© 2022 Journal of Pharmacy & Pharmacognosy Research, 10 (1), 39-51, 2022
ISSN 0719-4250
http://jppres.com/jppres

DOI: https://doi.org/10.56499/jppres21.1160_10.1.39

Original Article

Anticancer activity of *Piper cubeba* L. extract on triple negative breast cancer MDA-MB-231

[Actividad anticancerígena del extracto de *Piper cubeba* L. en el cáncer de mama triple negativo MDA-MB-231]

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Abstract

Context: *Piper cubeba* L. (family *Piperaceae*) is used traditionally for the treatment of many diseases, including cancer.

Aims: To determine the anticancer activity of *P. cubeba* crude extracts on cancer cells and identify chemical constituents of the active fraction.

Methods: Seeds of *P. cubeba* were sequentially extracted with dichloromethane followed by methanol and purified using column chromatography. Fractions were screened the cytotoxicity against triple negative breast cancer (MDA-MB-468) using MTT assay. Then, active fractions were evaluated cytotoxicity against breast cancer (MCF-7 and MDA-MB-231), colon cancer (HT-29), cholangiocarcinoma (KKU-M213) and normal fibroblast (L929) cells. Total phenolic, tannin, and flavonoid contents of active fraction were investigated, and the chemical composition was analyzed using GC-MS. Flow cytometry was applied to determine the cell cycle, apoptosis, and evidence of caspase activation.

Results: Fraction DE14 and DE15 showed cytotoxic activity against MDA-MB-468. Fraction DE15 exhibited the most potent cytotoxicity against MDA-MB-231, KKU-M213, HT-29 and L929. As the fraction DE15 was of dichloromethane extraction, major contents were nonpolar fatty acids and fatty acid esters, followed by propylene glycol and hydrocarbons. The phenolic compounds were however traceable, leading to their measurable antioxidant property. This extract did not cause cell cycle arrest on MDA-MB-231. However, fraction DE15 significantly increased apoptotic cells at 48 and 72 h, and significantly induced multi-caspases activity on MDA-MB-231 in a time-dependent manner.

Conclusions: Fraction DE15 exhibited cytotoxicity effect and induced apoptosis on triple negative breast cancer and showed less toxicity on normal fibroblast cells.

Keywords: *Piper cubeba*; anticancer; apoptosis; triple negative breast cancer.

Resumen

Contexto: *Piper cubeba* L. (familia *Piperaceae*) es utilizado tradicionalmente para el tratamiento de muchas enfermedades, incluido el cáncer.

Objetivos: Determinar la actividad anticancerígena de extractos crudos de *P. cubeba* sobre células cancerosas e identificar los componentes químicos de la fracción activa.

Métodos: Se extrajeron semillas de *P. cubeba* con diclorometano seguido de metanol y se purificaron mediante cromatografía en columna. Se examinó la citotoxicidad de las fracciones frente al cáncer de mama triple negativo (MDA-MB-468) usando un ensayo MTT. Luego, se evaluó la citotoxicidad de las fracciones activas frente a células de cáncer de mama (MCF-7 y MDA-MB-231), cáncer de colon (HT-29), colangiocarcinoma (KKU-M213) y fibroblasto normal (L929). Se investigó el contenido total de fenólicos, taninos y flavonoides de la fracción activa y se analizó la composición química mediante GC-MS. Se aplicó citometría de flujo para determinar el ciclo celular, la apoptosis y la evidencia de activación de caspasa.

Resultados: Las fracciones DE14 y DE15 mostraron actividad citotóxica contra MDA-MB-468. La fracción DE15 exhibió la citotoxicidad más fuerte contra MDA-MB-231, KKU-M213, HT-29 y L929. La composición de la fracción DE15, extraída con diclorometano, fue de ácidos grasos apolares y ésteres de ácidos grasos, seguidos de propilenglicol e hidrocarburos. Sin embargo, los compuestos fenólicos fueron detectados, lo que llevó a su propiedad antioxidante medible. Este extracto no provocó la detención del ciclo celular en MDA-MB-231. Sin embargo, la fracción DE15 aumentó significativamente las células apoptóticas a las 48 y 72 h, e indujo significativamente la actividad de caspasas múltiples en MDA-MB-231 de una manera dependiente del tiempo.

Conclusiones: La fracción DE15 exhibió efecto de citotoxicidad e indujo apoptosis en cáncer de mama triple negativo y mostró menos toxicidad en células de fibroblastos normales.

Palabras Clave: anticáncer; apoptosis; cáncer de mama triple negativo; *Piper cubeba*.

ARTICLE INFO
Received: July 29, 2021.
Received in revised form: August 24, 2021.
Accepted: August 28, 2021.
Available Online: September 7, 2021.

INTRODUCTION

Breast cancer is the most common and leading cause of death in the female population worldwide. The major obstacle for cancer treatment is the recurrence of the tumor and the side effects of chemotherapy drugs such as nausea and bone marrow suppression (Barreto et al., 2014). Therefore, the search for new potent chemotherapeutics from natural sources may provide alternative cancer treatment. Secondary metabolites isolated from plants have been reported to have anticancer properties both in *in vitro* and *in vivo*, including alkaloid, phenolic, flavonoid, quinone, coumarin, lignan, stilbene and tannin (Cai et al., 2004).

Piper species, belonging to the *Piperaceae* family, widely distributed in tropical and subtropical regions, are rich in bioactive compounds (Salehi et al., 2019). Among the *Piper* species, *P. cubeba* (tailed paper), a folkloric plant for the treatment of dysentery, syphilis, gonorrhea, abdominal pain, diarrhea, enteritis and asthma (Salehi et al., 2019), contains a large number of purified bioactive compounds with various biological activities, such as anti-inflammatory (Perazzo et al., 2013), anticancer (Graidist et al., 2015), antiproliferative (Yam et al., 2008) and anti-bacterial (Nahak and Sahu, 2011; Tamadher and Tememy, 2013). The study of the lignan compound from the fruit of *P. cubeba* found that the active substance was cubebin. This compound has several biological activities, including anti-inflammatory (Bastos et al., 2001), antiproliferation (Yam et al., 2008) and anticancer (Rajalekshmi et al., 2016). However, a high concentration of cubebin causes genotoxic in mice (Maistro et al., 2011). The high dose of *P. cubeba* seed extract also causes genotoxic in mice and rats (Junqueira et al., 2007).

Previously, we found that the methanol crude extract of *P. cubeba* induced apoptosis by causing DNA fragmentation on breast cancer cells (MCF-7, MDA-MB-468 and MDA-MB-231). This crude extract also exhibited a cytotoxicity effect against normal cells (L929) with equal IC_{50} values of breast cancer cells (Graidist et al., 2015). Due to the cytotoxicity effect of methanol crude extract of *P. cubeba* on normal cells, as previously reported, this present study aimed to find the extract that did not cause toxicity to normal cells. Therefore, the seed of *P. cubeba* was extracted with dichloromethane followed by fractionation using different solvents elution ratios. Then, we further explored the cytotoxic activity of *P. cubeba* crude extract on breast cancer, colon cancer, cholangiocarcinoma, and normal fibroblast cells to evaluate the specificity of the extract on cancer type. Cell cycle arrest and apoptosis were also determined to verify the activity of crude extract on the specific cancer cells.

