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Cytotoxic Activity of Ethyl-*para*-methoxycinnamate from *Kaempferia galanga* L. on A549 Lung Cancer and B16 Melanoma Cancer Cells

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Abstract

Kaempferia galanga L. belongs to the family of Zingiberaceae, an endangered medicinal plant with pharmacology activities. Ethyl-*p*-methoxycinnamate (EPMC) is an essential phytoconstituent of *K. galanga* rhizomes. Several studies have reported that EPMC has anticancer activities in several cancer cells, including CL-6 gallbladder cancer cells, HepG2 liver cancer cells, MCF-7 breast cancer cells, and Raji lymphoma cancer cells. However, studies on A549 lung cancer and B16 melanoma cancer cells have not been reported. This study aimed to determine the anticancer activity of EPMC against A549 lung cancer and B16 melanoma cancer cells. EPMC was obtained by extraction using *n*-hexane, then recrystallized with chloroform. The isolate was then analyzed by thin-layer chromatography (TLC), and the structure was characterized by Fourier Transform Infrared (FTIR) and Nuclear Magnetic Resonance (NMR) spectroscopy. Cytotoxic activity was determined under Presto Blue assay. Based on the result, EPMC from *K. galanga* showed the cytotoxic effect on B16 cells with an IC_{50} value of 97.09 µg/mL, whereas EPMC showed no significant cytotoxic effect on A549 with an IC_{50} value of 1407.75 µg/mL. It was concluded that EPMC has potential cytotoxic on B16 melanoma cancer cells, but it showed inactive activity against A549 lung cancer cells. Further molecular mechanism underlying EPMC cytotoxic activity needs to be conducted.

1. Introduction

Cancer is one of the leading causes of death in the world. For several decades, lung cancer has been the most common cancer globally. In 2018, the incidence was 12.3% of all cancer diagnose [1]. In Indonesia, lung cancer has the highest incidence in men, with 19.4 per 100,000 population with an average death rate of 10.9 per 100,000 population [2]. Similarly, the worldwide incidence of melanoma, a malignant skin cancer deriving from melanocytes, has increased more rapidly than other cancers [3]. The prognosis for patients with malignant melanoma is bleak, with an average survival time of 6–9 months. Melanoma is responsible for 80% of skin cancer patient mortalities [4].

There are several ways to treat cancer, including surgery, radiation therapy, chemotherapy, and a combination of those treatments. To date, the chemotherapeutic agent is the most common treatment [4]. However, chemotherapy drugs cause side effects such as hair loss, bone marrow suppression, drug resistance, gastrointestinal lesions, neurological dysfunction, and cardiac toxicity [5]. Besides, chemotherapy often causes failure due to the low selectivity of anticancer drugs [6]. Natural compounds represent attractive new drug leads. A recent report revealed that ~50% of all small molecule therapeutics are based on natural products or their derivatives [7]. Alkaloids such as vincristine, vinblastine, and taxol are examples of anticancer drugs that have long been used, and their molecular mechanisms are known [8]. Thus, exploring natural product isolates for cancer treatment needs to be increased to develop anticancer drugs.

Kaempferia galanga L., locally called kencur, is a medicinal plant that belongs to the Zingiberaceae family. It is mostly cultivated in south-east Asian countries, for example, China, Malaysia, Thailand, Indonesia, and India [9]. *K. galanga* rhizomes have been used in traditional medicine to treat several ailments such as fever, amoebiasis, asthma, rheumatism, indigestion, cold and headache, abdominal relief pain and, toothache [9, 10]. Besides, many studies reported that *K. galanga* has pharmacological activities such as antioxidant [11], antituberculosis [12], analgesic and anti-inflammatory [13, 14], hypopigmentation [15], and anticancer [16]. Pharmacological activities of *K. galanga* rhizomes are mainly due to secondary metabolites of different nature [17]. Ethyl-*p*-methoxycinnamate (EPMC) is the essential compound reported as a bioactive secondary metabolite of *K. galanga* rhizome [14].

