# Cytotoxic Saponin against Lung Cancer Cells from *Dioscorea birmanica* Prain & Burkill

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***Objective***: *To investigate the cytotoxic activity against lung cancer cells of Dioscorea birmanica Prain & Burkill extract and its compounds.*
***Material and Method***: *Cytotoxic activity was tested against two types of lung cancer cell line (A549 and CORL-23), one type of normal lung cell (MRC-5) by sulforhodamine B assay. Bioassay guide fractionation was used for isolating cytotoxic compounds. The structure elucidation of active ingredients was proven by spectrophotometry technique.*
***Results***: *The results found that the ethanolic extract of Dioscorea birmanica Prain & Burkill (DBE) showed high cytotoxic activity against lung cancer cells; A 549 and COR-L23 ($IC_{50}$ = 7.45 ± 0.31, 8.71 ± 0.29 µg/ml, respectively) but no cytotoxic activity against normal cancer cells MRC-5 ($IC_{50}$ = 94.76 ± 1.25 µg/ml). Six fractions from DBE were isolated by vaccuum liquid chromatography with ordering polarity of solvent and were coded as DB1-DB6, respectively. The fraction DB5 showed high cytotoxic against A 549 and COR-L23 ($IC_{50}$ = 6.14 ± 0.08 and 16.44 ± 1.23 µg/ml, respectively) but less toxic to normal cell. Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or Prosapogenin A of dioscin (DBS1) was isolated from DB5 fraction and had highest cytotoxic activity against those two types of lung cancer cells ($IC_{50}$ = 1.81 ± 0.03, 1.84 ± 0.05 µg/ml), respectively) but less cytotoxic against normal lung cells MRC-5 ($IC_{50}$ = 37.09 ± 0.67 µg/ml).*
***Conclusion***: *The steroid saponin from Dioscorea birmanica showed cytotoxic activity against human lung cancer cells but less toxic against normal lung cells.*

***Keywords:*** *Cytotoxic activity, Dioscorea birmanica Prain & Burkill, Lung cancer cell*

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Malignant disease is the leading cause of death in Thailand[1]. Among the several types of cancers that cause death around the world, lung cancer is the leading one. Plant-based systems have a long history of use in traditional health care[2]. World Health Organization estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care and at least 119 chemical substances derived from 90 plant species can be considered as important drugs currently in use in one or more countries[3]. 74% of these 119 compounds were discovered as a result of chemical studies directed at the isolation of the active substances from basic plants used for traditional medicine[4]. Therefore, the usage of ethnopharmacology, or traditional use, is channeled for discovery of new biologically-active molecules[2]. Thai traditional medicine has been used to treat cancer patients for a long time. From a selective interview of a southern folk doctor, it was found that *Dioscorea birmanica* Prain & Burkill is one plant used to treat cancer patients[5]. The previous report found that the ethanolic extract of *Dioscorea burmanica* rhizome showed high cytotoxic activity against lung, colon and breast cancer ($IC_{50}$ = 7.4, 22.6 and 16.3 µg/ml, respectively)[6]. Thus, the investigation be continued to investigate cytotoxic activity against at least two types of lung cancer cells compared with normal lung cells and also isolate the cytotoxic compounds from these extracts. The results from this research should support the use of Thai folk doctor's wisdom to treat lung cancer cells and solve problems for cancer patients throughout the world. Thus, the objective of this

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research is to test cytotoxic activity against two types of lung cancer cells and compare activities with normal lung cells and use the bioassay guide fractionation technique to isolate a cytotoxic compound. The ratio of lung cancer and normal lung cells would be compared for concluding selectivity.

## Material and Method

### *Plant Material*

The rhizomes of *Dioscorea birmanica* Prain & Burkill (Dioscoreaceae) were collected from Chantaburi province, Thailand. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where the herbarium voucher (SKP A062001002) is kept. Specimens are also kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkhla University, Songkhla, Thailand.

### *Isolation and purification of compounds*

Dried powdered of rhizome of *Dioscorea birmanica* (1 kg) was macerated with 95% ethanol, the extract concentrated to dryness under reduced pressure, to obtain 11.13% of crude extract. An aliquot of the ethanolic extract of *Dioscorea birmanica* (10 g) was separated by vacuum liquid chromatography (VLC), using hexane (1,000 ml), hexane: chloroform (1:1) (1,000 ml), chloroform (1,000 ml), chloroform: methanol (8: 2), chloroform: methanol (1:1) and methanol (1,000 ml), respectively and drying by rotary evaporation. Each fraction was dried and evaporated to yield 0.36, 0.63, 2.85, 9.77, 50.02 and 25.67%, respectively with these fractions being denoted as DB1, DB2, DB3, DB4, DB5 and DB6. All fractions were tested for cytotoxic activity by the SRB assay for leading to discovery a cytotoxic compound against lung cancer cells (Table 2).

