

# Dichloromethane Crude Extract of *Gymnanthemum extensum* Combined with Low Piperine Fractional *Piper nigrum* Extract Induces Apoptosis on Human Breast Cancer Cells

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Faisal *et al.*: *Gymnanthemum extensum* Combined with *Piper nigrum* Induces Apoptosis

This study aims to investigate the cytotoxicity of *Andrographis paniculata*, *Ziziphus spina-christi* and *Gymnanthemum extensum* crude extracts and their combination with low piperine fractional *Piper nigrum* extract. All plants were extracted with water and five organic solvents (methanol, ethanol, dichloromethane, ethyl acetate and hexane). Cytotoxicity was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay on three cancer types (breast, colorectal and ovarian cancers) and two non-cancerous cells. The combination among extracts with low piperine fractional *Piper nigrum* extract was separately conducted in several tests including cytotoxicity, apoptosis and multi caspase activity. We found that, the dichloromethane crude extract of *Andrographis paniculata*, *Ziziphus spina-christi* and *Gymnanthemum extensum* exhibited the strongest cytotoxicity on colorectal cancer cells SW-620 ( $7.49 \pm 0.04 \mu\text{g ml}^{-1}$ ), breast cancer cells Michigan Cancer Foundation-7 ( $13.35 \pm 0.30 \mu\text{g. ml}^{-1}$ ) and ovarian cancer cells A2780 ( $15.58 \pm 1.81 \mu\text{g ml}^{-1}$ ), respectively. Gas chromatography mass spectrometry study of dichloromethane crude extract of *Andrographis paniculata*, *Ziziphus spina-christi* and *Gymnanthemum extensum* identified major compounds including 1-heptatriacotanol (60.29 %) and palmitic acid (26.92 % for dichloromethane crude extract of *Ziziphus spina-christi* and 21.40 % for dichloromethane crude extract of *Gymnanthemum extensum*), respectively. The combination of dichloromethane crude extract of *Ziziphus spina-christi* and low piperine fractional *Piper nigrum* extract at ratio  $\text{IC}_{50} : 0.5 \text{IC}_{50}$  showed a moderate synergistic effect on Michigan Cancer Foundation-7 cells. Interestingly, the mixture of dichloromethane crude extract of *Gymnanthemum extensum* with low piperine fractional *Piper nigrum* extract at ratio  $\text{IC}_{50} : \text{IC}_{50}$ ,  $0.5 \text{IC}_{50} : \text{IC}_{50}$  and  $\text{IC}_{50} : 0.5 \text{IC}_{50}$  exhibited a synergistic effect on Michigan Cancer Foundation-7 cells. Moreover, combination of dichloromethane crude extract of *Gymnanthemum extensum* with low piperine fractional *Piper nigrum* extract induced the apoptosis and multi caspase activity in a time dependent manner. In conclusion, dichloromethane crude extract of *Andrographis paniculata*, *Ziziphus spina-christi* and *Gymnanthemum extensum* displayed potent anticancer activities and the combination of dichloromethane crude extract of *Gymnanthemum extensum* with low piperine fractional *Piper nigrum* extract can be a promising regimen for an alternative cancer treatment.

**Key words:** Apoptosis, combination, dichloromethane, *Gymnanthemum extensum*, *Piper nigrum*

The Global Cancer Observatory (GLOBOCAN) estimated more than 9 million death cases worldwide owing to cancer. Predictably, it would threaten people worldwide as 17 million cancer deaths per year in 2030<sup>[1]</sup>. Breast and colorectal cancer are the top three in death rates worldwide<sup>[2]</sup>. Moreover, ovarian cancer is the deadliest cancer type besides breast and colorectal cancers and causes over 50 % of the death rate<sup>[3]</sup>. Now

a days, several therapies to remedy cancers have been harnessed by clinicians such as surgery, radiotherapy and chemotherapy. Indeed, chemotherapy has been

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acknowledged as the most commonly used therapy. Otherwise, chemotherapy somehow might lead to being ineffective therapy due to drug resistance and cancer recurrence<sup>[4]</sup>.

To date, over 3000 plant species have been revealed their potency as an anticancer agent<sup>[5]</sup>. *Andrographis paniculata* (*A. paniculata*) (Acanthaceae) is a shrub plant grown annually and distributed to tropical and subtropical Asia and also Southeast Asia<sup>[6]</sup>. *Ziziphus spina-christi* (*Z. spina-christi*) (Rhamnaceae) is an endemic plant of the middle east and distributed from Saharan Oases to West Africa<sup>[7]</sup>. *Gymnanthemum extensum* (*G. extensum*) (Asteraceae) is a bitter leaf tree distributed to Northeastern Thailand<sup>[8]</sup>. Previously, *A. paniculata*, *Z. spina-christi* and *G. extensum* leaves showed high cytotoxicity on several cancer cells, indicated by half-maximal inhibitory concentration ( $IC_{50}$ ) lower than 20  $\mu$ g/ml.

The methanolic crude extract of *A. paniculata* leaves exhibited a strong cytotoxicity effect on nasopharynx (KB) and leukemic (P388) cells<sup>[9]</sup> and colorectal cancer cells<sup>[10]</sup>. In addition, ethanolic crude extract of *A. paniculata* leaves also showed a high cytotoxicity effect on leukemic cells (HL-60)<sup>[11]</sup>. Ethyl acetate crude extract of *A. paniculata* leaves inhibited breast cancer (T-47D) and colorectal cancer (WiDr) cells<sup>[12]</sup>. Furthermore, *Z. spina-christi* leaves extracted in ethanol showed notable cytotoxicity on Michigan Cancer Foundation-7 (MCF-7) cells<sup>[13]</sup>. Methylene chloride crude extract of *G. extensum* showed high cytotoxicity on liver cancer (HepG2) cells<sup>[14]</sup>.

According to these previous findings, we then carried out a study to investigate the cytotoxicity of these three medicinal plants in a different polarity of organic solvents. The selection of five different solvents used in this present study was referred to their polarity which correlated with the dielectric constant<sup>[15]</sup>. The polarity of solvent from highest to lowest are methanol (32.7), ethanol (24.6), dichloromethane (9.08), ethyl acetate (6.02) and hexane (1.9), respectively<sup>[16]</sup>. In addition, consideration of selective anticancer agents of these three medicinal plants was conducted on three cancer types and two non-cancerous cells (Vero and L-929). These normal cells were isolated from normal kidney cells of green African monkey and murine normal fibroblast cells, respectively. Due to the genetic stability and non tumorigenicity feature of Vero and L-929 cells, they are suitable to use in cytotoxicity tests<sup>[17,18]</sup>.

Previously, we reported that low piperine fractional *Piper nigrum* (*P. nigrum*) extract (PFPE) exhibited the highest

cytotoxicity compared to piperine and dichloromethane extract of *P. nigrum*<sup>[19]</sup>. This extract was able to inhibit various cancer cells including breast cancer (MCF-7, M.D. Anderson (MDA) Metastasis Breast cancer (MB)-468 and ZR-75-1) and cholangiocarcinoma (KKU-100, KKU-M213, KKU-M055 and human extrahepatic bile duct carcinoma cell line (TFK-1)<sup>[20]</sup>. Phytochemical compounds including kusunokinin and piperlongumine isolated from PFPE showed high cytotoxicity against breast cancer (MCF-7 and MDA-MB-468) and colorectal cancer (SW-620). PFPE degraded the proteins in cancer progression including signal transducer and activator of transcription 3 (STAT-3), cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF- $\kappa$ B), Topoisomerase II, Cellular myelocytomatosis oncogene (c-myc), cyclin D1 and B-cell lymphoma 2 (Bcl-2)). Moreover, PFPE contained pipericine, 2,4,14-eicosatrienamide, retrofractamide-a and piperanine showed cytoprotective on L-929 cells and hepatoprotective<sup>[20-22]</sup>. Furthermore, PFPE exhibited very low cytotoxicity against murine fibroblast cells (L-929 cells)<sup>[20]</sup>. Due to PFPE, showed high cytotoxicity on cancer cells and hepatoprotective activity, we here performed the combination of PFPE together with those three plant extracts to increase cytotoxicity which is higher than a single treatment.