The potential of EPMC from *K. galanga* rhizome against anticancer activity needs to be explored further. Several previous studies reported that EPMC has anticancer activity against CL-6 bile duct cancer cells [18], HepG2 liver cancer cells [19], MCF-7 breast cancer cells [20], and Raji's lymphoma cancer cells [10]. However, the anticancer activity of EPMC on lung and melanoma cancer cells has not been reported yet. This study aimed to isolate EPMC from *K. galanga* rhizomes and investigated its potential for anticancer activity against A549 lung cancer and B16 melanoma cancer cells. A549 cells are adenocarcinomic human alveolar basal epithelial cells in the lungs responsible for the diffusion of substances such as water and electrolytes in the alveoli. These cells are commonly used as model cells to research respiratory diseases such as lung cancer [12]. B16 cells are derived from epithelial mice that produce melanin. Melanin has many living systems functions, and its synthesis alterations occur in many disease states [21]. Although melanin's primary function is to protect skin from UV-induced damage, melanin pigment can also regulate epidermal homeostasis and thus can affect melanoma behavior [22].

2. Methodology

The rhizome part of *K. galanga* was obtained from Sukawening, Garut, West Java. The samples were authenticated in the Biology Department herbarium, Universitas Padjadjaran, Indonesia (Ref. No. 37/HB/02/2021). A549 lung cancer cell and B16 melanoma cancer cell were culture collection of Central Laboratory, Universitas Padjadjaran, solvent DMSO (Sigma-Aldrich Chemical Company, St. Louis, MO, USA); Complete medium of A549 cells consisting of RPMI (Rosewell Park Memorial Institute) 1640 (Gibco, Life Technologies, USA), Penicillin-Streptomycin 1% (v/v), FBS (Fetal Bovine Serum) 10% (v/v) (Gibco, Life Technologies, USA) and fungizone 0.5% (Sigma-Aldrich Chemical Company, St. Louis, MO, USA); Trypsin-EDTA 0.25% (Sigma-Aldrich Chemical Company, St. Louis, MO, USA), PBS (Phosphate Buffer Saline), distilled water, *n*-hexane, ethyl acetate, chloroform.

2.1. Isolation and Characterization of ethyl-p-methoxycinnamate

The rhizome of *K. galanga* was washed with water, then dried in an oven at 35°C. After drying, the sample was ground with a mechanical blender. *K. galanga* rhizome dry powder (820 g) was then extracted using 2 L of *n*-hexane solvent by maceration method for 3x24 hours at 25°C. The macerated extract was then filtered using a filter funnel and then concentrated using a vacuum rotary evaporator at 45°C. The concentrated *n*-hexane extract was purified by recrystallization. An impure EPMC crystal was mixed with hot chloroform as a solvent to form a saturated solution to perform recrystallization. The resulting crystals were tested for purity using Thin Layer Chromatography (TLC), Fourier Transform Infra-Red (FTIR) spectroscopy, and Nuclear Magnetic Resonance (NMR).

2.2. Cell Culture

These two-cancer cells, A549, and B16 cells were obtained from the Laboratorium Sentral of Universitas Padjadjaran. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were inoculated at a density of $1x10^5$ cells in a 6-well plate and were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

2.3. Cytotoxic Test with Presto Blue Assay Method

EPMC compound was tested for their cytotoxic activity against A549 and B16 cells using the Presto Blue assay method. A number of $1x10^4$ cells were distributed into the 96-well plates and incubated in a 5% CO_2 incubator at 37°C for 24 hours. EPMC was dissolved in 10% (v/v) of DMSO co-solvent, then it was added to 96-well plate with eight concentration series, namely 1000; 500; 250; 125; 63.5; 31.25; 15.625; 7.8125 µg/mL for 24 hours. Cisplatin was used as a positive control. At the end of incubation, the media and extract were discarded and then added with 100 µL of PrestoBlue ™ Cell Viability with a ratio of 1: 9. Cells were incubated again for 1–2 hours until a color change was seen in a 5% CO_2 incubator at 37°C. Living cells will reduce Presto Blue from the blue compound resazurin to the red compound resorufin. Absorption was measured at 570 nm and 600 nm (reference wavelength) using a multimode reader [23].

