

Cytotoxicity of *Etlingera pavieana* rhizome extract on resistant-cervical cancer cells

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Received: 10th April 2023, Revised: 14th May 2023, Accepted: 15th May 2023

Abstract - Cervical cancer is one of the most common malignancies in women worldwide. Cisplatin-based treatment is most often used in chemotherapy of the disease. However, serious adverse effects and drug-resistant development limit cisplatin in clinical practice. Crude extracts of edible plants can serve as an alternate source of novel anticancer agents that are safe and are able to face chemoresistance. Rhizome of *Etlingera pavieana* or Raew hawm, an aromatic plant commonly found in Southeast Asia, is a well-known spice and food ingredient used in the Eastern region of Thailand. In the previous studies, *E. pavieana* rhizome extract exhibited an inhibitory effect on the proliferation of various cancer cells including those resistant to doxorubicin, with much lower toxicity on non-cancer cells. In this ongoing study, the cisplatin-resistant cervical C-33A/R cells were established and were subjected to investigate the cytotoxic effect of the extract. MTT results demonstrated that the viability of C-33A/R cells was significantly reduced after extract treatment in both dose- and time-dependent manners. At 400 µg/mL extract, growth of C-33A/R cells was decreased by 77.32% at 72-hour incubation. The IC₅₀ value against the resistant cells was 269.36 µg/mL which was slightly higher than that of parental cells (230.99 µg/mL). The results suggest

Citation: Iawsipo, P., Panprasert, J., Poonbud, R., & Maneechot, R. (2023). Cytotoxicity of *Etlingera pavieana* rhizome extract on resistant-cervical cancer cells. *Food Agricultural Sciences and Technology*, 9(2), 23-34.

E. pavieana rhizome extract as a source of promising anticancer candidates for cervical cancer treatment.

Keywords: Cytotoxicity, *Etilingera pavieana*, cervical cancer, resistant cancer cells, rhizome extract

1. Introduction

The development of chemoresistance is a major obstacle to successful chemotherapy and also a main cause of cancer recurrence. Various molecular mechanisms underlying chemoresistance have been previously explored, including a reduction in the intracellular accumulation of the cytotoxic agents, an increase in drug efflux, induction of DNA damage repair, and inactivation of apoptosis (Haider *et al.*, 2020). Cisplatin-based treatment is a standard regimen to treat invasive or recurrent cervical cancer, and also other solid cancers from ovarian, bladder, and lung. Unfortunately, the development of chemoresistance often occurs after cisplatin treatment, leading to a chemotherapeutic failure (Chen *et al.*, 2015; Zhu *et al.*, 2016). Moreover, its clinical application is limited due to severe side effects, particularly nephrotoxicity, and there is no drug to prevent or treat cisplatin-induced kidney injury until now (Fang *et al.*, 2021). Due to these limitations, there is an urgent need to find new natural products with high efficacy in cancer elimination and mild side effects for patients.

Edible plants are considered to be a significant source of potential anticancer compounds that have been ensured to be safe, and less expensive. Extracts from numerous species in Zingiberaceae or ginger family have been previously reported to be rich in cytotoxic agents against cancer cells, including those of the cervix (Tuy-On *et al.*, 2020; Soumya *et al.*, 2021). Their

inhibitory activity targeting drug-resistant cancer cells has also been verified (Pereira *et al.*, 2011; Wu *et al.*, 2015; Mbese *et al.*, 2019).

Etilingera pavieana or Raew hawm is a locally well-known Zingiberaceae herb in Eastern Thailand, and commonly distributed throughout Southeast Asia. Its rhizome is generally used as a spice, freshly eaten, and also an important ingredient in the famous Chanthaburi “Moo Lieng” noodle (Naksang *et al.*, 2020; Srisook & Srisook, 2020). Previously, the cytotoxicity of ethanolic extracts from *E. pavieana* rhizome against various types of cancer cells, including hepatoma HepG2, colorectal carcinoma HCT116, breast adenocarcinoma MCF-7 and MDA-MB-231, and cervical carcinoma SiHa, HeLa and C33A was demonstrated (Iawsipo *et al.*, 2018). Low toxicity of the extract in non-cancer kidney Vero cells was also reported with the half maximal inhibitory concentration (IC₅₀) higher than 400 mg/mL, suggesting low nephrotoxicity induction by the extract. In addition, the extract was able to exert an inhibitory effect on doxorubicin-resistant breast cancer MDA-MB-231 cells (Iawsipo and Poonbud, 2019). In this ongoing study, the cisplatin-resistant cervical cancer C-33A cells were established and their response toward *E. pavieana* rhizome extract was investigated by 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