An aliquot (2g) of fraction DB5 which exhibited cytotoxic activity against CORL23 was separated by Column Chromatography (silica gel with a gradient of solvents, $CHCl_3$ (100%) (200 ml); $CHCl_3$: MeOH (9.5: 0.5) (400 ml); $CHCl_3$: MeOH (9: 1) (400 ml); $CHCl_3$: MeOH (8: 2) (400 ml): $CHCl_3$: MeOH (7: 3) (400 ml ); $CHCl_3$: MeOH (6:4) (400 ml); $CHCl_3$: MeOH (5: 5) (400 ml), $CHCl_3$: MeOH (4:6) (400 ml) ;$CHCl_3$:MeOH (3:7) (400 ml); $CHCl_3$: MeOH (2: 8) (400 ml); $CHCl_3$: MeOH (1:9) (400 ml) and finally MeOH (400 ml). 20 ml fractions were collected for each eluting solvent and fractions combined, following TLC examination (silica gel/$CHCl_3$: MeOH (7:3) detection with acidic anisaldehyde spray) to yield. Compounds 1 (9 mg, 0.225% w/w of crude extract). A pure compound was powdered by acetone.

### *Structure Elucidation*

The structure of the isolates (Fig. 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].

Compound 1 was identified as diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside (prosapogenin A of dioscin) by comparison of its spectral features with literature values and it was identical with published data for prosapogenin A of dioscin[7-9] (Table 1). This compound was also identical in chromatographic behavior when compared with authentic samples previously isolated[10].

DBS1 (Diosgenin-3-O-α-L-rhamnosyl (1→2)-β-D-glucopyranoside or Prosapogenin A of dioscin): white amorphous solid (9 mg, 0.225% w/w of 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) $cm^{-1}$ $^1$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 = 7.7 Hz, H-1') 5.19 (1H, d, J = 1.1 Hz, H-1") 5.36 (1H, br s, H-6) and $^{13}$C NMR (see Table 1).

### *In vitro Assay for Cytotoxic Activity*

#### *Human Cell Culture*

Two different types of human cancerous cell

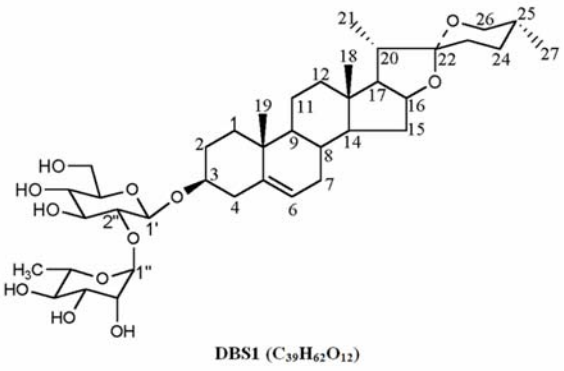


**Fig. 1** Diosgenin 3-O-α-L-rhamnopyranosyl (1→2)-β-D glucopyranoside

**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[7]

| $^{13}C$ | 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 |
| 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 $CD_3OD$

lines as the large cell lung carcinoma (COR-L23) and non small cell lung carcinoma (A549), normal human lung cell as normal lung fibroblast cell line (MRC-5) were used in the test. COR-L23 and A549 were cultured in RPMI 1640 medium supplement with 10% heated fetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin[6]. MRC-5 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 cells were maintained at 37°C in an incubator with 5% $CO_2$ and 95% humidity.

***Cytotoxic 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 P[11]. In brief, cells at the exponential growth phase were detached with 0.25% trypsin-EDTA to make single cell 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 $10^4$, 1 x $10^4$ and 5 x $10^4$ cells/ml for COR-L23, A549 and MRC-5, respectively. 100 ml/well of these cell suspensions would be seed in 96-well microtiter plates and 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 µM for pure compound. The final mixture was used for treating cells 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 were washed with medium. And 200 µl of fresh medium were added to each well. The plates were incubated for a recovery period for 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 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 4 times with 1% acetic acid until only dye adhering to the cells was left. The dry plates and 100 µl of 10 mM Tris base [tris (hydroxy methyl) aminomethane, pH 10.5] were added to each well to solubilize the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance (OD) of each well (4 replicate) was read on Microplate reader at 492 nm as an indication of cell number. Cell survival was measured as a percentage of absorbance compared with the control (non-treated cells). The $IC_{50}$ values were calculated from the Prism