3. Result and Discussion

3.1. Ethyl-p-methoxy cinnamate isolation

EPMC is found as a major ester and easily isolated compound from the rhizome of *K. galanga* [24]. Purification of *n*-hexane extracts of the rhizome of *K. galanga* produced a white crystal of EPMC (76.9 g). In case, the percentage of yield was 49.99%. EPMC isolate was then confirmed for purity using Thin Layer Chromatography (TLC). The stationary phase in TLC was in the form of a GF_{254} silica gel plate, while the mobile phase used was *n*-hexane and ethyl acetate with a ratio of 8:2.

3.2. FTIR Characterization

The Infra-red spectra of the isolated compound showed a very intensely broad peak at 3450 cm^{-1} for O–H bond vibrations, and moderately intense peaks at 1510 and 1600 cm^{-1} were observed for the aromatic part of C=C vibration. The unsaturated C–H vibration was observed at 2900 cm^{-1}. This is supported by the presence of absorption at wavenumbers 1367 cm^{-1} and 1417 cm^{-1}, which indicates the presence of CH_3 groups. The C–O ether group and C–O methoxy group's vibrations were observed at 1200 cm^{-1} and 1100 cm^{-1}. The para substitution of aromatic was shown at 820 cm^{-1}, which confirms the structure of EPMC.

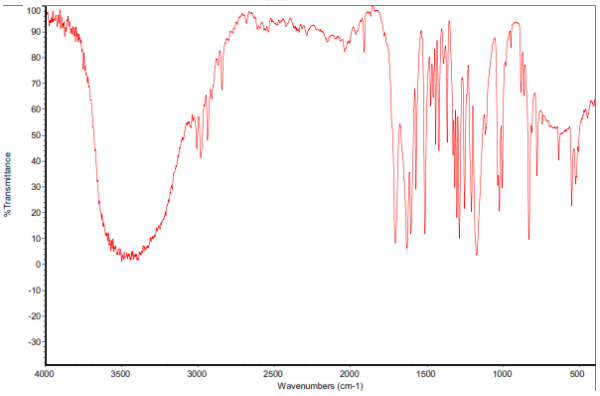

Figure 1. FTIR spectra of EPMC

3.3. NMR Characterization

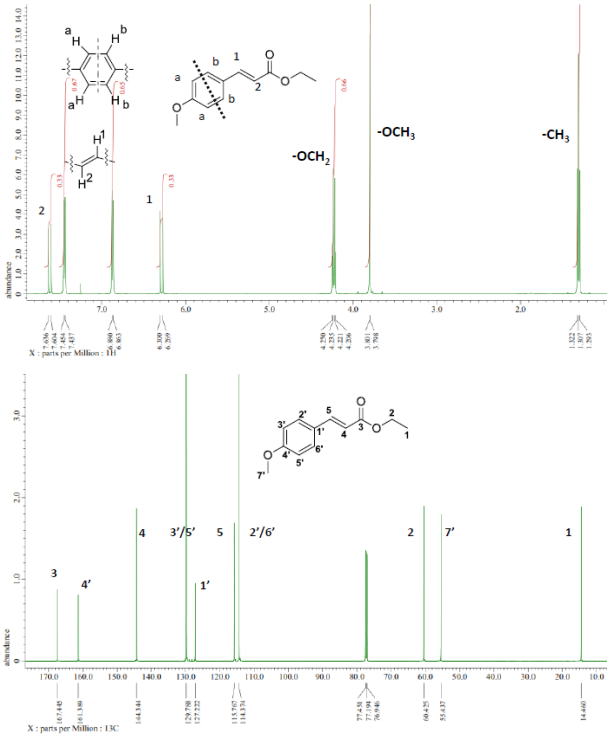

Figure 2. ^{1}H-NMR spectra (A), and ^{13}C-NMR spectra of EPMC (B)

Based on the ^{1}H-NMR spectrum (Figure 2A) of EPMC, two doublet peaks of proton signal indicate a para-substituted aromatic ring on δ 7.454 ppm and 6.880 ppm. The appearance of two doublet peaks at δ 7.636 ppm and 6.300 ppm shows an alkene proton's signal. Also, other proton signals at δ 4.250 ppm and 1.322 ppm indicate an ethoxy group (-OCH_2CH_3), and at δ 3.801 ppm, it indicates the methoxy group (-OCH_3).

