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FULL ARTICLE

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Cytotoxic effects of *Etlingera pavieana* rhizome on various cancer cells and identification of a potential anti-tumor component

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Abstract

Much effort over the last several decades has been made to identify new anticancer agents, preferably with reduced adverse side effects. In this study, cytotoxic activity of rhizome extract from *Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm. was assessed. We demonstrate that ethanolic extract (EE) of *E. pavieana* rhizome shows reduced viabilities of several cancer cell lines in dose- and time-dependent manners. Following cytotoxic-guided isolation of EE, the bioactive compound *trans*-4-methoxycinnamaldehyde (4-MCA) was identified. 4-MCA exhibited a dose-dependent inhibitory effect on cancer cell growth with the IC_{50} values ranging from 34 to 40 μM, which are approximately four times lower than that of noncancerous cells. This antiproliferative effect might be due to its cooperative actions of inducing apoptosis and arresting cell cycle at G2/M phase. To the best of our knowledge, this is the first report on the in vitro antitumor promoting activity of 4-MCA.

Practical applications

Rhizome of *E. pavieana* (Zingiberaceae) has been widely used as spices and a medicinal plant in Thailand, Cambodia, Laos, and Vietnam. In this study, we demonstrated the medicinal role of this plant on suppressing the growth of various cancer cells of common cancer types including those of breast, cervix, liver, and colon/rectum. Moreover, we verified that this action was, at least in part, due to one of its constituents, 4-MCA. Overall results demonstrated the potential cytotoxic activities of 4-MCA toward cancer cells, including inhibition of cell proliferation and colony formation, induction of apoptosis, and cell cycle arrest whereas it exhibited negligible effect on noncancerous cells. The comprehensive characterization of *E. pavieana* rhizome performed in this study, highlights the pharmaceutical potential of 4-MCA, one of its major constituents, to be a promising candidate for future development of new cancer regimen.

KEYWORDS

apoptosis induction, cell cycle arrest, cytotoxicity, *Etlingera pavieana*, 4-methoxycinnamaldehyde

1 | INTRODUCTION

Cancer is an important disease and imposes high mortalities on human populations. Chemotherapy is routinely used in cancer treatment in combination with surgery and/or radiotherapy (Devita & Chu, 2008). The efficiency of chemotherapeutic drugs is recognized, however, adverse side-effects from damage to normal cells often restrict their usage. Treatment with appropriate natural products may reduce this problem. Presently, extracts and bioactive compounds from a number of edible plants have demonstrated antitumor activity (Butt, Naz, Sultan, & Qayyum, 2013; Rao & Gan, 2014; Vallianou, Evangelopoulos, Schizas, & Kazazis, 2015).

The spices belong to a group of plant products which are globally consumed because of their accessibility and low price. Besides favoring

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food, many spices have a long history of use in traditional medicine due to their useful pharmaceutical properties such as anticancer, antimicrobial, antioxidant, and anti-inflammatory activities (Chen et al., 2008; Kumar, Asish, Sabu, & Balachandran, 2013; Stanić, 2017).

Etlingera pavieana (Pierre ex Gagnep.) R.M.Sm. is indigenous to Thailand, Cambodia, Laos, and Vietnam, and is among aromatic Thai spices in the Zingiberaceae or ginger family with useful pharmaceutical properties (Elkady, Abuzinadah, Baeshen, & Rahmy, 2012; Troselj & Kujundzic, 2014). The rhizome is used as an ingredient in noodle soup and curries, or boiled in water and taken a solution as a tonic drink and its young shoot as a component of salad (Poulsen & Phonsena, 2017). In traditional medicine, parts of *E. pavieana* are used to treat fever, cough, diuresis, and flatulence (Tachai & Nuntawong, 2016). A recent study has demonstrated the toxicity of dichloromethane extract and an essential oil of *E. pavieana* rhizome against human lung NCI-H187 cancer cells and the isolated compound, (*E*)-((*E*)-3-(4-methoxyphenyl)allyl) 3-(4-hydroxyphenyl) acrylate (or 4-methoxycinnamyl *p*-coumarate) on human oral cavity cancer KB, breast cancer MCF7, and lung NCI-H187 cancer cells as well as *Mycobacterium tuberculosis* (Tachai & Nuntawong, 2016). Recently, an anti-inflammatory activity of the ethyl acetate (EA) fraction from its ethanolic extract has been revealed. The extract has been shown to prevent the LPS-stimulated inflammation in RAW264.7 macrophage by inhibiting NF-κB signaling pathway, leading to down-regulated iNOS expression and decreased NO production (Srisook, Palachot, Mankhong, & Srisook, 2017). Four bioactive phenolic compounds (4-methoxycinnamyl alcohol, *trans*-4-methoxycinnamaldehyde, *p*-coumaric acid, and 4-methoxycinnamyl *p*-coumarate) were isolated from this EA fraction (Srisook et al., 2017).

In this study, the cytotoxic effects of *E. pavieana* rhizome extract were demonstrated in several types of common cancer cell lines including breast, colon, liver, and cervical cancer cell lines, in comparison with noncancerous cells. Following our cytotoxic-guided isolation, *trans*-4-methoxycinnamaldehyde (4-MCA) was identified as one of the major bioactive compounds. We have shown in vitro, potential antitumor promoting activities of the compound, including antiproliferation, inhibition of colony formation, blocking of cell cycle progression, and apoptotic inducing activity.

