CYTOTOXIC COMPOUNDS FROM *DIOSCOREA MEMBRANACEA* AGAINST THREE TYPES OF COLON CANCER CELLS

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ABSTRACT: Hua-Khao-Yen-Tai (*Dioscorea membranacea* Pierre) have been commonly used among ingredients in Thai traditional anticancer preparations. The rhizome of *D. membranacea* was found potently cytotoxic against colon cancer cells. Thus the objective of this research was to identify cytotoxic compounds from the extract of *Dioscorea membranacea* rhizomes by bioassay-guided isolation using three types of colon cancer cell lines (Caco-2, LS-174T and SW480) and the Sulforhodamine B (SRB) assay. The results found that the ethanolic extract of *D. membranacea* showed cytotoxic activity against only two types of colon cancer cell lines; LS-174T and SW480, with IC_{50} values 45.97±0.64 and 62.83±0.51 µg/ml, respectively, but there was no cytotoxic activity against Caco-2 cells. It water extract had no cytotoxicity against all types of colon cancer cells. Dioscorelide B and dioscoreanone were isolated from its ethanolic extract by bioassay guided isolation and both compounds exhibited high potency against three types of colon cancer cell line with the IC_{50} in range 0.67-4.36 µg/ml. This study supports the use of *D. membranacea* by Thai traditional doctors for colon cancer treatment.

Keywords: Cytotoxicity, colon cancer, *Dioscorea membranacea*, SRB assay

INTRODUCTION

Cancer is a leading cause of dead worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008 [1]. The World Health Organization estimates that 7.6 million people died of cancer in 2005 and 84 million people will die in the next 10 years if action is not taken. More than 70% of all cancer deaths occur in low-and-middle-income countries, where resource available for prevention, diagnosis and treatment of cancer are limited and nonexistent [2]. The main types of cancer were lung (1.4 million deaths), stomach (740,000 deaths), liver (700,000 deaths), colorectal (610,000 deaths) and breast (460,000 deaths) [1]. Colon and rectum cancers are rare in developing countries, the high incidence rates occur in countries of Europe, North America, Australia and Japan. Colorectal cancer was estimated 1,023,152 new cases occurring annually worldwide and was responsible for 528,978 deaths in 2007. The highest incidence rates of colon cancer for males was in Japan and for females in New Zealand. The highest incidence rates of rectal cancer for males was in Japan and for females in Singapore Chinese [3]. In Thailand, the incidence is rising, probably due to the acquisition of Western lifestyle. Incidence also increases with age: carcinomas are rare before the age of 40 years except in individuals with genetic predisposition or predisposing conditions. Although the incidence rate of colorectal cancer in Thailand is low when compared with other countries, it is the third in frequency in males after liver and bile duct and lung cancers, and the fifth for females after cancers of the cervix, breast, liver and bile duct and lungs. The highest incidence for both sexes is seen in Bangkok and the number of cases of colorectal cancer in both sexes is increasing. The precursor of advanced colorectal cancer is either an adenomatous polyp or a flat neoplastic lesion. The most common type of cancers arising in the colon and rectum is adenocarcinoma, which accounts for more than 90% of all large bowel tumors [4].

Dioscorea membranacea Pierre ex Prain & Burkill (Dioscoreaceae) is known in Thai as Hua-Khao-Yen. It has been long used as a common ingredient in several preparations, including those used in the treatments of inflammatory diseases e.g., arthritis, lymphopathy, dermopathy, venereal diseases, leprosy and cancers [5]. By following the cancer treatment program of Thai traditional doctors in Songkhla province, it was found that the formula with *D. membranacea* could extend lifespan by 2-3 years for elderly patients and more than ten years for the young patients [6]. Previous research has

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shown that *D. membranacea* rhizome extract was potently cytotoxic against cancer cells, but less toxic to normal cells, making possible a therapeutic effect [7].

