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Original Research Article

Extracts of edible, medicinal Thai plants inhibit the human breast cancer cells

Benjaporn Buranrat[1]*, Ampa Konsue[2], Pornpimon Wongsuwan[2]
[1]Faculty of Medicine, [2]Applied Thai Traditional Medicine, Faculty of Medicine, Mahasarakham University, Muang District, Maha Sarakham 44000, Thailand

For correspondence: **Email: buranrat@gmail.com; **Tel:** +66-4302-1021 ext 7848*

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Abstract

Purpose: *To evaluate the effects of ten edible, medicinal Thai plant extracts on MCF-7 cell viability and cell migration, as well as their mechanism(s) of action.*
Methods: *Ethanolic plant extracts of ten edible, medicinal plants were tested for their cytotoxicity against MCF-7 cells using sulforhodamine B (SRB). To investigate the cytotoxic mechanism(s) of action of these extracts, the study was examined gene expression and protein expression by reverse transcription polymerase chain reaction (RT-PCR) and Western blotting. Cell migration was studied by wound healing assay.*
Results: *Four of the ten test extracts were potently cytotoxic, Careya sphaerica (CS), Azadirachta indica (AI), Piper nigrum (PN) and Oroxylum indicum (OI) with half maximal inhibitory concentrations (IC_{50}) less than 100 µg/mL. All four extracts stimulated ROS overgeneration, increased caspase 3 activity and decreased growth-related gene expression including cdk2, cdk4, cdk6, cyclin D1 and cyclin E. Furthermore, the extracts significantly enhanced cyclin-dependent kinase inhibitor (CDKI) p21 levels and activated cancer cell death. The four extracts, CS, AI, PN and OI, also significantly reduced cancer cell migration, with PN being the most potent.*
Conclusion: *Extract of the edible plants CS, AI, PN and OI have in vitro anticancer activity and are promising starting points for the development of breast cancer drugs.*

Keywords: *Careya sphaerica (CS), Azadirachta indica (AI), Piper nigrum (PN), Oroxylum indicum (OI), Breast cancer, Cell death*

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INTRODUCTION

Compounds derived from herbal medicines have long attracted consideration as a starting point for drug development including anticancer drug. Anti-cancer agents are developed from plants and others natural products because they have low adverse effects [1,2]. Recently, research with medicinal plants has increased with a view to reducing drug resistance and toxicity in cancer treatment. Several plants have been shown to have anticancer activity but no harmful effects [3].

Several herbal medicines have been identified with anticancer properties. Many plant-derived anticancer compounds have been discovered

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through large-scale screening methods [4] including anti-breast cancer [2,5,6]. The present study screened extracts of ten edible Thai plants, *Clitoria ternatea* (CT), *Piper sarmentosum* (PS), *Morinda citrifolia* (MC), *Centella asiatica* (CA), *Thunbergia laurifolia* (TL), *Moringa oleifera* (MO), *Oroxylum indicum* (OI), *Azadirachta indica* (AI), *Piper nigrum* (PN) and *Careya sphaerica* (CS) for activity against breast cancer. All ten plants are edible medicinal plants used in Asian countries including Thailand and are consumed either fresh or cooked. Importantly, all of the plants tested have been reported to have activity against many types of cancer cells. Thai plants can be used to inhibit the growth of cancer cells or to kill them. In *Piper longum* and *Piper nigrum,* the main active constituents is piperine. Piperine significantly decreases breast cancer cell proliferation by increasing cells in the G2/M phase, by decreasing cell migration, and by inhibiting MMP 9 and MMP 13 expression [7]. Present study measured the inhibitory activities of ethanolic extracts of ten edible Thai plants on human breast cancer cells, MCF-7. A anti-migratory effects and mechanisms of action were also investigated.

