Selective cytotoxicity and antioxidant effect of compounds from *Dioscorea membranacea* rhizomes

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Bioassay-guided isolation was used to separate the active ingredients of the ethanolic extract of *Dioscorea membranacea* by testing cytotoxic activity against three human cancer cell lines, i.e. large cell lung carcinoma (COR-L23), colon cell line (LS-174T) and breast cancer cell line (MCF-7) and two normal human cell lines, keratinocytes (SVK-14) and normal human fibroblasts (HF), using the SRB assay. The DPPH test for antioxidant activity was also carried out as was a test for LDH release as an indicator of damage to the cell membrane. Eight compounds were isolated, two naphthofuranoxepins (dioscorealides A (1) and B (2)), one 1,4-phenanthraquinone (dioscoreanone (3)), three steroids (β-sitosterol (4), stigmasterol (5) and β-D-sitosterol glucoside (8)) and two sterol saponins (3-O-α-L-rhamnopyranosyl (1→2)-β-D-glucopyranoside (6) and diosgenin 3-O-β-D-glucopyranosyl (1→3)-β-D-glucopyranoside (7)). 2, 3 and 6 showed cytotoxic activity against three cancer cell lines, and 2 showed selective cytotoxic activity against lung and breast cancer, but was less active against the two normal cells and had no toxicity on cell membranes in the LDH assay. 3 showed the highest antioxidant activity.

**Keywords:** SRB cytotoxicity assay, LDH assay, antioxidant. *Dioscorea membranacea*

The rhizomes of *Dioscorea membranacea* Pierre (Dioscoreaceae) are commonly used as ingredients in Thai traditional anticancer preparations. The ethanolic rhizome extract showed high and selective cytotoxic activity against human cancer cell lines using the SRB assay [1]. A subsequent paper described the bioassay-guided isolation of its active ingredients by testing cytotoxic activity against three human cancer cell lines, i.e. large cell lung carcinoma (COR-L23), colon cell line (LS-174T) and breast cancer cell line (MCF-7), using the SRB assay [2]. Three compounds were isolated, being two novel naphthofuranoxepins (dioscorealide A and dioscorealide B), one novel 1,4-phenanthraquinone (dioscoreanone). The activity of these compounds had not been previously tested using normal cells to determine any selectivity, so this paper continues these studies by investigating these and other constituents against the three human cancer cell lines, i.e. large cell lung carcinoma (COR-L23), colon cell line (LS-174T) and breast cancer cell line (MCF-7) and two normal human cells; keratinocytes MCF-7 and two normal human cells; keratinocytes SVK-14 and human fibroblasts HF. The compounds that were isolated were also tested for their toxic effect on membranes by the LDH assay and for antioxidant activity using the DPPH assay, since this latter activity is related to cancer prevention.

The spectra of compound 1, 2 and 3 were identical with published data for dioscorealide A, dioscorealide B and dioscoreanone [2]. The three compounds were also identical in chromatographic behaviour when compared with authentic samples previously isolated [2]. Compound 4 gave identical spectra to an authentic sample of stigmasterol (Sigma) and with literature values...
Compounds against five cell types concluded that difference in effects on cancer cells and normal cells at exposure times 24, 48 and 72 h (Table 2). There is a significant cytotoxic against all cell lines at exposure times 24, 48 and 72 h (Table 2). There is a significant difference in effects on cancer cells and normal cells. The comparison of cytotoxicity activity of the compounds against five cell types concluded that dioscorealide B (2) showed high activity and selectivity with lung and breast cancer cell lines since it had less effect with normal cells, especially the SVK-14 keratinocytes. From the comparison of ratio of IC_{50} (µM) normal cells/ IC_{50} (µM) cancer cells of the three cytotoxic compounds and the crude extracts at exposure time 72 h it was found that 2 showed the highest ratio between MCF7 breast cancer cells and normal cells SVK14 and HF (85.3 and 15.5 respectively). A difference was also seen with the crude extract DME where the breast cancer cells were 5.9 times more sensitive then SVK14 (Table 2).

Three compounds dioscorealide B (2) showed high activity and selectivity with lung and breast cancer cell lines since it had less effect with normal cells, especially the SVK-14 keratinocytes. From the comparison of ratio of IC_{50} (µM) normal cells/ IC_{50} (µM) cancer cells of the three cytotoxic compounds and the crude extracts at exposure time 72 h it was found that 2 showed the highest ratio between MCF7 breast cancer cells and normal cells SVK14 and HF (85.3 and 15.5 respectively). A difference was also seen with the crude extract DME where the breast cancer cells were 5.9 times more sensitive then SVK14 (Table 2).

