounds **1, 3-6** in $CDCl_3$ and **2** in acetone-d_6 (δ_C in ppm)

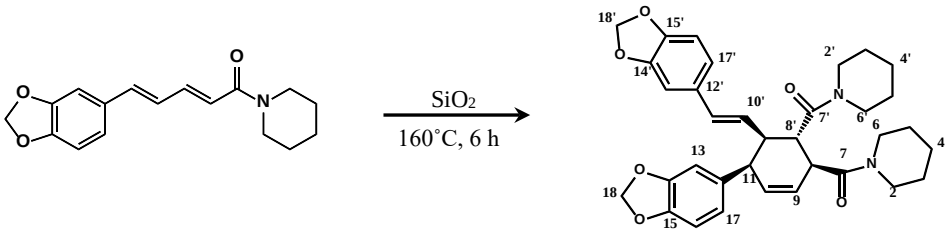
Position	1	2	3	4	5	6
1	165.4	167.1	165.1	166.3	166.3	166.5
2	120.1	120.6	121.4	122.6	120.4	121.3
3	142.5	145.1	142.0	140.9	142.5	143.8
4	125.4	124.8	125.3	124.8	125.5	125.3
5	138.2	140.1	138.9	138.8	138.5	138.8
6	131.0	131.0	131.1	131.0	131.1	131.0
7	105.6	105.7	105.8	105.8	105.8	105.8
8	148.2	148.7	148.3	148.3	148.3	148.3
9	148.1	148.5	148.3	148.3	148.2	148.3
10	108.4	108.4	108.6	108.6	108.6	108.5
11	122.5	123.1	122.7	123.5	122.6	122.7
12	101.3	101.6	101.4	101.4	101.4	101.4
1’	46.9	-	46.6	39.5	48.0	143.8
2’	43.2	-	46.1	31.9	32.0	127.5
3’	26.8	-	26.2	20.2	20.4	129.7
4’	25.7	-	24.4	13.9	14.0	127.6
5’	24.7	-	-	-	-	129.7
6’	-	-	-	-	-	127.5
1”	-	-	-	-	46.7	37.6
2”	-	-	-	-	29.3	-
3”	-	-	-	-	20.2	-
4”	-	-	-	-	14.0	-

Compound **3** (((*2E,4E*)-5-(benzo[*d*][1,3]dioxol-5-yl)-1-(pyrrolidin-1-yl)penta-2,4-dien-1-one)) was obtained as a brown solid with a yield of 45.6% from the reaction of piperic acid (**2**) with pyrrolidine. The ^{1}H and ^{13}C NMR spectroscopic data (Tables 2 and 3) indicated that this compound containing pyrrolidine moiety, proven by the existence of 8 protons at δ_H 3.55 (2H-1', t, *J*= 7 Hz), 3.54 (2H-2', t, *J*= 7 Hz), 1.97 (2H, m), 1.87 (2H, m) with 4 carbon at δ_C 46.6, 46.1, 26.2 and 24.4. Compound **4** (((*2E,4E)*-5-(benzo[*d*][1,3]dioxol-5-yl)-*N*-butylpenta-2,4-dienamide) was obtained from the reaction of piperic acid (**2**) with *n*-butylamine as a brown solid with a yield of 58.1%. The ^{1}H and ^{13}C NMR spectroscopic data (Tables 2 and 3) of compound **4** showed the aliphatic chain by signals at δ_H 3.35 (2H-1', t, *J*= 7 Hz), 1.53 (2H-2', m), 1.37 (2H-3', m), 0.93 (3H-4', t, *J*=7.5 Hz) and δ_C 39.5, 31.9, 20.2, 13.9. Compound **5** (*(2E,4E)*-5-(benzo[*d*][1,3]dioxol-5-yl)-*N*,*N*-dibutylpenta-2,4-dienamide) was obtained as a dark-yellow solid with a yield of 28.2% from the reaction of piperic acid (**2**) with di-*n*-butylamine. The ^{1}H and ^{13}C NMR spectroscopic data indicated aliphatic chain of this by signals of 18 proton at 3.39 (2H-1', t, *J*= 8 Hz), 3.31 (2H-1", t, *J*= 8 Hz), 1.56 (2H-2' and 2H-2", m), 1.34 (2H-3' and 2H-3", m), 0.96 (3H-4', t, *J*= 7.5 Hz), 0.93 (3H-4", t, *J*= 7.5 Hz) and 8 carbon at δ_C 48.0, 46.7, 32.0, 29.3, 20.4, 20.2, 14.0, 13.9 as shown in the Tables 2 and 3. Lastly, compound **6** (*(2E,4E)*-5-(benzo[*d*][1,3]dioxol-5-yl)-*N*-methyl-*N*-phenylpenta-2,4-dienamide) was obtained as a dark yellow solid with a yield of 32.5% from the reaction of piperic acid (**2**) with *N*-methylaniline. ^{1}H and ^{13}C NMR spectroscopic data of this structure are shown in Tables 2 and 3. The methyl at C-1" is clearly shown by signals at [δ_H 3.36 (3H, s); δ_C 37.6]. Meanwhile, the aromatic ring chain was proven by proton and carbon signals at δ_H 7.41 (1H-2' and 1H-6', d, *J*=8 Hz), 7.20 (1H-3' and 1H-5', d, *J*=7.5 Hz), 7.34 (1H-4', t, *J*=7 Hz), and δ_C 143.8, 127.5, 129.7, 127.6, 129.7, 127.5.