The ethanolic extract of *D. membranacea* rhizomes exhibited high cytotoxic activity against lung cancer cell line, MCF-7 breast cancer cell line and LS-174T colon cancer cell line with IC_{50} values of 6.2, 12.0 and 16.7 µg/ml, respectively but no no cytotoxic activity was noted when a SVK-14 keratinocyte normal cell line was used (IC_{50} > 70 µg/ml), indicating the specificity of the *D. membranacea* extract to cancer cell lines [8]. Bioassay-guided isolation was used to isolate two novel and selective cytotoxic compounds from *D. membranacea*, dioscorealide B and dioscoreanone [9]. Dioscorealide B exhibited high cytotoxicity against MCF-7 breast, COR-L23 lung and LS-174T colon cancer cell lines (IC_{50} = 0.92, 1.59 and 5.26 µg/ml, respectively), but little cytotoxicity towards SVK-14 keratinocyte normal cell line (IC_{50} = 43.5 µg/ml). Dioscoreanone exhibited high cytotoxic activity against COR-L23 lung and MCF-7 breast cancer cell lines (IC_{50} = 2.89, 3.76 µg/ml, respectively), but was less active against LS-174T colon cancer cell line (IC_{50} = 9.96 µg/ml) and SVK-14 keratinocyte normal cell line (IC_{50} = 16.5 µg/ml)[9] . The ethanolic and water extracts of *D. membranacea* showed no acute toxicity in rats (LD_{50} = 9 g/kg and LD_{50} >25 g/kg, respectively) [10]. In these previous studies, compounds were tested against only one cancer cell line for each type of cancer .The objective of this research was to investigate cytotoxicity of the extract against three types of colon cancer cell from colon and colorectal regions, followed by bioassay guided isolation using all three cell lines to determine the active compounds.

MATERIAL AND METHODS

Plant material and preparation of extract

The rhizomes of *D. membranacea* (Dioscoreaceae) were collected from Amphor Pa-tue, Chumporn Province, Thailand in July 2010. Authentication of plant material was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand, where the herbarium voucher has been kept. A duplicate set has been deposited in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The voucher number is SKPA062041305.

The rhizomes of *D. membranacea* were washed, cut into small pieces and dried in hot air oven at 50°C for 48 hours; they were then powdered and extracted by two methods corresponding to those practised by Thai traditional doctors i.e. a water and an ethanolic extract. The ethanolic extract was prepared by macerating dried plant powder (1 kg) with 95% ethanol for 3 days, filtering and evaporating the filtrate to dryness under reduced pressure. The water extract was prepared by boiling dried plant powder (1kg) in water for 30 minutes, filtering and drying using a lyophillizer.

Isolation of pure compounds

An aliquot of the ethanolic extract (40 g) was chromatographed over silica gel using hexane: chloroform (1:9, 2500 ml), chloroform (2000 ml), chloroform:methanol (9:1, v/v 2000 ml) and methanol (2500 ml), respectively to give four fractions FD1, FD2, FD3 and FD4. These were dried by evaporation at 40ºC to yield residues of 23.96, 10.52, 0.75, and 24.80 g, respectively. The chloroform extract (FD2, 2.28 g), the best active fraction giving the best activity against colon cancer cells. The chloroform fraction was separated by silica gel column chromatography using a step gradient of ethyl acetate:hexane (1:1), ethyl acetate, ethyl acetate: methanol (1:1) and methanol, respectively. Following TLC examination and detection with acidic anisaldehyde spraying reagent, fractions giving a similar profile were combined. The ethylacetate: hexane fraction was further purified by silica gel column chromatography eluted with ethylacetate: hexane (1:1) to get two compounds compound 1 (40.1 mg, 1.76 %w/w) and compound 2 (16.1 mg, 0.71% w/w).

Structure elucidation

The structure of the isolates were 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 [SPECORD S 100 (Analytikjena) spectrometer], IR spectra [Perkin Elmer FTS FT-IR spectrometer] AND EI mass spectra, where both HRMS and LRMS, were obtained from a Thermo Finnigan MAT 95XLmass spectrometer.