Three of the eight compounds isolated from the ethanolic extract of Dioscorea membranacea i.e. 2, 3 and 6, showed a strong cytotoxicity activity (Table 1) but had no effect on cell membranes in LDH assay. All compounds showed weak antioxidant activity by DPPH assay (EC_{50}>100µg/ml).

Three compounds dioscorealide B (2) dioscoreanone (3) and prosapogenin A (6) were cytotoxic against all cell lines at exposure times 24, 48 and 72 h (Table 2). There is a significant difference in effects on cancer cells and normal cells. The comparison of cytotoxicity activity of the compounds against five cell types concluded that

$$\text{compd}$$

<table>
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DME is the ethanolic extract of Dioscorea membranacea, NT= not tested

In the light of these results, studies on 2 could be continued for investigation of mechanism because it showed a distinct difference in effect on cancer cell and normal cells. The results for dioscoreanone (3) showed selective cytotoxicity activity, especially against lung cancer cells, although it showed a lower ratio than 2. It showed a greater activity against all three cancer cells compared with normal cells (SVK14) than the crude extract (DME). The prosapogenin A of dioscin (6) showed definite cytotoxic activity against all cell types, with in fact a higher cytotoxic activity against normal cells. Dioscorealide B (2) exhibited selective cytotoxicity activity, paralleled with antioxidant activity, but dioscorealide A had 20 times less activity than dioscorealide B. The hydroxyl group of 2 appears to have high potential for cell death since, when it is replaced by a methoxy as in 1, the cytotoxic activity is reduced considerably. In a similar way the anticancer drug 6-methyl-thiopurine is demethylated to give the active anticancer drug, 6-mercaptopurin [19]. The reason why dioscorealide B 2 and dioscoreanone 3 are specific for some cell lines could be that different types of cell have membranes which differ in lipid composition, as lung and breast cancer cells are the adeno-cell type or epithelial type from adipose tissue [20, 21], which have more
unsaturated fatty acids than some other cells. However, since they showed no activity in the LDH assay (Table 3), it is unlikely the cell membrane was affected in this way.

Dioscoreanone (3), a naphthoquinone derivative, showed the highest free radical scavenging activity (Table 1). 2, which displays a OH group, was significantly less active than dioscoreanone (P<0.005), although it showed more than twenty times scavenging ability (P<0.0001) than 1, which had a methoxy group (Table 1).

Halliwell and Gatteridge, [22] stated that the degree of hydroxylation and relative positions of –OH groups are of primary importance in determining antioxidant activity, so dioscoreanone (3) and dioscorealide B (2), which have one –OH group in their structure, should have more activity than dioscorealide A (1). There are no previous reports for the antioxidant activity of these three compounds.

Many studies on stigmasterol, β-sitosterol and β-sitosteryl-3-O-D glucopyranoside have found that they have no significant cytotoxicity activity [23-28]. In contrast, the cytotoxicity activity of steroidal saponins appears to be due to both the monosaccharides and their sequence in the sugar part, as well as the structure of the aglycone. If the aglycone is a spirosten type (diosgenin) it shows stronger cytotoxicity than the furostanol type and the sugar part contributes to the cytotoxicity [29]. There is a difference in activity between compounds 6 and 7, with 6 (diosgenyl-3-α-L-rhamnopyranosyl(1→2)-β-D-glucopyranoside), which has α-L-rhamnosyl attachment vertical to the plane of the diosgenin moiety, having by far the stronger activity (Table 1). 7 (Diosgenyl-3-β-D-glucopyranosyl (1→3)-β-D-glucopyranoside) has -β-D-glucopyranosyl attachment at C-3 which is in the same plane as the as diosgenin moiety and so should have little cytotoxic activity [29]. The prosapogenin A of dioscin or 6, from Dracaena draco (Agavaceae) showed cytostatic activity against HL-60 cell lines (IC\textsubscript{50}=1.3 μg/ml at exposure 72 h)[29]. This report shows the results of cytotoxicity activity of 6 against three cancer cell lines with cytostatic activity against lung, colon and breast cancer cell lines, because IC\textsubscript{50} values at exposure times of 24, 48 and 72 h were identical.