A combination in cancer treatment has been recognized as an effective way to improve successful treatment by increasing the cytotoxicity<sup>[23]</sup>. The combination of *A. paniculata* with *Silybum marianum* showed a higher percentage of cell inhibition on breast (MCF-7), ovarian (Human cervical tumor cell-SiHa) and liver (human liver cancer cell line-HepG2) cancer cells than each individual treatment<sup>[24]</sup>. Moreover, *Z. spina-christi* with termite shelter extract concentration at 300  $\mu$ g/ml exhibited stronger cytotoxicity on cervical cells (Henrietta Lacks-HeLa) than individual treatment<sup>[25]</sup>. Thus, we hypothesized that *A. paniculata*, *Z. spina-christi* and *G. extensum* would have stronger cytotoxicity once we combined them with PFPE rather than a single treatment. In this report, we conducted the cytotoxicity test of different extracts of *A. paniculata*, *Z. spina-christi* and *G. extensum* leaves individually and their combination with PFPE on cancer cells. Phytocompounds were observed by gas chromatography-mass spectrometry (GC-MS) and the cytotoxicity of three medicinal plants alone and their combination with PFPE on cancer cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Finally, to approach further results whether the regimen could

inhibit precisely cancer cells growth only, apoptosis and multi caspase analysis were performed by flow cytometry analysis.

## MATERIALS AND METHODS

### Plant materials collection:

Dried leaves of *A. paniculata* and dried fruits *P. nigrum* were harvested from Banten Province, Indonesia and Songkhla Province, Thailand, respectively. Both plant specimens were authenticated by Assistant Professor Dr. Supreeya Yuenyongsawad and deposited in the Herbarium of Southern Centre of Thai Traditional Medicine, Department of Pharmacognosy and Pharmaceutical Botany, Prince of Songkla University (PSU), Thailand as a voucher specimen number SKP 001011601 and SKP 146161401, respectively. Dried leaves of *Z. spina-christi* was harvested from East Java Province, Indonesia and taxonomically authenticated by Dr. Nurainas (Chief of ANDA Herbarium, Department of Biology, Andalas University) with letter No. 305/K-ID/ANDA/IX/2018. Dried leaves of *G. extensum* were harvested from Phatthalung Province, Thailand and identified by Professor Dr. Suchada Sukrong and deposited as a voucher specimen (No. SS-PG-001) and kept at the Museum of Natural Medicine, Faculty of Pharmaceutical Science, Chulalongkorn University, Thailand.

### Chemicals and reagents:

In extraction protocol, ethanol ( $C_2H_5OH$ ), methanol ( $CH_3OH$ ), dichloromethane ( $CH_2Cl_2$ ), ethyl acetate ( $C_4H_8O_2$ ) and acetone ( $C_3H_6O$ ) was purchased from J. T. Baker® (Phillipsburg, NJ, USA). Hexane ( $C_6H_{14}$ ) was purchased from Reagent Chemical Industry (RCI) LabScan Ltd (Bangkok, Thailand). Furthermore, to make 1x phosphate buffered saline (PBS) for cell culture, the chemical substance such as sodium chloride (NaCl) was purchased in Omnipur® (Idaho, ID, USA). Moreover, sodium phosphate dibasic anhydrous ( $Na_2HPO_4$ ), potassium chloride (KCl), potassium phosphate ( $KH_2PO_4$ ) and dimethyl sulfoxide ( $C_2H_6OS$ ) were purchased from Amresco® (Solon, OH, USA). In the cell culture study, the cell medium and supplements including Roswell Park Memorial Institute Medium (RPMI-1640) and Dulbecco's Modified Eagle Medium (DMEM) powder, L-Glutamine, Fetal Bovine Serum (FBS) and antibiotics (penicillin-streptomycin) were purchased from Gibco™ (Massachusetts, USA). MTT assay and dimethyl sulfoxide ( $C_2H_6OS$ ) were purchased from GibThai (Bangkok, Thailand) and Amresco®,

respectively. Apoptosis and multi caspase reagents were purchased from Becton, Dickinson (BD) Biosciences, Inc (San Jose, CA, USA).

### Maceration extraction:

3 g of pulverized dried leaves were soaked in 300 ml of five types of organic solvents consists of methanol, ethanol, dichloromethane, ethyl acetate and hexane. Afterward, the mixture was filtered using Whatman Paper No. 1 filter paper. The filtrates were evaporated using a rotor evaporator at 40-45°, 1-3 mbar of vacuum pressure and 40 rpm of rotation. Then, the crude extracts were stored at -20° until used. The stock solution was diluted with dimethyl sulfoxide (DMSO).

### Decoction extraction and lyophilization:

3 g of pulverized dried leaves were soaked in 300 ml of boiled water within 10 min. Next, the extracts were placed in room temperature. After the temperature of extracts was decreased, the extracts were lyophilized by the freeze-drying method as described by Seong and colleagues<sup>[26]</sup>. Then, the freeze- dried extracts were kept at -20° until used. The stock solution was diluted with dimethyl sulfoxide (DMSO).

### Cell culture:

Human breast adenocarcinoma MCF-7 and MDA-MB-231, colorectal (HT-29 and SW-620) and ovarian (A2780 and SKOV-3) were used as cancerous representative cells. MCF-7 (American Type Culture Collection (ATCC®) HTB-22), MDA-MB-231 (ATCC® HTB-26), SKOV-3 (ATCC® HTB-77) and Vero (ATCC® CCL-81) cells were purchased from American Type Culture Collection (Manassas, Virginia, USA). A2780 cells were purchased from AddexBio (San Diego, California, USA). SW-620 cells were kindly donated by Dr. Surasak Sangkhathat, MD from Department of Surgery, Faculty of Medicine, Prince of Songkla University (PSU). Meanwhile, HT-29 cells were kindly given by Dr. Ruadeekorn Wiwattanapatapee from Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, PSU. L-929 cells were kindly provided by Dr. Jasadee Kaewsichan (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, PSU). Vero, A2780 and SKOV-3 cells were cultured in RPMI-1640 culture medium. HT-29, SW-620, MDA-MB-231 and L-929 cells were grown in DMEM. Both media (RPMI-1640 and DMEM) were supplemented with 10 % fetal bovine serum and



1 % of penicillin and streptomycin. All cultures were maintained at an incubation condition of 37° in 5 % carbon dioxide and at 95 % relative humidity.

### Cytotoxicity of individual extract:

To investigate the cytotoxicity in a single extract, an MTT colorimetric assay was conducted. Cells in the medium were seeded into 96-well plates (SDL Biosciences, Thailand). MCF-7, MDA-MB-231, SW-620 and SKOV-3 cells were seeded at a density of  $2 \times 10^3$  cells/well. Vero, L-929 and A2780 cells were seeded at a density of  $1 \times 10^3$  cells/well and HT-29 cells were seeded at a density of  $1.5 \times 10^3$  cells/well. All seeded cells were incubated within 24 h for adhering. The cell medium was mixed with extracts with different concentrations (5, 10, 20, 40 and 80 µg/ml) and applied to the cells within 72 h. As a positive control, doxorubicin with different concentrations (0.625, 1.25, 2.5, 5 and 10 µM) was applied to the respective wells. Furthermore, 0.5 % v/v of DMSO (a final concentration) was added to the complete cell medium mentioned as a vehicle. A complete cell medium with cells was mentioned as a negative control. After 72 h, the cell medium was changed by 100 µl MTT for 30 min and then dissolved by 100 µl of DMSO per well to accumulate the formazan salt. The colour formation was generated and observed using a microplate reader spectrophotometer (Spectra Max M5, Molecular Devices) at 570 nm and 650 nm, then the  $IC_{50}$  values were calculated using the formula as previously described<sup>[19]</sup>. Refers to the National Cancer Institute (NCI), a plant extract is considered to possess *in vitro* activity with the  $IC_{50}$  value of  $\leq 20$  µg/ml<sup>[19]</sup>. The  $IC_{50}$  of the individual extract was used as the initial concentration of the combination study. The selective index (SI) was determined adapted by Sriwiriyan and colleagues<sup>[27]</sup> which divided the  $IC_{50}$  values of extracts of non-cancerous cells with the  $IC_{50}$  values of extracts on cancer cells.

### Gas Chromatography/Mass Spectrometry (GC/MS):

To confirm the presence of phytochemicals possessed of notable cytotoxicity, gas chromatography/mass spectrometry analysis was conducted using two analytical instruments, Gas chromatography Agilent 7890B and Agilent 5977A triple quadrupole mass spectrometer (Agilent Technologies Inc, USA). This protocol was performed as previously described<sup>[20]</sup>.

