Structure-Activity Relationship Study on the Ethyl *p*-Methoxycinnamate as an Anti-Inflammatory Agent

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ABSTRACT

*Ethyl p-methoxycinnamate (EPMC) (**1**) has been isolated as a major compound from the rhizome of* Kaempferia galanga *together with the other compound ethyl cinnamate (**2**). As reported in the literature, EPMC (**1**) exhibited a significant in vitro and in vivo anti-inflammatory activity. In this research, we have investigated the anti-inflammatory activity of compounds **1** and **2** by using anti-denaturation of heat bovine serum albumin (BSA) method. In order to analyze active sites that are responsible for the anti-inflammatory activity, therefore, it is necessary to conduct structural modification of EPMC (**1**). The structural modification was performed through re-esterification reaction by using conventional and assistance of the unmodified microwave oven. Evaluation of the results of the bioassay indicated that the ester and methoxy functional groups of EPMC (**1**) play an important role for the anti-inflammatory activity.*

Keywords: *Ethyl p-methoxycinnamate; anti-inflammatory; anti-denaturation; microwave assisted reaction; re-esterification*

ABSTRAK

*Etil p-metoksisinamat (EPMS) (**1**) telah berhasil diisolasi sebagai senyawa utama dari rimpang* Kaempferia galanga *(kencur), bersama-sama dengan senyawa lainnya etil sinamat (**2**). Penelitian sebelumnya telah melaporkan bahwa EPMS (**1**) memiliki aktivitas anti-inflamasi yang telah diujikan secara in vitro dan in vivo. Pada penelitian ini, kami melakukan investigasi terhadap aktivitas anti-inflamasi senyawa **1** dan **2** dengan menggunakan metoda anti-denaturasi bovine serum albumin (BSA). Dalam rangka menganalisis pusat aktif yang bertanggung jawab terhadap aktivitas anti-inflamasi dari EPMS (**1**), maka dipandang perlu untuk melakukan modifikasi struktur dari EPMS (**1**). Modifikasi struktur dilakukan melalui reaksi re-esterifikasi secara konvensional dan dengan bantuan oven microwave. Evaluasi hasil uji bioassay menunjukkan bahwa gugus fungsi ester dan metoksi dari EPMS (**1**) mempunyai peran penting untuk aktivitas anti-inflamasinya.*

Kata Kunci: *Etil p-metoksisinamat; anti-inflamasi; anti-denaturasi; reaksi berbantuan microwave; re-esterifikasi*

INTRODUCTION

Ethyl *p*-methoxycinnamate (EPMC) has been identified as a major compound of the rhizome of *Kaempferia galanga* [1] and has been reported to have various activity such as mosquito repellent and larvicidal [2], anti-tuberculosis [3], sedative [4], anticancer [5], analgesic and anti-inflammatory [1,6] and hypopigmentary [7]. *In vivo* study on the anti-inflammatory activity indicated that EPMC (**1**) dose-dependently inhibited carrageenan-induced rat paw edema with a MIC of 100 mg/kg. *In vitro* study showed that EPMC (**1**) non-selectively inhibited the activity of cyclooxygenases 1 and 2 with IC_{50} values of 1.12 µM and 0.83 µM, respectively [1]. Furthermore, Umar et al. [6] suggested that EPMC (**1**) exhibited significant anti-inflammatory by inhibiting pro-inflammatory cytokines and angiogenesis, thus inhibiting recruitment of blood the main function of endothelial cells.

Previously, in order to develop derivative of EPMC (**1**) as a sunblock, a conventional direct re-esterification of EPMC (**1**) has been carried out by reacting EPMC (**1**) with *n*-octanol to give octyl *p*-cinnamate in 87.4% yield [8]. The other structural modification was also performed in obtaining thiourea derivatives of EPMC (**1**), in which these derivatives

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were then evaluated for their activity against fibrosarcoma *in vivo* [9]. A literature search indicated that there have been no reports of the previously re-esterification of EPMC (**1**) in order to study the structure-activity relationship of its anti-inflammatory activity.