2 | MATERIALS AND METHODS

2.1 | Plant materials and extraction

Fresh rhizomes of *E. pavieana* (The Plant List Record 244738) were collected from a local farm in Khung district, Chantaburi province, Thailand in October 2015 and a voucher specimen (KS-SCBUU-0012-1) was deposited in the Faculty of Science, Burapha University. Plant identification was authenticated by B. Chewprecha, Department of Biology, Faculty of Science, Burapha University. Rhizomes were washed with tap water, cut into thin pieces, oven dried at 50°C and homogenized in a blender. The crude ethanolic extract (EE) was obtained by macerating the rhizome powder (1.88 kg) three times in 95% ethanol (1 g: 10 L) for 15 days followed by filtration (Buchner funnel) under vacuum. The solvent was removed by rotary evaporation.

The EE (167.07 g) was successively partitioned with hexane, ethyl acetate, and water to give hexane (HF, 7.61 g; 4.55% w/w), ethyl acetate (EF, 13.46 g; 8.06% w/w), and water (WF, 39.71 g; 23.77% w/w) soluble fractions. EF (13.46 g) which exhibited the highest growth inhibitory activity was then subjected to silica gel (0.040–0.063 mm) column chromatography using 0.5–10% (v/v) methanol in dichloromethane as a mobile phase to yield five sub-fractions (SF1 0.13 g, 0.97% w/w; SF2 0.26 g, 1.93% w/w; SF3 0.08 g, 0.59% w/w; SF4 0.07 g, 0.52% w/w, and SF5 5.60 g, 41.60% w/w). DMSO was used to dissolve all extracts and nontoxic concentration of 0.2% (v/v) DMSO was used as control for all biological tests.

2.2 | Identification of isolated compound from *E. pavieana*

Due to small amount of SF1 sub-fraction, identification of containing compounds in this sub-fraction was primarily evaluated by ^{1}H-NMR spectroscopy (Bruker AVANCE 400, Switzerland) in $CDCl_3$ solvent and compared with published data (Battistuzzi, Cacchi, & Fabrizi, 2003; Srisook et al., 2017). The major phytochemical compound in SF1 was confirmed by HPLC using a Waters 600 HPLC pump and controller in-line degasser coupled to a Waters 996 photodiode array detector, utilizing a RP-18 analytical column 250 mm × 4.6 mm Luna, Phenomenex, Inc. (Waters, The United States). Standard 4-methoxycinnamaldehyde (4-MCA; Pubchem CID: 641294) was purchased from Sigma-Aldrich (MO). The mixture was eluted with acetonitrile/water (30:70) for 40 min with a flow rate of 1.2 mL/min at ambient temperature. Standard or extract solution (20 μL) was injected and detected at 320 nm for each experiment. After that, 4-MCA was isolated from SF1 by HPLC technique, with the condition as described above. The chemical structure of the isolated compound was elucidated using ^{1}H-NMR spectroscopy.

2.3 | Cell culture

Seven human cancer cell lines (hepatoma HepG2, colorectal carcinoma HCT116, breast adenocarcinoma MCF-7 and MDA-MB-231, and cervical carcinoma SiHa, HeLa, and C33A), as well as noncancerous Vero cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in humidified air containing 5% CO_2. All culture reagents were purchased from Gibco (NY).

2.4 | Cell viability assay

Effects of *E. pavieana* extracts/compound on cell proliferation were determined by MTT assay in 96-well plate (2,500 or 5,000 cells/well) (Cheng, Tian, Tang, Shen, & Yao, 2016). After incubation for 24, 48, or 72 hr, the medium was changed into MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Applichem, Germany) containing medium (0.05 mg/mL of MTT in 100 μL DMEM) and incubated for additional 3 hr. Purple formazan products were dissolved in 200 μL DMSO and subjected to absorbance measurement at 540 nm. Cytotoxic effect of extracts/compound was expressed as % cell

