CYTOTOXIC COMPOUNDS AGAINST BREAST ADENOCARCINOMA CELLS (MCF-7) FROM PIKUTBENJAKUL

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ABSTRACT: Pikutbenjakul, a Thai Traditional medicine preparation, is composed of five plants: *Piper chaba* fruit, *Piper sarmentosum* root, *Piper interruptum* stem, *Plumbago indica* root and *Zingiber officinale* rhizome. It is a balanced health preparation in Thai traditional medicine. From selective interviews of folk doctors in Southern Thailand, it was found that Benjakul was used as an adaptogen drug for breast cancer patients. It was give them before using cancer drug. Thus, the objectives of this research were investigating cytotoxic activity against breast cancer cell (MCF-7) of Pikutbenjakul preparation and its components extracts by using the SRB assay. The extraction method imitated by folk doctors used by maceration in ethanol and boiling in water. The results were found that the ethanolic extract of *Piper chaba*, *Zingiber officinale* and Pikutbenjakul showed high cytotoxic activity against breast cancer cell (IC_{50}= 35.17, 31.15 and 33.20 μg/ml, respectively) but water extract showed no cytotoxic activity against breast cancer cells. Two compounds [piperine and 6-shogaol as 7.48 and 0.54% w/w of crude extract] were isolated from the ethanolic extract of Pikutbenjakul by bioassay guide fractionation and were also tested for cytotoxic activity. It was found that piperine and 6-shogaol had cytotoxicity against MCF7 with IC_{50} value of 9.80 and 10.18 μg/ml. These results can support using Pikutbenjakul to treat breast cancer patients of Thai folk doctors.

Keywords: Cytotoxic activity, Breast cancer, MCF7, SRB assay, Thai medicinal plants, Pikutbenjakul, *Piper chaba* , *Piper sarmentosum*, *Piper interruptum*, *Plumbago indica*, *Zingiber officinale*

INTRODUCTION: Cancer has been the first leading cause of death in Thailand for several years and the number of people died from cancer is still increasing every year. For specific types of cancer occurred only in women, breast and cervix cancers were the two highest causes of death in Thai women. Plant-based systems have a long history of use in traditional health care[1]. Sixty percents of currently used anticancer agents are derived in one way or another from natural sources[2]. Therefore, the usage of ethnopharmacology or traditional use is channel for discovery of new biologically-active molecules[1]. Investigation of indigenous wisdom on cancer treatment of Thai traditional doctors[3] revealed that a Pikutbenjakul, which is composed of five Thai medicinal plants (*Piper chaba* Linn, *Piper sarmentosum* Roxb, *Piper interruptum* Opiz., *Plumbago indica* Linn. and *Zingiber officinale* Roscoe) has been used as an adaptogen drug for cancer patients. Folk doctors would give Pikutbenjakul to treat breast cancer patients for 2 or 3 weeks before treatment with breast cancer preparation. It is claimed that Pikutbenjakul is balances elements in patient's body or increases their immunity. Pikutbenjakul also showed no toxicity changes when tested by a sub-chronic toxicity method[4]. In spite of this preparation is commonly used in Thai traditional Medicine before the treatment of many diseases, there are no reports on testing its pharmacological activity, such as cytotoxic activity against cancer cells. Only one record exists of for cytotoxicity against cancer cells of the ethanolic extract of *Piper chaba* which showed cytotoxic activity against human lymphocytes, ovarian cells from Chinese hamster and Dalton's lymphoma cells (IC_{50} = 0.13,0.145 and 0.3 μg/ml respectively)[5]. In the present study, the five Thai medicinal plant extracts which are ingredients of Pikutbenjakul formula and the Pikutbenjakul preparation were tested for their cytotoxic activity against breast adenocarcinoma cell cancer (MCF-7). The isolated compounds from the Pikutbenjakul extract were also isolated and tested cytotoxic activity against breast cancer cells. These results could also support the use of these plants by folk doctors to treat breast cancer patients.