2. Materials and methods

2.1 Extract preparation

Cleaned fresh rhizomes of *E. pavieana* (Figure 1) (The Plant List Record 244738) were obtained from a local farm in Khung district, Chanthaburi province, Thailand. The species was authenticated by Dr. Benchawan Chewprecha, a plant taxonomist at the Department of Biology, Faculty of Science, Burapha University. A voucher specimen (KS-SCBUU-0012-1) is deposited at the Faculty of Science, Burapha University.

Before being subjected to extraction, the rhizomes were sliced into small pieces (about 1 cm long), dried at 50°C in a hot-air circulation drying oven for 6

hours, and pulverized into fine powder. The extraction was performed as previously described (Iawsipo *et al.*, 2018) by maceration method using 95% ethanol as a solvent (1 g: 10 mL) for 5 days at room temperature. The extraction was repeated three times. After filtration, the collected filtrate was evaporated to dryness under vacuum using a rotary evaporator at 42°C. The extraction yield (%yield) was calculated by (weight of dry extract/ weight of dry rhizome powder) × 100. The dried extract was initially dissolved in dimethyl sulfoxide (DMSO) for a stock solution and subsequently dissolved in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) to obtain the desired extract concentrations which contained 0.2% DMSO (non-cytotoxic DMSO concentration).



Figure 1. Cleaned fresh rhizomes of *E. pavieana* for extract preparation.

2.2 Development of cisplatin-resistant C-33A cells (C-33A/R)

In order to establish the C-33A/R cells, 1×10^4 cells of the parental cervical cancer C-33A were seeded onto a 6-well plate in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (Thermo Fisher

Scientific, Waltham, MA, USA), and incubated in a 37°C humidified incubator with 5% CO₂. The treatment method was modified from Yang *et al.* (2017) and followed the suggestions from Coley (2004). Initially, C-33A cells were exposed to 0.5 μM cisplatin (MedChemExpress, Monmouth Junction, NJ, USA) for 7 days and recovered in a drug-free medium until

they reached 80-90% confluency. After that, the treatment of stepwise increasing concentrations of cisplatin (0.5 μ M interval; up to 10 μ M) was applied with the same procedure. Finally, the resultant cell lines that grew exponentially in the presence of 10 μ M cisplatin were designated as the resistant C-33A/R cells. Cells were maintained in the drug-free medium for 2 weeks before being used in the experiment. Cell morphology was visualized and photographed under a phase-contrast inverted microscope at 100X magnification.

The cisplatin-resistant phenotype of C-33A/R cells was subsequently confirmed by MTT assay as the method described below. In the experiment, 2 μ M of cisplatin was used because it is approximately the IC_{50} value of cisplatin against the parental C-33A cells at 72-hour incubation (Table 1).

The IC_{50} values of cisplatin against C-33A/R cells were calculated at different time points of treatment, and the resistance index (RI) was determined as a ratio of IC_{50} of the resistant cells to that of parental cells.

In addition, the C33A/R cells were also tested for *MDR1* gene expression by real-time RT-PCR technique (MyGo® Pro qPCR, Novacyt, UK) using forward primer: 5'-TCCATGCT CAGACAGGATGT-3' and reverse primer: 5'-AACTTGAGCAG-CATCATTGG-3'. PCR was amplified for 40 cycles using the following conditions: 95°C 15 sec (Denaturation), and 60°C 45 sec (Annealing/Extension) (Davoudi *et al.*, 2014). The signals of SYBR green (PCR Biosystem, UK) from *MDR1* amplified products in C-33A and C-33A/R cells were normalized to that of *GAPDH* control gene (conditions as reported in Ikeguchi *et al.*,

2002). The relative quantification of *MDR1* gene expression in C-33A/R cells to their parentals (fold change) was calculated by the $2^{-\Delta\Delta Ct}$ method.