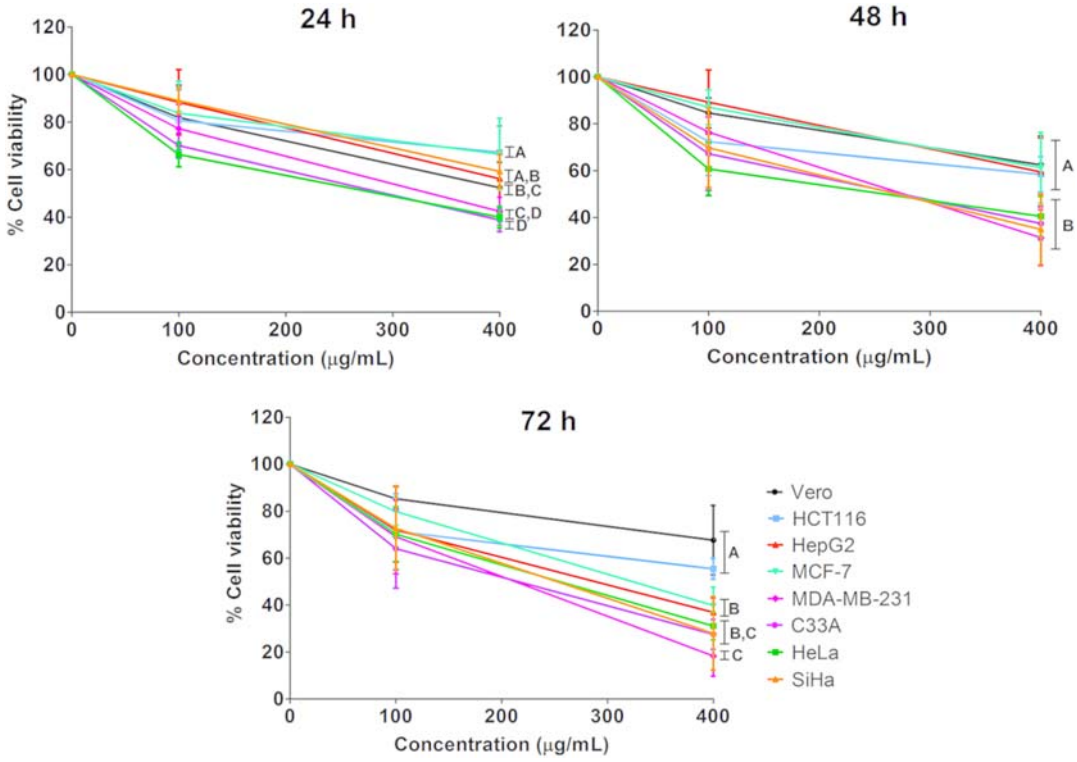

FIGURE 1 Effects of *E. pavieana* EE on cell viability as determined by MTT assay. Cells treated with 100 or 400 μg/mL of EE for 24, 48, or 72 hr were compared with those treated with 0.2% DMSO which was set to 100%. Data are presented as means ± *SD* of three independent experiments ($n = 9$). Groups of cells which do not share the same letter (A, B, C, or D) are significantly different ($p < .05$). Cytotoxic effects of additional doses of EE against all cell types are given in Supporting Information material (Supporting Information Figure S1)

viability which was normalized against cells treated with 0.2% (v/v) DMSO (100%) or as IC_{50} (concentration that could inhibit cell proliferation by 50%). Cisplatin and doxorubicin (Pfizer, Australia) were used as positive controls. All graphs were plotted using GraphPad Prism® version 5 (GraphPad Software, Inc., CA).

2.5 | Clonogenic cell survival assay

Capacity to produce cell progeny after treatment with 4-MCA was also assessed as described earlier with some modifications (Wanichwatanadecha, Sirisrimangkorn, Kaewprag, & Ponglikitmongkol, 2012). Cells,

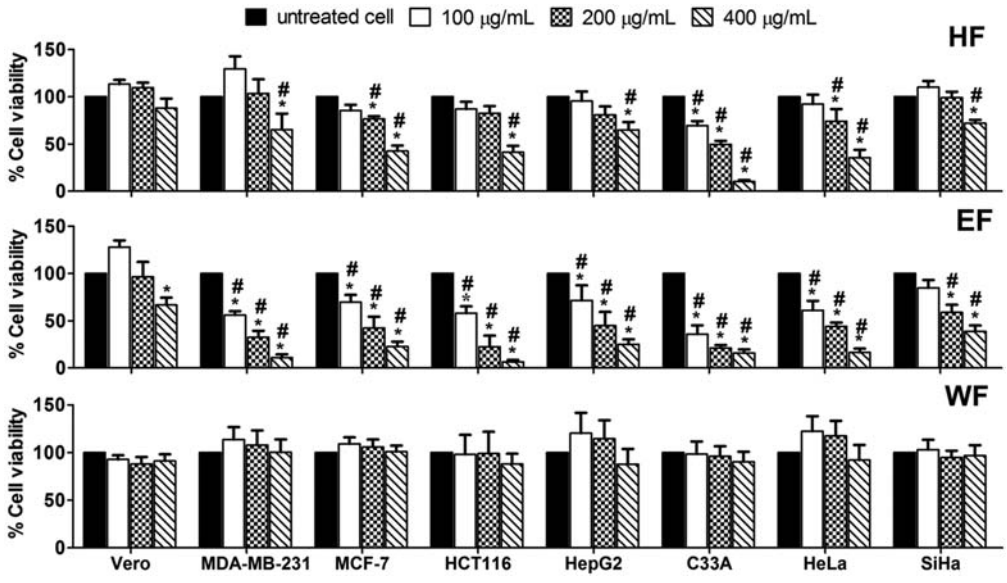

FIGURE 2 Viability of cells treated with HF, EF, or WF at 100, 200, or 400 μg/ml for 72 hr as determined by MTT assay. Results are shown as percentages of cell viability relative to untreated cell (0.2% DMSO). Data are expressed as means ± *SD*. Statistical significance was accepted at $p < .05$ when (*) was compared between treated and untreated cells, and (#) between cancer and Vero cells under the same treatment

