Original article

Cytotoxic Activity of *Dioscorea birmanica* Prain & Burkill extract and its Cytotoxic Compound against Breast and Ovarian Cancer Cells

Nuanjan Jaiarree*, Arunporn Itharat*, **, Saovapak Poomirat*

Abstract

Introduction: Breast and ovarian cancers in Thailand are becoming a significant health problem. *Dioscorea birmanica* Prain and Burkill (Dioscoreaceae) is one of the main medicinal herbs to cure the breast and ovarian cancers in Thailand. The objective were to investigate cytotoxic activity against breast and ovarian cancer cells of *D. birmanica* extract and to isolate its cytotoxic compound.

Method: The ethanolic extract of *D. birmanica* and its fractions were tested for its cytotoxic activity against two types of breast cancer cells (T47D and MCF-7), ovarian cancer cells (SKOV-3) and human keratinocyte cells (HaCaT) using sulforhodamine B assay and the criterion of the National Cancer Institute (NCI) guidelines for extracts with IC_{50} value < 30 µg/mL. Bioassay guided fractionation was used for isolating the cytotoxic compound. The structural elucidation of active ingredients was conducted by spectrophotometry.

Results: The results showed that diosgenin-3-O-α-L-rhamnosyl (1→2) -β-D-glucopyranoside or prosapogenin A of dioscin (DBS1) was isolated from the ethanolic extract of *D. birmanica*. It showed high cytotoxic activity against two types of breast cancer cells MCF-7 and T47D (IC_{50} = 5.36 ± 0.26 and 6.13 ± 0.33 µg/mL, respectively.) and ovarian cancer cells SKOV-3 (IC_{50} = 7.30 ± 0.05 µg/mL, but not cytotoxic activity against human keratocyte cells, HaCaT, (IC_{50}= 51.76±2.58 µg/mL).

Discussion and Conclusion: The cytotoxic compound, Prosapogenin A of dioscin (DBS1), showed high cytotoxic activity against ovarian and breast cancer cells; none against human keratocyte cells. These results can support further researches in molecular mechanisms and clinical trial to treat cancer patients, especially those with ovarian and breast cancers.

Key words: cytotoxic activity, *Dioscorea birmanica* Prain & Burkill, breast and ovarian cancer cells, keratinocyte cells

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Introduction

Breast cancer is now the leading cause of cancer-related death in women in the developing regions of the world. Breast and ovarian cancers in Thailand are becoming a significant health problems, and the leading causes of death[1]. Over recent years, using of ethnopharmacology, or traditional use, has been accepted for discovery of new biologically-active molecules. Thai medicinal herbs have been used to treat cancer patients for a long time, especially female reproductive system and breast cancer. *Dioscorea birmanica* Prain & Burkill (Dioscoreaceae) is one of the main medicinal herbs to cure cancer in Thailand[2]. *D. birmanica* has a nauseating taste. Thai traditional practitioner always use it for treating lung, colon, liver, breast, uterus and ovary cancers, in addition to lymphatic drainage and detoxification.[2] From the literature review of Thai traditional scripture, *D. birmanica* is one herb used to treat a variety of cancers, such as lung, breast, colon and liver cancers.[2] The previous research reported that the ethanolic extract of *D. birmanica* showed high cytotoxic activity and related potency, such as anti-microbial, antioxidant and anti-inflammation[3, 4]. The previous report from Jaiarree[5] found that *D. birmanica* showed high cytotoxic on two types of lung cancer cells, A 549 and COR-L23, with IC_{50} of 1.81 ± 0.03 and 1.84 ± 0.05 µg/ mL, respectively. But it was less cytotoxic to normal lung cells, MRC-5, (IC_{50} = 37.09. ± 0.67 µg/m[5]).The previous research also showed the toxic to Hela cells with IC_{50} value of 6.07± 0.02 µg/mL[6]. Considering the literature and the traditional use of *D. birmanica* used to treat breast and reproductive system; it is appropriate that the research should continue with investigating cytotoxic activity against at least two types of breast cancer cells and ovarian cancer cells. The results from this research are likely to support the use of Thai medicinal herbs to treat breast and ovarian cancers. For this reason, the objective of this research is to test cytotoxic activity against two types of breast and one type of ovarian cancer cells, as well as to compare toxicity on Human keratinocyte cells and using the bioassay guided a technique of fractionation

Method

Plant Material

The rhizomes of *D. birmanica* were collected from Chantaburi province, Thailand. The herbarium voucher (SKP A062001002) is kept at the herbarium of Faculty of Medicine, Thammasat University, Thailand. The plant powder shown in Figure 1 was identical with the specification reported by Itharat *et al*. 2002[7].

