Original Article

Cytotoxic Activity on Colorectal Cancer Cells and Antioxidant Activity of Crude Extracts of Pi-Kad-Tri-Khan-Tha-Wart Remedy and Its Components

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Abstract

Introduction: Pi-Kad-Tri-Khan-Tha-Wart (PK) is a Thai traditional remedy utilizing for gastrointestinal tract disorder such as nausea vomiting stomachache. It consists of three medicinal plants. Two ingredients of the remedy, nutmeg (seed of *Myristica fragrans* Houtt., MF) and clove (flower buds of *Syzygium aromaticum* (L.) Merr. & L.M. Perry., SA), had anti-inflammation, antioxidation and cytotoxic property against breast cancer cells (MCF-7). Fruit of *Amomum xanthioides* Wall. (AX) had cytotoxicity against ovarian cancer cells (SK-OV-3) and malignant melanoma cells (SK-MEL-2). This study investigated the cytotoxicity against colorectal cancer cells and antioxidant of the Pi-Kad-Tri-Khan-Tha-Wart remedy and its components.

Methods: The remedy and each plant ingredient were extracted using into 2 methods by 95% ethanol maceration and aqueous decoction. We investigated cytotoxic activity against colorectal adenocarcinoma cells (SW480) and normal keratinocyte cells (HaCaT) using SRB assay and the antioxidant activity by DPPH assay.

Results: PKE and PKA exerted antioxidant activity higher than positive control, BHT with EC_{50} value of 17.03 ± 10.29 µg/ml and 9.22 ± 1.51 µg/ml, respectively. SAA exerted the highest antioxidant activity with EC_{50} value of 5.29 ± 0.36 µg/ml. Both PK extracts did not show cytotoxic activity. MFE and SAA had the cytotoxic activity against SW480 cells by IC_{50} of 36.13 ± 2.87 µg/ml and 40.08 ± 1.24 µg/ml, respectively.SAE had cytotoxicity against HaCaT cells with IC_{50} = 39.55 ± 2.85 µg/ml.

Conclusions: PKE and PKA had no inhibitory effect on colorectal cancer cells but they exerted high antioxidant activity. SAA exerted the highest antioxidant among others. MFE and SAA exerted inhibitory effect on the cell growth of the tested cancer cells. MFE also showed cytotoxicity against the HaCaT normal cells.

Keywords: Colorectal cancer, Pi-Kad-Tri-Khan-Tha-Wart recipe, Cytotoxicity, Antioxidant

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Introduction

Colorectal cancer (CRC) is a threatening disease effecting from the uncontrollable growth of the colorectal cells and is a leading cause of death worldwide.[1] The cancer is induced by several factors and one of the important factors is oxidant or free radicals. The radicals can damage the cells and immune system causing cell degeneration until the normal cells transform to the carcinoma cells.[2] Many anti-cancer drugs were discovered from potent herbal chemicals. In addition, there were several studies showed that strong effectiveness of the plant extracts or phytochemicals against various cancer cells.[3,4]

Pi-Kad-Tri-Khan-Tha-Wart (PK) is one of the herbal remedies in Thai traditional pharmacy scripture. It consists of *Amomum xanthioides* Wall (AX), *Myristica fragrans* Houtt (MF) and *Syzygium aromaticum* (SA) in equal ratio. The PK remedy is used to cure the impairment of elements in the body, nausea, vomiting, hemorrhoids and stomach-ache.[5] In previous study, methanol AX extract had cytotoxicity against ovarian cancer cell (SKOV-3) and human melanoma cell (SK-MEL-2).[4] MF and SA extracts had antioxidant activity and anticancer against breast cancer cell (MCF-7).[3]

MF extract showed high antioxidant activity by inhibitory effect on PMA-induced superoxide radical in DMSO differentiated from HL-60 cells (NBT reduction assay) (IC_{50} values = 21.164 + 1.03 µg/ml).[6] The antioxidant activity of MF determined by DPPH radical scavenging assay revealed the IC_{50} value to be 63.76 ppm.[7] Additionally the MF extract showed excellent anticancer activity against MCF-7 cell lines with the IC_{50} value of 10.75 ppm.[8]

SA extract was screened for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay at 500 µg/ml concentration and showed 80.8% inhibition of DPPH free radical.[9] SA extract showed the potent cytotoxicity against MCF-7 cells by using MTT assay with IC_{50} of 16.71 µg/mL.[10,11]

Moreover, the PK remedy and its medicinal plants have never been studied for inhibitory effect on the colorectal adenocarcinoma cells. We investigated the cytotoxic activity against SW480 human colorectal cancer cells and antioxidant activity of the ethanol and the aqueous extracts of the remedy and its herbal ingredients.