2.3 Cytotoxic assay

The cytotoxic effect of *E. paviiana* rhizome extract against C-33A/R cells was determined by colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay in 96-well culture plate as previously described (Iawsipo *et al.*, 2018). 0.2% DMSO was used as a negative control. The parental C-33A and the resistant C-33A/R cells were treated with 50, 100, 200, and 400 μ g/mL of the extract for 72 hours (or 100, 200, and 400 μ g/mL at indicated incubation time in a time-course experiment). After MTT treatment, the insoluble purple formazan crystals formed in active cells were completely dissolved in 200 μ L DMSO, and the absorbance was measured at 540 nm by a microplate reader. The %cell viability was calculated by dividing the A540 of the treated group by the untreated group and multiplied by 100, then normalized against those of untreated cells or those of the beginning (0 h) which was set to 100%.

2.4 Statistical analysis

The experiment was at least performed in triplicate and replicated three times. The results were reported as mean \pm standard deviation (SD). The difference between values from individual treated cells and untreated cells was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's comparison test using Minitab 18 software (Minitab Inc.;

State College, PA, USA), and the data was considered as a significant difference when p -value < 0.05 .

3. Results and discussion

3.1 Cisplatin resistance of C-33A/R Cells

After the parental cervical cancer C-33A cells were exposed to stepwise incremental concentrations of cisplatin (0.5 - 10 μM) over a period of 15 months, the resistant C-33A/R cells were established. As shown in Figure 2, the resistant sublines were smaller in size and rounder in shape compared to their parental cells.

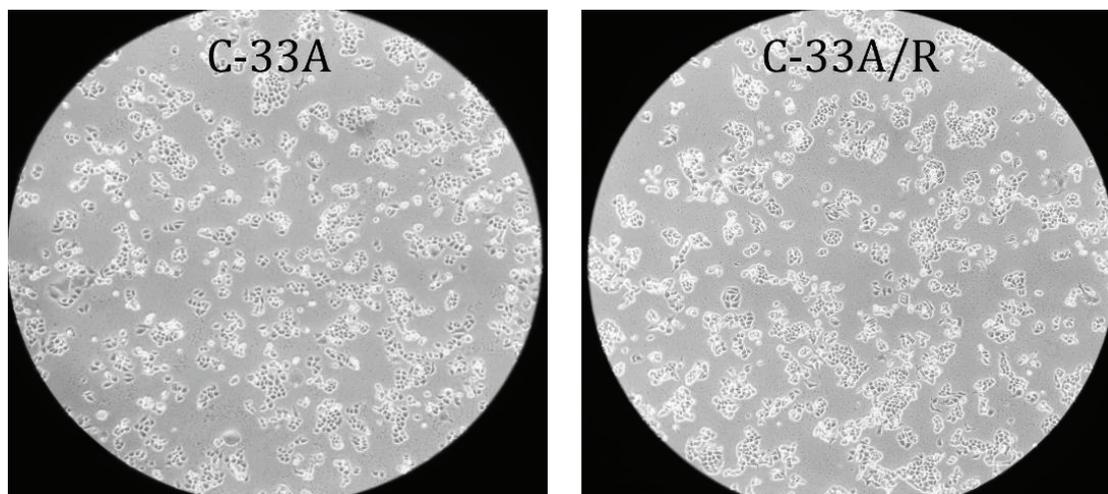


Figure 2. Cellular morphology of the parental cervical cancer C-33A and the developed cisplatin-resistant C-33A/R cells. The pictures were taken under a phase-contrast inverted microscope at 100X magnification.

Moreover, the C-33A/R cells were able to proliferate significantly slower than their sensitive parents (Figure 3; red and blue solid lines, respectively). The retarded growth of drug-resistant cancer cells was also detected in other previous studies (Han *et al.*, 2014; Cazzaniga *et al.*, 2017; Qi *et al.*, 2021). The higher cisplatin tolerance in C-33A/R cells was confirmed by MTT assay and shown in Figure 3. When exposed to 2 μM of cisplatin, the viability of the parental C-33A cells (blue dashed line) was decreased with longer incubation time and reduced nearly to 50%

after 72 hours of incubation compared to the untreated group (blue solid line). On the contrary, the proliferative capability of the resistant C-33A/R cells was unaffected by drug treatment (red dashed line), indicating the acquired cisplatin-resistant phenotype of these cells. The IC_{50} values of cisplatin to C-33A/R cells and their parents at different treatment time points and the resistance index (RI) values were shown in Table 1. Moreover, the expression of *MDR1* gene was increased in C-33A/R cells 2.98 ± 1.12 fold when compared to the parental cells.

