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ARTICLE

Isolation and Modification of Ethyl-*p*-Methoxycinnamate from *Kaempferia galanga* and Its Antioxidant Activity

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ABSTRACT

Oxidative stress has been associated with the development of many diseases. *Kaempferia galanga*, also known as aromatic ginger, is widely distributed in Asian countries, including Indonesia, and has been traditionally used as an herbal medicine to treat various ailments. Its main constituent, ethyl-*p*-methoxycinnamate (EPMC), has been reported to exhibit various biological activities, including anticancer, anti-inflammatory, and antituberculosis. However, there has been little research on the antioxidant activity of EPMC. Therefore, this study aimed to investigate the antioxidant properties of EPMC isolated from the rhizome of *K. galanga* and its hydrolyzed compound. EPMC was successfully isolated from the *n*-hexane extract of *K. galanga* in a 5.88% yield. Hydrolysis of EPMC under basic conditions resulted in *p*-methoxycinnamic acid (PMCA) in a 21% yield. Results of *in vitro* antioxidant assay showed that PMCA gave stronger scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals with an IC_{50} value of 518.58 ppm compared to EPMC (IC_{50} >1000 ppm). These results featured the importance of the hydroxyl group in enhancing the antioxidant properties of EPMC.

Keywords: Antioxidant activity, ethyl-p-methoxycinnamate, hydrolysis, Kaempferia galanga, p-methoxycinnamic acid.

ABSTRAK

Stres oksidatif telah dikaitkan dengan perkembangan berbagai jenis penyakit. *Kaempferia galanga*, atau yang biasa dikenal sebagai kencur, banyak tersebar di negara-negara Asia, termasuk Indonesia, dan secara tradisional telah banyak digunakan sebagai obat herbal untuk mengobati beragam penyakit. Komponen utama tanaman ini, yaitu etil-*p*-metoksisinamat (EPMS) telah dilaporkan menunjukkan beragam aktivitas biologis, diantaranya sebagai antikanker, antiinflamasi, dan antituberkulosis. Namun, penelitian terkait aktivitas antioksidan dari EPMS masih sangat terbatas. Oleh karena itu, studi ini bertujuan untuk menginvestigasi aktivitas antioksidan dari EPMS yang diisolasi dari rimpang *K. galanga* serta senyawa hasil hidrolisisnya. EPMS berhasil diisolasi dari ekstrak *n*-heksana *K. galanga* dengan rendemen sebesar 5,88%. Hidrolisis EPMS yang dilakukan pada kondisi basa menghasilkan asam *p*-metoksisinamat (APMS) dengan rendemen sebanyak 21%. Hasil pengujian aktivitas antioksidan menunjukkan bahwa APMS memiliki aktivitas peredaman radikal 2,2-difenil-1-pikrilhidrazil (DPPH) yang lebih kuat dengan nilai IC_{50} sebesar 518,58 ppm dibandingkan dengan EPMS (IC_{50} > 1000 ppm). Hasil penelitian ini menunjukkan pentingnya gugus hidroksil untuk meningkatkan ativitas antioksidan dari EPMS.

Kata Kunci: Aktivitas antioksidan; etil-p-metoksisinamat, hidrolisis, Kaempferia galanga; asam p-metoksisinamat.

1. INTRODUCTION

Oxidative stress, a condition in which there is an imbalance between the production of oxidants and antioxidant defence systems in the human body, has been linked to the development of various diseases, such as cancer, inflammation, diabetes, Alzheimer's, and Parkinson's disease [1-2]. During oxidative stress, different reactive species are generated abnormally, like reactive oxygen species (e.g. OH•, H_2O_2, $O_2^{\bullet -}$) and reactive nitrogen species (e.g. •NO, $\bullet NO_2$) that can damage macromolecules, including proteins, nucleic acids, and membrane lipids [1-3].