All of the synthesized compounds had relatively low to moderate percentage yields, ranging from 28.2-58.1%. Compound **6** had a less percentage yield than compounds **3** and **4** because *N*-methylaniline contains the aromatic ring, which decreases its nucleophilicity. Compound **5** had the least percentage yield because of its steric hindrance. Furthermore, the amidation reaction using the DCC coupling method was accompanied by the formation of by-product dicyclohexylurea (DCU) that was quite difficult to remove from the product, leading to multiple purifications and resulted in a lower yield. The addition of DMAP in this reaction aimed to reduce the production of DCU by the reaction of DMAP with *O*-acylisourea to form acyl pyridinium. However, the result showed that the formation of DCU could not be avoided. Therefore, another synthesis method is needed to obtain a better yield of the products.

4.3 Synthesis of piperine dimer

Piperine dimer (**7**) was reported to exhibit a broad spectrum of biological activities, including anti-cancer and anti-inflammatory activity (Ngo et al., 2017; Rao et al., 2011). Nevertheless, there is only one report regarding the evaluation of its anti-inflammatory activity tested to RAW 264.7 cell line. Therefore, this compound was synthesized to evaluate its anti-inflammatory activity toward the J774.A1 cells as compared with the other amide series of the piperine derivatives. Compound **7** was obtained as a yellow semisolid compound from the SiO_2-mediated Diels-Alder reaction of piperine in a 42.5% yield. The presence of silica gel in this reaction was reported to increase the reactivity and selectivity of piperine's dimerization (Wei et al., 2013). ^{1}H and ^{13}C NMR spectroscopic data of this structure (Table 4) are compared to previously reported data (Rukachaisirikul, Prabpai, Champung, & Suksamrarn, 2002).



Scheme 2 Synthesis of a piperine dimer (**7**)

Table 4 ^{1}H and ^{13}C NMR ($CDCl_3$) spectral data for compound **7** (δ in ppm, mult., *J* in Hz)

Position	^{1}H	^{13}C	Position	^{1}H	^{13}C
2	3.55 m	47.2	2’	3.35 m	42.9
3	1.65 m	26.8	3’	1.12 m	25.6
4	1.60 m	24.7	4’	1.35 m	24.6
5	1.65 m	25.8	5’	1.12 m	26.4
6	3.55 m	43.4	6’	3.26 m	47.0
7	-	171.2	7’	-	172.3
8	4.12 dd (14.0, 7.5)	42.9	8’	3.65 dd (12.0, 9.5)	37.7
9	5.85 dt (10.0, 2.0)	125.6	9’	2.92 ddd (12.0, 11.0, 5.5)	45.6
10	5.88 ddd (10.5, 5.0, 3.0)	130.4	10’	5.20 dd (15.5, 10.0)	128.2
11	3.45 m	46.0	11’	6.31 d (15.5)	131.1
12	-	133.6	12’	-	132.0
13	6.80 s	108.0	13’	6.65 d (1.6)	105.4
14	-	147.6	14’	-	147.8
15	-	146.5	15’	-	146.5
16	6.81 d (10.5)	110.8	16’	6.68 d (8.0)	108.3
17	6.81 d (11.0)	123.5	17’	6.63 dd (8.0, 2.0)	120.8
18	5.96 d (1.5)	101.0	18’	5.91 d (1.5)	101.0

4.4 Anti-inflammatory activity

Table 5 NO inhibitory effects of piperine and its derivatives

Compound	IC_{50} (μM)
1	44.4
2	>50.0
3	19.5
4	38.9
5	26.7
6	27.2
7	Toxic [b]
Indomethacin [a]	44.5