The ^{13}C-NMR spectrum (Figure 2B) shows seven C-sp^2 signals in a range of δC 167.445–114.375 ppm, which are specific signals for C=O in ester group (δ 167.445 ppm), oxyaryl (δ 161.389 ppm) and C=C in an aromatic ring (δ 144.344 ppm (C-4'), δ 129.788 ppm (C-3'/5'), δ 127.222 ppm (C-1'), δ 115.767 ppm (C-5) and δ 114.344 ppm (C-2'/6')). Besides, the ^{13}C-NMR spectrum also shows three C-sp^3 signals at δ 60.425 ppm (-OCH_2), δ 55.437 ppm (-OCH_3), and δ 14.460 ppm (-CH_3).

To ensure the appropriate obtained structure of EPMC, we compared our original spectrum values with other spectra that had been reported. The comparison between original and reference spectrum values can be seen in Table 1 and Table 2.

Table 1. Comparison between isolated and reference EPMC chemical shift of ^{1}H-NMR

Signal of H	δ_H (multiplicity) of isolated EPMC	δ_H (multiplicity) of reference EPMC [14]
CH alkene	7.636 (1H, d, 1xCH alkene)	7.65 (1H, d, 1xCH alkene)
CH benzylic	7.454 (2H, d, 2xCH benzylic)	7.42 (2H, d, 2xCH benzylic)
CH benzylic	6.880 (2H, d, 2xCH benzylic)	6.90 (2H, d, 2xCH benzylic)
CH alkene	6.300 (1H, d, 1xCH alkene)	6.31 (1H, d, 1xCH alkene)
-CH_2	4.250 (2H, q, 1x-OCH_2)	4.25 (2H, q, 1x-OCH_2)
-OCH_3	3.801 (3H, s, 1x-OCH_3)	3.82 (3H, s, 1x-OCH_3)
CH_3	1.322 (3H, t, 1xCH_3)	1.32 (3H, t, 1xCH_3)

Table 2. Comparison between isolated and reference EPMC chemical shift of ^{13}C-NMR

Signal of C	δ_C of isolated EPMC	δ_C of reference EPMC [14]
C=O	167.445	167.4
Ar-C	161.389	161.3
CH alkene	144.344	144.2
Ar-C	129.788	129.7
Ar-C	127.222	127.3
CH alkene	115.767	115.7
CH_2 benzylic	114.344	114.3
-CH_2	60.425	60.2
-OCH_3	55.437	55.3
-CH_3	14.460	14.5

Tables 1 and 2 showed that the NMR data for isolated EPMC had similarities with the NMR reference. It can be seen that the spectrum value of isolated EPMC approached to spectrum value of the reference. In order that we conclude that the structure of isolated EPMC was the same as the reference.

3.4. Cytotoxicity Activities of A549 Lung Cancer and B16 Melanoma Cancer Cells

To examine whether EPMC has anticancer activity, the cytotoxic activity of EPMC against A549 lung cancer and B16 melanoma cancer cells was performed. The analysis was done using the Presto Blue assay method described in the Methodology section. PrestoBlue has been developed for detecting cell-mediated cytotoxicity in vitro. It is a resazurin-based compound converted to the reduced form by mitochondrial enzymes of viable cells in the tested systems. Boncler *et al.* [25] stated that PrestoBlue assay offers a new alternative to MTT for analyzing cell viability. The assay was fast, simple, and allows continuous monitoring of cultures. Thus, we decided to use the Presto Blue assay method for cytotoxic activity assessment.