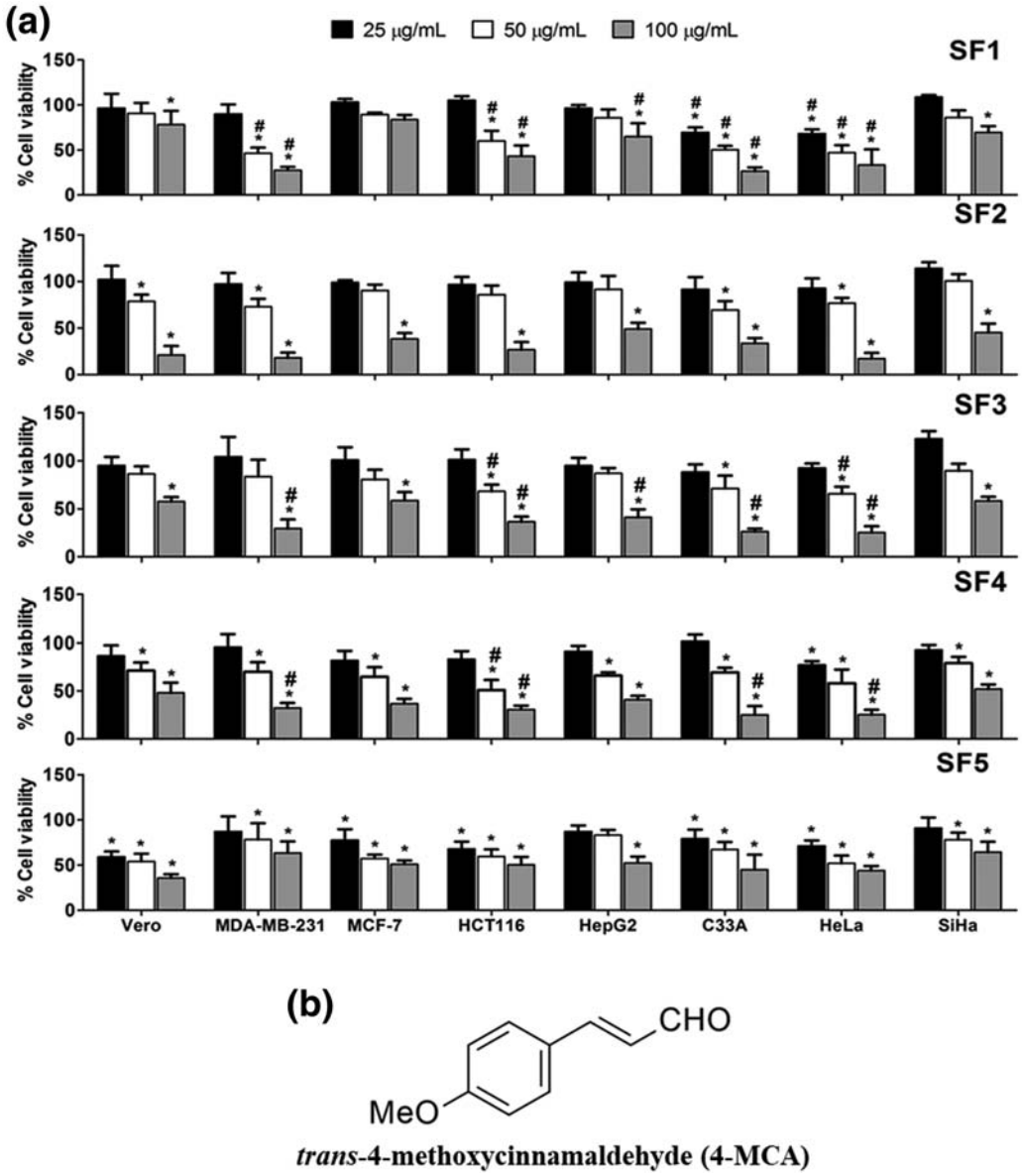

FIGURE 3 Effects of EF subfractions on cell survival and its bioactive compound. (A) % viability of cells exposed to various doses of SF1–SF5 for 24 hr relative to their untreated cells (100%, not shown) determined by MTT assay. Data are expressed as means ± *SD*. Statistical significance was accepted at $p < .05$ when (*) was compared between treated and untreated cells, and (#) between cancer and Vero cells under the same treatment. (B) Chemical structure of the bioactive compound (4-MCA) purified from SF1

500 each of MDA-MB-231 and C33A, were seeded onto a 6-cm plate and incubated in 12.5, 25, and 50 μM of 4-MCA for 24 hr. After replacement with compound-free medium, cells were grown for an additional 96 hr, stained with 0.4% trypan blue solution (Gibco; NY) and cell colonies (≥8 cells) were counted.

2.6 | Analysis of DNA fragmentation

Induction of apoptosis by 4-MCA was determined on the basis of internucleosomal DNA cleavage from the presence of DNA ladder using agarose gel electrophoresis. MDA-MB-231 cells of 1.5×10^6 in 6-cm plates were treated with 0.2% (v/v) DMSO (negative control), 1 mg/mL of SF1, or 1 mM of commercial 4-MCA for 72 hr. Genomic DNA was extracted using FavorPrep Tissue Genomic DNA Extraction Mini Kit (Favorgen; Changzhi, Taiwan) following the manufacturer's procedure and resolved in 1.5% agarose gel. After staining with ethidium bromide, DNA was visualized by UV light using InGenius gel documentation system (Syngene; Cambridge, The United Kingdom).

2.7 | Annexin V/7-AAD assay

In order to determine if 4-MCA induced toxicity through apoptotic process, dual cell staining with Annexin V-FITC and 7-amino-actinomysin (7-AAD) was performed. C33A and MDA-MB-231 cells (1×10^5) were treated with 4-MCA at their IC_{50} values or 0.2% (v/v) DMSO for 72 hr. Total cells were harvested by trypsinization and then live and apoptotic cells were evaluated by using the Muse® Annexin V and Dead Cell Kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer's