[a] Positive control
[b] Cell viability was less than 80% at 50 μM

Nitric oxide (NO) is one of the crucial mediators involved in regulating inflammatory diseases and is released locally within damages to facilitate the healing process. However, an excessive amount of NO has been implicated in many diseases, including cardiovascular diseases, neurodegenerative disorders, and cancers (Tazawa et al., 2013; Tripathi, Kartawy, & Amal, 2020). In this research, all compounds’ anti-inflammatory activity was determined by their efficacy in inhibiting the production of NO in LPS-activated macrophage J774.A1 cells (Sarigaputi et al., 2014). Indomethacin was used as a positive control with an IC_{50} value of 44.5 μM (Table 5). Indomethacin is known to produce an anti-inflammatory effect by inhibiting the cyclooxygenase (COX) enzyme, resulting in the suppression of prostaglandins’ production (Summ & Evers, 2013). Indomethacin was also reported to reduce the production of NO by the inhibition of inducible nitric oxide synthase in murine macrophage J774 cells (Ogawa et al., 2000).

Piperine as the parent compound showed a nitric oxide inhibitory effect relatively the same as the positive control, with an IC_{50} value of 44.4 μM. The functional group of piperine, such as methylenedioxy, butadiene chain, and the amide linkage were expected to bind with the enzyme involved in the production of NO, causing the inhibition of NO level. Among all compounds, compound **3** showed the most potent activity with an IC_{50} value of 19.5 μM. Compared with the parent compound **1**, the amide ring size of compound **3** is

smaller than the piperidine ring of the piperine structure. The smaller size of the ring may allow the ring to fit with the active site of the enzyme. The second compound showing good anti-inflammatory activity was compound **5,** followed by compound **6**, with IC_{50} values of 26.7 μM and 27.2 μM, respectively. The last compound showing moderate activity was compound **4**, with an IC_{50} value of 38.9 μM. The total of 2 butane chain of compound **5** showed an effect to anti-inflammatory activity compared with compound **4** that only have 1 butane chain, which might be because the more aliphatic chain contributed to higher lipophilicity of the compound, enabling the compound to penetrate the lipid layer of the membrane cell. Moreover, the aliphatic chain is flexible to rotate and adjust itself to bind tightly with the enzyme. As for compound **6**, it contained an aromatic ring that might contribute to the π- π stacking interaction, resulting in better activity than piperine. Meanwhile, the acid form of piperine showed no significant activity at a dose of 50 μM. This finding indicated that the amide moiety of piperine derivatives might play a key role in anti-inflammatory activity.

All compounds showed no cytotoxicity towards the cells as measured by MTT assay, except compound **7**, resulting in less than 80% cell viability at a dose of 50 μM. Consequently, compound **7** was not evaluated for its anti-inflammatory activity. Most piperine derivatives (**3-6**) exhibited higher anti-inflammatory activity than the parent compound (**1**) and indomethacin, a standard drug. However, more study is needed to reveal the SAR and mechanism of action of the piperine-amide derivatives.

5. Conclusion

In summary, piperine amide derivatives (**3-7**) were successfully synthesized. Among all compounds tested for their anti-inflammatory activity, compound **3** was the most active with an IC_{50} value of 19.5 μM. Piperine amide derivatives **4-6** showed better activity than indomethacin as the positive control whereas compound **7** was toxic to the cells. This finding indicated that the piperine amide derivatives showed good anti-inflammatory activity. Hence, the researcher will extend this series for studying their SAR to get more useful information in the development of new anti-inflammatory agents.

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7. References

Bai, R., Yao, C., Zhong, Z., Ge, J., Bai, Z., Ye, X., . . . Xie, Y. (2021). Discovery of natural anti-inflammatory alkaloids: Potential leads for the drug discovery for the treatment of inflammation. *European Journal of Medicinal Chemistry, 213*, 113165. doi:10.1016/j.ejmech.2021.113165

Chavarria, D., Silva, T., Magalhaes e Silva, D., Remiao, F., & Borges, F. (2016). Lessons from black pepper: piperine and derivatives thereof. *Expert Opinion on Therapeutic Patents, 26*(2), 245-264. doi:10.1517/13543776.2016.1118057

Dunetz, J. R., Magano, J., & Weisenburger, G. A. (2016). Large-Scale Applications of Amide Coupling Reagents for the Synthesis of Pharmaceuticals. *Organic Process Research & Development, 20*(2), 140-177. doi:10.1021/op500305s

Dvorakova, M., & Landa, P. (2017). Anti-inflammatory activity of natural stilbenoids: A review. *Pharmacological Research, 124*, 126-145. doi:10.1016/j.phrs.2017.08.002

Greenshields, A. L., Doucette, C. D., Sutton, K. M., Madera, L., Annan, H., Yaffe, P. B., . . . Hoskin, D. W. (2015). Piperine inhibits the growth and motility of triple-negative breast cancer cells. *Cancer Letters, 357*(1), 129-140. doi:10.1016/j.canlet.2014.11.017