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MATERIALS AND METHODS:

Plant materials

The relevant parts of the species, which are reported to be used against cancer by folk doctors in Thailand, were collected from all parts of Thailand in January to March 2006 (Table 1). Authentication of plant materials was carried out at the herbarium of the Department of Forestry Bangkok, Thailand where the herbarium vouchers have been kept. Another voucher has been deposited in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Songkhla, Thailand (for voucher numbers see Table1)**.**

Preparation of plant extracts

Plant materials were dried at 50°C, powdered and extracted in ways corresponding to those practised by Thai traditional doctors, i.e. water extraction and ethanolic extraction For the water extract of each plant, the dried ground plant material (100 g) was boiled for 30 minutes in distilled water (300 ml), filtered and freeze dried. For the ethanolic extracts dried ground plant material (100 g) was percolated with 95 % ethanol and the filtrate concentrated to dryness under reduced pressure. The percentage yields are shown in Table 4. The water extracts were dissolved in sterile water and the ethanolic extracts were dissolved in DMSO and all stock solution were filtrated by sterile filter paper (0.2 μm) before testing.

Isolation and purification of active compounds

An aliquot of the ethanolic extract of Pikutbenjakul (40 g) was separated by vacuum liquid chromatography (VLC), using silica gel and a gradient elution of hexane (10x200ml), hexane: chloroform (10x200ml), chloroform (10x200ml), chloroform:MeOH (1:1) (10x200ml), MeOH (10x200ml). Drying and evaporation of each fraction yielded residues of 0.22 g, 0.46, 5.61, 21.72 and 6.26 g, denoted as FA, FB, FC, FD and FE respectively. These five fractions were tested for cytotoxic activity against lung cancer by the SRB assay because it was found that Pikutbenjakul preparation showed the highest cytotoxicity against breast cancer. Thus, the five fractions were also tested against breast cancer cell line. It was found that FC showed the highest cytotoxic activity against MCF-7 (% cytotoxic = 82% at concentration 50 μg/ml)

An aliquot of the ethanolic extract of Pikutbenjakul was separated by CC (silica gel with a gradient of solvents: hexane:EtOAc (8:2); (350 ml); hexane:EtOAc (7:3) (100ml); hexane: EtOAc (7:3) (100ml); hexane:EtOAc (6:4) (200 ml);; hexane:EtOAc (1:1) (200ml); EtOAc:hexane (2:8) (300 ml) , EtOAc (200ml); EtOAc:MeOH (9.5;0.5) (200ml), EtOAc:MeOH (9:1) 200ml ; EtOAc:MeOH (1:1) 200ml and finally MeOH (300 ml). Ten milliter fractions were collected for each eluting solvent and fractions combined, following TLC examination (silica gel/ $CHCl_3$: MeOH (7:3) and detection with acidic anisaldehyde spray. Compound A (158.5 mg, 7.81 % w/w) was isolated as yellow crystals, designated F1, from FC dissolved in chloroform before CC. CC (silica gel) was carried out on the mother liquor with EtOAc: hexane (2:8) to get yellow crystals, which were recrystallized in MeOH. Compound B (9.6mg, 0.54 % w/w) as pale yellow oil was isolated from fractions 66-73 after TLC purification.

Table 1 The summarized data of the investigated plant species as the ingredients of Pikutbenjakul

Plants (Family)	Places for plant collection (Amphor, Province)	Voucher number	Part of used
Piper chaba Linn (Piperaceae)	Kaosaming, Chantaburi	SKP 146160301	Fruit
Piper sarmentosum Roxb. (Piperaceae)	Jombueng, Ratchaburi	SKP146161901	Root
Piper interruptum Opiz. (Piperaceae)	Phoopan, Sakonnakhon	SKP 146160901	Stem
Plumbago indica Linn (Plumbaginaceae)	Talingchan, Bangkok	SKP148160901	Root
Zingiber officinale Roscoe. (Zingiberaceae)	Khaokho, Petchaboon	SKP206261501	Rhizome
Pikutbenjakul	-	-	-

Structure elucidation

The structure of the isolates (Figure 1) was determined by their NMR data [^{1}H and ^{13}C on a Varian Unity Inova 500 spectrometer (500 MHz for ^{1}H; 125 MHz for ^{13}C)], UV spectra [a Hewlett Packard 8452A Diode array spectrometer], IR spectra [Jasco IR-810 spectrometer], EI mass spectra, Low resolution were obtained from a JEOL JMS-AX505W spectrometer.