Either B16 cell and A549 cell treated with EPMC with various concentration of 1000; 500; 250; 125; 63.5; 31.25; 15.625; 7.8125 µg/mL. Furthermore, the IC_{50} value was acquired as the EPMC concentration parameter to inhibit 50% A549 and B16 cell growth. Figures 3A and 4A showed the effect of EPMC on A549 and B16 cell viability for 24 h treatment. Linear regression of EPMC concentration against corrected absorbance (Fig. 3A) gave a 97.09 µg/mL value. Treatment of EPMC on B16 cells showed cell growth inhibition in a concentration-dependent manner. Interestingly, we did not observe the decrease of A549 cell growth treated with EPMC with an IC_{50} value of 1407.75 µg/mL. This IC_{50} value can be obtained even though the concentration tested was 1000 µg/mL due to poorly linear regression of EPMC concentration toward corrected absorbance of A549 cells (Fig. 4A). Meanwhile, we evaluated cisplatin's cytotoxic activity against A549 cells and B16 cells, given an IC_{50} value of 18 µg/mL 53 µg/mL, respectively. Prayong *et al.* [26] stated that cytotoxic activity was categorized into three depending on the IC_{50} value, which are potential cytotoxic (IC_{50} < 100 µg/mL), moderate cytotoxic (100 µg/mL < IC_{50} < 1000 µg/mL), and weak cytotoxic activity (IC_{50} > 1000 µg/mL). According to that statement, we suggest that EPMC had no cytotoxic activity on A549 cells. Simultaneously, it was cytotoxic on B16 cells, even though the IC_{50} value of EPMC against B16 cells was still higher than cisplatin. However, EPMC has the potential to be developed as an anticancer agent on B16 cells.