**Cytotoxicity of combination extract:** The present study was conducted to investigate the interaction of

this combination exhibited synergism, additive, or antagonism in inhibiting cancer cells. The combination extract protocol was conducted by MTT colorimetric assay. This investigation was performed by four ratios including  $IC_{50}$  extract:  $IC_{50}$  PFPE;  $0.5 \times IC_{50}$  extract:  $IC_{50}$  PFPE;  $IC_{50}$  extract:  $0.5 \times IC_{50}$  PFPE and  $0.5 \times IC_{50}$  extract:  $0.5 \times IC_{50}$  PFPE. The combination treatment results were computerized by “CompuSyn” software to procure the combination index values<sup>[28]</sup>. Manually, the combination index (CI) values were calculated by the Chou-Talalay equation<sup>[29]</sup>.

The interaction of combination was indicated by CI, which estimated from dose effect data, both single and combined drug treatments. The synergism was identified from a CI value based on the criteria as followed:  $CI < 0.1$  (synergism); 0.1-0.3 (strong synergism); 0.3-0.7 (synergism); 0.7-0.85 (moderate synergism); 0.85-0.9 (slight synergism); 0.90-1.10 (nearly additive); 1.10-1.45 (slight antagonism); 1.45-3.3 (antagonism); 3.3-10 (strong antagonism) and  $> 10$  (very strong antagonism)<sup>[30]</sup>.

### Apoptosis assay:

In the present study, apoptotic and necrotic cells were observed using an annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) detection kit (Catalog No. MCH100105, Merck Millipore), following the manufacturer's instruction. Briefly, MCF-7 cells ( $2 \times 10^5$  cells/well) were seeded in 12 well plates. Afterward, the cells were incubated with the combination of dichloromethane crude extract of *Gymnanthemum extensum* DEGE with PFPE at ratio  $IC_{50}$ : $IC_{50}$  at various times (0, 24, 48 and 72 h). Treated cells were harvested and cell pellets were incubated in 100 µl of Muse® FITC-Annexin V apoptosis staining kit and 100 µl of propidium iodide for 30 min. The signal of fluorescent was observed by flow cytometry using Muse® Cell Analyzer (Merck Millipore, Germany).

### Multi caspase assay:

The observation of multi caspase activity was performed by Muse® multi caspase assay kit (Catalog No. MCH100109, Merck Millipore), following the manufacturer's instruction. The MCF-7 cells were seeded into 12 well plates at a density of  $2 \times 10^5$  cells/well. Afterward, the cells were treated by the combination of DEGE and PFPE (ratio  $IC_{50}$ : $IC_{50}$ ) at various times (0, 24, 48 and 72 h). The cells were harvested and cell pellets were resuspended in 5 µl of

Muse® Multi caspase assay for 30 min followed by 150 µl of 7-Aminoactinomycin D (7-AAD) within 15 min. The fluorescent marks of multi caspase and 7-AAD were completely identified by the Muse® Cell Analyzer (Merck Millipore, Germany).

### Statistical analysis:

All experiments were done in triplicate of each experiment and the data was calculated from three independent experiments. The  $IC_{50}$  values were expressed as the mean±standard deviation (SD). The percentages of apoptosis and multi caspase were presented as an average and SD. All graphs were created by GraphPad Prism 8. The apoptosis and multi caspase data were statistically analyzed by the Student t-test to compare among the treated group and control group using Microsoft excel software.  $p < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

Cancer have been a health concern caused by free

radicals and carcinogens, which have been naturally and artificially established to harm Deoxyribonucleic acid (DNA)<sup>[31]</sup>. Until now, many attempts for precise and effective cancer anticancer therapy were investigated and recognized as complementary alternative medicine (CAM), due to the fear of society against synthetic drugs detrimental effects. One of interesting CAM is to use plant extracts as an anticancer therapy. *A. paniculata* leaf was reported to possess of anticancer activity.

According to results in Table 1, we selected dichloromethane extract of *A. paniculata* (DEAP) for the next experiment due to its cytotoxicity on both HT-29 ( $8.93 \pm 0.52 \mu\text{g ml}^{-1}$ ) and SW-620 cells ( $7.49 \pm 0.04 \mu\text{g. ml}^{-1}$ ). Even though methanolic extract of *A. paniculata* (MEAP) also showed strong cytotoxicity on SKOV-3 cells ( $7.60 \pm 0.72 \mu\text{g ml}^{-1}$ ), we rather considered the dichloromethane extract as the extract which can affect the two cell types, than methanolic one where only one cell type affected. In this present study, we also used high polar solvent such as methanol and ethanol. Empirically, organic solvents with higher

**TABLE 1: CYTOTOXICITY OF *A. paniculata* LEAVES PREPARED IN SIX SOLVENTS AGAINST DIFFERENT TYPE OF CANCER CELLS**

Cell Line	$IC_{50}$ value ( $\mu\text{g/ml}$ ) ( $\mu\text{M}$ ) <sup>a</sup>						
	FEAP ( $SI_{Vero}$ , $SI_{L-929}$ )	MEAP ( $SI_{Vero}$ , $SI_{L-929}$ )	EEAP ( $SI_{Vero}$ , $SI_{L-929}$ )	DEAP ( $SI_{Vero}$ , $SI_{L-929}$ )	EAAP ( $SI_{Vero}$ , $SI_{L-929}$ )	HEAP ( $SI_{Vero}$ , $SI_{L-929}$ )	DOX ( $SI_{Vero}$ , $SI_{L-929}$ )
<b>Breast cancer</b>							
MCF-7	Not inhibited (ND, ND)	$14.97 \pm 1.56$ (1.22, 0.72)	$18.44 \pm 1.71$ (0.77, 2.43)	$26.77 \pm 2.18$ (0.53, 0.93)	$21.64 \pm 0.93$ (0.69, 0.95)	$47.46 \pm 0.93$ (ND, ND)	$1.15 \pm 0.07$ (6.84, 1.38)
MDA- MB-231	Not inhibited (ND, ND)	$12.54 \pm 1.38$ (1.46, 0.86)	$21.49 \pm 0.21$ (0.66, 2.08)	$24.03 \pm 0.40$ (0.59, 1.04)	$19.06 \pm 1.15$ (0.79, 1.07)	Not inhibited (ND, ND)	$1.57 \pm 0.23$ (5.02, 1.01)
<b>Colorectal cancer</b>							
HT-29	Not inhibited (ND, ND)	$13.77 \pm 1.61$ (1.33, 0.78)	$12.45 \pm 0.05$ (1.13, 3.59)	$8.93 \pm 0.52$ (1.58, 2.80)	$13.17 \pm 0.52$ (1.14, 1.56)	Not inhibited (ND, ND)	$2.53 \pm 0.56$ (3.11, 0.63)
SW-620	Not inhibited (ND, ND)	$12.39 \pm 1.61$ (1.47, 0.87)	$12.08 \pm 0.82$ (1.17, 3.70)	$7.49 \pm 0.04$ (1.89, 3.34)	$10.31 \pm 0.32$ (1.46, 1.99)	$39.40 \pm 0.42$ (ND, ND)	$3.15 \pm 0.62$ (2.50, 0.5)
<b>Ovarian cancer</b>							
A2780	Not inhibited (ND, ND)	$15.29 \pm 0.68$ (1.19, 0.70)	$20.51 \pm 1.60$ (0.69, 2.18)	$10.98 \pm 0.48$ (1.29, 2.28)	$13.53 \pm 1.28$ (1.11, 1.51)	$34.27 \pm 2.31$	$2.33 \pm 0.18$ (3.38, 0.68)
SKOV-3	Not inhibited (ND, ND)	$7.60 \pm 0.72$ (2.40, 1.41)	$30.6 \pm 0.93$ (0.46, 1.46)	$19.34 \pm 0.15$ (0.74, 1.29)	$17.23 \pm 1.28$ (0.87, 1.19)	Not inhibited (ND, ND)	$1.64 \pm 0.19$ (4.80, 0.97)
<b>Non-cancerous</b>							
Vero	Not inhibited	$18.27 \pm 1.22$	$14.11 \pm 1.59$	$14.14 \pm 0.29$	$15.02 \pm 0.94$	Not inhibited	$7.88 \pm 0.43$
L-929	Not inhibited	$10.75 \pm 0.27$	$44.74 \pm 2.44$	$25.02 \pm 1.07$	$20.48 \pm 1.34$	Not inhibited	$1.59 \pm 0.18$

Data represented mean±SD from three independent experiments.  $SI_{Vero}$  or  $SI_{L-929}$  cells are selectivity index, calculated by dividing  $IC_{50}$  of Vero or L-929 with  $IC_{50}$  cancer cells. <sup>a</sup>Doxorubicin concentration was in a unit of  $\mu\text{M}$ , Not inhibited:  $IC_{50}$  not observed at the maximum concentration at  $80 \mu\text{g/ml}$ , ND: not determined, FEAP: freeze-dried extract of *A. paniculata*, MEAP: methanol extract of *A. paniculata*, EEAP: ethanol extract of *A. paniculata*, DEAP: dichloromethane extract of *A. paniculata*, EAAP: ethyl acetate extract of *A. paniculata*, HEAP: hexane extract of *A. paniculata*

dielectric constant prefer to dissolve and interact with polar compounds. Correlating with the extracted phytocompounds, methanol is capable to dissolve antioxidant compounds such as phenol and polyphenol which is proved by the total antioxidant contents<sup>[32]</sup>. This information led to an implication that the antioxidant may play a role in cytotoxicity from MEAP.