Many synthetic and dietary antioxidants have been developed as therapeutic agents to treat different human diseases [4]. However, previous studies have shown that some antioxidants were less effective, especially when tested in clinical trials and led to detrimental effects [4-5]. For example, supplementation of vitamin E was found to improve the risk of prostate cancer [5]. Other deleterious effects were also observed in patients with diabetes when exposed to a high intake of antioxidants due to the increase in blood glucose levels [6]. Therefore, antioxidant candidates that can be effective for treating diseases are still continuously sought.

Plants have been a great source of many bioactive compounds that can benefit humans for drug discovery and development. *Kaempferia*, a genus belonging to the family Zingiberaceae, is indigenous to tropical and subtropical Asia and commonly found in Southeast Asia, including Indonesia, Thailand, Malaysia, and East Asia, such as India, China, and Bangladesh [7-9]. *Kaempferia* plants have been traditionally used as herbal medicines to treat cough and cold, headache, fever, swellings, and wound healing in addition to their utility as cosmetics, spices, and flavouring agents [7,10]. *Kaempferia galanga*, commonly known as aromatic ginger, is one of the most widespread *Kaempferia* species in Asian countries and has been used as folk medicine to manage various ailments, including swellings, cough, stomachache, diarrhoea, rheumatic diseases, and infections [7,9-12]. *K. galanga* exhibits a wide range of biological activities such as anticancer [13], antimicrobial [14-15], anti-inflammatory [16], antioxidant [17], and antihypertensive effect [18]. Estimated 49 phytochemicals have been isolated and identified from the rhizome of *K. galanga*, including flavonoids, thiourea derivatives, polysaccharides, diarylheptanoids, phenolic glycoside, phenolic compounds, terpenoids, and esters [10]. The latter two are the major constituents of essential oils of the *K. galanga* rhizome and have been reported to exert therapeutic potential [10,19].

One of the most abundant compounds in essential oils extracted from the rhizome of *K. galanga* was ethyl-*p*-methoxycinnamate (EPMC), which made up 31.77% [9], [20]. EPMC represented promising anti-metastasis activity against melanoma cell B16F10- NFκB Luc2 by inhibiting NFκB and targeting the p38/Akt pathway. Furthermore, the combination of EPMC with paclitaxel could increase the sensitivity of paclitaxel against B16F10 and the toxicity against the SK-Mel 28 melanoma cell line [21]. Lakshmanan et al. documented that EPMC could inhibit *Mycobacterium tuberculosis* H37Ra, H37Rv, and its multi-drug resistant (MDR) strains [22]. Another study also revealed that EPMC significantly inhibited the pro-inflammatory cytokines, including TNF-α and IL-1 [22], thus demonstrating the potential of EPMC to treat inflammatory diseases [23]. Despite the promising biological activities of EPMC, reports concerning the antioxidant activity of EPMC are still limited. Hence, this study aimed to isolate and modify the structure of EPMC from the rhizome of *K. galanga* and evaluate its antioxidant activity.

2. METHODS

2.1 Plant Material

Aromatic ginger (*K. galanga*) was obtained from the local market in Bandung, Indonesia.

2.1 Chemicals and Equipment

Reagents used in this research were solvents for extraction and purification, including *n*-hexane, ethyl acetate, methanol, ethanol, chloroform, acetone, and distilled water. Purified products were checked using thin layer chromatography (TLC) using aluminum plates coated with silica gel GF_{254} and detected by short wavelength ultraviolet light (254 nm). Hydrolysis of ethyl-*p*-methoxycinnamate was carried out in a basic solution using sodium hydroxide and acidified with hydrochloric acid. Antioxidant activity was determined spectrophotometrically using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method and ascorbic acid was used as a reference. Melting points were measured using the Cole Parmer analogue melting point apparatus and are uncorrected. Infrared spectra were recorded using the FTIR spectrometer Shimadzu 8400. Antioxidant activity was measured using the UV-Vis spectrophotometer Shimadzu 1240. ^{1}H NMR spectrum was recorded in the designated solvent using the NMR benchtop Fourier 80 Bruker and chemical shifts are reported in ppm.