Table 1. IC₅₀ values of cisplatin against parental C-33A and resistant C-33A/R cells.

Cell type	IC ₅₀ values (μM) (Mean ± SD)		
	24 hours	48 hours	72 hours
C-33A	> 8	4.60 ± 0.35	2.53 ± 0.25
C-33A/R	> 8	5.75 ± 0.35	4.30 ± 0.85
Resistance index (RI)	N/A	1.15	1.70

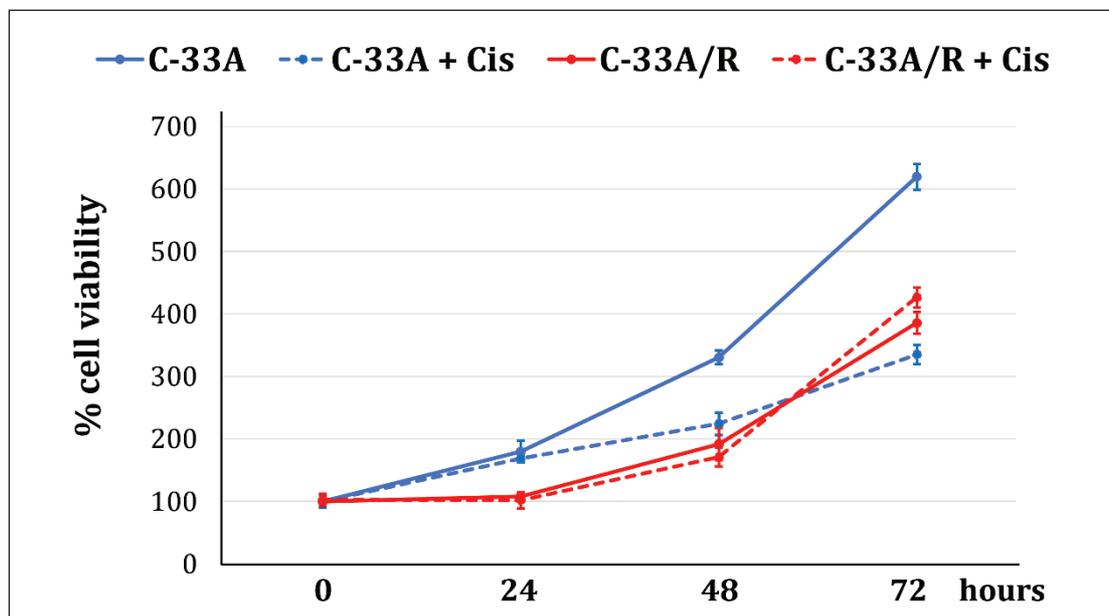


Figure 3. Survival response to cisplatin treatment of C-33A cells (blue lines) and C-33A/R cells (red lines). Cells were exposed to either 2 μM cisplatin (dashed lines) or 0.2% DMSO (negative control; solid lines) for 24, 48, and 72 hours before analyzing their viability by MTT assay. Cell viability was expressed as a percentage of the untreated control and normalized to those of 0 hours which was set to 100%. Values represent mean ± SD of triplicate samples of three independent experiments.

3.2 E. *pavieana* rhizome extract and its cytotoxic effect against C-33A/R cells

After macerating the dried powder *E. pavieana* rhizome in absolute ethanol for 15 days, the brownish sticky solid extract was obtained with a percentage yield of 8.14%. Previously, the inhibitory effect of *E. pavieana* rhizome extract was revealed in various cell types including cervical cancer C-33A cells (Iawsipo *et al.*, 2018). In addition, the extract was able to reduce

the viability of doxorubicin-resistant breast cancer MDA-MB-231 cells (Iawsipo & Poonbud 2019). In this study, the extract was ongoingly evaluated for its cytotoxic effect against cisplatin-resistant C-33A/R cells. By observation under an inverted microscope, the shape of extract-treated cells, both C-33A and C-33A/R, were changed to be more spherical and some were detached from the surface of culture plate, compared to the untreated cells. The MTT results demonstrated a dose-dependent