Therefore, in order to study the structure-activity relationship and analyze the anti-inflammatory active site of EPMC (**1**), here we are reporting the isolation of EPMC (**1**) and ethyl cinnamate (**2**) from the rhizome of *K. galanga* and structural modification of EPMC (**1**) through a re-esterification reaction. Re-esterification reaction was conducted in both conventional and microwave oven assisted reactions. The structure of compounds was established by a combination of extensive analysis of NMR, IR, and GCMS spectroscopic data. Anti-inflammatory activity of EPMC (**1**) and its derivatives were performed by using anti-denaturation of the heat Bovine Serum Albumin (BSA) assay.

EXPERIMENTAL SECTION

Materials

The rhizome of *K. galanga* was collected from BALITRO (Balai Penelitian Obat dan Rempah) Bogor, West Java, Indonesia in May 2014. This species was identified in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Sciences, Bogor, Indonesia.

The chemicals used in the research were methanol (Merck), *n*-propanol (Merck), *n*-butanol (Merck), H_2SO_4 (Smartlab), HCl (Merck), Na_2SO_4 (Merck), Na diclofenac (Sigma-Aldrich), Bovine serum albumin fraction V (Sigma-Aldrich), NaOH (Merck), Trizma Base (Sigma-Aldrich), NaCl (Merck), silica gel (Merck), and TLC plate 60 F254 (Merck).

Instrumentation

Melting point was measured by using DSC-60 Shimadzu and melting point apparatus Stuart SMP10 without correction. IR spectra were recorded on a Shimadzu FTIR Prestige-21 Shimadzu. The ^{1}H- and ^{13}C-NMR were measured on Jeol-500 MHz (^{1}H; 500 MHz, ^{13}C; 125 MHz) instruments. Chemical shift values were expressed in δ (ppm) downfield from TMS as an internal standard, and in δ 77.03 (ppm) from $CDCl_3$ a standard (^{13}C-NMR). Reactions were carried out by using a microwave oven assisted (Samsung). Column chromatography was performed on silica gel 60 (0.063-0.200 mm) (Merck). Re-esterification product was analyzed by using GCMS GC/MS-MSD 7890A/5975C (Agilent Technologies) under the following conditions: HP-5MS capillary column (30 m x 0.25 mm ID, 0.25 µm, film thickness) held at 70 °C for 2 min, raised to 285 °C, at a rate of 20 C/min and held for 20 min, injection temperature: 250 °C, 285 °C for MSD, carrier helium at a flow rate 1.2 mL/min.

Procedure

Extraction and isolation of EPMC (**1**) from the rhizome of *K. galanga* was carried out in accordance with our previous report [10]. *n*-Hexane and ethyl acetate extracts were stored in the refrigerator from which a colorless crystal of EPMC (**1**) crystallized, m.p. 50 °C [10] (lit. 49 °C) [1,6]. The residue of ethyl acetate extract that was not crystallized (22.9 g) was then purified by using column chromatography method. Silica gel was used as stationary phase and a mixture of *n*-hexane and ethyl acetate in stepwise was used as an eluent to give 16 fractions (A-P). Fraction D (2.7 g) was further purified by using silica gel column chromatography and eluted with a mixture of *n*-hexane and ethyl acetate in stepwise to give 9 fractions (I-IX). Fraction VIII obtained 305.8 mg of colorless oil of ethyl cinnamate (**2**) [11].

Hydrolysis of 1 to give p-methoxy cinnamic acid (3)

Hydrolysis of **1** was carried in accordance with our previously reported to give **3** as colorless crystals [10] m.p. 175 °C (lit. 169 °C) [9].