2.2 Extraction and Isolation

A total of 4 kg of aromatic ginger was washed, cut, air-dried, and crushed to give powder (0.7 kg). The aromatic ginger powder (0.5 kg) was macerated with *n*-hexane for 3x24 hours. Then, the powder was filtered and the filtrate was concentrated under vacuum to get the crude extract. It was then cooled in an ice bath until the crude EPMC crystals were formed. Subsequently, the resulting crystals were filtered off, dried, and recrystallized from *n*-hexane followed by further recrystallization using a two-solvent system of ethanol and water to afford the pure ethyl-*p*-methoxycinnamate (EPMC) as white crystals (29.40 g). ^{1}H NMR (80 MHz, $CDCl_3$): δ 7.65 (d, J = 16 Hz, 1H, H_β), 7.49 (d, J = 9 Hz, 2H, 2xCH aromatic), 6.91 (d, J = 9 Hz, 2H, 2xCH aromatic), 6.30 (d, J = 16 Hz, 1H, Hα), 4.26 (q, J = 7 Hz, 2H, O–CH_2), 3.84 (s, 3H, O–CH_3), 1.34 (t, J = 7 Hz, 3H, CH_3).

2.3 Hydrolysis Reaction of EPMC

To a suspension of EPMC (2.5 g) in ethanol (5 mL) was added sodium hydroxide solution (1.25 g in 20 mL of water). The mixture was heated to reflux for 30 minutes. The reaction mixture was cooled to room temperature and neutralized with dilute HCl. The precipitated white solid was filtered, washed with water, and dried. Then, it was recrystallized from methanol to give *p*-methoxycinnamic acid (PMCA) as a white powder (0.46 g, 21%).

2.4 Determination of Antioxidant Activities of EPMC and PMCA

Antioxidant activities of EPMC and PMCA were evaluated using DPPH assay according to the method described previously with modifications [3]. DPPH methanol solution (10 ppm; 2.5 mL) was added to EPMC solution in methanol (2.5 mL) at different concentrations (200, 300, 500, and 900 ppm). The mixture was then homogenized and incubated in the dark at room temperature for 30 minutes. The absorbance of the solution was recorded using a UV-Vis spectrophotometer at 515.5 nm. The procedure was repeated for measuring the antioxidant activity of PMCA at various concentrations (100, 400, 700, and 800 ppm). As the blank group, the sample was replaced with 1 mL of methanol mixed with 3 mL of 10 ppm DPPH solution. Ascorbic acid was used as a positive control. Inhibition of the DPPH free radical was obtained from the following formula:

$$Inhibition\ ratio\ (\%) = \frac{A_1 - A_2}{A_1} \times 100\%$$

where A1 is the absorbance of the blank, and A2 is the absorbance of the test compound. The concentration of antioxidants required to inhibit 50% of the DPPH free radical (IC_{50} value) was determined from the plot of inhibition ratio against the sample concentrations, which fit the linear regression.

3. RESULTS AND DISCUSSION

3.1 Isolation and Modification of EPMC

In this study, EPMC was readily isolated from the *n*-hexane extract of *K. galanga* rhizome with a yield of 5.88% after recrystallization. This yield was higher than the previously reported study using other nonpolar solvents, such as chloroform (0.026%) and petroleum ether (1.04%) [23]. The product had a sharp melting point of 49 °C and was unambiguously assigned as EPMC from the ^{1}H NMR spectrum (Figure 1), which indicated the presence of two doublet signals with a large *J* value of 16 Hz seen at δ_H 6.30 ppm and δ_H 7.65 ppm (d, *J* = 16 Hz, Hβ) corresponding to the olefinic protons Hα and Hβ respectively. The presence of oxygenated protons at δ_H 3.84 ppm (s, O–CH_3) and δ_H 4.26 ppm (q, O–CH_2), also two aromatic signals at δ_H 6.91 ppm (d, *J* = 9 Hz) and δ_H 7.49 ppm (d, *J* = 9 Hz) indicating *para*-substituted benzene further confirmed the isolation of pure EPMC crystals from *K. galanga* rhizome. These data also featured a close match to that reported in the literature [21,23].