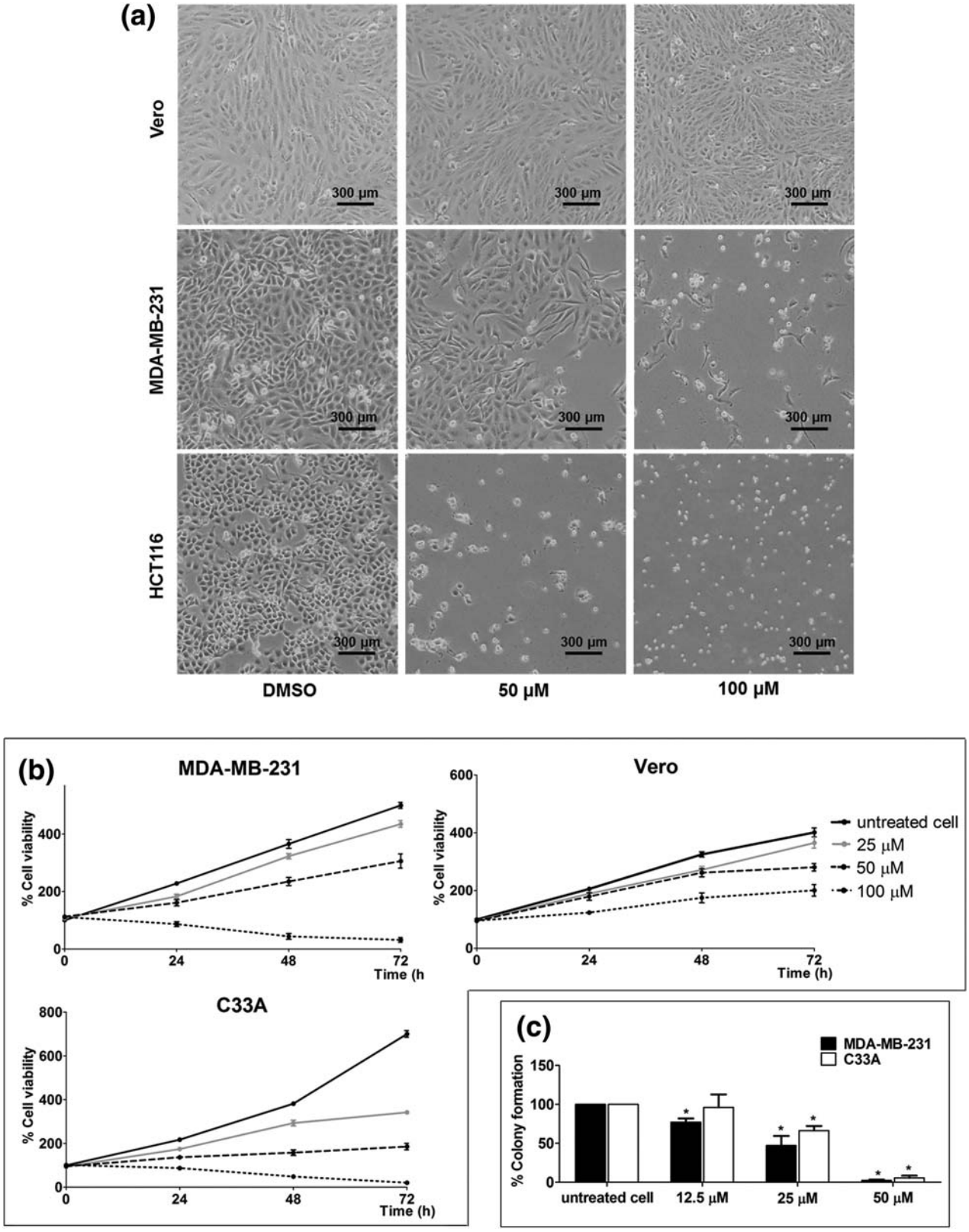

FIGURE 4 Effects of 4-MCA on Vero and cancer cells. (A) Cell morphology and cell number after 72 hr treatment with 50 or 100 μM 4-MCA. The photographs were taken under inverted microscope at 10× objective lens magnification. The scale bar represents 300 μm. (B) Cells were treated with various doses of 4-MCA for 0, 24, 48, or 72 hr and viable cells were determined by MTT assay having those of 0 hr exposure as control set to 100%. Data are presented as means ± *SD* (C) Colony forming potential of MDA-MB-231 and C33A after exposure to various doses of 4-MCA for 24 hr and recovery for 96 hr. * $p < .05$ (compared with untreated cells)

TABLE 1 IC_{50} values of 4-MCA and positive controls (cisplatin and doxorubicin) against various types of cancer cells and noncancerous Vero cells at 72 hr of treatment

	IC_{50} (mean ± SD, μM)		
Cell type	4-MCA	Cisplatin	Doxorubicin
MDA-MB-231	39.33 ± 1.53	41.00 ± 1.73	0.045 ± 0.0041
HCT116	38.67 ± 5.51	45.67 ± 1.16	0.048 ± 0.000
HepG2	40.67 ± 2.31	19.00 ± 1.73	0.046 ± 0.000
C33A	34.33 ± 5.86	7.00 ± 1.00	0.038 ± 0.001
Vero	143.67 ± 17.01	8.00 ± 0.00	0.058 ± 0.002

protocol. The samples were analyzed by Muse® Cell Analyzer (Merck KGaA, Darmstadt, Germany).

2.8 | Cell cycle analysis

Alteration in cell cycle distribution induced by 4-MCA was examined by analysis of DNA content using flow cytometry. Cells (1×10^6) were seeded into 60-mm dishes and incubated for 18–24 hr prior to exposure to compound. Cells were grown in medium containing 75 or 150 μM of 4-MCA, or 0.2% DMSO for 24 hr. After that, cells were harvested, washed twice with PBS, permeabilized with cold 70% ethanol, and incubated at −20°C for 3 hr. About 200 μL of cell suspension was then centrifuged at 13,000 rpm for 5 min and washed once with PBS. Cells were then treated with 200 μL Muse® Cell Cycle reagent at room temperature for 30 min before analysis. The distribution of cell cycle phases with different DNA contents was calculated by Muse® Cell Analyzer (Merck KGaA, Darmstadt, Germany) and reported as percentage.