Previously, the dichloromethane extract of *A. paniculata* leaves showed considerable cytotoxicity on HT-29 cells<sup>[33]</sup>. Another promising extract, dichloromethane extract of *Z. spina-christi* leaf (DEZSC), also showed notable cytotoxicity towards breast and ovarian cancer cells, MCF-7 cells ( $13.35 \pm 0.30 \mu\text{g. ml}^{-1}$ ) and A2780 cells ( $14.64 \pm 1.51 \mu\text{g. ml}^{-1}$ ), shown in Table 2. Apart from the dichloromethane extract, a previous investigation by Farmani and colleagues revealed that the methanol

extract of *Z. spina-christi* exhibited notable cytotoxicity on MCF-7 cells<sup>[13]</sup>. In addition, an endemic plant of Northeastern Thailand, *G. extensum*, extracted by dichloromethane (abbreviated as DEGE) demonstrated strong cytotoxicity against MCF-7 ( $15.58 \pm 1.81 \mu\text{g. ml}^{-1}$ ) and A2780 cells ( $10.08 \pm 0.04 \mu\text{g. ml}^{-1}$ ) (Table 3). The dichloromethane extract of *Vernonia extensa* (synonym of *G. extensum*) was additionally reported for strong cytotoxicity on HepG2 (liver carcinoma cells), HuCCA-1 (cholangiocarcinoma cells), A549 (lung cancer cells) and MOLT-3 (leukemic cells)<sup>[14]</sup>. However, the freeze-dried extract of these three plants has no cytotoxic effect on tested cells, which could be caused by freeze-drying process which damaged the phytocompounds structure and finally led to the losing activity of compounds after the

**TABLE 2: CYTOTOXICITY OF *Z. spina-christi* LEAVES PREPARED IN SIX SOLVENTS AGAINST DIFFERENT TYPE OF CANCER CELLS**

Cell Line	IC <sub>50</sub> value ( $\mu\text{g/ml}$ ) ( $\mu\text{M}$ ) <sup>a</sup>						
	FEZSC (SI <sub>Vero</sub> , SI <sub>L-929</sub> )	MEZSC (SI <sub>Vero</sub> , SI <sub>L-929</sub> )	EEZSC (SI <sub>Vero</sub> , SI <sub>L-929</sub> )	DEZSC (SI <sub>Vero</sub> , SI <sub>L-929</sub> )	EAZSC (SI <sub>Vero</sub> , SI <sub>L-929</sub> )	HEZSC (SI <sub>Vero</sub> , SI <sub>L-929</sub> )	DOX (SI <sub>Vero</sub> , SI <sub>L-929</sub> )
<b>Breast cancer</b>							
MCF-7	Not inhibited (ND, ND)	107.19 $\pm$ 1.64	Not inhibited (ND, ND)	13.35 $\pm$ 0.30 (1.58, 3.52)	57.63 $\pm$ 0.25 (0.92, ND)	Not inhibited (ND, ND)	1.15 $\pm$ 0.07 (6.84, 1.38)
MDA-MB-231	Not inhibited (ND, ND)	80.83 $\pm$ 1.65 (ND, ND)	Not inhibited (ND, ND)	38.12 $\pm$ 0.23 (0.55, 1.23)	55.13 $\pm$ 0.44 (0.96, ND)	Not inhibited (ND, ND)	1.57 $\pm$ 0.23 (5.02, 1.01)
<b>Colorectal cancer</b>							
HT-29	Not inhibited (ND, ND)	39.01 $\pm$ 2.65 (ND, ND)	82.35 $\pm$ 11.96 (ND, ND)	17.02 $\pm$ 0.69 (1.24, 2.76)	96.46 $\pm$ 12.08 (0.55, ND)	Not inhibited (ND, ND)	2.53 $\pm$ 0.56 (3.11, 0.63)
SW-620	Not inhibited (ND, ND)	77.91 $\pm$ 2.04 (ND, ND)	40.73 $\pm$ 0.73 (ND, ND)	23.2 $\pm$ 1.08 (0.91, 2.03)	39.67 $\pm$ 2.85 (1.34, ND)	Not inhibited (ND, ND)	3.15 $\pm$ 0.62 (2.50, 0.5)
<b>Ovarian cancer</b>							
A2780	Not inhibited (ND, ND)	18.54 $\pm$ 2.33 (ND, ND)	36.23 $\pm$ 1.27 (ND, ND)	14.64 $\pm$ 1.51 (1.44, 3.21)	53.86 $\pm$ 2.46 (0.99, ND)	Not inhibited (ND, ND)	2.33 $\pm$ 0.18 (3.38, 0.68)
SKOV-3	Not inhibited (ND, ND)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	44.45 $\pm$ 0.79 (0.47, 1.06)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	1.64 $\pm$ 0.19 (4.80, 0.97)
<b>Non-cancerous</b>							
Vero	Not inhibited	Not inhibited	Not inhibited	21.05 $\pm$ 0.18	53.11 $\pm$ 0.16	Not inhibited	7.88 $\pm$ 0.43
L-929	Not inhibited	Not inhibited	Not inhibited	47.03 $\pm$ 2.91	Not inhibited	Not inhibited	1.59 $\pm$ 0.18

Data represented mean $\pm$ SD from three independent experiments. SI<sub>Vero</sub> or SI<sub>L-929</sub> cells are selectivity index, calculated by dividing IC<sub>50</sub> of Vero or L-929 with IC<sub>50</sub> cancer cells. <sup>a</sup>Doxorubicin concentration was in a unit of  $\mu\text{M}$ , Not inhibited: IC<sub>50</sub> not observed at the maximum concentration at 80  $\mu\text{g/ml}$ , ND: not determined, FEZSC: freeze-dried extract of *Z. spina-christi*, MEZSC: methanol extract of *Z. spina-christi*, EEZSC: ethanol extract of *Z. spina-christi*, DEZSC: dichloromethane extract of *Z. spina-christi*, EAZSC: ethyl acetate extract of *Z. spina-christi*, HEZSC: hexane extract of *Z. spina-christi*

rehydration process<sup>[34]</sup>. In this study, Vero and L-929 cells were used for determining SI score by dividing  $IC_{50}$  of non-cancerous cell lines with  $IC_{50}$  of cancer cell lines. Our results demonstrated SIL929 values of DEAP and DEZSC more than 2 on HT-29, SW-620, A2780 and MCF-7 cells. According to Koch and colleagues, SI score of less than 2.0 indicates toxicity to non-cancerous cell lines<sup>[35]</sup>. However, several extracts could not be calculated for the cytotoxicity at the maximum concentration because the extract did not show an inhibitory effect on cancer cells. These results were noted as “Not determined (ND)”, shown in Tables 1-3.