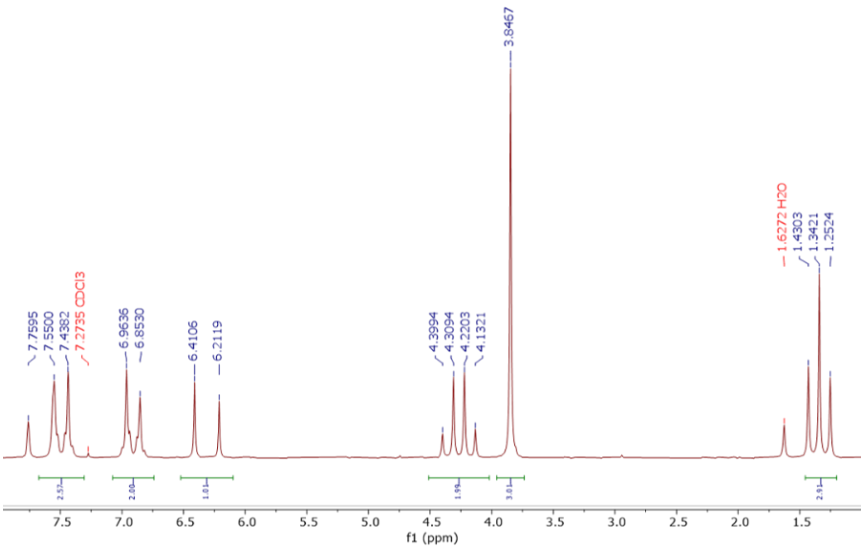
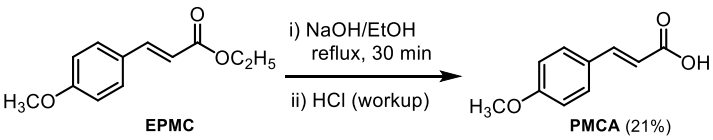


Figure 1. ^{1}H NMR (80 MHz, $CDCl_3$) spectrum of EPMC.

The modification of EPMC was achieved by hydrolyzing it under basic conditions to transform the ester group into a carboxylic acid. Refluxing EPMC in ethanolic sodium hydroxide solution followed by acidification resulted in *p*-methoxycynnamic acid (PMCA) in 21% yield (Scheme 1). Both IR spectra of EPMC and PMCA (Figure 2) exhibit peaks at around 2900 cm^{-1} corresponding to C–H stretching of sp^3 carbons and strong peaks at 1500-1600 cm^{-1} are assigned to C=C stretching, representing aromatic benzene ring. A strong peak at 1712.85 cm^{-1} has been attributed to carbonyl group vibration of the conjugated ester of EPMC. The vibration of the carbonyl group of PMCA appears at 1637.62 cm^{-1}, indicating the conjugated carboxylic acid. Overall, the IR spectrum of PMCA resembles with EPMC, except for the appearance of a broad peak at 3418 cm^{-1} attributed to vibration of O–H stretching.



Scheme 1. Hydrolysis reaction of EPMC under basic conditions.