MATERIAL AND METHODS

Plant material and extraction

The dried seeds of *P. cubeba* (1 kg) were purchased from a traditional medicine shop in Bangkok province, Thailand (latitude 13.76636 and longitude 100.64541). Plant specimen was identified by an expert (Dr. Supreeya Yuenyongsawad) in the field as previously described, and a specimen was deposited in the Southern Centre of Thai Traditional Medicine, Department of Pharmacognosy and Pharmaceutical Botany, Prince of Songkla University (PSU), Thailand (voucher number SKP146160302) (Sriwiriyajan et al., 2014). Seeds were grounded into powder and extracted with dichloromethane, and then the residue was further extracted with methanol. The powder was initially soaked in dichloromethane for four cycles (2.5 L of dichloromethane each, at 7 days intervals), followed by extraction of remaining residues similarly using methanol. The extract of each step was filtered using Whatman filter paper (No. 1), pooled, and then concentrated to give dark brown viscous extract under vacuum by rotary evaporation at 40°C. The dried extracts (158.6 g of dichloromethane and 34.4 g of residue-methanol extract) were protected from light and stored in a desiccator at room temperature until used (Fig. 1).

Fractionation of extract

The 34 g of dichloromethane extract (fraction D) and 34.4 g of residue-methanol extract (fraction RD-M) were subjected to silica gel column chromatography (silica gel 60, 230-400 Mesh ASTM, Merck) followed by gradient elution. The gradient elution of fraction D was the ratio of hexane:dichloromethane followed by the ratio of dichloromethane:methanol. The gradient elution of fraction RD-M was the ratio of dichloromethane:methanol. All fractions were analyzed by thin-layer chromatography (TLC). Then, fractions with the same TLC profile were combined. Fifteen fractions (DE1 to DE15) were obtained from fraction D. Thirteen fractions (RD-ME1 to RD-ME13) were obtained from methanol extract (Fig. 1).

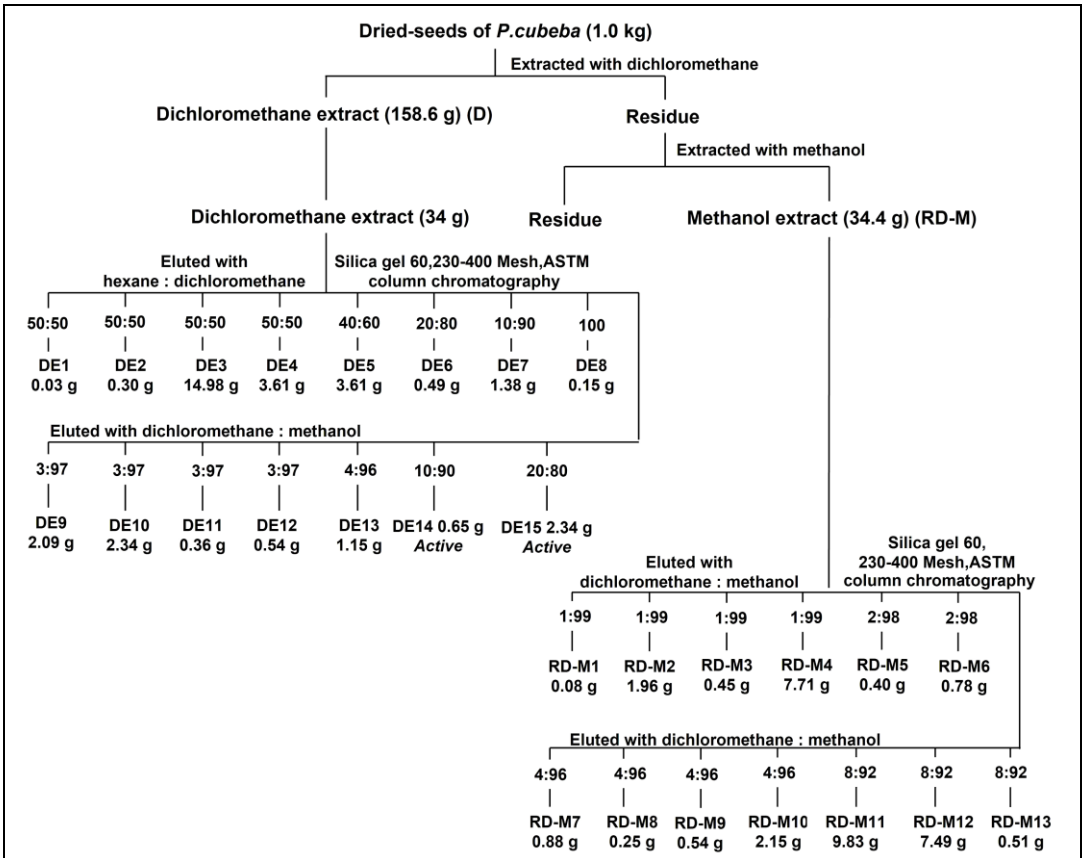

Figure 1. Fractionation process of the extract of *P. cubeba*.

Determination of total phenolic, flavonoid, condensed tannin and antioxidant

The total phenolic content of the extracts was determined using Folin-Ciocalteu assay. In brief, 20 µL of plant extract was mixed with 100 µL of Folin-Ciocalteu reagent. The mixture was incubated for 1 min followed by adding 80 µL of 7.5% Na_2CO_3. After incubation for 30 min, absorbance was measured with a spectrophotometer at 765 nm. The total phenolic content was calculated as mg gallic acid equivalent (mg GAE/mg extract) using a gallic acid calibration curve (Singleton and Rossi, 1965).

The total flavonoids were determined using aluminum chloride. Briefly, 25 µL of plant extracts were mixed with 75 µL of 95% ethanol. Then, 5 µL of $AlCl_3$, 5 µL of 1 M potassium acetate, and 140 µL of distilled water were added. Following incubation for 30 min, the absorbance of the reaction mixture was measured at 415 nm against a methanol blank. The total flavonoid content was calculated as mg quercetin equivalent (mg QE/mg extract) using a quercetin calibration curve (Chang et al., 2002).

Condensed tannins (proanthocyanidins) were determined according to the method of Singleton and Rossi (1965). A volume of 20 µL of plant extract was mixed with 50 µL of 1% vanillin solution and 50 µL of 25% H_2SO_4. The mixture was allowed to stand for 15 min, and the absorbance was measured at 500 nm against methanol as a blank. The amount of total condensed tannins was expressed as catechin equivalents (CE, milligram catechin per gram sample).

The DPPH assay was done according to the method of Brand-Williams et al. (1995) and Thaipong et al. (2006) with some modifications. In brief, 10 µL of plant sample was mixed with 190 µL of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and incubated in the dark for 30 min. Then the absorbance was taken at 515 nm. The results were expressed as Trolox equivalent in milligrams per milligram sample (mg TEAC/g extract).