**Table 2.** Cytotoxic activity showed as $IC_{50}$ value (μg/ml) and SEM of *Dioscorea birmanica* Prain & Burkill extract and its fraction against lung cancer cell lines (COR-L23 and A549) and normal cell line (MRC-5) at exposure time (72 hrs) (n = 3)

| Code of Extract | Solvent | % yield | Cytotoxic activity ($IC_{50}$ μg/ml ± SEM) | | |
|---|---|---|---|---|---|
| | | | COR-L23 | A549 | MRC-5 |
| DBE | Crude ethanolic extract | 11.13 | 8.71 ± 0.29<br>10.9 times | 7.45 ± 0.31<br>12.7 times | 94.76 ± 1.25<br>- |
| DB1 | Hexane | 0.36, | 96.75 ± 1.25 | 100 ± 0 | 100 ± 0 |
| DB2 | Hexane: Chloroform 1: 1 | 0.63, | 75.66 ± 1.48 | 100 ± 0 | 100 ± 0 |
| DB3 | Chloroform | 2.85, | 67.52 ± 2.045 | 23.01 ± 0.43 | 83.51 ± 1.20 |
| DB4 | Chloroform:methanol 8: 2 | 9.77, | 20.22 ± 1.67 | 6.07 ± 0.25 | 38.77 ± 1.63 |
| DB5 | Chloroform:methanol 1:1 | 50.02 | 16.44 ± 1.23 | 6.14 ± 0.08 | 85.57 ± 1.70 |
| DB6 | Methanol | 25.67 | 100 ± 0 | 89.34 ± 1.74 | 100 ± 0 |
| Compound 1 (DBS1) | Isolated from DB5 | 0.225 | 1.84 ± 0.05 (2.54 μM)<br>20.2 times of normal cells | 1.81 ± 0.03 (2.49 μM)<br>20.6 times of normal cells | 37.09 ± 0.67 (51.21 μM) |
| Vinblastine sulphate (nM) | | | 1.38 ± 0.16 nM | 1.45 ± 0.16 nM | > 100 nM |

n = number of independent experiments which was performed in 3 replications

program.

## Results

The percentage of yield and cytotoxic activity against all cells is shown in Table 2.

The ethanolic extract of *Dioscorea birmanica* Prain & Burkill showed high activity against A549 and COR-L23 ($IC_{50}$ = 7.45 and 8.71 μg/ml, respectively) but this extract showed no cytotoxic activity against normal lung cell ($IC_{50}$ = 94.76 μg/ml) or it can killed A549 and COR-L23 as 12.7 and 10.9 times of normal lung cells (MRC-5). These results showed that this extract had specific cytotoxic activity against cancer cells only but could not kill normal cells. In addition, DB4 and DB5 showed high potency against COR-L23 and A549 and were less active for normal cell MRC-5. DB4 showed the highest cytotoxic against A549 and DB5 showing the highest activity against A549 and COR-L23 (6.07 and 16.44 μg/ml, respectively). These two were a fraction less active against normal cells. Thus, DB5, which showed high cytotoxic activity and high yields, was isolated pure compound. The compound as a steroid saponin was diosgenyl-3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside (DBS1), its synonyms being protosaponin A of dioscin, progenin III, ophigogenin C[7-9]. It was elucidated by spectrophotometry and the authentic sample obtained from Assoc. Prof. Arunporn Itharat, Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University (Thailand) was compared. It showed high activity against A 549 and COR-L23 ($IC_{50}$ are 1.81 ± 0.03 and 1.84 ± 0.05 μg/ ml or 2.49 and 2.54 μM, respectively) but less cytotoxic activity against the normal cell line (MRC-5) ($IC_{50}$ values is 37.09 ± 0.67 μg/ ml or 51.21 μM). The comparison of ratio of $IC_{50}$ (μM) normal cells/ $IC_{50}$ (μM) of two types of cancer cells (COR-L23 and A549) was 20.6 and 20.2 times, respectively.