Deterioration of cancer cells, especially breast cancer, when exposed to three cytotoxic compounds differed. Cells exposed to dioscorealide B (2) and prosapogenin A of diosgenin (6) were spherical, small with a disappearing nucleus whilst cells exposed to dioscoreanone have a granular appearance around the nucleus and vacuole. However, the LDH experiment showed that the cell membrane remained intact with exposure to all three compounds (Table 3).

Table 3. Damage to membranes of different cell lines by compounds 2, 3 and 6 expressed as percentage leakage of LDH compared with that given by Triton X-100.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration ( \mu g/mL (\mu M) )</th>
<th>COR-L23 ( % )</th>
<th>LS-174T ( % )</th>
<th>MCF-7 ( % )</th>
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</thead>
<tbody>
<tr>
<td>2</td>
<td>0.1 (0.33)</td>
<td>-2.3</td>
<td>-1.8</td>
<td>-0.5</td>
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<td>1.0 (3.3)</td>
<td>-4.4</td>
<td>0.8</td>
<td>-1.7</td>
</tr>
<tr>
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<td>10 (33)</td>
<td>-4.8</td>
<td>1.8</td>
<td>-1.7</td>
</tr>
<tr>
<td>1</td>
<td>50 (165)</td>
<td>-4.4</td>
<td>6.5</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>0.1 (0.35)</td>
<td>-6.6</td>
<td>2.3</td>
<td>5.0*</td>
</tr>
<tr>
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<td>1.0 (3.5)</td>
<td>-5.6</td>
<td>0.8</td>
<td>5.8**</td>
</tr>
<tr>
<td>1</td>
<td>10 (35)</td>
<td>-6.9</td>
<td>1.0</td>
<td>9.4**</td>
</tr>
<tr>
<td>1</td>
<td>50 (175)</td>
<td>-2.3</td>
<td>-8.3</td>
<td>8.4**</td>
</tr>
<tr>
<td>6</td>
<td>0.1 (0.14)</td>
<td>-4.1</td>
<td>-3.5</td>
<td>2.7</td>
</tr>
<tr>
<td>1</td>
<td>1.0 (1.4)</td>
<td>-3.3</td>
<td>-1.3</td>
<td>1.9</td>
</tr>
<tr>
<td>1</td>
<td>10 (14)</td>
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<td>1</td>
<td>50 (70)</td>
<td>6.3*</td>
<td>9.8*</td>
<td>6.1*</td>
</tr>
</tbody>
</table>

* P<0.05; ** P < 0.005 using non-parametric unpaired T-test

The five compounds that showed little cytotoxic activity have been studied for other activities such as anti-inflammatory or immunomodulatory effects, which are relevant in the treatment of cancer. For example, β-sitosterol, which is the principal phytosterol in most higher plants, and commercially used to decrease the absorption of cholesterol in the digestive system and decrease the amount of cholesterol produced by the liver, was shown to be a chemopreventive agent [30, 32]. β-sitosteryl (5) and β-sitosteryl-β-D-glucoside (8) showed analgesic activity in the acetic acid-induced writhing test and hot plate method and they also exhibited antimutagenic activities [33], antipyretic, anti-inflammatory, anti-neoplastic and immune-modulating activity [34-36]. This indicates that β-sitosterol and β-sitosteryl-β-D-glucoside, which are found in Dioscorea membranacea and have no cytotoxicity or antioxidant activity, may be useful in adjuvant treatment of cancer by action on the immune system and reducing some effects such as pain, fever and inflammation.

In summary, three compounds (2, 3 and 6) which have been isolated from the ethanolic extract of Dioscorea membranacea showed a high level of selective cytotoxicity with no effect on cell membrane permeability and some antioxidant activity.
Experimental

The rhizomes of *D. membranacea* Pierre (Dioscoreaceae) were collected from Amphor Pa-tue Chumporn province, Thailand. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where the herbarium voucher (SKP A062041305) is kept. Specimens are also kept in the herbarium of Southern Center of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand.

Dried powdered of rhizome of *Dioscorea membranacea* (1 kg) was percolated with 95% ethanol, the extract concentrated to dryness under reduced pressure, to give 31 g of crude extract. An aliquot of the ethanolic extract of *Dioscorea membranacea* (10 g) was separated by vacuum liquid chromatography (VLC), using chloroform (10x100ml), chloroform:MeOH (1:1) (10x100ml), MeOH (10x100ml). Drying and evaporation of each fraction yielded residues of 2.816g, 0.1876g and 6.78g, denoted as FA, FB and FC respectively.