decrease in the cell number of C-33A/R cells after extract treatment (Figure 4). The growth reduction was prominent when cells were exposed to 400 µg/mL of *E. pavieana* extract for 72 hours (22.68%). However, the extract exhibited a slightly stronger effect in the cisplatin-sensitive C-33A cells, consistent with other studies using natural extracts (Lin *et al.*, 2018; Francisco Fernandez *et al.*, 2019). The IC₅₀ values for the parental and resistant cells were 230.99 µg/mL and 269.36 µg/mL, respectively, similar to that of doxorubicin-resistant breast cancer MDA-MB-231 cells (248 µg/mL) (Iawsipo & Poonbud, 2019). Moreover, a time dependent response of the resistant C-33A/R cells to the extract was also observed (Figure 5). Increasing the exposure time could enhance the cytotoxic of the *E. pavieana* extract even at 100 µg/mL.

The reduction in cancer cell viability by *E. pavieana* extract detected in the MTT assay could be a result of either inhibition of cell proliferation or activation of cell death, or both. For the C-33A cells, the cytotoxic effect of *E. pavieana* rhizome was earlier reported to be mainly due to the activities of its active constituent, *trans*-4-methoxycinnamaldehyde, (4-MCA)

to antiproliferation, cell cycle arrest, and apoptosis induction (Iawsipo *et al.*, 2018). 4-MCA might also be a key compound responsible for the cytotoxic effect of the extract against C-33A/R cells. Nevertheless, the possibility that other bioactive phenolics in *E. pavieana* rhizome such as 4-methoxycinnamyl alcohol, *p*-coumaric acid as well as 4-methoxycinnamyl *p*-coumarate might also be involved in the activity could not be excluded (Srisook & Srisook, 2020).

By the time-course experiment (Figure 5 yellow line), the finding of decreased number of C-33A/R cells treated with 400 µg/mL extract at 72 hours which was lower than that of the initial (at time 0) suggests some cells underwent cell death. This indicates the possible action of the extract in inducing C-33A/R cell death by a yet unidentified pathway to exert its cytotoxic effect. Several *Etilingera* extracts also triggered apoptotic cell death and induced cell cycle arrest to exhibit inhibitory activity in various cancer cells (Sabli *et al.*, 2012; Krajarng *et al.*, 2017; Wahyuni *et al.*, 2021). However, additional experiments are needed to understand the cytotoxic mechanism of the *E. pavieana* extract in C-33A/R cells.