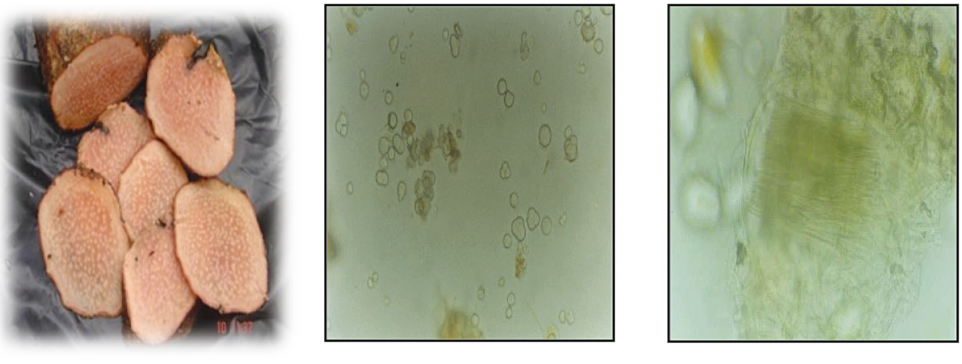

Figure 1 Rhizomes, Starch grains and Rhaphide crystals in powder from *Dioscorea birmanica* Prain and Burkill

Isolation and Purification of Compounds

Dried powder rhizome of *D. birmanica* (1kg) was macerated with 95% ethanol. The extract was concentrated to dryness under reduced pressure, to obtain 11.13% of crude extract. Ten grams aliquot of the ethanolic extract of *D. birmanica* (DBE) was separated by vacuum liquid chromatography (VLC) using C_6H_{14}, C_6H_{14}: $CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$: MeOH (8:2), $CHCl_3$: MeOH (1:1) and MeOH, respectively and drying by rotary evaporation. DBCM fraction of C_6H_{14}: $CHCl_3$ (1:1), was selected to identify a cytotoxic compound because the previous research showed it had high potency against lung and cervical cancer cells[5,6]. An aliquot (2g) of DBCM fraction was separated by column chromatography (silica gel with a gradient of solvents as follows - $CHCl_3$, $CHCl_3$: MeOH (9.5:0.5), $CHCl_3$: MeOH (9:1), $CHCl_3$: MeOH (8:2), $CHCl_3$: MeOH (7:3), $CHCl_3$: MeOH (6:4), $CHCl_3$: MeOH (5:5), $CHCl_3$: MeOH (4:6), $CHCl_3$: MeOH (3:7), $CHCl_3$: MeOH (2:8), $CHCl_3$: MeOH (1:9) and finally MeOH. Each 20 mL fraction was collected for each eluting solvent and combined fractions, following TLC examination (silicagel/$CHCl_3$: MeOH (7:3) and detected with acidic anisaldehyde spray.

Structure Elucidation

The pure compound of the isolate was determined by its 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]. The pure compound was identified as diosgenin-3-O-α-L-rhamnosyl(1→2)-β-D-glucopyranoside (prosapogenin A of dioscin, DBS1) by comparison of its spectral features with literature values, and it was identical with published data for prosapogenin A of dioscin[4, 8, 9]. This compound was also identical in chromatographic behavior when compared with authentic samples previously isolated[10]. DBS1 was white amorphous solid (9 mg, 0.225% w/w of crude extract): mp 240 - 243°C (dec), IR (KBr disc) 3,420 (broad), 2,950, 2,900, 2,875, 1,650, 1,450, 1,380, 1,240 (acetate carbonyl), 1,140, 1,050, 980, 960, 920, 900 (intensity of 900 > 920,25 (R)-spiroketal) cm-[11] H-NMR ($CDCl_3$ and CD_3OD, 400 MHz) ppm 0.79 (3H, d, J=5.4 Hz, H-27), 0.85 (3H, s,H-18), 1.04 (3H,s,H-19), 1.25 (3H, d, J=6.3Hz, H-21),1.75 (3H, d, J=7.3 Hz, H-6'' of rhamnose), 3.2-3.7 (peak of sugar proton, H-3, H-16, H-26) 3.86 (1H, dd, J = 2.9,9.9 Hz, H-6') 4.1(1H, dd, J=1.8, 3.3 Hz, H-2''), 4.44 (1H, m, H-3''), 4.5 (1H, d, J=7.7Hz, H-1') 5.19 (1H, d, J=1.1 Hz, H-1") 5.36 (1H, br s, H-6) and ^{13}C NMR (Table 1 and Figure2).The extract was concentrated to dryness under reduced pressure and kept in freezer (-20°C) for further use. It was studied for cytotoxic activity.