Compound A (Piperine): $C_{17}H_{19}NO_3$ (158.5 mg, 7.81%w/w); light yellow needle crystal solids; EIMS (low resolution) m/z (% relative intensity) 285 (M+, 75), 201 (100), 173 (19), 143 (17), 115 (45). Compound A was the major compound isolated from the ethanolic extract of Pikutbenjakul preparation. The TLC analysis of this compound was compared with authentic sample piperine (Merck) by TLC using 3 solvent systems and gave identical behavior. The 1H NMR spectrum, compared with the previous ^{1}H-NMR data of piperine, was the same as the spectrum recorded for piperine[6] (see table 2). It was identified as piperine by comparison on chromatography and spectral features with an authentic sample purchased from Merck and was the major product isolated. The structure was shown in Figure 1.

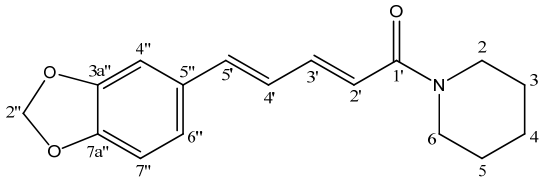

Figure 1 Structure of compound A (piperine)

Table 2 ^{1}H-NMR spectral data (500 MHz) of compound A isolated from the ethanolic extract of Pikutbenjakul compare with piperine in $CDCl_3$ and CD_3OD

Position	δ_H (mult., J in Hz) of Piperine	δ_H (mult., *J* in Hz) of Compound A
2	3.48 (*br.s*; 2H)	3.64 (*dd*; 2H, 11.1, 5.7)
3	1.49 (*m*; 2H)	1.62 (*m*; 2H)
4	1.56 (*m*; 2H)	1.71 (*m*; 2H)
5	1.49 (*m*; 2H)	1.62 (*m*; 2H)
6	3.48 (*br.s*; 2H)	3.64 (*dd*; 2H, 11.1, 5.7)
2'	6.36 (*d*, 14.6)	6.65 (*d*, 14.7)
3'	7.31 (*m*)	7.34 (*dd*, 14.7, 9.6)
4'	6.64 (*m*)	6.88 (*dd*, 14.7, 9.9)
5'	6.65 (*m*)	6.89 (*d*, 14.7)
2"	5.86 (*s*; 2H)	5.98 (*s*; 2H)
4"	6.88 (*d*, 1.6)	7.11 (*d*, 1.5)
6"	6.79 (*dd*, 1.6, 8.0)	6.98 (dd, 8.1, 1.5)
7"	6.67 (*d*, 8.0)	6.81 (*d*, 8.1)

Compound B (6-gingerol): $C_{17}H_{26}O_4$ (9.6 mg, 0.54%w/w); orange needle crystal solids; EIMS (low resolution) m/z (% relative intensity) 294 (M^+, 50), 150 (55), 137 (100). Compound B was isolated from the ethanolic extract of Pikutbenjakul preparation, obtained as orange needle crystal solids. The TLC analysis of this compound was compared with authentic sample 6-gingerol (Wako) by TLC using 3 solvent systems and gave identical behavior. The ^{1}H NMR spectrum, compared with the previous ^{1}H-NMR data of 6-gingerol, was the same as the spectrum recorded for 6-gingerol[7] (Table 3). Thus, it was strongly supported that compound B to be 6-gingerol. The structure was shown in Figure 2.