Due to strong cytotoxicity exhibited by DEAP, DEZSC and DEGE, we decided to perform a phytochemical characterization. Two major DEAP compounds were 1-heptatriacotanol (60.29 %, alcohol) and androsta-1,4-dien-3-one,6,17-dihydroxy-, (6 $\beta$ ,17 $\beta$ ) (32.27 %, steroid). Furthermore, the highest amount in our DEZSC was palmitic acid (26.92 %, fatty acid). This chemical compound contained anticancer activity through intercalating with DNA topoisomerase-I<sup>[36]</sup>. DEGE displayed similar chemical composition with DEZSC, however, several compounds only found

in DEGE were neophytadiene, phytol, linoleic acid, hentriacontane, phytol acetate and heneicosane, in which these compounds were reported for anticancer property<sup>[37-42]</sup>. The limitation of characterization was marked since GC-MS is able to detect only volatile compounds. However, other chemical contents can be analyzed through other spectroscopic methods with a library of spectra to compare with the observed compounds<sup>[43]</sup>. We suggest phytochemical screening techniques such as UltraViolet-Visible spectroscopy<sup>[44]</sup>, Infrared (IR) spectroscopy<sup>[45]</sup> and Liquid Chromatography Mass Spectroscopy (LC-MS)<sup>[46]</sup> to analyze other phytochemicals of DEAP, DEZSC and DEGE for further study.

Our individual extract cytotoxicity test revealed that DEAP, DEZSC and DEGE possessed high cytotoxicity. In this study, we attempted to enhance the cytotoxicity of DEAP, DEZSC and DEGE by combining them with PFPE. In the previous study using PFPE, *P. nigrum* dichloromethane crude extract and piperine showed  $IC_{50}$  values of 7.45, 23.46 and  $>20 \mu\text{g. ml}^{-1}$ , respectively<sup>[19]</sup>. Based on our previous results, *P. nigrum* extract containing piperine increased

**TABLE 3: CYTOTOXICITY OF *G. extensum* LEAVES PREPARED IN SIX SOLVENTS AGAINST DIFFERENT TYPE OF CANCER CELLS**

Cell Line	$IC_{50}$ value ( $\mu\text{g/ml}$ ) ( $\mu\text{M}$ ) <sup>a</sup>						
	FEGE ( $SI_{Vero}$ , $SI_{L-929}$ )	MEGE ( $SI_{Vero}$ , $SI_{L-929}$ )	EEGE ( $SI_{Vero}$ , $SI_{L-929}$ )	DEGE ( $SI_{Vero}$ , $SI_{L-929}$ )	EAGE ( $SI_{Vero}$ , $SI_{L-929}$ )	HEGE ( $SI_{Vero}$ , $SI_{L-929}$ )	DOX ( $SI_{Vero}$ , $SI_{L-929}$ )
<b>Breast cancer</b>							
MCF-7	Not inhibited (ND, ND)	26.34 $\pm$ 0.53 (1.27, 1.72)	23.67 $\pm$ 1.81 (1.09, 2.01)	15.58 $\pm$ 1.81 (0.59, 1.18)	25.56 $\pm$ 0.60 (1.06, 1.20)	Not inhibited (ND, ND)	1.15 $\pm$ 0.07 (6.84, 1.38)
MDA- MB-231	Not inhibited (ND, ND)	51.18 $\pm$ 2.07 (0.65, 0.88)	57.84 $\pm$ 1.31 (0.45, 0.82)	20.78 $\pm$ 2.01 (0.44, 0.88)	23.15 $\pm$ 1.57 (1.17, 1.33)	Not inhibited (ND, ND)	1.57 $\pm$ 0.23 (5.02, 1.01)
<b>Colorectal cancer</b>							
HT-29	Not inhibited (ND, ND)	25.35 $\pm$ 1.94 (1.32, 1.78)	29.68 $\pm$ 2.04 (0.87, 1.60)	19.86 $\pm$ 0.61 (0.46, 0.92)	33.63 $\pm$ 2.16 (0.81, 0.91)	Not inhibited (ND, ND)	2.53 $\pm$ 0.56 (3.11, 0.63)
SW-620	Not inhibited (ND, ND)	23.27 $\pm$ 1.54 (1.43, 1.94)	17.03 $\pm$ 0.09 (1.52, 2.79)	20.09 $\pm$ 2.03 (0.46, 0.91)	27.83 $\pm$ 2.20 (0.97, 1.10)	Not inhibited (ND, ND)	3.15 $\pm$ 0.62 (2.50, 0.5)
<b>Ovarian cancer</b>							
A2780	Not inhibited (ND, ND)	16.12 $\pm$ 1.40 (2.07, 2.81)	41.21 $\pm$ 1.23 (0.63, 1.15)	10.08 $\pm$ 0.04 (0.91, 1.82)	15.22 $\pm$ 0.58 (1.78, 2.02)	43.16 $\pm$ 1.51 (ND, ND)	2.33 $\pm$ 0.18 (3.38, 0.68)
SKOV-3	Not inhibited (ND, ND)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	29.95 $\pm$ 1.12 (0.31, 0.61)	Not inhibited (ND, ND)	Not inhibited (ND, ND)	1.64 $\pm$ 0.19 (4.80, 0.97)
<b>Non-cancerous</b>							
Vero	Not inhibited	33.39 $\pm$ 1.59	25.90 $\pm$ 0.47	9.16 $\pm$ 0.8	27.08 $\pm$ 3.03	Not inhibited	7.88 $\pm$ 0.43
L-929	Not inhibited	45.23 $\pm$ 2.43	47.57 $\pm$ 0.35	18.37 $\pm$ 0.66	30.73 $\pm$ 3.32	Not inhibited	1.59 $\pm$ 0.18

Data represented mean $\pm$ SD from three independent experiments.  $SI_{Vero}$  or  $SI_{L-929}$  cells are selectivity index, calculated by dividing  $IC_{50}$  of Vero or L-929 with  $IC_{50}$  cancer cells. <sup>a</sup>Doxorubicin concentration was in a unit of  $\mu\text{M}$ , Not inhibited:  $IC_{50}$  not observed at the maximum concentration at 80  $\mu\text{g/ml}$ , ND: not determined, FEGE: freeze-dried extract of *G. extensum*, MEGE: methanol extract of *G. extensum*, EEGE: ethanol extract of *G. extensum*, DEGE: dichloromethane extract of *G. extensum*, EAGE: ethyl acetate extract of *G. extensum*, HEGE: hexane extract of *G. extensum*



IC<sub>50</sub> which attenuated the cytotoxic effect on cancer cells. Moreover, phytocompounds of PFPE such as piperanine, pipericine, 2,4,14-eicosatrienamide and retrofractamide-A showed hepatoprotective activity which may protect non-cancerous cell lines from cytotoxic compounds<sup>[20]</sup>. Thus, we combined those three extracts with PFPE. The results showed that DEAP combination with PFPE provided antagonistic effect for the whole ratio against HT-29 and SW-620 cells. The combination of DEZSC with PFPE had an antagonistic effect on MCF-7 and A2780 cells. The observation on antagonistic effect emphasizes that phytocompounds of DEAP and DEZSC were not able to collaborate with compounds of PFPE. Steroids, fatty alcohols and fatty acids were detected in DEAP and DEZSC, from GC-MS results (fig. 1). Cholesterol is an initial form for synthesizing steroids. Meanwhile, fatty alcohols and fatty acids are the derivatives of lipids that can generate cholesterol. In contrast, caryophyllene which contained in PFPE exhibited anti-hypercholesterolemic or hyperlipid preventive activity<sup>[47]</sup>. Caryophyllene could additionally attenuate lipid accumulation on hepatic tissue which linked to the stimulation of AMP-activated protein kinase (AMPK) phosphorylation, a crucial enzyme in lipid metabolism. Caryophyllene also induces degradation of fatty acid

synthase (FAS)<sup>[48]</sup>. The different roles in caryophyllene and lipid-derivatives (fatty acids, steroids and fatty alcohols) could pinpoint the antagonistic effect of this combination (Tables 4-7).

Surprisingly, our investigation revealed that a combination of DEGE with PFPE remarkably showed synergistic interaction on MCF-7 cells. We had tried to identify possible synergic chemical compounds. As we mentioned previously, palmitic acid and kusunokinin showed intercalation to DNA topoisomerase-I together with PFPE, enabling degradation of DNA topoisomerase-II<sup>[36,49]</sup>. Due to their important role as a DNA controller, DNA topoisomerase-I and DNA topoisomerase-II are targeted for promising anticancer treatment<sup>[50,51]</sup>. A synergistic effect against MCF-7 cells from a PFPE-combined DEGE led to a further study for the relevant information to strengthen our hypothesis that MCF-7 cells could not escape from cell death since it was attacked by several death pathways of this combination. These expected mechanisms accordingly convinced us to conduct a study regarding an apoptotic (cell death) process.