Table 1 ^{13}C chemical shift (MHz, in ppm) of DBS1 compared with ^{13}C chemical shift of prosapogenin A of dioscin (A) from literature[4, 8, 9, 10]

^{13}C	A[a]	DBS1[b]
1	37.4	38.9
2	30.2	31.2
3	78.4	77.9
4	39.2	39.9
5	140.9	142.2
6	121.7	123.2
7	32.2	33.7

Table 1 ^{13}C chemical shift (MHz, in ppm) of DBS1 compared with 13C chemical shift of prosapogenin A of dioscin (A) from literature[4, 8, 9, 10]

^{13}C	A[a]	DBS1[b]
8	31.6	31.9
9	50.2	51.9
10	37.0	38.5
11	21.1	22.5
12	39.9	41.4
13	40.4	41.9
14	56.6	58.2
15	32.2	33.2
16	81.1	82.7
17	62.8	63.2
18	16.3	17.6
19	19.4	20.6
20	41.9	43.3
21	15.0	15.7
22	109.4	111.2
23	32.2	32.9
24	29.2	30.3
25	30.5	31.9
26	66.9	68.4
27	17.3	18.3
Glu-1’	100.6	101.1
C-2’	79.6	79.9
C-3’	78.0	79.5
C-4’	72.1	72.8
C-5’	78.0	79.6
C-6’	62.9	63.9
Rha-1”	101.9	102.6
C- 2”	72.5	72.2
C- 3”	72.9	72.8
C-4”	74.2	74.3
C- 5”	69.4	70.2
C6”	18.6	18.6

Note: [a]in pyridine -d_5, [b] in $CDCl_3$ and CD_3OD

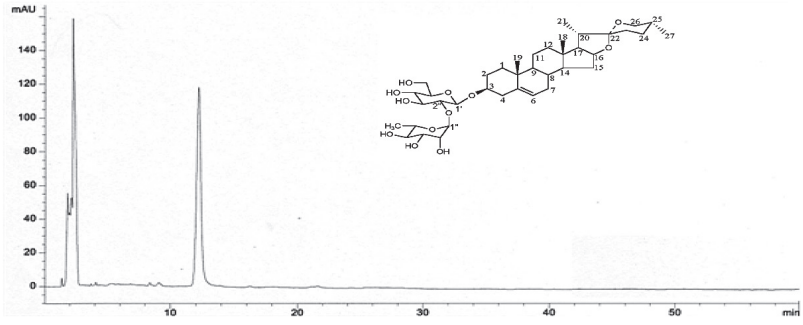

Figure 2 Chemical finger print of Diosgenin 3-O-α-L-rhamnopyranosyl (1→2)- β-D glucopyranoside, DBS1 ($C_{39}H6_2O_{12}$)

In vitro Assay for Cytotoxic Activity

Cell lines Culture

Two different types of breast cancer cells, MCF-7 and T47D, ovarian cancer cells, SKOV-3 and human keratinocyte cells, HaCaT, were used in the test. MCF-7,T47D and SKOV-3 cells were cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/mL penicillin and 50 ug/mL streptomycin[11]. HaCaT was cultured in DMEM culture medium containing 10% heated fetal bovine serum and 1% of 10,000 U penicillin and 10 mg/mL streptomycin. The cell lines were maintained at 37°C in an incubator with 5% CO_2 and 95% humidity.