EXPERIMENTAL

Preparation and extraction of herbs

Ten Thai plants including *Clitoria ternatea* (CT), *Piper sarmentosum* (PS), *Morinda citrifolia* (MC), *Centella asiatica* (CA), *Thunbergia laurifolia* (TL), *Moringa oleifera* (MO), *Oroxylum indicum* (OI), *Azadirachta indica* (AI), *Piper nigrum* (PN) and *Careya sphaerica* (CS) were collected in 2016 from Udon Thani Province. The plants were identified by Pornpimon Wongsuwan, an Assistant Professor of Applied Thai Traditional Medicine, at Mahasarakham University Faculty of Medicine. Voucher specimens (Table 1) were deposited at Mahasarakham University Herbarium. All of the samples were dried and sliced, with 250 g of each extracted twice with 1000 mL 95 % ethanol (v/v) ethanol for 7 days. The plant extracts were then filtered, evaporated, lyophilised and stored at -20 °C.

Cell culture and assessment of cell viability

Cells of the human breast cancer cell line MCF-7 (1 x 10^4 cells/well) were added to the various doses of extract (0 - 250 µg/mL) for 24 and 48 h. Cell viability was examined by SRB assay as described previously [1]. In brief, cells were fixed with 10% trichloroacetic acid, stained with SRB solution and lysed with the buffer, with optical density (O.D.) measured by spectrophotometer at 540 nm.

Assessment of anti-migratory activity of extracts by wound healing method

The migration of cancer cells was measured by wound healing assay [1]. In brief, a wound was made in each MCF-7 cell monolayer using sterile 0.2 mL pipette tips. These were then incubated with different doses of extract (0 - 100 µg/mL) for 48 h. After that, the cells were stained with 0.25% crystal violet, and air dried. The size distance of the wounds was then compared between control and treatment groups.

Assessment of extracts-induced ROS generation

Cells were exposed to various doses of extract (0 - 250 µg/mL) and a fixed concentration of fluorescent probe (25 µM DHE) in serum-free medium for 1.5 h in the dark [1]. ROS fluorescence intensity was then measured at an excitation wavelength of 518 nm and an emission wavelength of 605 nm.

Assessment of the extract effects on caspase 3 activity by caspase 3 assay kit method

Cell was exposed to the various doses of extract (0 - 250 µg/mL) for 24 h, and caspase 3 activity was measured as described previously [1]. In brief, a crude protein extract was prepared from cells and this was incubated with buffer containing Ac-DEVD-AMC (caspase 3 substrate) for 90 min in the dark. Fluorescence intensity was then measured at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The caspase 3 activity was calculated by using AMC.

Assessment of extract effect on gene expression

Cell were exposed to the various doses of extract (0 - 100 µg/mL) for 24 h, and gene expression (*cdk2, cdk4, cdk6, cyclin D1* and *cyclin E*) was measured by RT-PCR as described previously [1]. In brief, mRNA was extracted and converted to cDNA. PCR was then performed using the primers shown in Table 1 with ACTB as an internal control. The final reaction volume (20 µL) consisted of target gene or internal control and Master Mix.

Determination of extract effect on protein expression

Cells were exposed to a fixed doses of the extract (100 µg/mL) for 24 h, and protein expression was determined by Western blotting

as described previously [1]. In brief, a crude protein extract was prepared from the cells, this protein was loaded for SDS-PAGE and transferred to a PVDF membrane. After this, the membrane was treated with primary antibodies (p21, beta-actin) overnight, followed by secondary antibody for 2 h to detect the protein bands.

Statistical analysis

Student's t-test was used to identify statistically significant differences between non-treated and treated groups. Differences were considered statistically significant if $p < 0.05$. Statistical analysis was performed using the GraphPad Prism 5 program (GraphPad Software, San Diego, CA).

RESULTS

Effect of solvent on extraction yield and polyphenol content

Total flavonoid and total phenolic contents of the ten plant extracts were determined using standardized colorimetric methods with rutin and gallic acid. The results are shown in Table 2. *Careya sphaerica* (CS) extract was found to have the highest phenolic and flavonoid content, 112.31 ± 2.63 mg/g and 105.65 ± 5.08 mg/g, respectively.