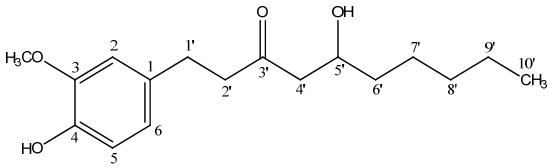

Figure 2 Structure of compound B (6-gingerol)

Table 3 ^{1}H-NMR spectral data (500 MHz) of BENS3 in $CDCl_3$

Position	δ_H (mult.,*J* in Hz) (6-Gingerol)	Position	δ_H (mult.,*J* in Hz) (Compound B)
2	6.69 (*d*, 2.0)	2	6.68 (*br.s*)
3-OCH_3	3.68 (*s*; 3H)	3-OCH_3	3.87 (*s*; 3H)
4-OH	-	4-OH	5.51 (*s*)
5	6.60 (*d*, 8.4)	5	6.82 (*d*, 8.4)
6	6.53 (*dd*, 8.4, 2.0)	6	6.65 (*dd*, 8.4, 2.1)
1′-2′	2.69 (*s*; 4H)	1′	2.74 (*br.d*,2H 7.2)
		2′	2.71 (*dd*, 6.6, 2.1)
			2.85 (*dd*, 6.6, 2.1)
4′	2.44 (*dd*; 2H, 8.4, 2.0)	4′	2.48 (*dd*, 17.4, 3.3)
			2.58 (*dd*, 17.4, 8.7)
5′	3.86 (*m*)	5′	4.04 (*m*)
6′-9′	1.21–1.32 (*m*; 8H)	6′-9′	1.25–1.50 (*m*; 8H)
10′	0.83 (*t*; 3H)	10′	0.89 (*t*; 3H, 6.6)

Note: 6-gingerol from previous report[7]

***In vitro* Assay for Cytotoxic Activity**

Human cell lines

The human breast adenocarcinoma cells (MCF-7) (ECACC No:86012803) were obtained from the European Collection of Animal Cell Culture (PHLS Center for applied Microbiology Research, Porton Down, Salisbury, UK). MCF-7 cells were cultured in Minimum Essential Media (MEM) with Earle Salt without glutamine medium supplement with 10% heated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 μg/ml streptomycin and 1% non-essential

amino acid and maintained at 37°C in a 5% CO_2 atmosphere with 95% humidity[8]. According to their growth profiles, the optimal plating densities of breast cancer cell line was determined $3x10^3$ cells/well to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay.

Cytotoxicity Assay

For the assay, cells were washed with magnesium and calcium free phosphate buffer saline (PBS) (Oxoid Ltd.,UK) PBS were decanted and cells detached with 0.025% trypsin-EDTA (sigma) PBS were added to a volume of 50 ml .The cell pellet obtained by centrifugation (1000g , 5 min) were resuspensed in 10 ml of medium to make single cell suspension and viable cells were counted by trypan blue exclusion in haemocytometer and diluted with medium to give a final concentration of $3x10^3$ cells/well. One hundred µl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24h the cells were treated with the extracts and pure compounds. Each extract was initially dissolved in an amount of DMSO for the ethanolic extracts and sterile distilled water for the water extracts and vincristine sulphate (Sigma, Lot No. 34H0 447) was used as the positive control. The extracts were diluted in medium to produce 8 concentrations and 100 µl of each concentration was added to each well of the plates in 6 replicates to obtain final concentrations of 100, 50, 25, 10, 5, 1.5, 0.5, 0.1 µg/ml for extract and 100, 50, 25, 10, 5, 1.5, 0.5, 0.1, 0.05 nM for vinblastine sulphate (positive control). The final mixture used for treating the cell contained not more than 1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure times of 72 hours. as indicated. At the end of each exposure time, the medium was removed. The wells were then washed with medium, and 200 µl of fresh medium were added. The plates were incubated for recovery period of 6 days and cell number were analyzed by SRB assay[8,9].

Sulphorhodamine B (SRB) assay

The anti-proliferative assay, SRB (sulphorhodamine B) assay,was performed according to method of Skehan *et al*[9] was used to assess growth inhibition This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB[9]. For the procedure of this assay is described in previous report[8]. The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines[10] extracts with IC_{50} values < 20 µg /ml were considered active.