Reaction of 1 with methanol to give methyl p-*methoxycinnamate (4)*

Conventional method. In 100 mL Erlenmeyer flask, NaOH (0.1 g) was dissolved in methanol *pa*, and then 2.5 g of compound **1** was added to the mixture. The mixture was stirred at a room temperature until the compound **1** had completely reacted, as indicated by TLC analysis (about 20 h). The reaction product was then washed with distilled water and added HCl 15% until the final pH should be 4. The precipitated product was then air-dried to give 1.34 g of compound **4** as colorless crystals (53.6% yield), m.p. 87 °C (lit. 87-88 °C) [12].

Microwave assisted reaction. In 100 mL Erlenmeyer flask with the cup, NaOH (0.36 g) was dissolved in 15 mL methanol, then compound **1** (1.03 g, 5 mmol) was added to the mixture. The reaction mixture in Erlenmeyer flask was put in an ice bath and then irradiated by using the unmodified microwave oven at 300 W for 6 min. The product of the reaction was partitioned by using ethyl acetate and H_2O. Ethyl acetate fraction was further purified by using column chromatography to give 728 mg of compound **4** as colorless crystals (70.7% yield), m.p. 87 °C (lit. 87–88 °C) [12].

Reaction of 3 with* n-*propanol to give propyl* p-*methoxycinnamate (5)

In 100 mL Erlenmeyer flask with the cup, compound **3** (890 mg, 5 mmol) was dissolved in 30 mL of *n*-propanol and then 0.2 mL H_2SO_4 was added. The reaction mixture in Erlenmeyer flask was put in an ice bath and then irradiated by using the unmodified microwave oven at 300 W for 30 min. Product of reaction was partitioned by using ethyl acetate and H_2O. Ethyl acetate fraction was further purified by using silica gel using column chromatography to give 396 mg of **5** as colorless oil (44.5% yield); FTIR (KBr) ν_{max} 2966, 2839, 1705, 825; ^{1}H-NMR ($CDCl_3$, 500): 0.97 (3H, t), 1.71 (2H, m), 3.80 (3H, s), 4.13 (2H, t), 6.29 (1H, d, *J* = 16 Hz), 6.87 (2H, d, *J* = 9 Hz), 7.44 (2H, d, *J* = 9 Hz), 7.62 (1H, d, *J* = 16 Hz). ^{13}C-NMR ($CDCl_3$, 125): 10.5, 22.2, 55.4, 66.0, 114.4, 115.8, 127.3, 129.8, 144.3, 161.4, 167.5; GCMS (m/z): 220 $[M]^+$, 178, 161 (base peak), 147, 134, 118, 103, 89, 77, 63, 41.

Reaction of 3 with* n-*butanol to give butyl* p-*methoxycinnamate (6)

In 100 mL Erlenmeyer flask with the cup, compound **3** (890 mg, 5 mmol) was dissolved in 30 mL *n*-butanol and then 0.2 mL H_2SO_4 was added. The reaction mixture in Erlenmeyer flask was put in an ice bath and then irradiated by using the unmodified microwave oven at 300 W for 30 min. The product of the reaction was partitioned by using ethyl acetate and H_2O. Ethyl acetate fraction was further purified by using silica gel column chromatography to give 484 mg of compound **6** as colorless oil (54.4% yield). ^{1}H-NMR ($CDCl_3$, 500): 0.95 (3H, t), 1.42 (2H, m), 1.67 (2H, m), 3.81 (3H, s), 4.19 (2H, t), 6.30 (1H, d, *J* = 16 Hz), 6.88 (2H, d, *J* = 9 Hz), 7.45 (2H, d, *J* = 9 Hz), 7.63 (1H, d, *J* = 16 Hz). ^{13}C-NMR ($CDCl_3$, 125): 13.8, 19.3, 30.9, 55.4, 64.4, 114.4, 115.9, 127.3, 129.8, 144.3, 161.5, 167.5; GCMS (m/z): 234 $[M]^+$, 178, 161(base peak), 134, 121, 89, 77.