All samples were analyzed in triplicate. All four procedures were performed at the Center of Excellence in Natural Products Innovation, Mae Fah Luang University, Chiang Rai, Thailand.

Gas chromatography-mass spectrometry (GC-MS) analysis

The analysis of the composition of the dichloromethane extract and fraction DE15 were performed using The Agilent 7890B gas chromatography combined with an Agilent 7000D triple quadrupole mass spectrometer (Agilent Technologies Inc, USA) with a

VF-WAXms column (length 30 m, film thickness 0.25 µm, ID 0.25 mm). An inlet temperature of 250°C with a split ratio of 50:1 was employed. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature was initially maintained at 50°C for 5 min and then increased at a rate of 3°C/min to 250°C, at which the temperature was held for 10 min. For MS detection, an electron ionization mode was used with ionization energy of 70 eV, ion source temperature of 230°C, scan mass range m/z 35 to 500. The components were identified based on a correlation of the recorded fragmentation patterns of mass spectra with those provided with the software of the GC-MS system version Wiley 10 and NIST14. All procedures were performed at Scientific Equipment Center, PSU, Thailand.

Cell culture conditions and *in vitro* cytotoxicity testing

Three breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-468) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Colorectal carcinoma (HT-29) cell lines were provided by Dr. Surasak Sangkhathat (Faculty of Medicine, PSU, Thailand). Cholangiocarcinoma (KKU-M213) cell lines were kindly donated by Dr. Mutita junking (Faculty of Medicine, Mahidol University, Thailand). Normal fibroblast cells (L929) cell lines were provided by Dr. Jasadee Kaewsrichan (Faculty of Pharmaceutical Sciences, PSU, Thailand). MCF-7 cells were grown in RPMI 1640 (Invitrogen). MDA-MB-231, MDA-MB-468, HT-29, KKU-M213, and L929 cells were grown in DMEM (Invitrogen). Each medium contained 10% fetal bovine serum (Invitrogen) supplemented with 50 units/mL of penicillin (Invitrogen) and 50 µg/mL of streptomycin (Invitrogen). All cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The cytotoxicity of the studied extracts and fractions were measured by MTT assay as previously described. An IC_{50} value less than 20 µg/mL was considered to have *in vitro* cytotoxic activity (Sriwiriyajan et al., 2014). Doxorubicin, a chemotherapeutic drug, was used as a positive control.

The selectivity index (SI) was used to determine the selectivity of the extract as previously described (Bézivin et al., 2003). The SI calculated the IC_{50} value of the extract on normal cells (L929) divided by the IC_{50} value of the extract on each cancer cells. SI value higher than 3 was considered as a prospective anticancer.

Cell cycle analysis

Cell cycle assay was performed as previously described (Rattanaburee et al., 2019). Briefly, MDA-MB-231 cells were seeded at a density of 1 × 10^5 cells/well in a 12-well plate and exposed to active fraction DE15 at various concentrations and incubation times. Cells were harvested and stained with propidium iodide (PI) dye (Millipore's Muse® Cell Cycle Kit, Merck Millipore). Cell cycle distribution (G0/G1, S and G2/M phase) was analyzed by Muse Cell Analyzer (Merck Millipore).

Apoptosis and multi-caspase activity assay

Apoptosis and multi-caspase activity assays were performed as previously described (Rattanaburee et al., 2019). MDA-MB-231 cells were seeded in 12-well plates at a density of 1 × 10^5 cells/well overnight. Cells were treated with fraction DE15 at an IC_{50} concentration for 72 h. Cells were then harvested by trypsinization. For apoptosis assay, treated cells were analyzed live, early/late apoptosis, and cell death using Annexin V and Dead Cell kit (Merck Millipore). The percentage of apoptotic cells was calculated from duplicate samples by statistical analysis of dot plot using Muse Cell Analyzer (Merck Millipore).

For the multi-caspase assay, activities of caspase-1, 3, 4, 5, 6, 7, 8, and 9 were measured using a Muse Multi-caspase kit (Merck Millipore). The cell pellet was incubated with 5 µL of Muse Multi-Caspase reagent at 37°C for 30 min. Then, 150 µL of Muse Caspase 7-AAD working solution was added to each sample. The multi-caspase assay was performed with Muse Cell Analyzer (Merck Millipore).

Statistical analysis

The data were statistically analyzed using the Microsoft Excel software and the student's t-test was carried out. The student's *t*-test was used to analyze intergroup differences. Experiments were repeated at least two times, and data were represented as the mean ± SD. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Cytotoxicity of fraction DE14 and DE15 on different types of cancer cells and normal fibroblast cells

Fraction D of *P. cubeba* showed cytotoxic activity against MDA-MB-468 cells with an IC_{50} value of 23.77 ± 4.14 µg/mL, which stronger than fraction RD-M (31.96 ± 0.16 µg/mL) (p = 0.013). After fractionation of fraction D and fraction RD-M, we found that fraction DE14 and DE15, which were isolated from fraction D showing a strongly inhibited growth of MDA-MB-468 cells with an IC_{50} value corresponding to 8.74 ± 1.61 µg/mL (p = 0.002) and 6.59 ± 1.08 µg/mL (p = 0.001), respectively, which were lower than 20 µg/mL. All

fractions from fraction RD-ME exhibited cytotoxicity on MDA-MB-468 cells more than 80 µg/mL (the maximum concentration in this experiment) (Table 1). Therefore, fraction DE14 and DE15 were further investigated for cytotoxic activity on other types of cancer cells. Results reveal that fractions DE14 and DE15 exhibited an IC_{50} value less than 20 µg/mL. Fraction DE15 exhibited stronger cytotoxicity on breast cancer, colon cancer, and cholangiocarcinoma than fraction DE14. Surprisingly, fraction DE15 showed the strongest cytotoxic effect against MDA-MB-231 with an IC_{50} value of 4.43 ± 0.16 µg/mL ($p = 0.004$). Fraction DE15 showed less toxicity on normal fibroblast (L929) when compared with cancer cells and doxorubicin treatment. However, fraction DE14 represented a lower cytotoxic effect against L929 than fraction DE15 (Table 2). According to these results, we then performed GC-MS in the next experiment to identify the chemical compounds in fraction DE15.

In addition, fraction 14 has SI values more than 3 on cells MCF-7, MDA-MB-231 and KKU-M213 cells. Fraction 15 had a SI value more than 3 only with MDA-MD-231 cells. Meanwhile, doxorubicin had SI values less than 3 in all tested cells (Table 2).

Total phenolic, flavonoid, condensed tannin and antioxidant containing in fraction D and fraction DE15

Phenolic, flavonoid and tannin are secondary metabolites in the plant, which respond to antioxidant (Muniyandi et al., 2019) and anticancer activities (Tietbohl et al., 2017). The fraction D showed the amount of total phenolic, flavonoid, and tannin content lower than fraction DE15 ($p<0.001$). The amount of antioxidant of fraction D was also lower than fraction DE15 (Table 3) ($p<0.001$). Taken together, fraction DE15 exhibited strong cytotoxicity against cancer cells, probably due to the phytochemical components.