Table 4 Cytotoxicity activity (IC_{50} µg/ml ± SEM) of plant extracts and isolated compounds against human breast adenocarcinoma cell line (MCF7) at exposure time 72 hr (n=3).

Plants	Part of used	Extract	Code	%yield	MCF-7 (IC_{50} µg/ml ± SEM)[µM]
Piper chaba Linn	Fruit	EtOH	PCE	12.3906	35.17 ± 1.91
		Water	PCW	15.8965	>100
Piper sarmentosum Roxb	Root	EtOH	PSE	1.7449	69.53 ± 9.09
		Water	PSW	8.5603	>100
Piper interruptum Opiz.	Stem	EtOH	PIE	0.6596	62.35 ± 5.23
		Water	PIW	5.0254	>100
Plumbago indica Linn	Root	EtOH	PLE	5.0071	40.81 ± 3.62
		Water	PLW	20.3808	>100
Zingiber officinale Roscoe	Rhizome	EtOH	ZOE	8.5651	31.15 ± 1.40
		Water	ZOW	13.7434	>100
Pikutbenjakul	-	EtOH	BENE	7.7332	33.20 ± 0.80
		Water	BENW	16.2897	>100
Compound A (Piperine)	-	-	-	7.81	10.18 ± 1.29 [35.72]
Compound B (6-gingerol)	-	-	-	0.54	9.80 ± 2.37 [33.33]

RESULTS AND DISCUSSION: The summarized data of the investigated plant species showed in Table 1. The results of cytotoxicity evaluation of all plant extracts as IC_{50} (μg/ml) were shown in Table 4. This data showed that the ethanolic extract of *Piper chaba*, *Zingiber officinale* and Pikutbenjakul were the extracts possessing the highest activity against the MCF7 cell line (IC_{50} = 35.17, 31.15 and 33.20 μg/ml, respectively). Piperine (Compound A) and 6-gingerol (Compound B) were cytotoxic compounds against MCF7 that isolated from ethanolic extract of Pikutbenjakul (Table 4). Piperine is a type of alkaloid which showed the highest percentage of yield of Benjakul extraction (7.81%) and was isolated from *Piper longum*[11] and many species of Piper. Piperine showed cytotoxic activity against MCF-7 (IC_{50} = 35.72μM).The previous report showed that piperine inhibited the solid tumor development in mice induced with DLA cells and increased the life span of mice bearing Ehrlich ascites carcinoma tumor to 58.8%[12]. Although piperine is a well-defined compound, its cytotoxic mechanisms on cancer cells especially breast cancer cells have not yet been examined. 6-Gingerol was isolated from *Zingiber officinale*[7], which is one of the five components of Pikutbenjakul Preparation. It showed cytotoxic against breast cancer cells (IC50 = 33.3 μM). This result is consistent with the other previous report of 6-gingerol. It was found that it showed cytotoxic against human cancer cells (HL-60 cells)[13] and inhibits cell adhesion, invasion, motility and activities of MMP-2 and MMP-9 in MDA-MB-231 human breast cancer cell lines[14]. However there is no report on its cytotoxicity against MCF-7. The results revealed that Pikutbenjakul prepara-tion possessed high cytotoxic activity against breast cancer cells. Its ethanolic extract should be promoted for industry. Piperine and 6-gingerol should be used as markers for standardization of chemical and biological fingerprints because piperine was present in high amount and high cytotoxic activity.

CONCLUSION: In summary, Pikutbenjakul as a Thai traditional medicine which was normally used to be adaptogen for cancer treatment showed cytotoxic against breast cancer cell. Two compounds, piperine and 6-gingerol, were isolated from the ethanolic extract of Pikutbenjakul preparation which also showed cytotoxic against breast cancer cells. Therefore, the present study supports the use of Benjakul to treat breast cancer by Thai traditional doctors. Further studies involved the investigation about cytotoxic acitivity against other human cancer cell lines and molecular mechanism of isolated compound against breast cancercomparative with Pikutbenja-kul extract

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