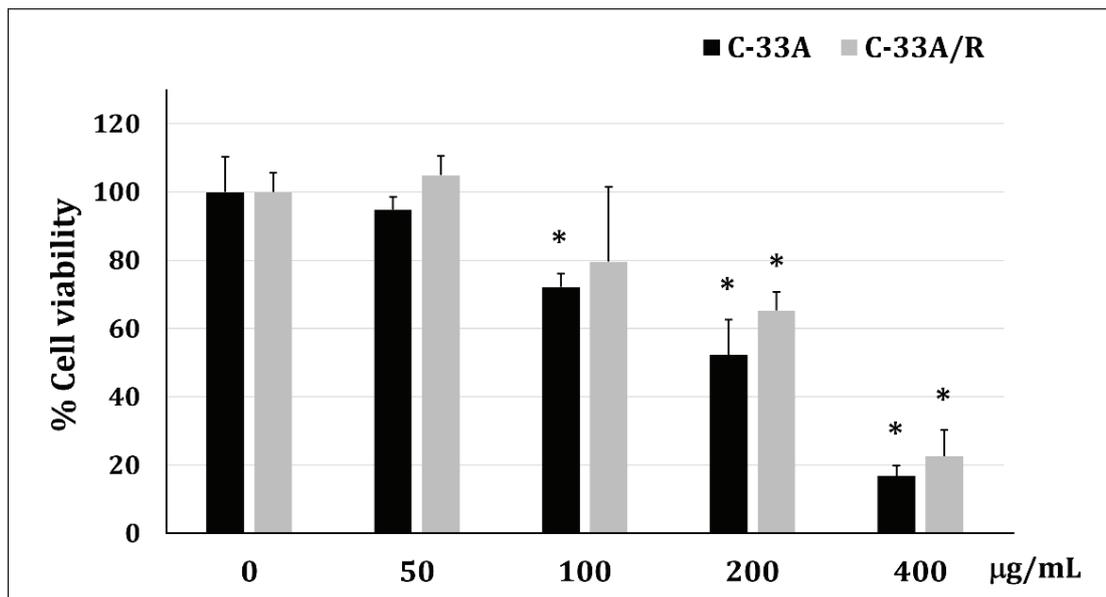


Figure 4. Cell viability after *E. pavieana* extract treatment. Cells were treated with various concentrations (50, 100, 200, 400 µg/mL) of rhizome extract for 72 hours and their survival was examined by MTT assay. Cell viability was expressed as a percentage of untreated control which was set to 100%. Values are mean ± SD of triplicate samples of three independent experiments. Asterisk (*) represents a significant difference between treated group and untreated group, $p < 0.05$.

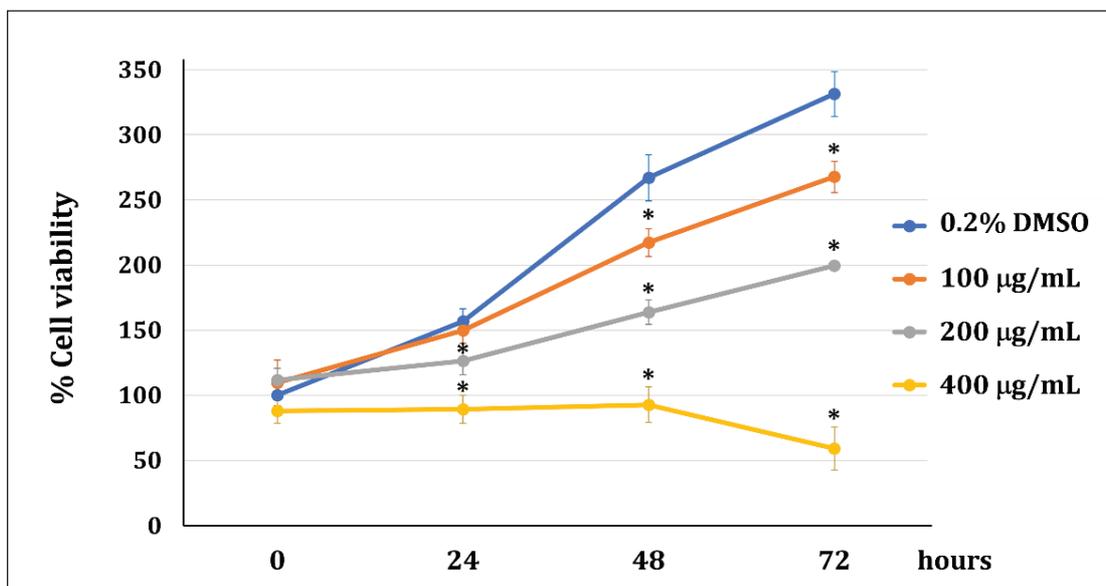


Figure 5. Time-course incubation of *E. pavieana* extract in C-33A/R cells. Cells were treated with various concentrations (100, 200, 400 µg/mL) of rhizome extract for 0, 24, 48, and 72 hours. Cell viability was evaluated by MTT assay and was expressed as a percentage of untreated control (the value of cells treated with 0.2% DMSO at 0 h was set to 100%). Values are mean ± SD of triplicate samples of three independent experiments. Asterisk (*) represents a significant difference between the treated group and control group at the same time point, $p < 0.05$.

4. Conclusion

This study demonstrated the cytotoxic effect of *E. pavieana* rhizome extract on the cisplatin-resistant C33A/R cells for the first time. The results suggest that *E. pavieana* rhizome extract can serve as a source of promising anticancer candidates for cervical cancer treatment including those of cisplatin resistance, to overcome the problems in chemoresistance and serious side effects caused by cisplatin treatment. The present finding of anticancer activity increases the medical value of *E. pavieana*, and in combination with other biological activities reported by other researchers, *E. pavieana* rhizome has the potential to be developed into a functional food.

5. Acknowledgement

This work was financially supported by the Research Grant of Burapha University through National Research Council of Thailand (Grant no. 180/2561 and 13.4/2562). The Research Unit of Natural Bioactive Compounds for Healthcare Products Development, and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation, Thailand, are gratefully acknowledged. The authors also would like to acknowledge the support from Science Innovation Facility, Faculty of Science, Burapha University (SIF-IN-55300056).

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