Cytotoxic activity

To measure the cytotoxic activities of the ten Thai plant extracts, the SRB method was used. All the extracts induced cancer cell death in a concentration- (0-250 µg/mL) and time-dependent manner (24 to 48 h) (Figure 1A-1J). The four most active extracts, most potent first, were CS > AI > PN > OI. All four extracts, reduced MCF-7 cell viability, with low IC_{50} values. CS extract had the most potent activity, with an IC_{50} value of 21.03±0.92 µg/mL after 24 h and 12.85±1.65 µg/mL after 48 h (Table 3). All subsequent work in this study focused on these four extracts.

Effect of extracts on ROS production and caspase 3 activity in cancer cells

To help elucidate the mechanism(s) of actions of the four plant extracts (CS, AI, PN, and OI), their effects on ROS generation and caspase 3 activity were studied. Excessive ROS production can activate MCF-7 cell apoptosis via both extrinsic and intrinsic pathways in mitochondria, and cause apoptosis by stimulating caspase 3 activity. Treatment with various concentrations of plant extract induced significantly ROS production, especially high concentrations, except for PN which increased ROS formation only slightly (Figure 2). Low concentration of PN (10 - 50 µg/mL) induced significant caspase 3 activity. The other three extracts also induced significant induced caspase 3 activity, especially at high doses (Figure 2).

Effect of extracts on cell cycle-related gene expression in cancer cells

To examine the effects of the extracts on cancer cell gene expression, RT-PCR assay was used. Plant extracts AI, CS and PN reduced expression of all five of the cell cycle-related genes, except *cyclin E* expression in the case of CS and PN extracts (Figure 3A). Plant extract OI, by contrast induced a significant increase in the expression of all five gene.

Effect of extracts on p21-related cell cycle inhibitor expression in cancer cells

To examine the effects of the extracts on p21 protein expression, Western blotting was used. Four of the extracts significantly increased p21 protein levels, with PN extract inducing the greatest activity (Figure 3B).

Anti-migratory effect of the extracts on cancer cells

To examine the anti-migratory effects of the four extracts on cancer cells, the wound healing method was used. All extracts were found to suppress the migration of cancer cells in a dose-dependent manner. PN was the most potent, with an IC_{50} value of 3.19 ± 0.46 µg/mL, followed by CS with an IC_{50} value of 9.16 ± 3.21 µg/mL, AI with an IC_{50} value of 18.16 ± 1.61 µg/mL and OI with an IC_{50} value of 126.60 ± 7.21 µg/mL (Figure 4). Thus, the four crude extracts demonstrated anti-migratory activity with low IC_{50} values.

DISCUSSION

Plant-based traditional medicine has played a crucial role in anticancer therapy, and recent research has focused on screening such medicines for potently anticancer compounds with low toxicity. The present study screened ten edible Thai plants for their anti-growth and anti-migratory activities on the human breast cancer cell lines MCF-7. For anti-proliferative activity, data indicated that the four of these plant extracts were particularly potent. *Careya sphaerica* (CS) showed the highest anti-

proliferative activity. CS is a vegetable and the young leaves are used as a food in Thailand.

Natesan *et al* have previously shown that CS methanolic extract significantly decreased solid tumour volume activated by DLA cells [8], and CS bark methanolic extract reduces body weight, viable tumour cell count and packed cell volume [8] Also, the ethanol extract from the young leaves of *Azadirachta indica* (AI) have been shown to stimulate cancer cell death in HCT116, MCF-7 and Hep-G2 cell lines [9]. PN ethanolic extracts are known to induce breast cancer cell death too, by up-regulating the p53 tumour suppressor gene, and down-regulating vascular endothelial growth factor (VEGF), estrogen receptor (ER), E-cadherin (E-cad), matrix metalloproteinase 2 (MMP 2) and MMP 9 [10].

Elucidating the mechanism of action of novel anticancer extracts and compounds is of crucial importance. The present study showed CS, AI, PN and OI plant extracts significantly increase ROS formation, with AI having the highest activity. The induction of ROS may cause cancer cell apoptosis by increasing caspase 3 activity. PN extract stimulated less ROS production than other three extracts but, unexpectedly induced the highest levels of caspase 3 activity. This anomaly will be explored further in the future study.