An aliquot (2g) of fraction FA was separated by CC (silica gel with a gradient of solvents, hexane:CHCl₃ (6:4); (1000ml); hexane:CHCl₃ (8:2) (1000ml); CHCl₃ :MeOH (95:5) (1000 ml); CHCl₃ :MeOH (9:1) (500 ml); CHCl₃ :MeOH (7:3) (500ml ) and finally MeOH (500 ml). 15ml fractions were collected for each eluting solvent and fractions combined, following TLC examination (silica gel/ CHCl₃ :MeOH (7:3) detection with acidic anisaldehyde spray) to yield FA1 To FA6 Compounds 1 (11.1mg, 0.00484 % w/w) , 2 (31mg, 0.01353 % w/w) 3 (8.4mg, 0.00367 % w/w) and 8 (16.6 mg., 0.00725 % w/w ) were isolated from FA1 (fractions 56-63), FA4 (fractions 124-128) FA3 (fractions 100-102) and FA5 (fractions 209-212) respectively. Two mixtures of two compounds were obtained (10 mg, 164 mg) from fractions FA2 (fractions 74-78) and FA6 (fractions 239-250) respectively. All the pure compounds and the mixture from FA2 were crystallized from methanol and the mixture from

![Figure 1: Structures of the isolated compounds.](image-url)
FA6 was crystallized as white powder from acetone.

FA6 (164 mg) was separated by CC silica gel/CHCl₃: MeOH: H₂O (10:3:0.5) collecting 10 ml fractions to obtain two compounds 6 (32.1mg, 0.0140 % w/w) and 7 (15mg, 0.0065 %w/w) from fractions 30-33 (FA6A) and fractions 45-48 (FA6C) respectively and both were recrystallized from acetone. Fraction 34-44 (92 mg) gave a mixture of 6 and 7 and this was also separated by HPLC to give the fraction named FA6B. The HPLC-UV system used was a Jasco PU-1580 pump, a spectrophotometric UV-Vis variable–wavelength detector Jasco UV-1575 and recorder Waters 745 Data module) using a C8 column (250 x 4.6 mm.) (Rexchrom™) under isocratic conditions with an acetonitrile-water (86:14) mixture at flow rate of 0.5 ml /min.

Final isolation of compounds from FA2 and FA6 was accomplished by dissolving them in HPLC-grade acetonitrile (Merck) and using the HPLC system quoted above but eluting with acetonitrile 1000 µl/min.Components of FA2 (10 mg) yielded 4 (4.5 mg, 0.00196% w/w) and 5 (3.2 mg, 0.001397 % w/w), seen at retention times 16.87 and 19.16 min respectively. FA6A (10mg) was separated using a C18 column (250x4.6mm) (Hypersil™) under isocratic conditions with acetonitrile-water (50:50) at flow rate of 1.0 ml /min .The operating wavelength was set at 210 nm and the injection volume was 1 µl . 7 and 6 was collected at retention times 6.12 and 19.09 min respectively.

The structures of the isolates (Figure 1) were determined by their NMR data [¹H and ¹³C on a Varian Unity Inova 500 spectrometer (500 MHz for ¹H; 125 MHz for ¹³C)], UV spectra [a Hewlett Packard 8452A Diode array spectrometer], IR spectra [Jasco IR-810 spectrometer], EI mass spectra, both HR- and LR-, were obtained from a JEOL JMS-AX505W spectrometer. HR-FAB mass spectra were measured on KRATOS MS890MS spectrometer.

Three different kinds of human cancerous cell lines and two normal cell lines were used and the technique was similar to that described earlier [1]. The cancer cell lines were large cell lung carcinoma (COR-L23), the human colon adenocarcinoma (LS174T) (ECACC No:86060401), the human breast adenocarcinoma (MCF-7) (ECACC No:86012803), and the two normal cell lines were human keratinocyte cell line SVK-14 and human keratinocyte HF, kindly provided by Dr. H. Navasaria of Department of Experimental Dermatology, St Bartholomews and Royal London School of Medicine, Queen Mary and Westfield College, London UK, and were grown in an incubator with 10% CO₂ at 37°C in DMEM culture medium containing 10% foetal bovine serum.and 1% of 10,000 U penicillin and 10 mg/ml streptomycin [15]. According to their growth profiles, the optimal plating densities of each cell line were determined (1x10⁶, 1.5x10⁶, 3x10⁶, 5x10⁶ and 4 x10⁷cells/well for COR-L23, LS-174T, MCF-7, SVK-14 and HF respectively) to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analysed by SRB assay.