The spectra of compound 1 and 2 were identical with published data for dioscorealide B and dioscoreanone [11] . The two compounds were also identical in chromatographic behavior when compared with authentic samples previously isolated [11] (Figure 1).

In vitro assay for cytotoxic activity

Human cell culture

Three human colon cancer cells; human epithelial adenocarcinoma Caco-2 (ATCC No. HTB-37), human caucasian colon adenocarcinoma LS-174T

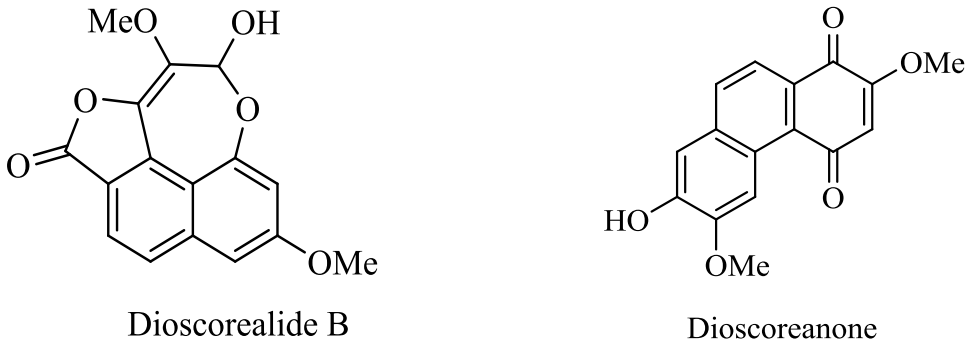


Figure 1 Chemical structure of the isolated compounds from the ethanolic extract of *D. membranacea*

(ECACC No. 87060401) and colorectal adenocarcinoma SW480 (ATCC No. CCL-228) were also used. Caco-2 and LS-174T were cultured in MEM (Biochrom) supplement with 10% heat-inactivated fetal bovine serum (FBS) (Biochrom), 1% of 100 µg/ml penicillin and 100 µg/ml streptomycin (Sigma). SW480 was cultured in RPMI 1640 medium (Biochrom) supplement with 10% heat-inactivated fetal bovine serum, 1% of 100 µg/ml penicillin and 100 µg/ml streptomycin. All cells were maintained at 37°C in a humidified incubator containing 5% CO_2. According to their growth profiles, the optimal plating densities of each cell line were determined (3×10^3, 3×10^3 and 1×10^3 cells/well for Caco-2, LS-174T and SW480, respectively) 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 phosphate buffer saline (PBS) (Biochrom) free of magnesium and calcium. The PBS was decanted and cells detached with 0.025% trypsin-EDTA (Sigma) and PBS was added to a volume of 50 ml. The cell pellet, obtained by centrifugation (1000×g, 5 min) was resuspended in 10 ml of medium to make a single cell suspension, viable cells density being counted by trypan blue exclusion (Sigma) in a haemocytometer and then diluted with medium to give the determined optimal plating densities for Caco-2, LS-174T and SW480, respectively. 100 µl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated at 37°C to allow for cell attachment. After 24 hours the cells were treated with various concentrations of the samples. Each sample was initially dissolved in dimethyl sulfoxide (DMSO) (RCI Labscan) at a concentration of 10 mg/ml and then re-diluted in medium to produce the required concentrations. Vincristine sulphate (Sigma) was used as a positive control. 100 µl/well of each concentration was added to the plates in four replicates. The final dilution used for treating the cells contained not more than 1% of the initial solvent, this concentration being used in the solvent control wells. The plates were incubated for selected exposure times of 72 hours. At the end of each exposure time the medium was removed and the wells were then washed with medium, and 200 µl of fresh medium were then added. The plates were incubated at 37°C for a recovery period of 6 days and cell growth was then analyzed using the SRB assay. Three replicate plates were used to determine the cytotoxicity of each extract.