Table 1: Edible Thai plant materials used

Plant name	Family	Part used	Yield (%)	Voucher specimen no.
Clitoria ternatea	Fabacae	Flower	23.53	MSUT_7230
Piper sarmentosum	Piperaceae	Leaf	15.62	MSUT_7224
Morinda citrifolia	Rubiaceae	Leaf	12.23	MSUT_7225
Centella asiatica	Umbelliferae	Leaf	16.52	MSUT_7231
Thumbergia laurifolia	Acanthaceae	Leaf	11.52	MSUT_7233
Moringa oleifera	Moringaceae	Leaf	13.69	MSUT_7232
Oroxylum indicum	Bignoniaceae	Leaf	12.56	MSUT_7226
Azadirachta indica	Meliaceae	Leaf	15.67	MSUT_7228
Piper nigrum	Piperaceae	Fruit	19.45	MSUT_7234
Careya sphaerica	Lecythidaceae	Leaf	12.54	MSUT_7227

Table 2: Primer sequences for RT-PCR

Gene	Primer sequence
Cdk2	Forward primer 5' CCA-GGA-GTT-ACT-TCT-ATG-CCT-GA 3'
	Reverse primer 5' TTC-ATC-CAG-GGG-AGG-TAC-AAC 3'
Cdk4	Forward primer 5'ATG-GCT-ACC-TCT-CGA-TAT-GAA-C 3'
	Reverse primer 5' CAT-TGG-GGA-CTC-TCA-CAC-TCT 3'
Cdk6	Forward primer 5' GCT-GAC-CAG-CAG-TAC-GAA-TG 3'
	Reverse primer 5' GCA-CAC-ATC-AAA-CAA-CCT-GAC-C 3'
Cyclin D1	Forward primer 5' GCT-GCG-AAG-TGG-AAA-CCA-TC 3'
	Reverse primer 5' CCT-CCT-TCT-GCA-CAC-ATT-TGA-A 3'
Cyclin E	Forward primer 5' ACT-CAA-CGT-GCA-AGC-CTC-G 3'
	Reverse primer 5' GCT-CAA-GAA-AGT-GCT-GAT-CCC 3'
ACTB	Forward primer 5' GTG-ACG-TTG-ACA-TCC-GTA-AAG-A 3'
	Reverse primer 5' GCC-GGA-CTC-ATC-GTA-CTC-C 3'

Table 3: IC_{50} and Emax values for cytotoxicity

Group	IC_{50} (µg/mL)		Emax	
Clitoria ternatea (CT)	>250	>250	ND	ND
Piper sarmentosum (PS)	>250	>250	ND	ND
Morinda citrifolia (MC)	>250	>250	ND	ND
Centella asiatica (CA)	>250	>250	ND	ND
Thunbergia laurifolia (TL)	>250	>250	ND	ND
Moringa oleifera (MO)	>250	>250	ND	ND
Oroxylum indicum (OI)	139.47±21.32	74.21±5.42	79.29±5.10	90.40±1.01
Azadirachta indica (AI)	25.75±0.18	15.34±0.44	90.49±0.83	95.13±1.38
Piper nigrum (PN)	47.35±2.71	18.16±0.96	100.59±4.17	92.31±1.20
Careya sphaerica (CS)	21.03±0.92	12.85±1.65	66.96±1.11	83.81±1.06