Phytochemical screening of fraction DE15

In this study, the phytochemical analysis using GC-MS was carried out to identify the phytochemical component in fraction DE15. The predicted constituents are shown in Fig. 2 and Table 4. The fraction DE15 contained 62 chemical compounds. Fraction DE15 mainly comprised of dodecanoic acid (24.05%, fatty acid), propylene glycol (23.82%, alcohol), n-hexadecanoic acid (11.37%, fatty acid), and 9-octadecanoic acid (10.00%, fatty acid) compounds with a high percentage of peak area.

Fraction DE15 inhibited cell cycle progression

To investigate the role of fraction DE15 in the inhibition of cell cycle progression, the studies were conducted on breast cancer MDA-MB-231 cells using flow cytometry. Results showed that percentages of G0/G1, S, and G2/M phases were no changed when treated MDA-MB-231 cells with various concentrations of fraction D and incubation times compared with non-treated cells and control group at 0 hour (Fig. 3).

Fraction DE15 induced apoptosis and multi-caspases activity

In this experiment, we determined the anticancer activity of fraction DE15 through the induction of apoptosis and multi-caspase activity. We first study the induction of apoptosis by fraction DE15. Results showed that live cells were significantly decreased when treated MDA-MB-231 with an IC_{50} concentration of fraction DE15 for 72 h. Moreover, the percentage of late apoptosis/dead was also significantly increased to 37.75 ± 0.78 and 36.95 ± 1.77% after treatment with fraction DE15 for 48 and 72 h (Fig. 4). These results indicated that fraction DE15 inhibited triple negative breast cancer through the induction of apoptosis.

In order to confirm the apoptosis induction by fraction DE15, a multi-caspase activity assay was performed on MDA-MB-231 cells. The results of live cells in this experiment were similar to the apoptosis experiment. Live cells were significantly decreased after treatment with fraction DE15 for 72 h. Moreover, the multi-caspases activity was significantly increased at 24-72 h after treatment with fraction DE15 compared to control (0 h) (Fig. 5). These data revealed that fraction DE15 induced apoptosis on triple negative breast cancer (MDA-MB-231) through caspases activation in a time-dependent manner.

DISCUSSION

Triple negative breast cancer is more aggressive than other types of breast cancer. Due to the absence of hormone receptors, triple negative breast cancer is not responding to hormone therapy. Medicinal plants may be an alternative treatment for triple negative breast cancer. There are many compounds of plants (such as *Allium* spp., *Glycine* spp., *Curcuma longa* and *Panax ginseng*) that can inhibit triple negative breast cancer through the specific pathway including HIF-I, MAPK, MTAP, NF-κB, PI3K, p53 and STAT3. The inhibition of these pathways causes inhibition of tumor growth and induction of apoptosis (Webb and Kukard, 2020).

In a previous study, we reported that methanol crude extract of *P. cubeba* showed anticancer activity against breast cancer (MCF-7 and MDA-MB-468) and less effect on normal breast cells. However, the fraction of CE showed strong cytotoxicity against MCF-7,

Table 1. Yields and cytotoxicity of *P. cubeba* extract on MDA-MB-468 cells.

Fractions	**Yield (%)**	**IC_{50} (µg/mL)**
D	15.86	23.77 ± 4.14
RD-M	3.87	31.96 ± 0.16
Doxorubicin (µM)	-	1.04 ± 0.20
Fraction D		
DE1	0.03	>80
DE2	0.03	>80
DE3	3.01	>80
DE4	14.98	>80
DE5	3.61	>80
DE6	0.49	>80
DE7	1.38	>80
DE8	0.15	>80
DE9	2.09	>80
DE10	2.34	>80
DE11	0.36	>80
DE12	0.54	>80
DE13	1.15	>80
DE14	0.65	8.74 ± 1.61
DE15	2.34	6.59 ± 1.08
Fraction RD-M		
RD-M1	0.08	>80
RD-M2	1.96	>80
RD-M3	0.45	>80
RD-M4	7.71	>80
RD-M5	0.40	>80
RD-M6	0.78	>80
RD-M7	0.88	>80
RD-M8	0.25	>80
RD-M9	0.54	>80
RD-M10	2.15	>80
RD-M11	9.83	>80
RD-M12	7.49	>80
RD-M13	0.51	>80

Half maximum concentrations (IC_{50}) are represented as mean ± SD (n = 3). D: Dichloromethane extract; RD-M: Residue-methanol extract; DE1-15: Fraction of dichloromethane extract number 1-15; RD-M1-13: Fraction of residue-methanol extract number 1 to 13.

Table 2. Cytotoxicity of fraction DE14, DE15 and doxorubicin on different types of cancer cells and normal fibroblast cells.

Cells	IC_{50}		
	Fraction DE14 (µg/mL)	**Fraction DE15 (µg/mL)**	**Doxorubicin (µM)**
Breast cancer			
MCF-7	7.99 ± 1.39 (6.69)[a]	7.95 ± 1.41 (1.76)[a]	1.49 ± 0.13 (1.15)[a]
MDA-MB-231	12.46 ± 2.85 (4.29)[a]	4.43 ± 0.16 (3.17)[a]	2.71 ± 0.13 (0.63)[a]
Colon cancer			
HT-29	26.62 ± 1.74 (2.01)[a]	10.82 ± 1.55 (1.30)[a]	2.63 ± 1.08 (0.65)[a]
Cholangiocarcinoma			
KKU-M213	9.45 ± 0.70 (5.65)[a]	6.76 ± 0.52 (2.08)[a]	1.53 ± 0.15 (1.12)[a]
Normal fibroblast			
L929	53.43 ± 1.66	14.03 ± 1.13	1.71 ± 0.32

Half maximum concentrations (IC_{50}) are expressed as mean ± SD (n = 3). SI > 3 was considered as a prospective anticancer. [a]Selectivity index; DE14: Fraction of dichloromethane extract number 14; DE15: Fraction of dichloromethane extract number 15.

Table 3. Total phenolic, tannin, flavonoid and antioxidant contents in Fraction D and DE15.

Phytochemical analysis	Fractions	
	D	**DE15**
Total phenolics (mg GAE/mg extract)[a]	0.175 ± 0.003	0.276 ± 0.010[e]
Flavonoids (mg QE/mg extract)[b]	0.033 ± 0.005	0.139 ± 0.021[e]
Condensed tannin (mg CE/mg extract)[c]	0.201 ± 0.006	0.497 ± 0.040[e]
Antioxidant (mg TEAC/mg extract)[d]	0.249 ± 0.010	1.347 ± 0.114[e]

Data are represented as mean ± SD (n = 3). [e]p-value of less than 0.001. D: Dichloromethane extract; DE15: Fraction of dichloromethane extract number 15. [a]mg of gallic acid equivalence by mg of extract; [b]mg of quercetin equivalence by mg of extract; [c]mg of catechin equivalence by mg of extract; [d]mg of Trolox equivalence by mg of extract; GAE: Gallic acid equivalent; QE: Quercetin equivalent; CE: Catechin equivalent; TEAC: Trolox equivalent antioxidant capacity.