Cytotoxic activity by SRB Assay

The sulforhodamine B (SRB) assay was used to estimate cell numbers indirectly by staining total cellular protein with the SRB. The protocol was based on that originally described by Skehan[12]. In brief, cell lines at the exponential growth phase were detached with 0.25% trypsin-EDTA to make single cell line suspensions. The viable cells were counted by trypan blue exclusion using a haemocytometer and diluted with medium to give a final concentration of 1 x 10^4 cells /mL for SKOV-3, MCF-7 and T47D cell lines (ATCC® CCL-2 ™). 100 µL/well of these cell suspensions were seeded in 96-well plates as well as incubated to allow cell attachment. After 24 hours, the cells were treated with various concentrations of the extracts. The extracts were diluted in medium to produce the required concentrations and 100 µL/well of each concentration was added to the plates to obtain final concentrations of 1, 10, 50, 100 µg/mL for the extract and 0.1, 1, 10, 50 µg/mL for the pure compound. The final mixture was used for treating cell lines containing not more than 1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure time of 72 hours. At the end of each exposure time, the medium was removed. The wells which were washed with medium and 200 µL of fresh medium were added to each well. The plates were incubated for a recovery period of 72 hours. On the seventh day of culture period, cells were fixed by 100 µl of ice-cold 40 % trichloroacetic acid (TCA) per well, incubated at 4°C for 1 hour in the refrigerator and washed 5 times with tap water to wash away non-viable cells, so viable cells were fixed as monolayer in each well. 50 µl of SRB solution (0.4% w/v in 1% acetic acid) was added to each well and left in contact with the cells for 30 min; then the plates were washed for 4 times with 1 % acetic acid until only dye adhering to the cells which were left. The dry plates and 100 µL of 10 mMTris base [trishydroxy methyl) aminomethane, pH 10.5] were added

to each well to dissolve the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (4 replicates) was read on a microplate reader at 492 nm as an indication of cell number. The cell survival was measured as a percentage of absorbance compared with the control (non-treated cell lines). The IC_{50} values were calculated from the Prism program.

Results

The 95% ethanolic extract of *D. birmanica* (DBE) and DBCM separated by column chromatography (silica gel with a gradient of solvents ($CHCl_3$:MeOH 1:1), showed no activity against two types of breast cancer cells, one type of ovarian cancer cell and human keratinocyte cells. However, the steroid saponin (DBS1) that isolated from DBCM fraction, it showed high activity against MCF-7, T47D and SKOV-3 (IC_{50} = 5.36 ± 0.26, 6.130 ± 0.33 and 7.30 ± 0.05 µg/mL, respectively). But It was not toxic to normal keratinocyte cells (IC_{50} = 52.76 ± 2.58 µg/ml). These results suggest that DBS1 had specific cytotoxic activity against two type of breast and one type of ovarian cancer cells but not toxic to human keratinocyte cells.

Discussion and Conclusion

These results showed that the pure compound, *D. birmanica* (Prosapogenin A or DBS1) can kill two types of breast and one type of ovarian cancer cell, but it did not kill human keratinocyte cells. It conforms with the previous research[4, 5, 6] and agrees with the result of Jaiarree N and Ittharat A 2010[5] who found that Prosapogenin A exhibited cytotoxicity against two types of lung cancer cells, A549 and COR-L23, with IC_{50} of 1.81 ± 0.03 and 1.84 ± 0.05 µg/mL but not against normal lung cells, MRC-5, with IC_{50} of 37.09 ± 0.67 µg/mL[5]. In addition, this result confirms the report of Jaiarree N and Ittharat A, 2016[6]. who found that Prosapogenin A or DBS1 exhibited cytotoxicity against Hela cells with IC_{50} value of 6.07+0.02 ug/mL[6].

Regarding DBE extract, even though the IC_{50} of SKOV-3 cells is more than 30 µg/mL (IC_{50} value of 33.75 ± 1.21 µg/mL), yet it is very close to 30 µg/mL (the criterion of the National Cancer Institute guidelines for extracts with IC_{50} value < 30 µg/mL[13]). This result is positive in that it agrees with ancient Thai traditional practice of treating cancer patients effectively with a 40% ethanolic extract of *D. birmanica wiea*, and this result is correlated with the previous research of Jaiarree N, (2012)[5]. which showed the 95 % ethanolic extract of *D. birmanica* (DBE) was toxic on two types of lung cancer cells (A549 and COR-L23) with IC_{50} of 7.45 ± 0.31 and 8.71 ± 0.29 µg/mL, respectively. But it was not toxic to normal lung cells (MRC-5) with IC_{50} of 94.76 ± 1.25 µg/mL[5] Such result are consistent with those of Ittharat A, 2004[4] who found that the 95% ethanolic extract of *D. birmanica* (DBE) was toxic to colon cancer cells (LS174-T) with IC_{50} of 22.6 ± 3.9 µg/mL, yet was not toxic to normal colon cells[4]. It can be noted that the present and the past result may support the wisdom of using *D. birmanica* in a macerated form. However, all the extracts are safe on human keratinocyte and normal lung cells. It is said to be a security when considered in conjunction with the actual use of traditional Thai medicine that has been used for over 100 years. Prosapogenin A of dioscin or DBS1 has also been extracted from *Draecaena draco* (Agavaceae) and showed cytostatic activity against HL-60 cells with IC_{50} of 1.3 ug/mL at exposure 72 hours[14]. In conclusion, the cytotoxic compound, DBS1 showed high cytotoxic activity against ovarian and breast cancer cells but not against human keratocyte cells. These results support further researches in molecular mechanisms and clinical trials to treat cancer patients, especially those with ovarian and breast cancers. DBS1 should be a marker for analysis in drug development processes.