Anti-denaturation of heat BSA assay

A Stock solution of 0.2% (w/v) BSA, fraction V of 96% purity (Sigma Chemical Co) was prepared in a mixture of 0.05 M Tris-buffer saline, which was adjusted to pH 6.3 with glacial acetic acid. Ethyl *p*-methoxycinnamate and its derivatives were prepared in methanol at various concentrations. From each stock solution of the sample, of the 0.2% (w/v) stock solution of BSA to produce concentration 0.1, 1, 10, 100 ppm. Each mixture sample was heated for 5 min at 70 °C in a test tube placed in a water bath, then cooled for 20 min under laboratory conditions and its turbidity measured at 660 nm using Hitachi U-2910 spectrophotometer. Sodium diclofenac (Sigma-Aldrich) was used as a standard. The degree of inhibition of denaturation or precipitation of the BSA from the solution by each extract was calculated by using following equation [13-14].

$$\text{Percentage of Inhibition}(\%) = \frac{(\text{OD control} - \text{OD Sample})}{\text{OD control}} \text{ x100\%}$$

RESULT AND DISCUSSION

Isolation and Structural Modifications

Purification of *n*-hexane and ethyl acetate extracts of the rhizome of *K. galanga* resulted in the isolation a major compound ethyl *p*-methoxycinnamate (EPMC) (**1**) [1,10]. Extracts that have been stored in the refrigerator produce a colorless crystal of EMPC (**1**) easily. The residue of extracts that was not crystallized was further purified by using column chromatography to obtain ethyl cinnamate (**2**) as the structures were shown in Fig. 1. Due to the compound **1** was isolated as major compound and has been reported to have a significant anti-inflammatory activity, therefore, this compound was further modified in order to analyze active sites that are responsible for its anti-inflammatory effect. Structural modifications were carried out through re-esterification reaction.

Re-esterification was performed by reacting EPMC (**1**) with methanol, *n*-propanol, and *n*-butanol. In an initial reaction as shown in Fig. 2, EPMC (**1**) was reacted with MeOH using NaOH as a catalyst in a conventional method. This reaction was completed for about 20 h to produce methyl *p*-methoxycinnamate (**4**) in 53.6% yield. An alternative method was then performed in order to re-esterify EPMC (**1**) with MeOH. The reaction was conducted by using the irradiation microwave oven at 300 W for 6 min and NaOH was used as a catalyst. This reaction successfully produced methyl *p*-methoxycinnamate (**4**) in 70.6% yield. So, it can be concluded that re-esterification reaction of EPMC (1) with methanol, which was conducted by using the assistance of microwave oven showed a better