Montalbetti, C. A. G. N., & Falque, V. (2005). Amide bond formation and peptide coupling. *Tetrahedron, 61*(46), 10827-10852. doi:10.1016/j.tet.2005.08.031

Ngo, Q. M., Tran, P. T., Tran, M. H., Kim, J. A., Rho, S. S., Lim, C. H., . . . Min, B. S. (2017). Alkaloids from Piper nigrum Exhibit Antiinflammatory Activity via Activating the Nrf2/HO-1 Pathway. *Phytotherapy Research, 31*(4), 663-670. doi:10.1002/ptr.5780

Ogawa, O., Umegaki, H., Sumi, D., Hayashi, T., Nakamura, A., Thakur, N. K., . . . Iguchi, A. (2000). Inhibition of inducible nitric oxide synthase gene expression by indomethacin or ibuprofen in β-amyloid protein-stimulated J774 cells. *European Journal of Pharmacology, 408*(2), 137-141. doi:https://doi.org/10.1016/S0014-2999(00)00721-4

Rao, V. R. S., Suresh, G., Babu, K. S., Raju, S. S., Vishnu vardhan, M. V. P. S., Ramakrishna, S., & Rao, J. M. (2011). Novel dimeric amide alkaloids from Piper chaba Hunter: isolation, cytotoxic activity, and their biomimetic synthesis. *Tetrahedron, 67*(10), 1885-1892. doi:10.1016/j.tet.2011.01.015

Rukachaisirikul, T., Prabpai, S., Champung, P., & Suksamrarn, A. (2002). Chabamide, a Novel Piperine Dimer from Stems of Piper chaba. *Planta Medica, 68*(09), 853-855. doi:10.1055/s-2002-34410

Sarigaputi, C., Sommit, D., Teerawatananond, T., & Pudhom, K. (2014). Weakly anti-inflammatory limonoids from the seeds of Xylocarpus rumphii. *Journal of Natural Products, 77*(9), 2037-2043. doi:10.1021/np5003687

Sharma, S., Kumar, D., Singh, G., Monga, V., & Kumar, B. (2020). Recent advancements in the development of heterocyclic anti-inflammatory agents. *European Journal of Medicinal Chemistry, 200*, 112438. doi:10.1016/j.ejmech.2020.112438

Summ, O., & Evers, S. (2013). Mechanism of action of indomethacin in indomethacin-responsive headaches. *Curr Pain Headache Rep, 17*(4), 327. doi:10.1007/s11916-013-0327-x

Tazawa, H., Kawaguchi, T., Kobayashi, T., Kuramitsu, Y., Wada, S., Satomi, Y., . . . Okada, F. (2013). Chronic inflammation-derived nitric oxide causes conversion of human colonic adenoma cells into adenocarcinoma cells. *Experimental Cell Research, 319*(18), 2835-2844. doi:10.1016/j.yexcr.2013.08.006

Tripathi, M. K., Kartawy, M., & Amal, H. (2020). The role of nitric oxide in brain disorders: Autism spectrum disorder and other psychiatric, neurological, and neurodegenerative disorders. *Redox Biology, 34*, 101567. doi:10.1016/j.redox.2020.101567

Umar, S., Golam Sarwar, A. H., Umar, K., Ahmad, N., Sajad, M., Ahmad, S., . . . Khan, H. A. (2013). Piperine ameliorates oxidative stress, inflammation and histological outcome in collagen induced arthritis. *Cellular Immunology, 284*(1-2), 51-59. doi:10.1016/j.cellimm.2013.07.004

Wei, K., Wang, S., Liu, Z., Du, Y., Shi, X., Qi, T., & Ji, S. (2013). A solvent-free Diels–Alder cycloaddition of piperine on silica gel: synthesis of dimeric amide alkaloid from the roots of Piper nigrum. *Tetrahedron Letters, 54*(18), 2264-2266. doi:10.1016/j.tetlet.2013.02.080

Ying, X., Yu, K., Chen, X., Chen, H., Hong, J., Cheng, S., & Peng, L. (2013). Piperine inhibits LPS induced expression of inflammatory mediators in RAW 264.7 cells. *Cellular Immunology, 285*(1-2), 49-54. doi:10.1016/j.cellimm.2013.09.001

Zarai, Z., Boujelbene, E., Ben Salem, N., Gargouri, Y., & Sayari, A. (2013). Antioxidant and antimicrobial activities of various solvent extracts, piperine and piperic acid from Piper nigrum. *LWT - Food Science and Technology, 50*(2), 634-641. doi:10.1016/j.lwt.2012.07.036