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บทคัดย่อ

ฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งเต้านมและรังไข่ของสมุนไพรหัวข้าวเย็นและสารบริสุทธิ์ที่เป็นองค์ประกอบ

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** ศูนย์แห่งความเป็นเลิศทางวิชาการด้านการแพทย์แผนไทยประยุกต์ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์

ผู้ที่ให้ติดต่อ: ผู้ช่วยศาสตราจารย์ ดร.นวลจันทร์ ใจอารีย์ ๙๕ หมู่ ๘ คณะแพทยศาสตร์ มหาวิทยาลัยธรรมศาสตร์ ตำบลคลองหนึ่ง อำเภอคลองหลวง จังหวัดปทุมธานี ๑๒๑๒๐ **อีเมล:** nuanjan_j@yahoo.com

บทนำ: มะเร็งเต้านมและมะเร็งรังไข่เป็นปัญหาทางสุขภาพที่สำคัญของประเทศไทย หัวข้าวเย็นจัดเป็นสมุนไพรที่มีการใช้เป็นหลักในการรักษามะเร็งเต้านมและรังไข่ วัตถุประสงค์ในงานวิจัยนี้ เพื่อศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งเต้านมและรังไข่ของสมุนไพรหัวข้าวเย็นและสารบริสุทธิ์ที่แยกได้

วิธีการศึกษา: ทดสอบฤทธิ์ความเป็นพิษของสารสกัดชั้น 95% เอทานอลหัวข้าวเย็น และ สารที่แยกได้จากหัวข้าวเย็นในเซลล์มะเร็งเต้านมสองชนิดคือ T47D, MCF-7 และมะเร็งรังไข่ชนิด SKOV-3 เปรียบเทียบกับเซลล์ผิวหนังมนุษย์ชนิด HaCaT โดยใช้วิธี sulforhodamine B และประเมินความเป็นพิษต่อเซลล์ เมื่อสารสกัดมีค่าน้อยกว่า ๓๐ ไมโครกรัมต่อมิลลิลิตร พร้อมทั้งใช้หลักการ guided fractionation ในการสกัดสารบริสุทธิ์จากหัวข้าวเย็นและใช้ spectrophotometry ในการพิสูจน์สารบริสุทธิ์ที่แยกได้จากหัวข้าวเย็น

ผลการศึกษา: พบว่าสารบริสุทธิ์จากหัวข้าวเย็นชื่อ Diosgenin-3-O-α-L-rhamnosyl (1→2) -β-D-glucopyranoside หรือ Prosapogenin A of dioscin (DBS1) มีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งเต้านมสองชนิด คือ MCF-7 และ T47D เท่ากับ ๕.๓๖ ± ๐.๒๖ และ ๖.๑๓ ± ๐.๓๓ ไมโครกรัมต่อมิลลิลิตรตามลำดับ และมีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งรังไข่ชนิด SKOV-3 เท่ากับ ๗.๓๐ ± ๐.๐๕ ไมโครกรัมต่อมิลลิลิตร แต่ไม่มีพิษต่อเซลล์ผิวหนังมนุษย์ชนิด HaCaT (ค่า IC_{50} เท่ากับ ๕๑.๗๖ ± ๒.๕๘ ไมโครกรัมต่อมิลลิลิตร)

สรุปวิจารณ์และผลการศึกษา: สารบริสุทธิ์ DBS1 แสดงฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งเต้านมทั้งสองชนิดและมะเร็งรังไข่ และปลอดภัยในเซลล์ผิวหนังมนุษย์ งานวิจัยนี้สามารถนำไปสนับสนุนการวิจัยเพิ่มทั้งระดับโมเลกุลและงานวิจัยทางคลินิกโดยเฉพาะมะเร็งเต้านมและมะเร็งรังไข่

คำสำคัญ: ฤทธิ์ความเป็นพิษต่อเซลล์, หัวข้าวเย็น, เซลล์มะเร็งเต้านมและรังไข่, เซลล์ผิวหนัง