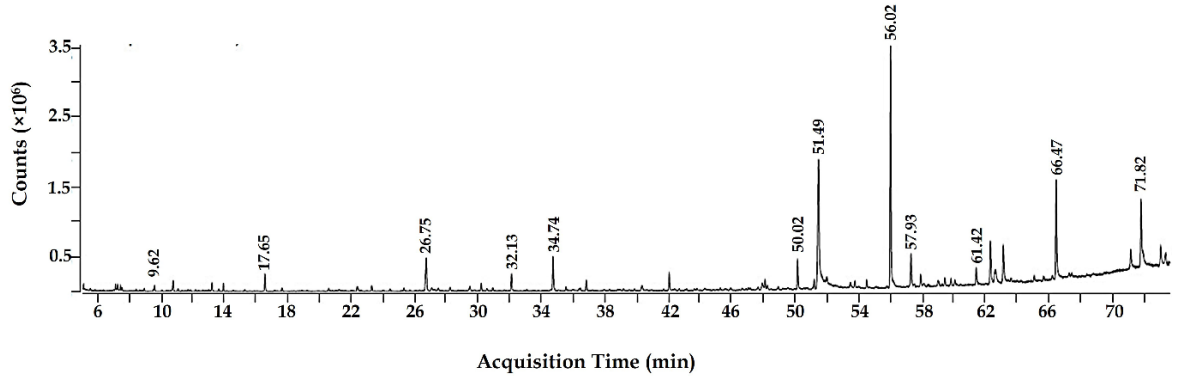

Figure 2. GC-MS chromatographic chromatogram of fraction DE15.

Table 4. Phytochemical constituents of fraction DE15.

Compound group	Identified compounds	Formula	Molecular mass (g/mol)	Area (%)	RT (min)
Fatty acid and fatty acid ester	Propanoic acid	$C_8H_{14}O_2$	74.08	0.74	17.18
	2-pentenoic acid	$C_6H_{12}O_2$	100.12	0.16	39.18
	Hexanoic acid	$C_6H_{12}O_2$	116.16	0.95	36.84
	Octanoic acid	$C_8H_{16}O_2$	144.21	0.18	43.80
	Ethyl-2-oxohexadecaoate	$C_8H_{34}O_3$	158.19	0.50	60.08
	Ethyl-(R,E)-4-hydroxy-3-methylpent-2- enoate	$C_8H_{14}O_3$	158.19	1.12	57.93
	Nonanoic acid	$C_9H_{18}O_2$	158.24	0.16	47.06
	Geranic acid	$C_{10}H_{16}O_2$	168.23	0.39	52.02
	Trans-2-decenoic acid	$C_{10}H_{18}O_2$	170.25	0.49	53.77
	Decanoic acid	$C_{15}H_{20}O_2$	172.26	2.62	50.02
	Dodecanoic acid	$C_{12}H_{24}O_2$	200.32	24.05	56.02
	Methyl decanoate	$C_{13}H_{26}O_2$	214.34	1.80	34.73
	Tetradecanoic acid	$C_{12}H_{28}O_2$	229.36	1.66	61.42
	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	11.37	66.47
	Palmitic acid methyl ester	$C_{17}H_{34}O_2$	270.50	0.65	48.12
	9,12-Octadecadienoic acid (Z,Z)	$C_{18}H_{32}O_2$	280.40	2.50	73.06
	Octadecanoic acid	$C_{18}H_{36}O_2$	282.50	2.08	71.17
	9-Octadecenoic acid	$C_{18}H_{34}O_2$	282.50	10.00	7.82
	Methyl-9-octadecanoate	$C_{19}H_{36}O_2$	296.50	0.60	54.52
Phenolic compound	4-Vinylphenol	C_8H_8O	120.15	0.44	53.49
	2,4-Bis(1,1-dimethylethyl)-Phenol	$C_{14}H_{22}O$	266.38	0.76	51.21
Hydrocarbon	2-Pentene	C_5H_{10}	86.13	0.50	5.140
	Ethynylbenzene	C_8H_8	102.13	0.71	13.23
	1,6-Octadiene	$C_{10}H_{16}$	136.23	0.25	9.62
	Cyclohexene	$C_{10}H_{16}$	136.23	0.82	10.79
	Undecane	$C_{11}H_{24}$	156.31	0.31	7.29
Aldehyde/ketone	Hexanal	$C_6H_{12}O$	100.16	0.38	7.15
	4-Hydroxy-4-methyl-2-pentanone	$C_6H_{12}O_2$	116.16	0.29	17.65
	(2R,2''R)-(-)-Tetrahydro-2,2-'-biuranyl-5,5'-dione	$C_8H_{10}O_4$	116.12	1.30	73.37
	Citral	$C_{10}H_{16}O$	152.23	0.60	30.22
	3,7-Dimethyl-2,6-octadienal	$C_{10}H_{16}O$	152.23	1.42	32.13
Alcohol	Propylene glycol	$C_3H_8O_3$	92.09	23.82	51.49
	(2S)-3-Methyl-3-buttene-1,2-diol	$C_5H_{10}O_2$	102.13	0.15	51.11
	3-Cyclohexene-1-methanol	$C_{10}H_{18}O$	154.25	0.22	30.94
	Phytol	$C_{20}H_{40}O$	296.50	0.44	59.05
Terpenoids	3-Carene	$C_{10}H_{16}$	136.23	0.18	8.96
	Trans beta-caryophyllene	$C_{15}H_{24}$	204.35	1.90	26.75
	Caryophyllene oxide	$C_{15}H_{24}O$	220.35	0.65	40.34

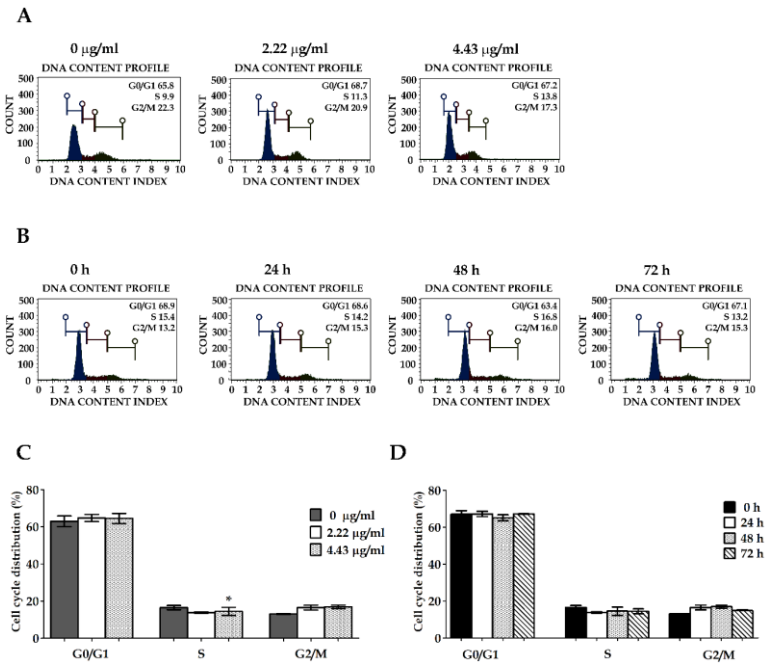

Figure 3. Effect of fraction DE15 on cell cycle.