ND = not detected

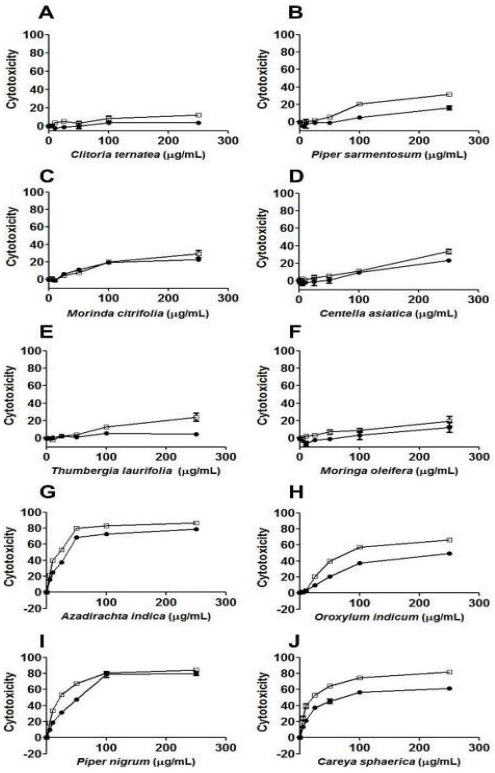

Figure 1: Effect of the four plant extracts on MCF-7 cell viability (A-J). The data represent mean ± SEM from three independent experiments ●; 24 h treatment, □; 48 h treatment.

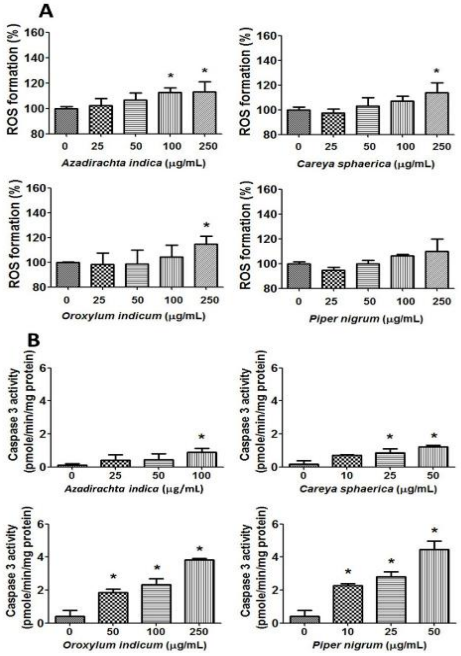

Figure 2: Effects of the four plant extracts on ROS production (A) and caspase 3 activity (B). Error bars represent the mean ± SEM from three independent experiments; *$p < 0.05$ vs. untreated control groups

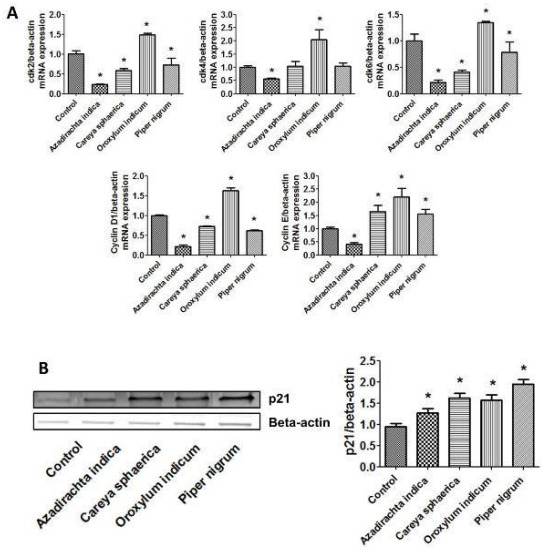

Figure 3: Effects of four plant extracts on *cdk2*, *cdk4*, *cdk6*, *cyclin D1* and *cyclin E* mRNA expression (A) and p21 protein expression (B). Error bars represent SEM (n = 3); *$p < 0.05$ vs. untreated control groups