Apoptosis and multi caspase activity experiments were investigated using annexin V/propidium iodide assay and multi caspase/7-AAD assay. In the assay, the total

**TABLE 4: CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF COMPOUNDS OF DICHLOROMETHANE CRUDE EXTRACT OF *A. paniculata* (DEAP) IDENTIFIED THROUGH GC-MS ANALYSIS**

RT (min)	Identified compound	Formula	Molecular mass (g mol <sup>-1</sup> )	Area (%)	Nature of compound
17.34	Furfuryl alcohol	C <sub>5</sub> H <sub>10</sub> O <sub>2</sub>	102.13	0.01	Alcohol
21.28	Octadecane	C <sub>18</sub> H <sub>38</sub>	254.5	0.02	Hydrocarbon
29.72	Neophytadiene	C <sub>20</sub> H <sub>38</sub>	278.5	1.2	Terpene
29.83	6,10,14-trimethyl pentadecane-2-one	C <sub>18</sub> H <sub>36</sub> O	268.5	0.53	Terpene
31.24	2-Methyltetradecanal	C <sub>15</sub> H <sub>30</sub> O	226.4	0.03	Aldehyde
32.17	Palmitic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.42	1.24	Fatty acid
33.3	2-octylacolein	C <sub>11</sub> H <sub>20</sub> O	168.28	0.01	Hydrocarbon
34.74	3,7-Dimethyl-2,3-epoxyoctanal	C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	170.25	0.01	Aldehyde
38.16	Tricosane	C <sub>23</sub> H <sub>48</sub>	324.6	0.03	Hydrocarbon
42.09	1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	390.6	0.25	Phthalate
47.09	2-oxobutanoic acid neopentyl ester	C <sub>9</sub> H <sub>16</sub> O <sub>3</sub>	172.22	0.01	Carboxylic acid
49.91	1-Heptatriacotanol	C <sub>37</sub> H <sub>76</sub> O	537	60.29	Alcohol
50.14	α-Tocopherol	C <sub>29</sub> H <sub>50</sub> O <sub>2</sub>	430.7	0.05	Tocopherol
50.45	Androsta-1,4-dien-3-one, 6,17-dihydroxy-, (6B,17 B)	C <sub>19</sub> H <sub>26</sub> O <sub>3</sub>	302.4	32.27	Steroid
51.59	Stigmasterol	C <sub>29</sub> H <sub>48</sub> O	412.7	0.98	Steroid
52.29	γ-Sitosterol	C <sub>29</sub> H <sub>50</sub> O	414.7	3.04	Terpene
52.65	β-Amyrin	C <sub>30</sub> H <sub>50</sub> O	426.7	0.02	Terpene

RT: Retention time



**TABLE 5: CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF COMPOUNDS OF DICHLOROMETHANE CRUDE EXTRACT OF *Z. spina-christi* (DEZSC) IDENTIFIED THROUGH GC-MS ANALYSIS**

RT (min)	Identified compound	Formula	Molecular mass (g mol <sup>-1</sup> )	Area (%)	Nature of compound
28.06	Myristic acid	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	228.37	1.33	Fatty acid
29.64	Neophytadiene	C <sub>20</sub> H <sub>38</sub>	278.52	7.91	Terpene
29.75	6,10,14-trimethyl pentadecane-2-one	C <sub>18</sub> H <sub>36</sub> O	268.48	1.79	Terpene
30.14	Phytol, acetate	C <sub>22</sub> H <sub>42</sub> O <sub>2</sub>	338.57	1.28	Terpene
30.49	Phytol	C <sub>20</sub> H <sub>40</sub> O	296.53	4.07	Terpene
32.22	Palmitic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.42	26.92	Fatty acid
35.31	Linoleic acid	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	280.46	6.76	Fatty acid
35.41	Linolenic acid	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	278.43	11.22	Fatty acid
35.81	Stearic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	284.48	3.8	Fatty acid
38.93	4,8,12,16-Tetramethylheptadecan-4-olide	C <sub>21</sub> H <sub>40</sub> O <sub>2</sub>	324.54	0.71	Furan hydrocarbon
41.99	Bis(2-ethylhexyl) phthalate	C <sub>24</sub> H <sub>38</sub> O <sub>4</sub>	390.56	0.88	Phthalate
46.04	Squalene	C <sub>30</sub> H <sub>50</sub>	410.72	1.68	Terpene
46.37	α-Tocospino A	C <sub>29</sub> H <sub>50</sub> O <sub>4</sub>	462.7	1.34	Tocopherol
46.67	α-Tocospino B	C <sub>29</sub> H <sub>50</sub> O <sub>4</sub>	462.7	1.44	Tocopherol
46.99	n-Tetracosanol-1	C <sub>24</sub> H <sub>50</sub> O	354.65	6.07	Fatty alcohol
48.97	γ-Tocopherol	C <sub>28</sub> H <sub>48</sub> O <sub>2</sub>	416.68	1.11	Tocopherol
49.63	Octacosanol	C <sub>28</sub> H <sub>58</sub> O	410.76	5.26	Fatty alcohol
50.01	Vitamin E	C <sub>29</sub> H <sub>50</sub> O <sub>2</sub>	430.71	0.77	Tocopherol
51.08	Campesterol	C <sub>28</sub> H <sub>48</sub> O	400.68	1.04	Steroid
51.49	Stigmasterol	C <sub>29</sub> H <sub>48</sub> O	412.69	4.91	Steroid
52.19	Stigmast-5-en-3-ol	C <sub>29</sub> H <sub>50</sub> O	414.7	5.24	Steroid
52.83	Lupenone	C <sub>30</sub> H <sub>48</sub> O	424.7	2.91	Terpene
53.09	Lupeol	C <sub>30</sub> H <sub>50</sub> O	426.72	1.91	Terpene

RT: Retention time

**TABLE 6: CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF COMPOUNDS OF DICHLOROMETHANE CRUDE EXTRACT OF *G. extensum* (DEGE) IDENTIFIED THROUGH GC-MS ANALYSIS**

RT (min)	Compound name	Formula	Molecular mass (g mol <sup>-1</sup> )	Area (%)	Compound nature
15.68	1,2-Di-tert-butylbenzene	C <sub>14</sub> H <sub>22</sub>	190.32	0.08	Phenylpropane
17.92	2-Chlorohexylacetate	C <sub>8</sub> H <sub>15</sub> ClO <sub>2</sub>	178.66	0.02	Carboxylic acid
22.1	Pentadecane	C <sub>15</sub> H <sub>32</sub>	212.41	0.02	Hydrocarbon
22.49	5-Isopropyl-4-(trifluoromethyl)-1H-pyrimidin-2-one	C <sub>8</sub> H <sub>9</sub> F <sub>3</sub> N <sub>2</sub> O	206.17	0.04	Alkaloid
22.85	Dihydroactinidiolide	C <sub>11</sub> H <sub>16</sub> O <sub>2</sub>	180.24	0.47	Benzofuran
27.44	Heptanoic acid, tridecafluoro-heptyl ester	C <sub>14</sub> H <sub>15</sub> F <sub>13</sub> O <sub>2</sub>	462.25	0.02	Fatty acid ester
28.14	Loliolide	C <sub>11</sub> H <sub>16</sub> O <sub>3</sub>	196.24	1.64	Benzofuran
29.62	1-dodecanol	C <sub>12</sub> H <sub>26</sub> O	186.33	0.05	Fatty alcohol
29.72	Neophytadiene	C <sub>20</sub> H <sub>38</sub>	278.5	19.36	Terpene
29.83	6,10,14-trimethyl pentadecane-2-one	C <sub>18</sub> H <sub>36</sub> O	268.5	1.93	Terpene
30.21	Phytol	C <sub>20</sub> H <sub>40</sub> O	296.5	13.36	Terpene
31.23	2-Methyloctanal	C <sub>9</sub> H <sub>18</sub> O	142.24	0.04	Aldehyde
32.12	1,2-Benzenedicarboxylic acid, dibutyl ester	C <sub>16</sub> H <sub>22</sub> O <sub>4</sub>	278.34	0.19	Benzoic acid ester
32.24	Palmitic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.42	21.4	Fatty acid
34.42	Pentatriacontane	C <sub>35</sub> H <sub>72</sub>	492.9	0.34	Hydrocarbon
35.36	Linoleic acid	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	280.4	6.7	Fatty acid
35.48	Linolenic acid	C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	278.4	12.08	Fatty acid
36.52	4-Tridecen-2-ynal, (Z)-	C <sub>13</sub> H <sub>20</sub> O	192.3	0.06	Hydrocarbon
43.99	3-Penten-2-one	C <sub>5</sub> H <sub>8</sub> O	84.12	0.05	Ketone