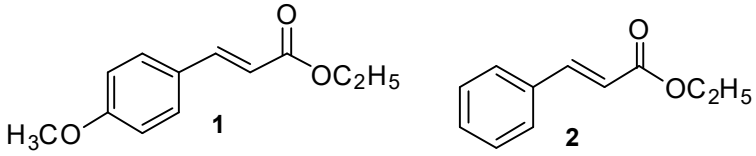

Fig 1. Isolated compounds from Rhizome of *Kaempferia galanga*

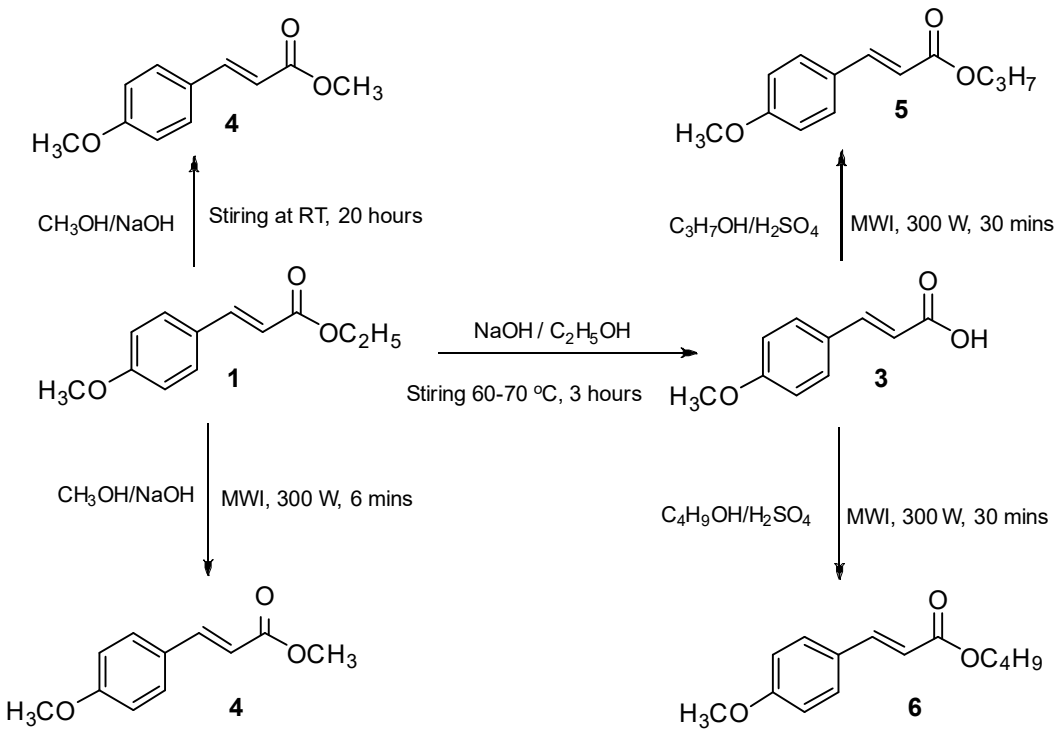

Fig 2. Structural modifications of EPMC (**1**); MWI = Microwave irradiation; RT = room temperature

result in yield and shorter time. Therefore, the further esterification reaction of EPMC (**1**) with *n*-propanol and *n*-butanol were conducted by using the irradiation microwave oven and concentrated H_2SO_4 was used as a catalyst. NaOH was not used as a catalyst due to the limited solubility in *n*-propanol and *n*-butanol. Unfortunately, in this reaction, the starting material does not change completely to the desired products of **5** and **6**, instead, it produced a mixture product that was difficult to be separated chromatographically. Hence, then the re-esterification reaction of EPMC (**1**) was conducted through 2 steps, which was started by hydrolyzing EPMC (**1**) to produce *p*-methoxy cinnamic acid (**3**), and then the hydrolyzed product was esterified with *n*-propanol and *n*-butanol to give compounds **5** and **6**, respectively. Scheme of the reactions was given in Fig. 2. The product of all reactions was monitored and analyzed by using TLC plate and GCMS. The structure of EPMC (**1**) and its derivatives were clarified by using ^{1}H- and ^{13}C NMR and IR spectroscopy. For the known compounds, spectroscopic data were compared to the previously reported.

Structure-Activity Relationship of the Anti-Inflammatory Activity

Isolated compounds and derivatives of EPMC (**1**) were further examined for their anti-inflammatory activity by using anti-denaturation BSA method. This is a simple and inexpensive method for assessing anti-inflammatory properties. In this assay, compounds that are able to inhibit denaturation of BSA greater than 20% over the range of concentrations were considered as having anti-inflammatory properties [13]. As results of bioassay that was shown in Table 1, it indicates that EPMC (**1**) has the ability to inhibit protein denaturation in the concentration range 0.1–100 µg/mL (percentage of inhibition > 20%). This result further supports the previously reported [1,6] which indicates that EPMC (**1**) has anti-inflammatory activity. Hence, it also supports the validity of the use BSA anti-denaturation method as a parameter for finding anti-inflammatory compounds in the early stage of screening. The result of assay indicated that the activity of anti-denaturation of compound **2** was lower than EPMC (**1**). Compound **2** showed activity in the concentration range of 1–100 µg/mL, and its percentage of inhibition at the same concentration was also lower than EPMC (**1**) as shown in Table 1. Hence, it could be suggested that the presence of a methoxy group in the *p*-position of EPMC (**1**) has a contribution to the anti-inflammatory activity.