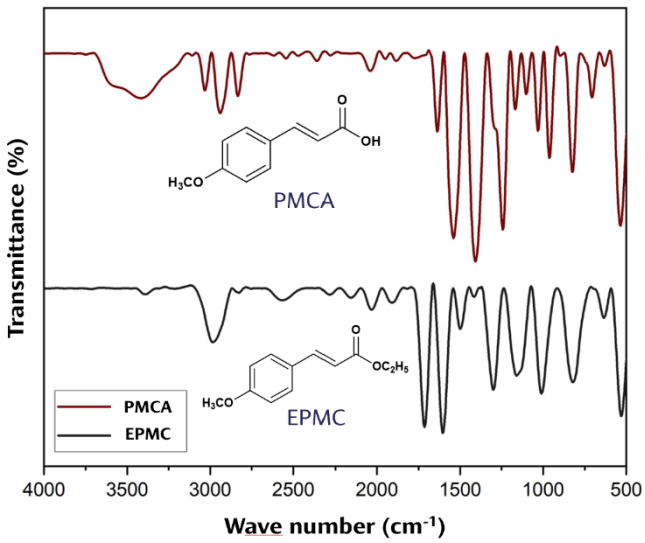


Figure 2. FTIR spectra of EPMC and PMCA.

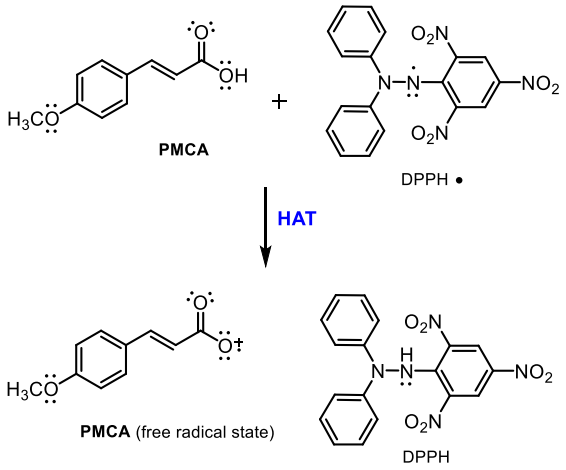
3.2 *In Vitro* Antioxidant Activity of EPMC and PMCA

The antioxidant properties of EPMC and PMCA were evaluated according to their ability to scavenge DPPH radicals. As shown in Table 1, PMCA exhibited stronger antioxidant activity against DPPH radical with an IC_{50} value of 518.58 ppm compared to EPMC (IC_{50} >1000 ppm). Although the antioxidant property of PMCA was approximately 68 times less potent than the ascorbic acid (IC_{50} = 7.66 ppm), this result highlighted the importance of an electron-rich group, such as the hydroxyl group to increase the scavenging effect against DPPH radicals.

The presence of a hydroxyl group in PMCA can enhance the hydrogen-donating ability of this compound, presumably through a mechanism known as hydrogen atom transfer (HAT), thus resulting in stronger antioxidant properties compared to EPMC in the DPPH assay (Scheme 2). This study was in agreement with previous findings on the antioxidant activity of *K. angustifolia* and its chemical constituents reported by Yeap et al., in which hydroxyl groups played an essential role in promoting the antioxidant properties [3]. In another study, Faroosh *et al*. also reported that extra hydroxyl groups led to better antioxidant activity as observed in gallic acid compared to protocatechuic acid [24].

Table 1. Antioxidant activity of EPMC and PMCA measured by the DPPH assay.

Sample	Linear Regression	IC_{50}/ppm
EPMC	y = 0.0022x + 28.633 r^2 = 0.9861	>1000
PMCA	y = 0.0162x + 41.593 r^2 = 0.9901	518.58
Ascorbic acid	y = 8.2412x – 13.156 r^2 = 0.992	7.66



Scheme 2. The proposed mechanism for the reaction between PMCA and DPPH via HAT mechanism.

4. CONCLUSIONS

In conclusion, EPMC has been successfully isolated from the *n*-hexane extract of *K. galanga*. The transformation of the ester group in EPMC through hydrolysis reaction resulted in PMCA bearing a carboxyl group with enhanced antioxidant activity as measured by the DPPH assay. This result enforces the importance of hydroxyl groups to promote antioxidant properties. Further research may involve the introduction of more hydroxyl groups to the EPMC structure to improve its antioxidant activity.

5. AUTHOR CONTRIBUTION

VAN analyzed the data and wrote the original draft. FAH performed the experiments. IM designed the research and reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

6. ACKNOWLEDGMENTS

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