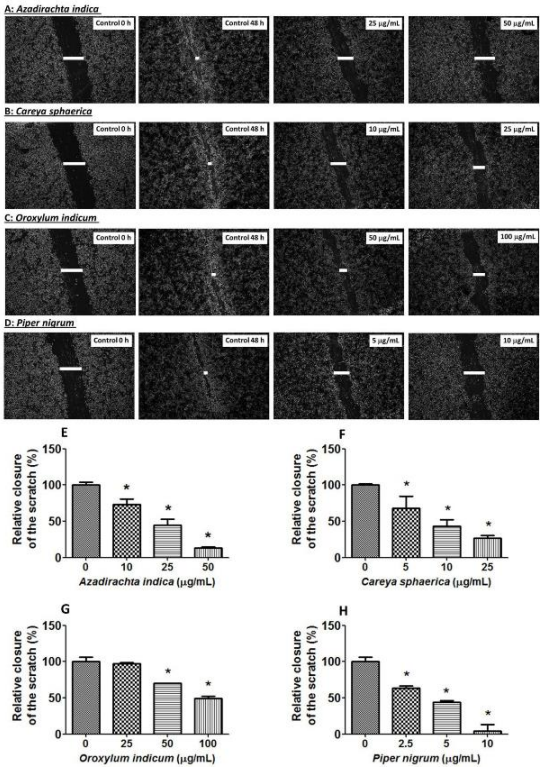

Figure 4. Effects of four plant extracts on cell migration (A-H). Error bars represent mean ± standard error from three independent experiments; *$p < 0.05$ vs. untreated control groups

In a previous work with AI leaf extract, an induction of ROS production was also detected, with this then stimulating oocyte apoptosis by increasing caspase, caspase 3 activities, cytochrome c concentration and DNA fragmentation [11]. Moreover, *Piper nigrum* (PN) and its constituent compounds (eg. Piperamides) have been shown to be cytotoxic and anti-proliferative to MCF-7 cells, and to have anti-tumour effects due to ROS overgeneration. This activated oxidative stress affects important proteins involved in cell cycle arrest at the G1/S stage, thereby stimulating apoptosis [12].

The present models of cell cycle control suggest that transition between different cell cycle states is controlled at checkpoints by genes *cdk2*, *cdk4* and *cdk6* [13]. Different cdk isoforms have crucial roles in cancer cell growth via loss of regulation of the cell cycle, a hallmark feature of cancer. These results show that AZ, CS and PN extracts tend to suppress *cdk2* and *cdk6*, whereas OI extract significantly induces all of the CDKs. OI extract is also less the cytotoxic towards MCF-7 cells than AI, CS and PN extracts. Cyclin D/cdk complexes are functionally active in G1, allowing progression to S phase. The accumulation of cyclin E at the G1/S phase boundary activates *cdk2* and *cdk1* successively, stimulating progression to the G2 phase [14]. These data found that only AI extract significantly suppresses *cdk2*, *cdk4*, *cdk6*, *cyclin D1* and *cyclin E* in breast cancer cells. OI extract, by comparison, increases cdk and cyclin and is less cytotoxic to cancer cells.

In addition to cytotoxic effects, this study suggests that Thai plant extracts could be used to suppress metastasis since they inhibit cell migration. In the present study, all four test extracts inhibit MCF-7 cells migration, and induced morphological changes indicative of an arrest in cell migration. These anti-metastatic effects were detected from the four Thai plant extracts at low concentrations which only minimally suppressed cell growth. The greatest anti-migratory activity was detected from PN extract. It has been reported that some Thai plants, for example, Curcuma longa (tumeric) inhibit invasion and migration because constituent curcuminoids reduce NF-κB in breast cancer cells, MDA-MB-231 [15]. *Cratoxy formosum* extract also exerts an anti-migratory effects by inhibiting NF-κB. *C. formosum* stimulates STAT3 too [16], this correlating with the reduction in MMP 2 and MMP 9 levels [1,5]. Thai plants could be source of precursors suitable for use in the targeted therapy of breast cancer.

CONCLUSION

The findings of this work show that CS, AI, PN and OI extracts exhibit cytotoxic activity against human breast cancer cells. This activity is attributable, in part, to an anti-proliferative effect by induction of ROS formation and caspase 3 activity. The four plant extracts also inhibit the migration of MCF-7 cells. These results suggest that CS, AI, PN and OI extracts contain compounds that can be developed as therapeutic agents for the management of breast cancer.

DECLARATIONS

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. BB performed the experiments, analyzed the data. AK and PW identified and extracted plants. BB, AK, and PW read and approved the final manuscript. The authors thank Dr. Tim Cushnie (MSU Faculty of Medicine) for language-editing the manuscript.

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