Methods

1. Plant Materials

Dry *A. xanthioide* (AX) fruits, *M. fragrans* (MF) seeds and *S. Aromaticum* (SA) flower buds were purchased from Thammasat Primary Health Care Center (Kukot) at Pathum Thani, Thailand. All plants crude drugs were identified by comparing with the authentic voucher specimens at the Faculty of Pharmaceutical Science, Prince of Songkla University, Songkla, Thailand. The herbarium voucher specimen numbers of AX, MF and SA were SKP 206 01 24 01, SKP 121 13 06 01, and SKP 206 26 03 01, respectively.

2. Extract Preparation

All plant materials were cleaned, sliced, dried and grinded into coarse powder. The PK remedy formula comprised equal ratio of AX: MF: SA (1:1:1) in the scripture. AX, MF, SA, and PK crude powders were extracted with two methods.

2.1 Decoction: Prepared 15 grams (g) of each crude powder and then added into 45 ml of water (1:3). Boiled for 15 minutes (min) and filtered. Simmered filtered water extract down to 15 ml (one-third) at 60 degrees Celsius (°C). Each extract was dried by freeze dry machine. The aqueous extract was weighed and kept at -20°C until used.

2.2 Maceration: 25 g of each powdered materials and the PK powder were macerated with 95% ethanol for 3 days. Filtered and then removed solvent by using a rotary evaporator to obtain dry ethanolic extract. The process was repeated twice. These four ethanol extracts were weighed and kept in containers at -20°C until used.

Table 1 Plant materials and code

Scientific name	Common name	Used Part	Solvent	Code
Amomum xanthioides Wall.	Big Chewy	Fruit	95% Ethanol Aqueous	AXE AXA
Myristica fragrans Houtt.	Nutmeg, Mace	Mace	95% Ethanol Aqueous	MFE MFA
Syzygium aromaticum (L.)	Clove	Flower buds	95% Ethanol Aqueous	SAE SAA
AX: MF: SA (1:1:1)	Pi-Kad-Tri-Khan-Tha-Wart	-	95% Ethanol Aqueous	PKE PKA

3. Cell Culture

Human colorectal SW480 cancer cells (No. CCL-228) and normal keratinocyte cells (HaCat) were obtained from American Type Culture Collection (Manassas, VA, USA). The SW480 cells were cultured in RPMI-1640 media (GIBCO/BRL Life Technologies, USA) with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA) and the HaCat cells were maintained in DMEM (GIBCO/BRL Life Technologies, USA) with 10% FBS. The SW480 cells and the HaCat cells were seeded in 96-well plates with an initial concentration of 3×10^3 cells/well and 5×10^3 cells/well, respectively, in 100 µl medium. The cells were maintained at 37°C under 75% relative humidified atmosphere of 5% CO_2 for 24 h. The cells were treated with various concentrations (1, 10, 50, or 100 µg/ml) of the extracts in the completed medium for 72 h. The medium was replaced with 200 µl new media and the cells were cultured for another 72 h. After incubation, the SW480 and HaCaT cells were measured for cell viability.

4. Cytotoxic Assay

Sulphodoramine B (SRB, Sigma-Aldrich, St. Louis, MO, USA) assay was preformed to measure cell viability.[4] The viable cells were fixed with 100 µl of 40% (w/v) trichloroacetic acid (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 1 h and then stained with 50 µl of 0.4% (w/v) SRB solution in 1% acetic acid (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. After staining, the color was dissolved with 10 mM Trisma base solution (Sigma-Aldrich, St. Louis, MO, USA). The absorbance was measured with a microplate reader (Bio-Tek Instruments) at wavelength 492 nm.

$$\% \text{ inhibition} = (OD_{control} - OD_{sample})/OD_{control} \times 100$$

The half inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA).