(A) MDA-MB-231 cells were treated with 2.22 and 4.43 µg/mL of fraction DE15 for 24 h. **(B)** MDA-MB-231 cells were treated with 4.43 µg/mL of fraction DE15 for 72 h.

To determine the cell distributions in each phase of the cell cycle, treated cells were stained with PI and measured DNA content using MUSE® Cell Analyzer. Data are represented as mean ± SD of two independent experiments. P<0.05 was considered to indicate statistically significant differences compared to the control group at 0 h.

MDA-MB-468, and MDA-MB-231 with IC_{50} values of 2.69 ± 0.09, 2.97 ± 0.15 and 3.98 ± 0.12 µg/mL, respectively, and also normal cells MCF-12A and L929 with IC_{50} values of 2.91 ± 0.15 and 4.17 ± 0.77 µg/mL (Graidist et al., 2015).

In this study, dichloromethane crude extract of *P. cubeba* exhibited cytotoxicity against MDA-MB-468 with an IC_{50} value of 23.77 ± 4.14 µg/mL, which was stronger than dichloromethane crude extracts of our previously reported. This result should be due to a soaking step that took for 7 days. Fraction DE15 exhibited the most potent cytotoxic effect against MDA-MB-231 with an IC_{50} value of 4.43 ± 0.16 µg/mL, which was stronger than the effect on normal cells (14.03 ± 1.13 µg/mL) (3.17 folds compared to MDA-MB-231). At present, breast cancer is classified into 4 types, including luminal A [estrogen receptor-positive (ER+)], progesterone receptor-positive (PR+), human epidermal growth factor receptor 2-negative (HER2-), luminal B (ER+, PR+, HER2+), non-luminal with HER2+ (ER-, PR-, HER2+) and basal-like or triple negative (ER-, PR-, HER2-) (Perou et al., 2000). MCF-7 cells have been classified as luminal A type. In addition, MDA-MB-468 and MDA-MB-231 have been classified as triple negative A and triple negative B (claudin-low) types. The luminal tumor has a better prognosis than the triple negative tumor (Dai et al., 2017). According to the results of our present study, we summarized that fraction DE specified to triple negative breast cancer.

Here, we further studied the phytochemical activity of dichloromethane *P. cubeba* crude extract and purified fraction DE15 and found that fraction DE15 represented higher levels of total phenolics, flavonoids, and condensed tannin than dichloromethane *P. cubeba* crude extract for 1.57, 4.21 and 2.47 folds, respectively. Phenolic compounds, such as phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes and tannins, are rich in antioxidant properties (Cotelle et al., 1996; Zheng and Wang, 2001). These antioxidant compounds possess anti-inflammatory, anti-atherosclerotic, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial and anti-viral activities (Mitscher et al., 1996; Owen et al., 2000; Sala et al., 2002). The total phenolic content of Chinese medicinal plants showed a positive significant linear relationship with antioxidant activity (Cai et al., 2004). High phenolic compounds in *Artemisia absinthium* extract showed antioxidant effect and cytotoxic activity on the colon (DLD-1) and endometrium (ECC-1) cancer cells (Koyuncu, 2018).

In Table 3, antioxidant property from the extracted fraction DE15 was detected. Gas chromatography was applied to identify the components of the extracted fraction. In Table 4, the phenolic contents as mentioned could be from 4-vinylphenol (0.44%), and 2,4-bis(1,1-dimethylethyl)-phenol (0.76%), even though they were to a lesser extent compared to other nonpolar compound groups. The major components of the extracted fraction DE15 were fatty acid and fatty acid esters, propylene glycol, and hydrocarbon, respectively. This was not surprising for dichloromethane if one considered its polarity index (PI) of 3.7, a nonpolar solvent (Snyder, 1978). The phenolic compounds preferred an extraction by higher polar solvents such as methanol (PI = 6.6), ethanol (5.2), or water (PI = 9.0) (Abarca-Vargas et al., 2016). Therefore, dichloro-

methane would not dissolve phenolic compounds as much as relatively nonpolar groups, in good agreement with Table 4.

Many active phytochemicals were founded in fraction DE15, including dodecanoic acid, propylene glycol, n-hexadecanoic acid, and 9-octadecenoic acid with a percentage of peak area at 24.05, 23.82, 11.37 and 10.00%, respectively. Dodecanoic acid or lauric acid, a saturated medium-chain fatty acid, demonstrated an anticancer effect on HER2+ breast cancer (SkBr3) cells through the increase of reactive oxygen species (ROS) levels, stimulates phosphorylation of EGFR, ERK and c-Jun, and induction of c-fos (Lappano et al., 2017). Palmitic acid or n-hexadecanoic acid from *Kigelia pinnata*, a common fatty acid from plants, demonstrated anticancer activity against colorectal cancer HCT-116 cells (Ravi and Krishnan, 2017). Moreover, palmitic acid from a marine red alga (*Amphiroa zonata*) induces apoptosis on the human leukemic MOLT-4 cells but no cytotoxicity to normal human dermal fibroblast (HDF) cells. Palmitic acid also shows *in vivo* anti-tumor activity in mice. This compound targets DNA topoisomerase I but has no effects on DNA topoisomerase II (Harada et al., 2002).

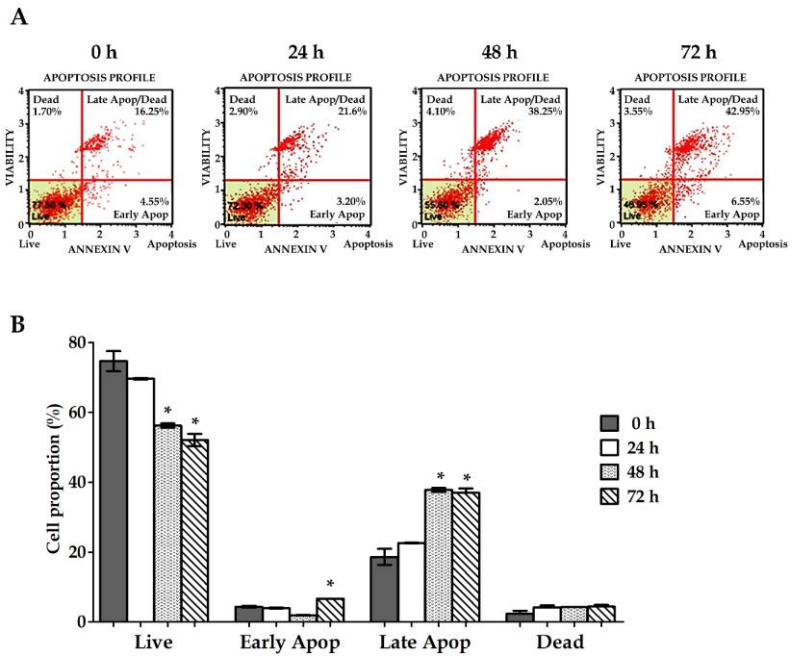

Figure 4. Anticancer effect of fraction DE15 on the induction of apoptosis.