In order to analyze the effect of an ethyl ester functional group of EPMC (**1**) for its anti-inflammatory activity, then EPMC (**1**) was hydrolyzed to produce compound **3** (Fig. 2). Results of bioassay indicates that compound **3** did not have anti-denaturation activity. So, it can be concluded that ethyl ester is the most important functional group in providing anti-inflammatory activity of

Table 1. Percentage of inhibition denaturation of ethyl *p*-methoxycinnamate and its derivatives

No	Compounds	Concentration (µg/mL)	% Inhibition
1	Ethyl *p*-methoxycinnamate (**1**)	0.1	32.9 ± 0.3
		1	41.3 ± 1.8
		10	44.7 ± 0.5
		100	51.6 ± 1.2
2	Ethyl cinnamate (**2**)	0.1	12.8 ± 1.5
		1	22.1 ± 1.7
		10	22.4 ± 0.7
		100	30.7 ± 1.6
3	*p*-Methoxy cinnamic acid (**3**)	0.1	-
		1	-
		10	-
		100	-
4	Methyl *p*-methoxycinnamate (**4**)	0.1	10.9 ± 1.1
		1	1.2 ± 1.3
		10	-
		100	-
5	Propyl *p*-methoxycinnamate (**5**)	0.1	32.9 ± 1.5
		1	29.7 ± 1.3
		10	22.3 ± 0.5
		100	18.3 ± 0.3
6	Butyl *p*-methoxycinnamate (**6**)	0.1	27.8 ±1.4
		1	21.0 ±1.3
		10	15.7± 1.0
		100	-

Percentage of inhibition denaturation value is represented as mean±SD (n = 3)
(-): Percentage of inhibition denaturation value ≤ 0

EPMC (**1**). Structural modification of EPMC (**1**) was further carried out in order to investigate the kind of ester group that will influence the anti-inflammatory activity. Re-esterification was conducted to produce methyl, propyl and butyl ester of EPMC (**1**). The result of assay indicated that methyl *p*-methoxycinnamate (**4**) did not show anti-denaturation activity. Propyl *p*-methoxycinnamate (**5**) showed anti-denaturation activity in the concentration range of 0.1–10 µg/mL, while butyl *p*-methoxycinnamate (**6**) showed activity in the concentration range of 0.1–1 µg/mL. Interestingly, compounds **5** and **6** did not show activity in the higher concentration (100 µg/mL). For the lower concentration (0.1 µg/mL) both compounds showed almost similar activity to EPMC (**1**). For both compounds **5** and **6,** there is a tendency of decrease activity due to increased concentration. This is likely caused by increased lipophilicity of both compounds **5** and **6** due to increase in the number of C atoms of these compounds. Therefore, both compounds have low solubility in the solution of BSA in the higher concentration and resulted in the increased turbidity in the anti-denaturation assay. Based on this phenomenon, we can suggest that both compounds **5** and **6** will be active as an anti-denaturation agent in the lower concentration. This phenomenon is also found in the previous research which was conducted by Williams et al. which indicated that the anti denaturation activity of the testing compound is greatest at the lower concentration. Normally, anti-denaturation activity was tested in the range concentration of 50 µg/mL to 0.0035 µg/mL [13].

CONCLUSION

In this research, we evaluated the ability in inhibiting of BSA denaturation of EPMC (**1**) and its derivatives in order to analyze the active sites that are responsible for the anti-inflammatory activity. A study on the structure-activity relationship suggested that the ethyl ester functional group of EPMC (**1**) is a most important functional group that is contributing to the anti-inflammatory activity. The other important functional group of EPMC (**1**) is the presence of a methoxy group in the *p*-position. When this functional group is absent, as the structure of ethyl cinnamate (**2**), the anti-inflammatory activity was reduced. A re-esterification reaction that was conducted by using the assistance of microwave oven showed a better result in the yield and shorter time.

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