5. Antioxidant Assay

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is a chemical reaction method for measuring antioxidant activity.[12] A 6×10^{-5} M of DPPH (Sigma-Aldrich, St. Louis, MO, USA) solution in absolute ethanol was freshly prepared. The extracts were dissolved in absolute ethanol at different concentrations (1, 10, 50, or 100 µg/ml) and a 100 µl of each was added into a 96-well plate with 100 µl. A 100 µl of The DPPH solution were mixed with each extract. The antioxidant activity was determined by a microplate reader (Bio-Tek Instruments) at 520 nm and Butylated hydroxytoluene (BHT) was screened as an antioxidant standard with DPPH assay to define the half effective concentration (EC_{50}) parameter. The ability of DPPH radical scavenging was calculated as percentage of inhibition by the following equation:

$$\% \text{ inhibition} = (OD_{control} - OD_{sample})/OD_{control} \times 100$$

The half effective concentration (EC_{50}) was calculated using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA).

6. Statistical Analysis

All experiments were repeated three times to confirm the results. SPSS version 16.0 (SPSS lnc., USA) was used to calculate mean and standard

error of the mean (SEM), analyzed the difference of the data with ANOVA, and tested the difference between the groups with student *t* test with a significance level of 0.05.

Results

1. Percent of Yield of the Extracts

The percent yields of the extracts were shown in Table 2. In the ethanolic extracts, SAE and AXE showed the highest and lowest percent yield as 18.75% and 0.83%, respectively. In group of the aqueous extracts, SAA and MFA showed the highest and lowest %yield as 9.86% and 6.53%, respectively. As the results on Table 2 could implied that the different methods of extraction probably affect the percent yield of the extracts.

Table 2 Percent yield of the extracts

Extracts	%yield
AXE	0.83
AXA	9.02
MFE	14.05
MFA	6.53
SAE	18.75
SAA	9.86
PKE	12.21
PKA	7.26

2. Antioxidant Activity of the Extracts

The antioxidant activities of the extracts were presented in Figure 1. SAA showed the highest antioxidant activity by EC_{50} value of 5.29 ± 0.36 µg/ml. The results of antioxidant activity showed the potential antioxidant activity of five extracts ranked from the strongest to the weakest as SAA, PKA, SAE, PKE, and AXA. The EC_{50} values of the five extracts were 5.29 ± 0.36, 9.22 ± 1.51, 9.54 ± 7.12, 17.03 ± 10.29, and 17.74 ± 0.62 µg/ml, respectively. SAA, PKA, SAE had better antioxidation than positive control, BHT (EC_{50} = 21.57 ± 10.58 µg/ml, $P < .05$. MFE, MFA, and AXE exerted less potent antioxidant activity by EC_{50} values of 35.38 ± 26.98, 49.21 ± 2.10 and > 100 µg/ml, respectively. Furthermore, PKA had potent DPPH radical scavenging activity better than PKE by EC_{50} values were 9.22 ± 1.51 and 17.03 ± 10.29 µg/ml, respectively.