Sulforhodamine B (SRB) assay

The antiproliferative SRB assay [12] was used to assess growth inhibition by a colorimetric assay which estimates cell number indirectly by staining total cellular protein with the dye SRB. In brief, cells were fixed by layering 100 µl of ice-cold 40% trichloroacetic acid (TCA) (Merck) on top of the growth medium. Cells were incubated at 4°C for 1 hour, after which plates were washed five times with cold water, the excess water drained off and the plates left to dry in air. SRB stain (50 µl; 0.4 in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 min, after which they were washed with 50 ml 1% acetic acid, rinsed four times until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM Tris base pH 10.5 (Sigma) were added to each well to solubilise the dye. The plates were shaken gently for 20 min on a gyratory shaker and the absorbance (OD) of each well was read on a Power Wave X plate reader (Bio-Tek instrument, Inc.) at 492 nm. The percentage of cytotoxicity compared to the control (untreated cells) was determined with the equation given below.

$$\%\ \text{Cytotoxicity} = \frac{[(\text{OD control} - \text{OD sample}) \times 100]}{[\text{OD control}]}$$

Table 1 The percentage inhibition (at concentration 50 µg/ml) and cytotoxicity (IC_{50} µg/ml) against three types of human colon cancer cell (Caco-2, LS-174T and SW480) by the ethanolic extract from *D. membranacea* and two isolated compounds (dioscorealide B and dioscoreanone).

Extract/fraction/compound	%yield	Caco-2		LS-174T		SW-480		MRC5	
		% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}	% inhibition	IC_{50}
DME	4.25	21.64±0.96	>100	55.57±0.95	45.97±0.64	36.52±0.32	62.83±0.51	78.54±3.35	34.49±1.77
DMW	18.08	2.89±0.60	>100	2.83±0.60	>100	6.09±1.33	>100	9.08±1.74	>100
F1	23.96	0.99±0.43	>100	4.23±1.07	>100	4.27±0.51	>100	2.57±0.26	>100
F2	10.52	35.02±0.62	71.75±0.81	89.51±0.56	22.10±0.79	28.04±1.27	>100	23.28±1.63	>100
F3	0.75	32.38±0.26	74.22±0.32	77.01±0.94	28.24±0.30	32.50±1.60	>100	34.71±0.66	>100
F4	24.80	7.68±0.80	>100	18.23±1.22	77.64±0.27	5.01±0.21	>100	13.08±1.31	>100
Dioscorealide B	1.76	86.72±0.91	3.08±0.11 (10.26±0.11 µM)	99.59±0.02	0.67±0.02 (2.23± 0.02 µM)	90.16±0.11	4.36±0.55 (14.53±0.55 µM)	52.32±0.82	45.11±0.85 (150.34±0.85 µM)
Dioscoreanone	0.71	90.54±0.53	2.47±0.07 (8.66±0.07 µM)	98.84±0.48	0.73±0.14 (2.56±0.14 µM)	99.57±0.05	1.42±0.03 (4.98±0.03 µM)	81.59±1.19	32.02±0.54 (112.32±0.54 µM)
Vincristine sulphate	-	96.55±0.63	0.0019±0.00 (0.002±0.00 µM)	97.53±0.76	0.008±0.10 (0.009±0.10 µM)	99.18±0.17	0.006±0.004 (0.007±0.004 µM)	45.10±1.08	3.24±0.19 (3.51±0.19 µM)

Data were expressed as the means of three determinations (n=3)

The IC_{50} values were calculated from the Prism program obtained by plotting the percentage of cytotoxicity versus the sample concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines [13] extracts with IC_{50} values < 30 µg/ml and compounds with < 4 µg/ml were considered active.