2.9 | Statistics

Each experiment was performed in triplicate of at least three independent tests and all data were expressed as averages ± standard deviation (*SD*). One-way ANOVA with Tukey comparison test was applied to determine significant difference between treated and untreated cells, or cancer and noncancerous cells at $p < .05$ using Minitab 17 software (Minitab, Inc., PA).

3 | RESULTS AND DISCUSSION

3.1 | Rhizome extracts of *E. pavieana* are toxic to cancer cells

Exposure of cancer cells to *E. pavieana* EE (100–400 μg/mL) for 24, 48, and 72 hr reduced cell survivals in dose- and time-dependent manners as shown by MTT assay (Figure 1; Supporting Information Figure S1). The longer incubation time emphasized the more difference in percentage of cell viability between groups of cancer cells (Figure 1, color lines) and control Vero cells (Figure 1, black line). Inhibition of cell proliferation was most evident for MDA-MB-231, HeLa, HepG2 and C33A cells with the IC_{50} values of 160, 182, 190, and 192 μg/mL, respectively,

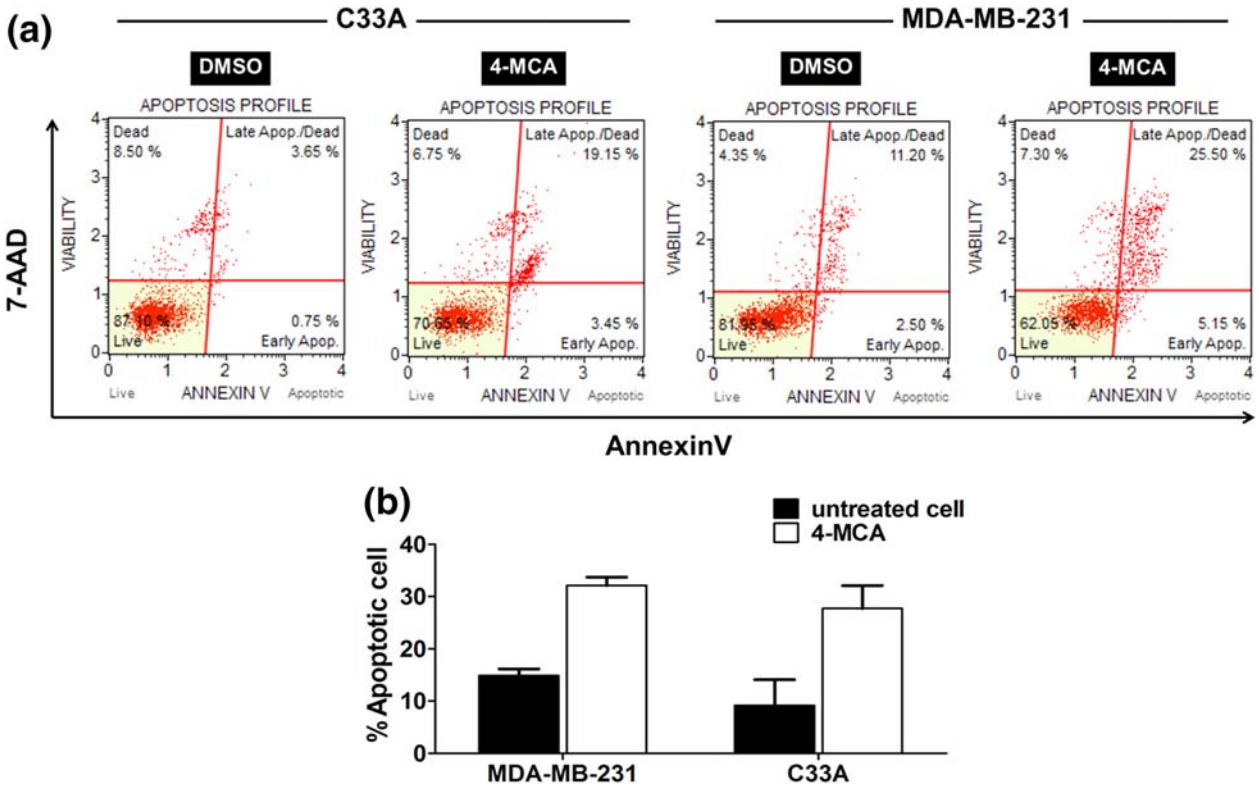

FIGURE 5 Effect of 4-MCA on apoptosis induction. (A) Apoptosis profiles of cells treated with or without 4-MCA at their IC_{50} for 72 hr as shown by staining with Annexin V-FITC and 7-AAD. (B) Percentage of total apoptotic cells treated with 4-MCA as compared with 0.2% DMSO

TABLE 2 Cell cycle distribution of two cancer cells (C33A and MDA-MB-231) and Vero cells after 4-MCA treatment as analyzed by flow cytometry