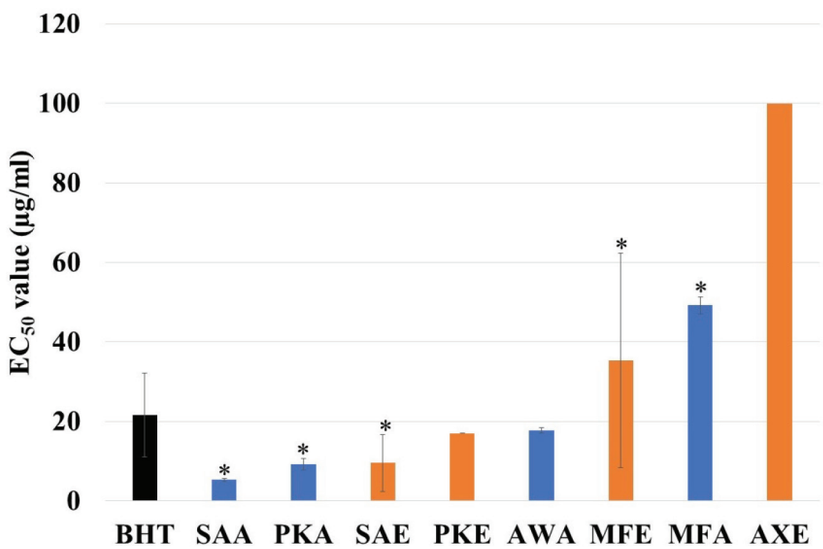

Figure 1 EC_{50} values of the extracts on DPPH radical scavenging inhibition.
* $P < .05$ vs. BHT.

3. Cytotoxicity of the Extracts on SW480 Cancer Cells and HaCaT Normal Cells

The cytotoxic activities of the extracts against the SW480 colorectal adenocarcinoma cells and the HaCaT keratinocyte cells. After screening the extracts at 50 µg/ml, we found that MFE and SAA inhibited the cell proliferation of the SW480 cancer cells while other extracts had no inhibitory effect on the SW480 cells. PKE and PKA showed inhibitory effect 10.43 ± 0.05% and 0.93 ± 0.13%, respectively (Table 3). However, the MFE and SAA showed the comparable inhibitory activity against SW480 cells (P = .17) as shown in Figure 2(A). Afterwards we investigated the suppression on the HaCaT cell growth of the two extracts, i.e., MFE and SAA. The result showed that MFE significantly inhibited the proliferation of HaCaT cells (IC_{50} = 39.55 ± 2.85 µg/ml) when compare with SAA (> 100 µg/ml), as shown in Figure 2(B). Our results showed that SAA had inhibitory effect on the SW480 adenocarcinoma cells without inhibitory effect on the normal cells, HaCaT.

Table 3 Cytotoxic activity of the extracts on SW480 colorectal cancer cells and HaCaT normal cells. (n = 3)

Types of Cells	Extracts	% inhibition of various concentrations				IC_{50} : µg/ml (Mean ± SEM)
		1 µg/ml	10 µg/ml	50 µg/ml	100 µg/ml	
SW480 cells	AXE	-	-	4.72 ± 2.28	-	> 50
	AXA	-	-	0.56 ± 0.35	-	> 50
	MFE	-2.77 ± 1.16	7.56 ± 0.99	72.52 ± 5.78	98.69 ± 0.25	36.13 ± 2.87
	MFA	-	-	0.38 ± 1.18	-	> 50
	SAE	-	-	42.45 ± 1.99	-	> 50
	SAA	0.82 ± 0.49	1.28 ± 0.33	69.37 ± 3.11	100.39 ± 0.53	40.08 ± 1.24
	PKE	-	-	10.43 ± 0.05	-	> 50
	PKA	-	-	0.93 ± 0.13	-	> 50
HaCaT cells	AXE	-	-	-	-	-
	AXA	-	-	-	-	-
	MFE	6.34 ± 1.21	14.40 ± 3.10	61.85 ± 3.20	99.72 ± 0.04	39.55 ± 2.85
	MFA	-	-	-	-	-
	SAE	-	-	-	-	-
	SAA	-13.66 ± 4.00	-11.21 ± 1.57	1.09 ± 3.95	33.22 ± 1.76	> 100
	PKE	-	-	-	-	-
	PKA	-	-	-	-	-