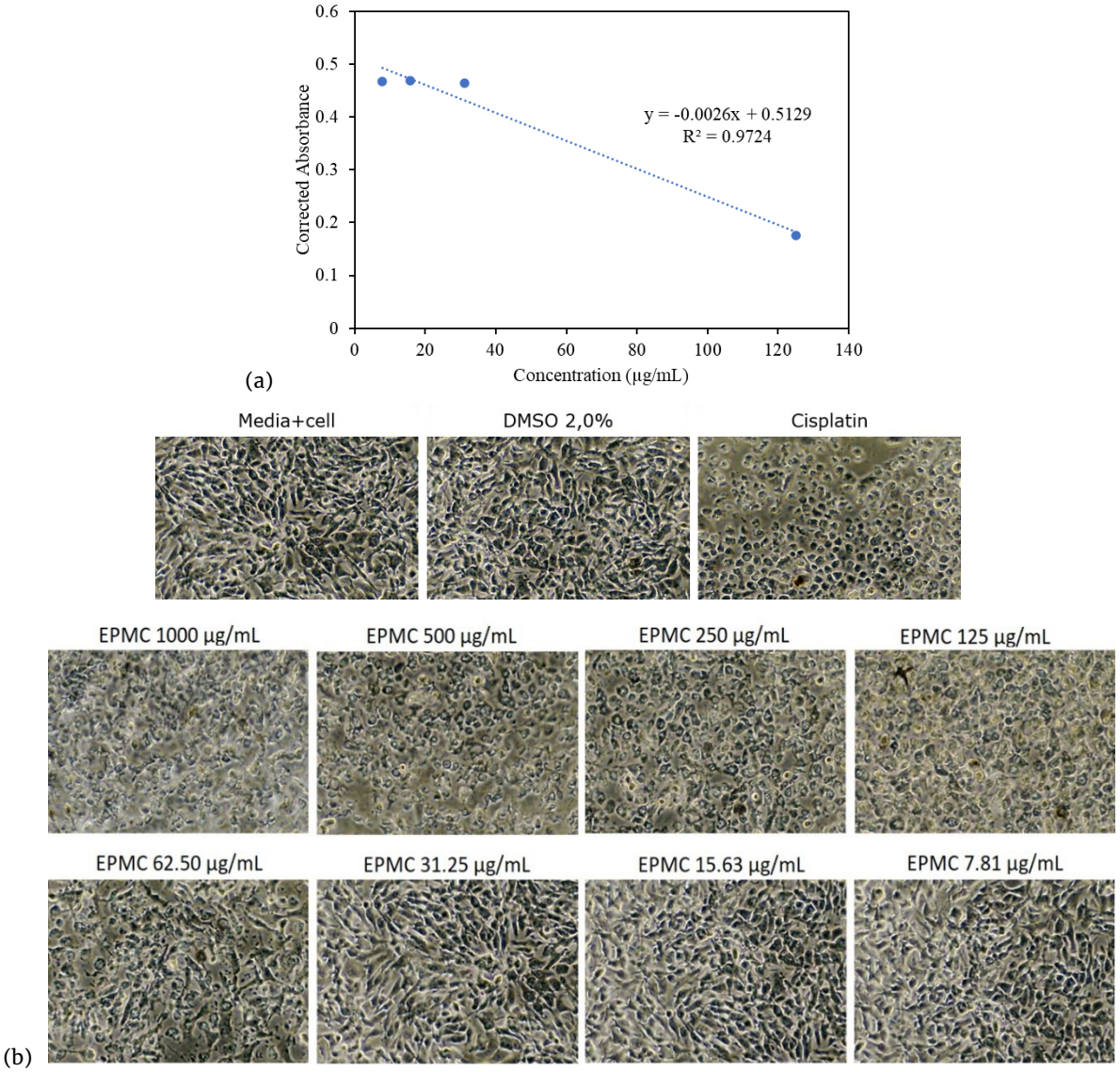

Figure 3. Cytotoxic effects of EPMC on B16 cells. (A) B16 cells were treated using EPMC for 24 h and determined by Presto Blue assay. (B) Morphological changes of B16 cells were observed using an inverted microscope.