## Discussion

These results showed differences from the previous report [10] which found that Prosapogenin A exhibited non selective cytotoxicity against cancer cell line and normal cells, but in this study, this compound can kill two types of lung cancer cells but not kill normal lung cells more than 20 times. Prosapogenin A of dioscin (DBS1) was also found in *Dioscorea bulbifera*[12] *D. colletii* [13] *D. panthaica*[14]and *D. colletii*[13] *D. gracillima*[15] *D. quinqueloba*[16] and *D. composita* tubers[17]. It has also been isolated from *Ophiopogon japonicus* tuber (Liliaceae) which is also named Ophiopogonin C[18]. Prosapogenin A of dioscin or DBS1

was isolated from *Draecaena draco* (Agavaceae) and showed cytostatic activity against HL-60 cell lines[19] ($IC_{50}$=1.3 µg/ml at exposure 72 hours)[19]. This report related the result of Ittharat[10] on cytotoxicity activity against three cancer cell lines. It showed cytostatic activity against lung, colon and breast cancer cell lines because $IC_{50}$ at exposure time of 24, 48, 72 and 144 hours were identical Ittharat A[10]. From these investigations, it was indicated that DBS1 could be continued for further study as a mechanism for cancer, especially lung cancer, because it showed a different effect on cancer cell and normal cells distinctly and it should be a marker for analysis of the ethanolic extract.

## Conclusion

It was concluded that the ethanolic extract and the ingredient of this plant showed cytotoxic activity against human lung cancer cells but no cytotoxic against normal lung cells. A steroid saponin should be used as a marker for analysis of *Dioscorea birmanica* extract.


## Acknowledgements

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## ซาโปนินเป็นพิษต่อเซลล์มะเร็งปอดแยกที่ได้จากหัวข้าวเย็นชนิด *Dioscorea birmanica*

**นวลจันทร์ ใจอารีย์, อรุณพร อิฐรัตน์, กัมมาล กุมารปาวา**

***วัตถุประสงค์:*** *เพื่อศึกษาฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดของสารสกัดหัวข้าวเย็นชนิด Dioscorea birmanica Prain & Burkill และสารที่แยกได้*

***วัสดุและวิธีการ:*** *การศึกษาฤทธิ์ใช้เซลล์มะเร็งปอด 2 ชนิด (A549 และ CORL-23) และเซลล์ปกติ 1 ชนิด (MRC5) ด้วยวิธีใช้สีย้อมซัลโฟโรดามีน บี (sulforhodamine B assay) วิธีการ Bioassay guide fractionation ใช้สำหรับแยกสารที่เป็นพิษต่อเซลล์มะเร็งการพิสูจน์สูตรโครงสร้างใช้เทคนิคสเปคโตรสโคปี*

***ผลการศึกษา:*** *สารสกัดขั้นเอทธานอลของหัวข้าวเย็นชนิด Dioscorea birmanica Prain & Burkill (DBE) แสดงฤทธิ์ต้านเซลล์มะเร็งปอดชนิด A549 และ COR-L23 ($IC_{50}$ = 7.45 ± 0.31 และ 8.71 ± 0.29 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ) แต่ไม่มีพิษต่อเซลล์ปกติของปอดชนิด MRC5 ($IC_{50}$ = 94.76 ± 1.25 ไมโครกรัมต่อมิลลิลิตร) สารสกัดย่อย 6 สารสกัดที่แยกจาก DBE ถูกแยกด้วยวิธี vaccuum liquid chromatography โดยการเรียงลำดับความมีขั้วของตัวทำละลายได้สารสกัดที่เป็นแต่ละส่วนที่มีรหัสเป็น DB1-DB6 ตามลำดับ DB5 แสดงฤทธิ์ความเป็นพิษต่อเซลล์ดีที่สุด โดยออกฤทธิ์ต้านมะเร็งปอดชนิด A549 และ COR-L23 มีค่า $IC_{50}$ เป็น 6.14 ± .08 และ 16.44 ± 1.23 ไมโครกรัมต่อมิลลิลิตร ตามลำดับแต่พิษน้อยต่อเซลล์ปกติ Diosgenin-3-O-$\alpha$-L-rhamnosyl (1 $\rightarrow$ 2)-$\beta$-D-glucopyranoside หรือ Prosapogenin A of dioscin (DBS1) ถูกแยกออกมาจาก DB5 มีฤทธิ์เป็นพิษต่อเซลล์สูงสุด โดยมีฤทธิ์ต้านมะเร็งปอด ชนิด A549 และ COR-L23 ($IC_{50}$ = 1.81 ± 0.03 และ 1.84 ± 0.05 ไมโครกรัมต่อมิลลิลิตรตามลำดับ) แต่ไม่มีฤทธิ์ ต้านเซลล์ปกติที่ปอดหรือ MRC-5 ($IC_{50}$ = 37.09 ± 0.67 ไมโครกรัมต่อมิลลิลิตร)*

***สรุป:*** *สารในกลุ่มเสตียรอยด์ซาโปนินจากหัวข้าวเย็นชนิด Dioscorea birmanica มีฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งปอดแต่มีพิษน้อย ต่อเซลล์ปอดปกติ*