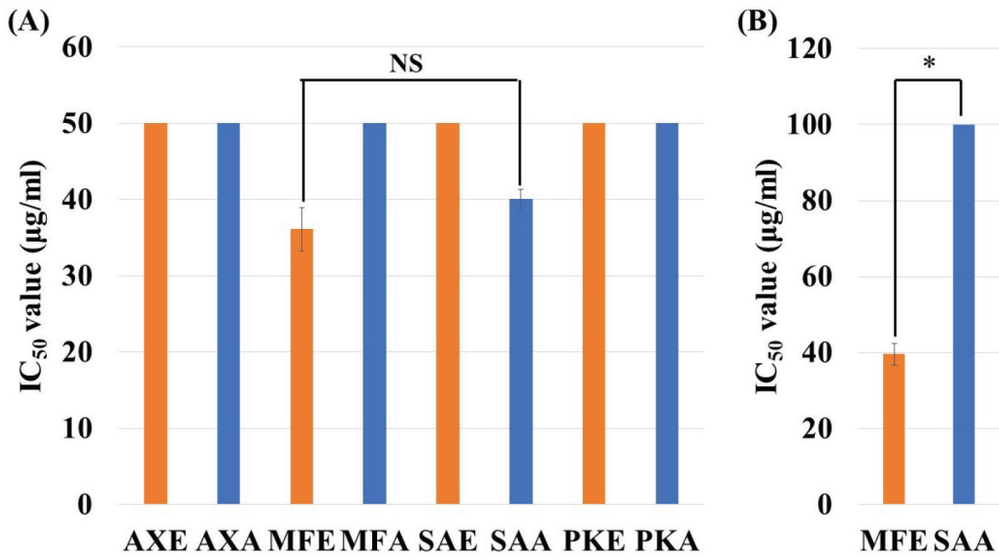

Figure 2 IC_{50} values of the extracts on growth inhibition of SW 480 colorectal cancer cells (A) and HaCaT keratinocyte cells (B). NS = not significant. * $P < .05$ vs. SAA.

Discussion

This study addressed the cytotoxicity of colorectal cancer cells and antioxidant activity of the Pi-Kad-Tri-Khan-Tha-Wart remedy and its plant extracts. The PK remedy, consisting of *A. xanthioides* fruits, *M. fragrans* seeds and *S. aromaticum* flower buds, as specified in Thai pharmacy textbook. The PK remedy has been utilized to restore the impairment of elements in the body, nausea, vomiting, hemorrhoids and stomachache. These properties may relate to the cancer treatment such as colorectal cancer. While antioxidants are able to decrease the risk of cancer formation by quenching ROS that are involved in cancer initiation and progression and assist in reducing cancer cells when the transformation of malignant occurred.[10]

With regard to antioxidant activity, aqueous PK extract showed greater antioxidant activity than ethanol PK extract and the positive control, BHT. The comparison between the PK extract and extracts of each ingredient, the aqueous *S. aromaticum* extract (SAA) showed the best antioxidant activity. Previous study investigated the antioxidant effect by DPPH radical scavenging assay of MF which were extracted by acetone, ethanol, methanol, aqueous and butanol. The results showed that the acetone extract had the highest antioxidant activity with IC_{50} value of 0.66 ± 0.01 µg/ml.[13]

Our study was the first report on the cytotoxic activity against the SW480 colorectal cancer cells of the PK remedy and its plant ingredients. The results showed that PKE and PKA did not inhibit the growth of the SW480 cells. Moreover, we found that cytotoxic activity against the SW480 cancer cells by 95% ethanol *M. fragrans* extract or MFE (IC_{50} = 36.13 ± 2.87 µg/ml) showed cytotoxicity against the SW480 cancer cells superior to aqueous *S. aromaticum* extract (SAA) (IC_{50} = 40.08 ± 1.24 µg/ml). The two extracts were studied for the cytotoxicity on the normal cells, HaCaT, which was used to confirm that the extracts were safe for the normal cells. The results found that SAA did not exhibit cytotoxic effect on HaCaT, but MFE was toxic to HaCaT with a IC_{50} value of 39.55 ± 2.85 µg/ml. Previous research presented that *M. fragrans* and *S. aromaticum* extracts had an anti-inflammatory effect, antioxidant activity and inhibited MCF-7 type breast cancer.[3,8]

In conclusion, PKE and PKA had potent antioxidant activity but showed inactive cytotoxicity on colorectal cancer cells. The aqueous SA extract was a potent extract on inhibition of colorectal cancer cells without inhibitory effect on the normal cells and it can reduce the damaging free radicals. Therefore, the proportion of PK ingredient may be modified by increasing amount of SA for further

development of PK extract for colorectal cancer and other colorectal cancerous cell lines should be investigated.

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