MDA-MB-231 cells were treated with fraction DE15 at IC_{50} concentration value. Treated cells were harvested at 24, 48 and 72 h. **(A)** After treatment, apoptotic cells were analyzed using the Muse® Annexin V Dead Cell kit. The scatter plots are shown the percentage of apoptotic cells. **(B)** The graph represents the summary of mean percentages ± SD of apoptotic cells of two independent experiments. The statistical analysis of the data was carried out by Student *t*-test. *P<0.05 was considered to indicate a statistically significant difference compared to the control group at 0 h.

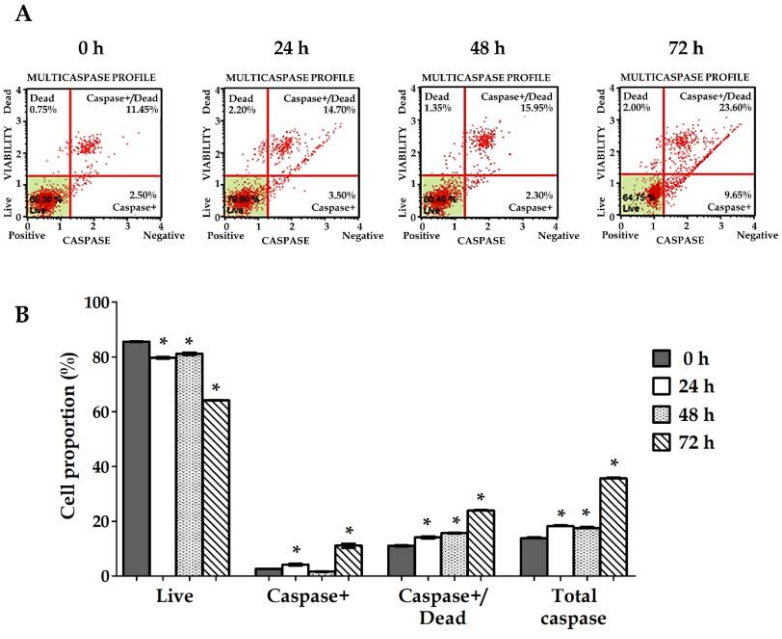

Figure 5. Effect of fraction DE15 on multi-caspase activity.

MDA-MB-231 cells were treated with fraction DE at IC_{50} concentration for 72 h. The multi-caspase activity was determined using the Muse® MultiCaspase Kit. **(A)** The plot depicted the effect of fraction DE. Each plot is a representative figure of the two replicates of each determination. **(B)** The graph depicts the percentage of cells with multi-caspase activity. Values represent mean ± SD. The statistical analysis of the data was carried out by Student *t*-test. *P<0.05 was considered to indicate a statistically significant difference compared to the control group at 0 h.

9-Octadecenoic acid (or oleic acid), the main ingredient of *Brucea javanica* oil, showed effectively inhibits tongue squamous cell carcinoma (TSCC) proliferation in a dose- and time-dependent manner. This compound inhibited tumor cells by induction of cell cycle arrest at G0/G1 phase and increasing the expression of p53 and cleaved caspase-3, decreasing cyclinD1, Bcl-2 and p62, and inhibition of Akt/mTOR signaling pathway. 9-Octadecenoic acid also inhibits the growth of CAL27 xenograft tumors in nude mice (Jiang et al., 2017).

Moreover, the dichloromethane extract of *Protaetia brevitarsis* larvae consisted of three free fatty acids, such as palmitic acid, (Z)-9-octadecenoic acid and octadecenoic acid. These compounds induce apoptosis by stimulation of DNA fragmentation and caspase-3 activation leading to apoptosis in colon 26 marine carcinoma cells (Yoo et al., 2007). Our previous results on fraction CE from methanol extract of *P. cubeba* also demonstrated anticancer activity through the induction of DNA fragmentation with a ladder pattern characteristic of apoptosis in MCF-7, MDA-MB-468 and MDA-MB-231 cells (Graidist et al., 2015). In this present study, faction DE increased multi-caspase activity and apoptosis in MDA-MB-231. However, fraction DE did not induce cell cycle arrest.

CONCLUSION

Fraction DE15 from dichloromethane extract of *P. cubeba* seed, composed of many active compounds (such as dodecanoic acid, n-hexadecanoic acid and 9-octadecenoic acid), and had cytotoxicity against breast, colon cancer, and cholangiocarcinoma, especially triple negative breast cancer type, MDA-MB-231. Fraction DE15 showed less cytotoxicity on normal fibroblast cells. This fraction also induced apoptosis by the stimulation of multi-caspases activity. According to the obtained results, this extract can be considered as an alternative treatment with a chemotherapeutic drug for triple negative breast cancer. Besides, fraction DE15 would also be a further study in the mechanism of action and the anticancer effect and genotoxic in an animal model.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This work was supported by grants from the Faculty of Medicine (REC 57-004-04-8), Prince of Songkla University; Research Center for Cancer Control in Thailand (MEDRC59036), and the Postdoctoral Fellowship from Prince of Songkla University, Songkhla, Thailand.

REFERENCES

Abarca-Vargas R, Pena Malacara CF, Petricevich VL (2016) Characterization of chemical compounds with antioxidant and cytotoxic activities in *Bougainvillea* × *buttiana* Holttum and Standl, (var. Rose) extracts. Antioxidants 5: 45.

Barreto JN, McCullough KB, Ice LL, Smith JA (2014) Antineoplastic agents and the associated myelosuppressive effects: a review. J Pharm Pract 27: 440–446.

Bastos JK, Carvalho JC, de Souza GH, Pedrazzi AH, Sarti SJ (2001) Anti-inflammatory activity of cubebin, a lignan from the leaves of *Zanthoxyllum naranjillo* Griseb. J Ethnopharmacol 75: 279–282.

Bézivin C, Tomasi S, Lohézic-Le Dévéhat F, Boustie J (2003) Cytotoxic activity of some lichen extracts on murine and human cancer cell lines. Phytomedicine 10: 499–503.

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of free radical method to evaluate antioxidant activity. LWT-Food Sci Tech 28: 25–30.

Cai Y, Luo Q, Sun M, Corke H (2004) Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci 74: 2157–2184.

Chang CC, Yang MH, Wen HM, Chern JC (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 10: 178–182.

Cotelle N, Bernier JL, Catteau JP, Pommery J, Wallet JC, Gaydou EM (1996) Anti-oxidant properties of hydroxyl-flavones. Free Radic Biol Med 20: 35–43.

Dai X, Cheng H, Bai Z, Li J (2017) Breast cancer cell line classification and its relevance with breast tumor subtyping. J Cancer 8: 3131–3141.