RESULTS AND DISCUSSION

The percentage of cytotoxic activity against three types of colon cancer cell at concentration 50 µg/ml and IC_{50} values are shown in Table 1. The ethanolic extract of *D. membranacea* exhibited high cytotoxic activity against only two of the types of colon cancer cell; LS-174T and SW480, (IC_{50} values 45.97±0.64 µg/ml and 62.83±0.51 µg/ml respectively) but it had no activity against Caco-2 (IC_{50} value > 100 µg/ml). Vacuum liquid chromatography was used for fractionation using a solvent gradient and four fractions obtained. Only fractions FD2 and FD3 showed high cytotoxic activity against Caco-2 and LS-174T cell lines, with the IC_{50} ranging from 22.10-35.02 µg/ml, while fraction FD4 showed weak cytotoxic activity against LS-174T cells only (IC_{50} value 77.64±0.27 µg/ml). Fraction FD1 displayed no cytotoxic activity against any of the colon cancer cell line. In addition, all fractions had no cytotoxic activity against SW480 cells (IC_{50} > 100 µg/ml). FD2 was chromatographed by column chromatography by ethyl acetate: hexane as solvent elution and two known compounds were obtained. They were proven to be dioscorealide B and dioscoreanone by comparison with chromatographic and spectral characteristics with authentic samples [11]. The strongest cytotoxic activity against LS-174T was given by dioscorealide B (IC_{50} value 0.67±0.02 µg/ml)but the highest activity against two types of colon cancer cell line was shown by dioscoreanone (IC_{50} value 1.42±0.03 µg/ml for SW480 and , 2.47±0.07 µg/ml for Caco-2. However, these results concur with the previous report which found that dioscorealide B also showed higher cytotoxicity against LS-174T than dioscoreanone [11]. The selective index (SI value), which is the ratio of IC_{50} (µM) value of normal cells to IC_{50} (µM) value of Dioscorealide, showed that this compound gave the highest value i.e. > 67. Therefore, dioscorealide B at an appropriate dose kill cancer cells but not kill normal cells. This compound also showed some specificity for cancer cells e.g. it was more specific for LS-174T but less and less for Caco-2 and SW 480 (SI 67.3, 14.7 and 10.4, respectively). Dioscoreanone also showed a high SI value

Table 2 The ratio of IC_{50} (µM) values of normal cells/ IC_{50} (µM) values of cancer cells of compounds and crude extract.

Sample/compound	SI		
	Caco-2	**LS-174T**	**SW480**
DME	NT	0.75	0.55
Dioscorealide B	14.65	67.33	10.35
Dioscoreanone	12.96	43.86	22.55
Vincristine sulphate	1705.26	405.00	540.00

DME is the ethanolic extract of *Dioscorea membranacea*, NT=not tested

against LS-174T (SI 43.9) and showed the highest activity against SW480 colorectal cancer cells (Table 2). A high SI value is a good criteria for identifying potential cancer drugs because the objective of cancer chemotherapy is to kill cancer cells with as little damage as possible to normal cells [7], so novel anticancer agents should be defined and developed as active at a low dose for specific typical cancer cells, but needing a much higher dose to kill normal cells. Alternatively a good lead molecule will have a unique mechanism of action for specific type of cancer [14]. Dioscoralide B and dioscoreanone showed specificity against colon cancer (LS174T) but were less active against epithelial colorectal adenocarcinoma lines (Caco-2 and SW480). Although two these compounds were less cytotoxic than viscristine sulphate (the positive control) their IC_{50} values for all cell lines were less than 4 µg/ml, which was considered active according the National Cancer Institute guidelines [13]. Thus, the studies on these two compounds should be continued to determine their molecular mechanisms against different types of colon cancer.

CONCLUSION

The ethanolic extract of *D. membranacea* showed *in vitro* cytotoxic activity against two different types of colon cancer cell lines (LS-174T and SW480). Two isolated compounds from the ethanolic extract of *D. membranacea*, dioscorealide B and dioscoreanone, showed cytotoxic activity against three types of colon cancer cell lines (Caco-2, LS-174T and SW480). In summary the results give support to the use by Thai folk doctors of *D. membranacea* for cancer treatment.

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