Cell lines	Treatment	SubG1 (%)	G1/G0 (%)	S (%)	G2/M (%)
Vero	0.2% DMSO	0.97 ± 0.5	58 ± 3.82	20.7 ± 1.11	20.33 ± 4.70
	4-MCA	0.88 ± 0.42	58.42 ± 3.55	19.0 ± 1.91	21.7 ± 4.51
C33A	0.2% DMSO	2.4 ± 0.87	49.97 ± 2.22	24.9 ± 1.31	22.73 ± 2.14
	4-MCA	5.8 ± 3.08*	40.73 ± 2.90*	22.26 ± 3.39	31.2 ± 4.23*
MDA-MB-231	0.2% DMSO	2.33 ± 0.95	56.27 ± 3.50	14.5 ± 1.61	26.56 ± 4.96
	4-MCA	4.20 ± 0.72*	42.80 ± 5.60*	17.37 ± 2.42	35.63 ± 3.89*

Note: Asterisk (*) represents the significant difference between untreated and treated groups at $p < .05$ ($n \geq 3$).

but to a lesser extent for Vero cells whose IC_{50} was greater than 400 μg/mL at 72 hr exposure.

With the relatively lower toxic effects of *E. pavieana* EE on normal cells, the extract was partitioned with hexane and ethyl acetate to produce three soluble fractions; HF, EF, and WF. Results from MTT showed that HF and EF, but not WF, exhibited the antiproliferative activities (Figure 2). Cell viability of HCT116, MDA-MB-231, C33A, and HeLa was reduced by 85–95% and of HepG2 and SiHa cells by 65–75% after 72 hr exposure to 400 μg/mL of EF when compared with a much lower cytotoxicity on Vero cells. HF also showed toxicity to most types of cancer cells but with weaker effects.

3.2 | 4-MCA is one of the bioactive compounds in EF

Purification of EF using column chromatography has identified five sub-fractions: SF1 to SF5. The reduction of cancer cell viability could be obviously observed since the cells were exposed to each sub-fraction for only 24 hr (Figure 3A). Interestingly, treatment of Vero cells with the highest concentration (100 μg/mL) of SF1 exhibited negligible growth inhibitory effect on their proliferation but showed strong effects on cancer cells. Exposure of cancer cells to a lower concentration of SF1 (50 μg/mL) for a longer incubation time (48 or 72 hr) augmented its inhibitory effects to cancer cells but not to Vero cells (Supporting Information Figure S2).

The double peak at δ9.62 ppm exhibited by the proton NMR spectrum of SF1 fraction indicated a peak of aldehyde proton being adjacent to a proton on double bond moiety. A high singlet signal at δ3.80 ppm indicated a methoxy group on an aromatic ring. By comparison with a reported chemical shift analysis (Battistuzzi et al., 2003; Srisook et al., 2017), it was found that SF1 fraction contained *trans*-4-methoxycinnamaldehyde (4-MCA) or (2E)-3-(4-methoxyphenyl) prop-2-enal as a major component (Figure 3B). The presence of 4-MCA as a major compound in the SF1 fraction was confirmed by HPLC analysis (Supporting Information Figure S3). Due to a small amount of SF1 (0.13 g), 4-MCA was further isolated by HPLC technique using condition as mentioned in Materials and methods. The chemical structure of purified yellow compound (0.0172 g, 13.23% w/w) eluted at 21.6 min was finally characterized and confirmed by ^{1}H-NMR spectroscopy as 4-MCA. The doublet peak at δ7.43 ($J = 16$ Hz) of the ^{1}H-NMR spectrum exhibited the existence of *trans*-isomer of 4-MCA. This compound was previously detected in essential oil and dichloromethane extract of *E. pavieana* rhizome (Tachai, Wangkarn, & Nuntawong, 2014; Tachai & Nuntawong, 2016), however, its role as antitumor promoting compound has, until this study, not been explored. Since a low quantity of isolated 4-MCA was obtained from SF1, commercial 4-MCA was used to study its activity.

3.3 | 4-MCA is a promising antitumor agent selective for cancer cells

Since MDA-MB-231 and C33A were two cancer cell lines which were most affected by SF1 sub-fraction, these cells were selected for further study. In order to assess the potential of 4-MCA as an antitumor promoting candidate, several in vitro tests including the MTT assay, colony forming assay, DNA fragmentation analysis, Annexin V/7-AAD staining, and cell cycle analysis were performed.

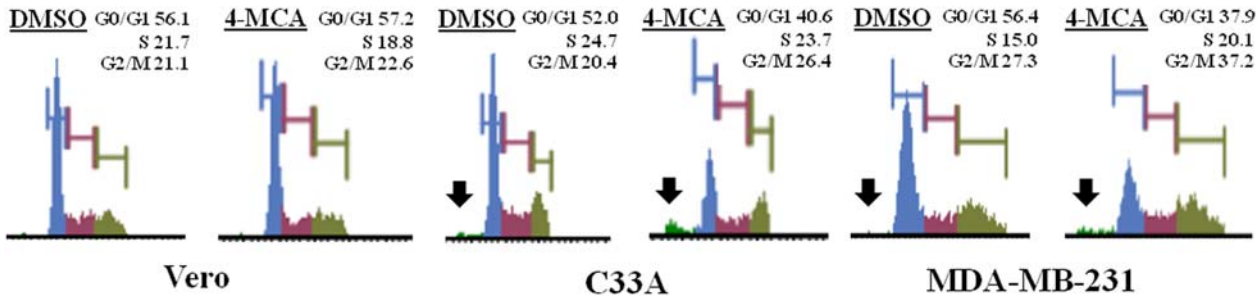

FIGURE 6 Representative DNA histogram of cells treated with 4-MCA at 1/3 IC_{50} or 0.2% (v/v) DMSO for 24 hr. Sub-G1 peaks were indicated by arrows