RT: Retention time

TABLE 7: COMBINATION INDEX OF EXTRACT WITH PFPE AGAINST CANCER CELL LINES

Combination	Cell line	Ratio Extract:PFPE	Extract Conc. ( $\mu\text{g/ml}$ )	PFPE Conc. ( $\mu\text{g/ml}$ )	% inhibition	CI	Interaction
DEAP+PFPE	HT-29	$\text{IC}_{50}:\text{IC}_{50}$	8.93	17.63	$82.35\pm1.13$	$1.12\pm0.04$	Slight antagonism
		$0.5 \text{ IC}_{50}:\text{IC}_{50}$	4.46	17.63	$54.89\pm1.34$	$1.86\pm0.05$	Antagonism
		$\text{IC}_{50}:0.5 \text{ IC}_{50}$	8.93	8.82	$47.37\pm2.45$	$1.74\pm0.08$	Antagonism
		$0.5 \text{ IC}_{50}:0.5 \text{ IC}_{50}$	4.46	8.82	$34.21\pm0.24$	$1.73\pm0.01$	Antagonism
	SW-620	$\text{IC}_{50}:\text{IC}_{50}$	7.49	11.3	$81.20\pm1.55$	$1.09\pm0.06$	Nearly additive
		$0.5 \text{ IC}_{50}:\text{IC}_{50}$	3.74	11.3	$67.68\pm1.25$	$1.29\pm0.04$	Slight antagonism
		$\text{IC}_{50}:0.5 \text{ IC}_{50}$	7.49	5.65	$65.58\pm0.77$	$1.14\pm0.01$	Slight antagonism
		$0.5 \text{ IC}_{50}:0.5 \text{ IC}_{50}$	3.74	5.65	$43.00\pm1.2$	$1.33\pm0.03$	Slight antagonism
DEZSC+PFPE	MCF-7	$\text{IC}_{50}:\text{IC}_{50}$	13.35	21.06	$78.65\pm4.33$	$1.19\pm0.16$	Slight antagonism
		$0.5 \text{ IC}_{50}:\text{IC}_{50}$	6.675	21.06	$50.4\pm2.65$	$1.86\pm0.11$	Antagonism
		$\text{IC}_{50}:0.5 \text{ IC}_{50}$	13.35	10.53	$82.66\pm2.65$	$0.72\pm0.07$	Moderate synergism
		$0.5 \text{ IC}_{50}:0.5 \text{ IC}_{50}$	6.675	10.53	$37.07\pm1.05$	$1.52\pm0.04$	Antagonism
	A2780	$\text{IC}_{50}:\text{IC}_{50}$	14.64	21.12	$72.46\pm5.09$	$1.39\pm0.07$	Slight antagonism
		$0.5 \text{ IC}_{50}:\text{IC}_{50}$	7.32	21.12	$13.59\pm4.36$	$4.77\pm0.91$	Strong antagonism
		$\text{IC}_{50}:0.5 \text{ IC}_{50}$	14.64	10.56	$48.7\pm8.73$	$1.66\pm0.30$	Antagonism
		$0.5 \text{ IC}_{50}:0.5 \text{ IC}_{50}$	7.32	10.56	$14.15\pm9.69$	$3.21\pm1.36$	Strong antagonism
DEGE+PFPE	MCF-7	$\text{IC}_{50}:\text{IC}_{50}$	15.58	21.06	$97.29\pm0.33$	$0.36\pm0.03$	Synergism
		$0.5 \text{ IC}_{50}:\text{IC}_{50}$	7.79	21.06	$93.65\pm3.72$	$0.44\pm0.14$	Synergism
		$\text{IC}_{50}:0.5 \text{ IC}_{50}$	15.58	10.53	$95.32\pm1.5$	$0.34\pm0.06$	Synergism
		$0.5 \text{ IC}_{50}:0.5 \text{ IC}_{50}$	7.79	10.53	$37.69\pm4.12$	$1.46\pm0.13$	Antagonism
	A2780	$\text{IC}_{50}:\text{IC}_{50}$	10.08	21.12	$80.41\pm1.97$	$1.22\pm0.08$	Slight antagonism
		$0.5 \text{ IC}_{50}:\text{IC}_{50}$	5.04	21.12	$10.11\pm4.19$	$6.70\pm1.61$	Strong antagonism
		$\text{IC}_{50}:0.5 \text{ IC}_{50}$	10.08	10.56	$51.22\pm4.84$	$1.63\pm0.16$	Antagonism
		$0.5 \text{ IC}_{50}:0.5 \text{ IC}_{50}$	5.04	10.56	$5.54\pm2.14$	$5.55\pm1.18$	Strong antagonism

Data represented mean $\pm$ SD from three independent experiments. CI is combination index, analyzed by compusyn software. DEAP: dichloromethane extract of *A. paniculata*, DEZSC: dichloromethane extract of *Z. spina-christi*, DEGE: dichloromethane extract of *G. extensum*, PFPE: low piperine fractional *P. nigrum* extract

apoptotic cells were obtained from a summation of early and late apoptotic cells. The early and later apoptosis stage could be distinguished by intercalation among Annexin V to Phosphatidylserine and intercalation of Annexin V together with PI inside permeable membrane of late apoptotic cells. Meanwhile, dead or necrotic cells were observed in this study. The different aspect of these two basic types of cell death is that the necrosis is caused by excessive external damage, while apoptosis occurs when the cells are aged and undergo progressive morphological alteration by internal molecular involvement<sup>[52]</sup>. Dot plots in fig. 2 reflected all cell conditions including live cells, early and late apoptotic cells and dead cells (necrotic cells).

A significant decrease of live cells was found in MCF-7 cells at 72 h after the incubation with the combination of DEGE and PFPE. Moreover, late apoptotic and total apoptotic cells were increased statistically significantly at 72 h after the treatment and responded in a time-dependent manner.

The apoptosis insight can be harnessed as a key strategy for novel targeted therapies that lead to the arrest of cancer cell growth and spread<sup>[53]</sup>. Moreover, the correlation between apoptosis and caspase activation is the loss of mitochondrial membrane potential. An activation of cytochrome c (cyt-c) would be then followed by caspase activation<sup>[54]</sup>. The caspases are activated when the cell inactivates zymogens, provoking

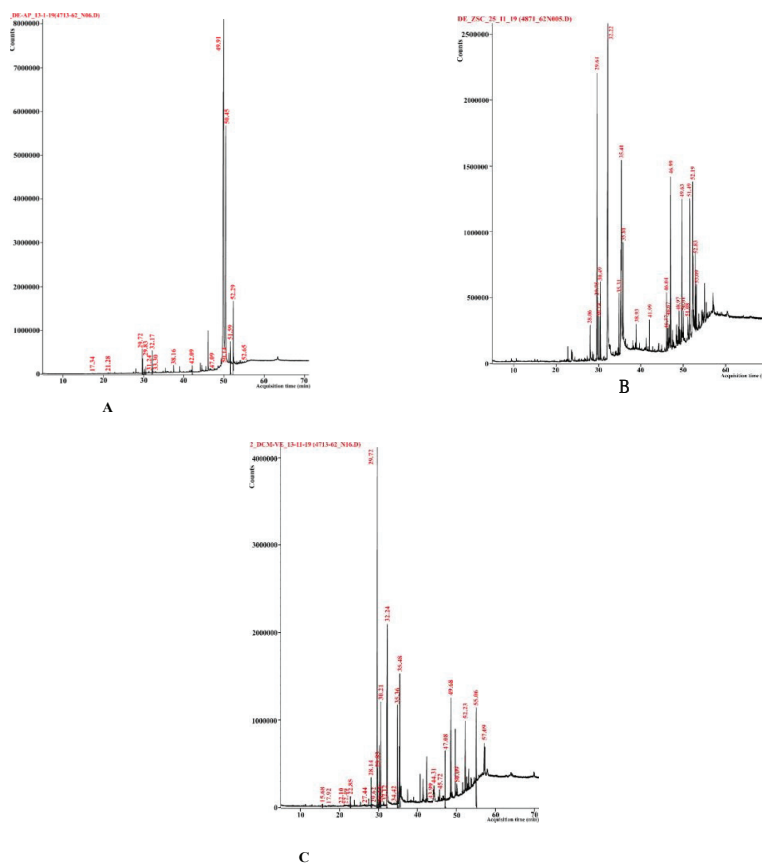


Fig. 1: GC-MS chromatographic profile of extracts: (A) DEAP, (B) DEZSC and (C) DEGE