The SRB assay was carried out as previously described [1, 16]. The cell pellets were resuspended and diluted with medium to give a final concentration of 1x10⁶, 1.5x10⁶, 3x10⁶, 5x10⁶ and 4x10⁷ cells/well for COR-L23, LS-174T, MCF7, SVK-14 and HF respectively. Each isolated compound was initially dissolved in an amount of DMSO. Vincristine sulphate (Sigma, Lot No.34H0447) was the positive control. The compounds were diluted in medium to produce 8 concentrations. The plates were incubated for selected exposure times of 24, 48 and 72 hours. At the end of each exposure time, the medium was removed. The wells were then washed with medium, and 200 µl of fresh medium were added. The plates were incubated for recovery period of 6 days and cell numbers were analysed by SRB assay. According to National Cancer Institute guidelines [17] compound with IC₅₀ values < 4 µg /ml (~ 10mM) were considered active.

LDH (lactate dehydrogenase) is a cytosolic enzyme that is not normally released from intact cells except under conditions involving cell death. A high level of LDH in the medium is commonly used as an indicator of damage to the cell membrane. The LDH is quantified by an oxidation reaction of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD). Formation of reduced nicotinamide adenine dinucleotide (NADH) results in an increase in absorbance at 340 nm, this being directly proportion to LDH activity in the sample (Sigma, 1995).

Three cancer cell lines were treated with the cytotoxic compounds at different concentrations (50, 10, 1 and 0.1µg/ml) and exposed for 72 h. 10 µl of supernatant or medium was then pipetted in duplicate into the 96 well plate, followed by 200 µl
of Sigma Diagnostic® Lactate Dehydrogenase (LD-L) reagent, which was freshly prepared by reconstituting with Millipore water to give 50 mmol/L lactate, 7 mmol/L nicotinamide adenine dinucleotide (NAD), and buffer pH 8.9. The plate was immediately read at 340 nm at interval of 9 sec over a period of 5 min in the Spectromax® 190 microplate spectrometer after initially shaking for 5 sec. The OD results recorded at 5 min were compared with those given by Triton X-100, as positive control.

The antioxidant assay used was the DPPH assay [18]. Samples were dissolved in absolute ethanol to obtain the highest concentration of 200 µg/ml. Each sample was further diluted two-fold for at least five dilutions. Each concentration was tested in triplicate. A portion of sample solution (500 µl) was mixed with an equal volume of 6x10⁻⁵ M DPPH (in absolute ethanol) and allowed to stand at room temperature for 30 min. The absorbance (A) was then measured at 520 nm. BHT (butylated hydroxytoluene), a well-known synthetic antioxidant, was a positive control. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The result was expressed as percentage inhibition in the formulae:

% inhibition = [(Acontrol - A sample)/Acontrol] x 100.

The EC50 value was obtained by linear regression analysis of the dose-response curve, plotting between % inhibition and concentration.

Acknowledgements
We are grateful to Prince of Songkla University for financial support for this project.

References

Table 1: Cytotoxicity of compounds 1-8 (IC50 value µM) with antioxidant activity (percentage of inhibition for concentration 100 µg/ml in DPPH assay) for compounds 1-8 (6 replicates, n=3, mean ± SEM)

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<th>Compd</th>
<th>CORL23 24h</th>
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<th>CORL23 72h</th>
<th>LS174T 24h</th>
<th>LS174T 48h</th>
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<th>MCF-7 24h</th>
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<th>SVK14 24h</th>
<th>SVK14 48h</th>
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<td>7.13±0.59</td>
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<tr>
<td>8</td>
<td>&gt;160</td>
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<td>&gt;160</td>
<td>&gt;160</td>
<td>&gt;160</td>
<td>12.95±1.05</td>
<td></td>
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</tr>
<tr>
<td>VS</td>
<td>±0.11</td>
<td>±0.26</td>
<td>±0.17</td>
<td>±0.20</td>
<td>±0.13</td>
<td>±0.15</td>
<td>±0.42</td>
<td>±0.10</td>
<td>±0.14</td>
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</tr>
</tbody>
</table>

VS = Vincristine sulfate (positive control), NT=no test