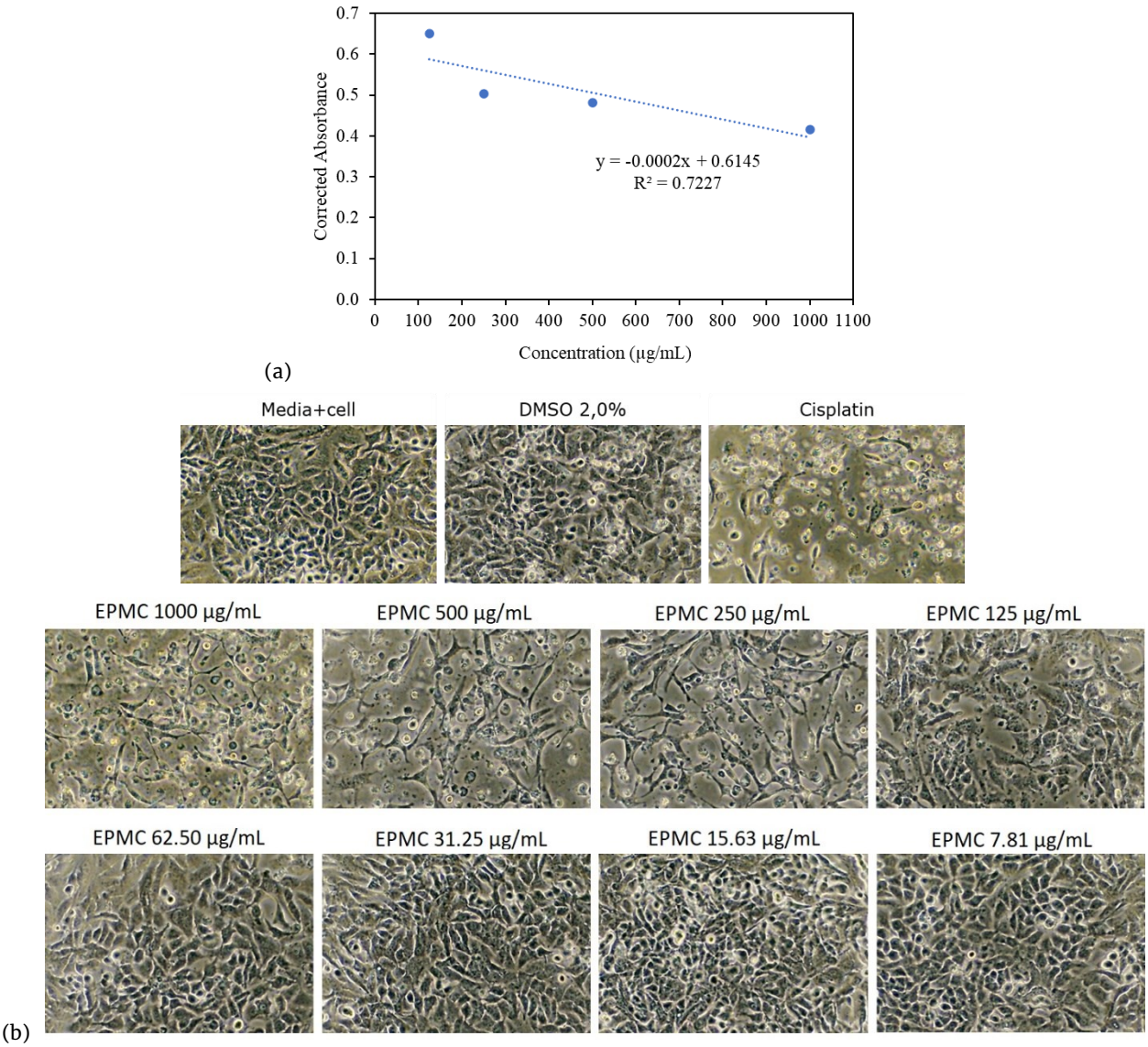

Figure 4. Effects of EPMC on cell viability and morphological changes on A549 cells. (A) A549 cells were treated using EPMC for 24 h. There is no significant decrease in A549 cell growth. (B) Morphological changes of A549 cells were observed using an inverted microscope.

Based on the observation under an inverted microscope, we found that EPMC altered cell morphology on B16 cells. An increase in EPMC concentration caused more cells to undergo morphological alteration. Viable cells exhibited epithelial shape, but after being treated with a specific concentration of samples, the cells exhibited cell shrinkage and rounding (Figure 3B). Supports its noncytotoxic activity, treated cells of A549 did not show any changes in cell morphology (Figure 4B). A549 cells retain their original morphology that showed a long fusiform shape, small size, clear cell boundaries, well-adherent pebble-like growth, placental cytoplasm, and less cytoplasmic granules.

Several studies of EPMC cytotoxic activities on different cancer cells have been reported. Amuamuta *et al.* [18] reported the IC_{50} value of EPMC against CL-6 and OUMS cancer cells were 49.16 µg/mL and 103.18 µg/mL. Anticancer activity of EPMC against the HepG2 cell line given IC_{50} value of 27.1 µg/mL exhibits antiproliferative properties, inducing the significant increase of subG0 cell population [19]. In addition, EPMC against PC-3 and HCT-116 cells showed IC_{50} value of 39.0 µg/mL and 42.1 µg/mL, respectively [27]. EPMC gave the IC_{50} value on various types of cancer cells was quite different. We suggest it was caused due to the difference in the genotype and phenotype of these cancer cells.

4. Conclusion

In this research, we have isolated ethyl-*p*-methoxycinnamate from *K. galanga,* with the percentage of yield was 49.99%. ^{1}H-NMR and ^{13}C-NMR analysis confirmed the structure of EPMC, and the spectrum was similar to the reference. The cytotoxic activity of EPMC was evaluated against two cancer cells, B16 cells, and A549 cells. EPMC showed a cytotoxic effect on B16 cells with an IC_{50} value of 97.09 µg/mL. However, EPMC showed no significant cytotoxic activity of A549 lung cancer cells with an IC_{50} value of 1407.75 µg/mL. Considering that EPMC can be an anticancer agent against B16 cells, further research on the cytotoxic mechanism of EPMC against B16 cells and its selectivity on normal cells needs to be investigated.

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