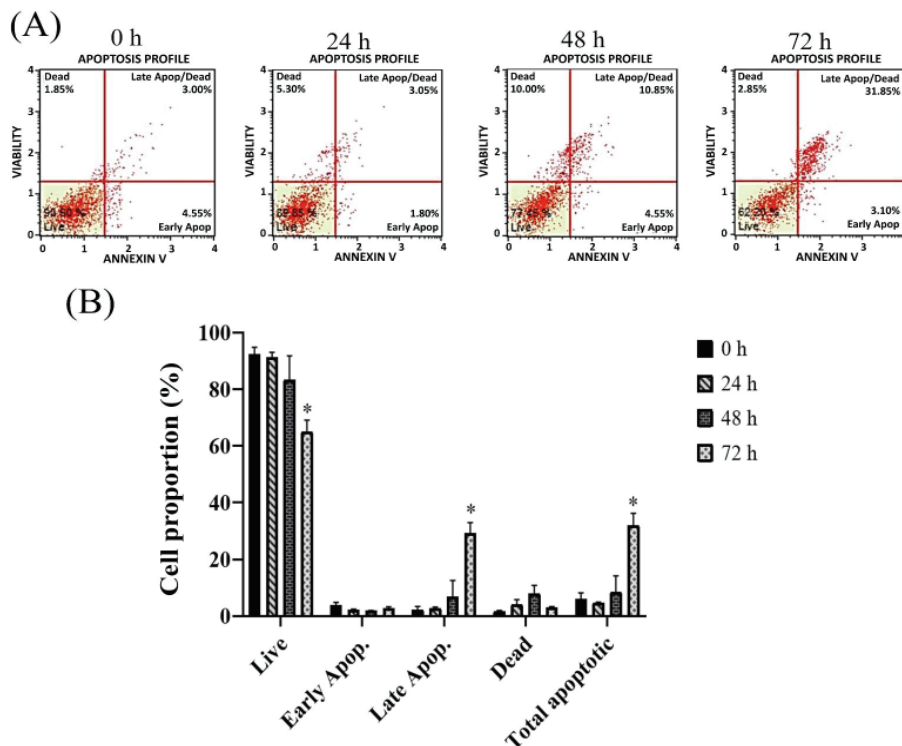
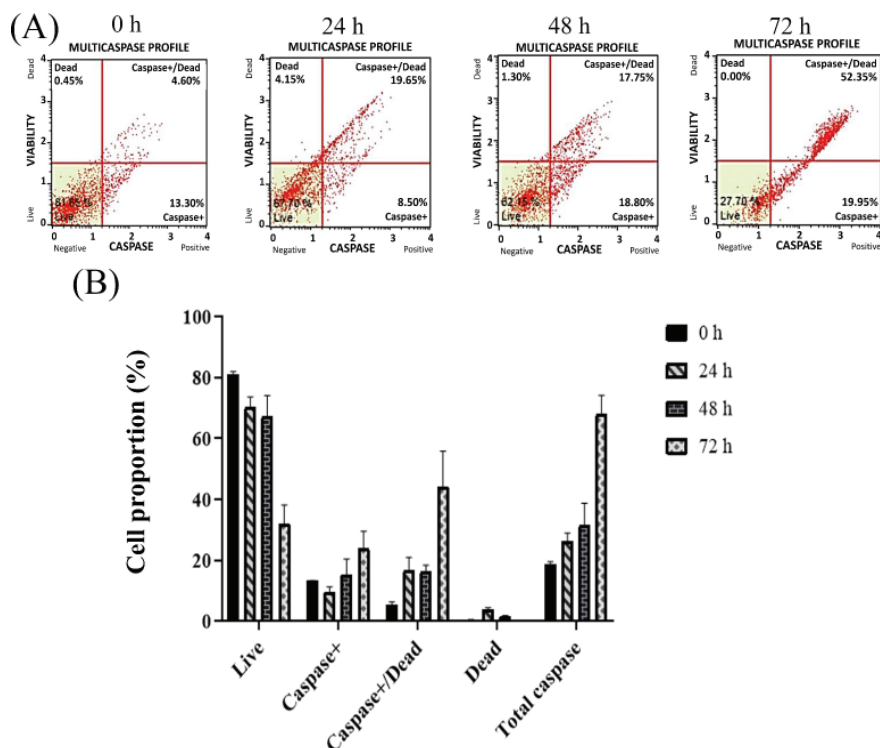


Fig. 2: The combination of DEGE with PFPE induces cell apoptosis

(A) MCF-7 cells were treated with the combination of DEGE with PFPE at  $IC_{50}$  concentration value of 15.58  $\mu\text{g/ml}$  and 21.06  $\mu\text{g/ml}$ , respectively and incubated for 24, 48 and 72 h. After treatment, apoptotic cells were analyzed by the Muse® Annexin V-FITC assay and Propidium Iodide (dead cell kit). (B) The graph represented the summary of average percentages  $\pm$  SD of live, early apoptotic, late apoptotic, dead and total apoptotic of three independent experiments. The statistical analysis of the data was tested by Student t test where p values less than 0.05 was statistically considered as significant differences compared to control group at 0 h



**Fig. 3: The combination of DEGE with PFPE induces multi caspase activity**

(A) MCF-7 cells were treated with the combination of DEGE with PFPE at  $IC_{50}$  concentration value of 15.58  $\mu$ g/ml and 21.06  $\mu$ g/ml, respectively and incubated for 24, 48 and 72 h. After treatment, multi caspase activity was analyzed by the Muse® Multi caspase assay and 7-AAD (dead cell kit). (B) The graph is represented the average percentages  $\pm$  SD of live, caspase+, caspase+/dead and total caspase of three independent experiments. The statistical analysis of the data was tested by Student t-test where p values less than 0.05 was statistically considered as significant differences compared to control group at 0 h

a low number of protease activity<sup>[55]</sup>. The dot plots, in fig. 3, represented the proportion of live, caspase+, caspase+/dead and dead cells of MCF-7 cells treated with the combination of DEGE and PFPE. We found that multi caspase activity was significantly increased at 72 h ( $p < 0.05$ ) after treatment and responded in a time-dependent manner similar to the apoptosis experiment. However, further analysis of proteins in the apoptosis pathway would be encouraged.

To the best of our insight, we analyzed and linked to the compounds observed by GC-MS. Several previous findings revealed that the chemical compositions of DEGE could affect apoptotic associated proteins and caspases. The most abundant phytocompound of DEGE was palmitic acid, which can promotes caspase -3, -8 and -9 activities in Pheochromocytoma cells (PC12)<sup>[56]</sup>. Furthermore, linoleic acid, phytol and stigmasterol up regulate apoptotic proteins, such as bax, caspase -3 and -9. These three compounds also down-regulate bcl-2 in colorectal, liver and Non-small cell lung cancer (NSCLC) cells, respectively<sup>[38,39,57]</sup>. Surprisingly, Western blot analysis of PFPE reflected that PFPE was able to regulate the apoptosis associated proteins including p53, bax and suppress bcl-2 expression on cholangiocarcinoma cells (KKU-M213 and TFK-1)

<sup>[20]</sup>. Merged all results together, this report points out that DEGE and PFPE would be a potential regimen for breast cancer treatment in the future. Nevertheless, an *in vivo* study using breast cancer and clinical trials remain necessary to support supplementary information.

In conclusion, dichloromethane extract of *A. paniculata*, *Z. spina-christi* and *G. extensum* showed strong cytotoxicity against colorectal, breast and ovarian cancer, respectively, with an  $IC_{50}$  value less than 14  $\mu$ g/ml. A combination of dichloromethane crude extract of *G. extensum* with *P. nigrum* crude extract interestingly enhanced a synergistic anticancer activity on breast cancer through the apoptosis induction.

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### Conflict of Interests:



The authors declared no conflict of interest.

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