Graidist P, Martla M, Sukpondma Y (2015) Cytotoxic activity of *Piper cubeba* extract in breast cancer cell lines. Nutrients 7: 2707–2718.

Harada H, Yamashita U, Kurihara H, Fukushi E, Kawabata J, Kamei Y (2002) Antitumor activity of palmitic acid found as a selective cytotoxic substance in a marine red alga. Anticancer Res 22: 2587–2590.

Jiang L, Wang W, He Q, Wu Y, Lu Z, Sun J, Liu Z, Shao Y, Wang A (2017) Oleic acid induces apoptosis and autophagy in the treatment of tongue squamous cell carcinomas. Sci Rep 7: 11277.

Junqueira APF, Perazzo FF, Souza GHB, Maistro EL (2007) Clastogenicity of *Piper cubeba* (Piperaceae) seed extract in an *in vivo* mammalian cell system. Mol Biol 30: 656–663.

Koyuncu I (2018) Evaluation of anticancer, antioxidant activity and phenolic compounds of *Artemisia absinthium* L. Extract. Cell Mol Biol (Noisy-le-grand) 64: 25–34.

Lappano R, Sebastiani A, Cirillo F, Rigiracciolo DC, Galli GR, Curcio R, Malaguarnera R, Belfiore A, Cappello A, Maggiolini M (2017) The lauric acid-activated signaling

prompts apoptosis in cancer cells. Cell Death Discov 3: 17063.

Maistro EL, Marcondes Natel AV, De Souza GHB, Perazzo FF (2011) Genotoxic effects of (-)- cubebin in somatic cells of mice. J Appl Toxicol 31: 185–189.

Mitscher LA, Telikepalli H, McGhee E, Shankel DM (1996) Natural antimutagenic agents. Mutat Res 350: 142-143.

Muniyandi K, George E, Sathyanarayanan S, George BP, Abrahamse H, Thamburaj S, Thangaraj P (2019) Phenolics, tannins, flavonoids and anthocyanins contents influenced antioxidant and anticancer activities of Rubus fruits from Western Ghats, India. Food Sci Hum Wellness 8: 73–81.

Nahak G, Sahu RK (2011) Phytochemical evaluation and antioxidant activity of *Piper cubeba* and *Piper nigrum*. J Appl Pharm Sci 1: 153–157.

Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H (2000) The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer 36: 1235–1247.

Perazzo FF, Rodrigues IV, Maistro EL, Souza SM, Nanaykkara NPD, Bastos JK, Carvalho JCT, de Souza GHB (2013) Anti-inflammatory and analgesic evaluation of hydroalcoholic extract and fractions from seeds of *Piper cubeba* L. (Piperaceae). Pharmacogn J 5: 13–16.

Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge Ø, Pergamenschikov A, Williams C, Zhu SX, Lønning PE, Børresen-Dale AL, Brown PO, Botstein D (2000) Molecular portraits of human breast tumours. Nature 406: 747–752.

Rajalekshmi DS, Kabeer FA, Madhusoodhanan AR, Bahulayan AK, Prathapan R, Prakasan N, Varughese S, Nair MS (2016) Anticancer activity studies of cubebin isolated from *Piper cubeba* and its synthetic derivatives. Bioorg Med Chem Lett 26: 1767–1771.

Rattanaburee T, Thongpanchang T, Wongma K, Tedasen A, Sukpondma Y, Graidist, P (2019) Anticancer activity of synthetic (±)-kusunokinin and its derivative (±)-bursehernin on human cancer cell lines. Biomed Pharmacother 117: 109115.

Ravi L, Krishnan K (2017) Cytotoxic potential of N-hexadecanoic acid extracted from *Kigelia pinnata* leaves. Asian J Cell Biol 12: 20–27.

Sala A, Recio M, Giner RM, Manez S, Tournier H, Schinella G, Ríos JL (2002) Anti-inflammatory and antioxidant properties of *Helichrysum italicum*. J Pharm Pharmacol 54: 365–371.

Salehi B, Zakaria ZA, Gyawali R, Ibrahim SA, Rajkovic J, Shinwari ZK, Khan T, Sharifi-Rad J, Ozleyen A, Turkdonmez E, Valussi M, Tumer TB, Fidalgo LM, Martorell M, Setzer WN (2019) Piper species: A comprehensive review on their phytochemistry, biological activities and applications. Molecules 24: 1364.

Singleton VL, Rossi JA (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic 16: 144–158.

Snyder LR (1978) Classification of the solvent properties of common liquids. J Chromatogr Sci 16:223–234.

Sriwiriyajan S, Ninpesh T, Sukpondma Y, Nasomyon T, Graidist P (2014) Cytotoxicity screening of plants of genus *Piper* in breast cancer cell lines. Trop J Pharm Res 13: 921–928.

Tamadher MK, Tememy AL (2013) Antibacterial activity of *Piper cubeba* linn. fruit extracts against selected bacterial pathogens in Basrah City. Bas J Vet Res 12: 142–151.

Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne DH (2006) Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J Food Compos Anal 19: 669–675.

Tietbohl LA, Oliveira AP, Esteves RS, Albuquerque RD, FollyD, Machado FP, Correa AL, Santos MG, Ruiz AL, Rocha L (2017) Antiproliferative activity in tumor cell lines, antioxidant capacity and total phenolic, flavonoid and tannin contents of *Myrciaria floribunda*. AABC 89: 1111–1120.

Webb MJ, Kukard C (2020) A review of natural therapies potentially relevant in triple negative breast cancer aimed at targeting cancer cell vulnerabilities. Integr Cancer Ther 19: 1534735420975861.

Yam J, Kreuter M, Drewe J (2008) *Piper cubeba* targets multiple aspects of the androgen-signaling pathway. A potential phytotherapy against prostate cancer growth. J Planta Med 74: 33–38.

Yoo YC, Shin BH, Hong JH, Lee J, Chee HY, Song KS, Lee KB (2007) Isolation of fatty acids with anticancer activity from *Protaetia brevitarsis* larva. Arch Pharm Res 30: 361–365.

Zheng W, Wang SY (2001) Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem 49: 5165–5170.

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Contribution	Maungchanburi S	Rattanaburee T	Sukpondma Y	Tedasen A	Tipmanee V	Graidist P
Concepts or ideas	x		x			x
Design	x		x			x
Definition of intellectual content	x					x
Literature search	x		x			x
Experimental studies	x	x	x	x	x	x
Data acquisition	x	x	x	x	x	x
Data analysis	x	x	x	x	x	x
Statistical analysis	x	x		x	x	x
Manuscript preparation	x	x	x	x	x	x
Manuscript editing	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x

Citation Format: Maungchanburi S, Rattanaburee T, Sukpondma Y, Tedasen A, Tipmanee V, Graidist P (2022) Anticancer activity of *Piper cubeba* L. extract on triple negative breast cancer MDA-MB-231. J Pharm Pharmacogn Res 10(1): 39–51. https://doi.org/10.56499/jppres21.1160_10.1.39