Cytotoxic responses of cancer cells after treatment with 4-MCA were observed as cells were more round in shape, smaller in size, and lower in number compared with Vero and untreated cells (Figure 4A). MTT results reflected a dose-dependent reduction in cell viability of 4-MCA on C33A and MDA-MB-231 cells (Figure 4B). Agreeing with their IC_{50} values shown in Table 1, those of 4-MCA against cancer cells (ranging from 34 to 40 μM at 72 hr) were approximately four times higher than that on Vero cells (143.67 μM). On the contrary, IC_{50} values of positive drugs toward cancer cells were higher or comparable to that of Vero cells, indicating the cytotoxicity of these drugs on normal cells. The decrease in cancer cell survival after 4-MCA treatment was also confirmed by clonogenic cell survival assay. As shown in Figure 4C, dose-dependent effect of 4-MCA on inhibition of colony formation was observed on C33A and MDA-MB-231 cells. Almost 100% inhibition of single cancer cells to form colonies was observed at 50 μM of 4-MCA.

Results from flow cytometry supported the potential lethality of 4-MCA (Figure 5). Incubation of C33A and MDA-MB-231 cells with 4-MCA at their IC_{50} values increased both early apoptotic (Annexin V^+/7-AAD^-) and late apoptotic cells (Annexin V^+/7-AAD^+) compared with untreated cells (Figure 5A). As shown in Figure 5B, about 30% of the two cell lines underwent apoptosis upon the treatment. The induction of apoptosis in MDA-MB-231 cancer cells after exposure to SF1 or 4-MCA was confirmed by fragmented DNA analysis while no such damage was detected in untreated cells (Supporting Information Figure S4).

Since the decrease in cell number observed in Figure 4A and by MTT assay (Figure 4B) can be resulted from either cell death or slow cell growth or both, the distribution of cell population at each phase of cell cycle was then analyzed. In this experiment, the effect of 4-MCA was investigated at 24 hr and one-third of the IC_{50} value was used to minimize its lethal effect (Chang, Cheng, Wang, Chou, & Shih, 2017). Flow cytometric data revealed the accumulation of both cancer cells at G2/M phase after 4-MCA treatment compared with their solvent treatment. We could not detect significant change in the cell cycle profile between treated and untreated Vero cells. However, the results confirmed the specific action of 4-MCA to cancer cells over noncancerous cell (Table 2). Previous studies have been noticed that *trans*-cinnamaldehyde and its derivatives, for example, 2′-benzoyloxycinnamaldehyde, 5-fluoro-2-hydroxycinnamaldehyde, 5-fluoro-2-benzoyloxycinnamaldehyde, and 2′-hydroxycinnamaldehyde derivative (CB403), also reduce cell viability via inducing both apoptosis and cell cycle arrest at G2/M phase at 24 hr in HSC-3, HCT116, MCF-7, SW620, and HUSMC cancer cells (Chang et al., 2017; Han et al., 2004; Jeong et al., 2003; Nagle et al., 2012) Many anticancer agents such as etoposide exhibit capability in blocking cells at G2 phase related to their function as topoisomerase inhibitor (Zhang et al., 2016). Although the underlying mechanism of 4-MCA is unknown, a structurally similar compound, 2-MCA, has been previously shown to inhibit topoisomerase I and II activities (Perng et al., 2016), suggesting the possible action of 4-MCA as a topoisomerase inhibitor.

Moreover, cells with lower DNA content than those of G1 phase (hypodiploid) represent those having fragmented DNA and could be counted as apoptotic cells (Plesca, Mazumder, & Almasan, 2008). As shown in DNA histogram (Figure 6), exposure of both C33A and MDA-MB-231 cancer cells to 4-MCA led to an increase of the sub-G1 peak (left of G1 peak) rendering 2- to 2.5-fold increase of sub-G1 population compared with those treated with DMSO (Table 2). These results clearly confirmed the apoptotic cell death induced by this compound.

Previously, only a few biological activities of 4-MCA have been reported. The 4-MCA exhibited the antihuman respiratory syncytial virus (RSV) activity by inhibiting viral infection to a human larynx carcinoma cell line (Wang, Chang, Chiang, & Lin, 2009) and selectively activated transient receptor potential ankyrin1 (TRPA1). This process was shown to be involved in inflammation, delayed gastric emptying, cold sensation, and chemosensation (Moon et al., 2015). Recently, anti-inflammation activity of 4-MCA has been recognized (Srisook et al., 2017). Although anticancer effect of several cinnamaldehyde derivatives have been noticed (Han et al., 2004; Jeong et al., 2003; Lin, Wu, & Lin, 2013; Perng et al., 2016; Wong et al., 2016), this is the first report on in vitro antitumor activity of 4-MCA.

In conclusion, this study demonstrated the presence of 4-MCA in *E. pavieana* rhizome and its inhibitory effects on various types of cancer cells. Our results suggested that the antiproliferative, and apoptosis and cell cycle arrest inducing activities of 4-MCA mainly contributed to its toxic effects. This scientific data increases pharmaceutical value to *E. pavieana* rhizome. Further underlying antitumor mechanisms of 4-MCA are still under investigation. With its low toxicity and reduced undesirable side-effects to noncancerous cells, 4-MCA is a good candidate for further in vivo tests and cancer drug development.

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CONFLICT OF INTEREST

All authors declared that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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