Abstract. Xanthorrhizol is a natural sesquiterpene compound isolated from the rhizome of Curcuma xanthorrhiza Roxb (Zingiberaceae). Recent studies of xanthorrhizol in cell cultures strongly support the role of xanthorrhizol as an antiproliferative agent. In our study, we tested the antiproliferative effect of xanthorrhizol using different breast cancer cell lines. The invasive breast cancer cell line, MDA-MB-231, was then selected for further investigations. Treatment with xanthorrhizol caused 50% growth inhibition on MDA-MB-231 cells at 8.67±0.79 μg/ml as determined by sulforhodamine B (SRB) assay. Hoechst 33258 nuclear staining assay showed the rate of apoptosis of MDA-MB-231 cells to increase in response to xanthorrhizol treatment. Immunofluorescence staining using antibody MitoCapture™ and fluorescein isothiocyanate (FITC)-labeled cytochrome c revealed the possibility of altered mitochondrial transmembrane potential and the release of cytochrome c respectively. This was further confirmed by Western-blotting, where cytochrome c was showed to migrate from mitochondrial fraction to the cytosol fraction of treated MDA-MB-231 cells. Caspase activity assay showed the involvement of caspase-3 and caspase-9, but not caspase-6 or caspase-8 in MDA-MB-231 apoptotic cell death. Subsequently, cleavage of PARP-1 protein is suggested. These data suggest treatment with xanthorrhizol modulates MDA-MB-231 cell apoptosis through the mitochondria-mediated pathway subsequent to the disruption of mitochondrial transmembrane potential, release of cytochrome c, activation of caspase-3 and caspase-9, and the modulation of PARP-1 protein.

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Key Words: Antiproliferative property, apoptotic effect, xanthorrhizol, MDA-MB-231.
protective ability, antimitastatic activity, and modulation of anticancer-associated proteins such as COX-2, NF-kappaB, MMP-9, ERK, p53, Bcl-2, Bax, caspases and PARP (5-20), xanthorrhizol may serve as an alternative chemotherapeutic agent for cancer if its mechanism of action can be well elucidated.

For these reasons, we decided to further explore the tremendous biological activities of xanthorrhizol by evaluating its growth inhibitory action and potential mechanism of action on human breast cancer cell lines, particularly MDA-MB-231 cells. To date, no studies have been carried out to determine the antiproliferative potential of xanthorrhizol towards MDA-MB-231 cells. MDA-MB-231 cells have high invasive ability that can contribute to metastasis (21, 22). In addition, they tend to establish resistance against programmed cell death stimuli (23, 24). Thus, the ability of substances to halt their proliferation is definitely beneficial for the prevention and treatment of invasive cancer.

Materials and Methods

Cell culture. Human breast cancer cell lines, namely MDA-MB-231, MCF-7, T-47D, MDA-MB-453, SK-BR-3, and normal cell lines, Vero and MDBK, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231, MCF-7 and SK-BR-3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Co., Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen Co., Carlsbad, California, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Flowlab, Sydney, Australia) whereas T-47D, MDA-MB-453, Vero and MDBK cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Invitrogen Co., Carlsbad, California, USA) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ in air at 37°C.

In vitro panel of human breast cancer cell lines screening assay. The antiproliferative activity of xanthorrhizol, curcumin (Sigma Chemical Co., St. Louis, MO, USA), and tamoxifen (Sigma Chemical Co.) towards MDA-MB-231, MCF-7, T-47D, MDA-MB-453 and SK-BR-3 human breast cancer cell lines was evaluated by using the sulforhodamine B (SRB) method, as described elsewhere (25-29). Cells were seeded 24 hours prior to treatment in a 96-well plate at plating densities ranging from 10,000-20,000 cells/well depending on the doubling time of individual cell lines in order to obtain semi-confluent cultures. One plate from each cell line was fixed in situ with trichloroacetic acid (TCA) (Sigma Chemical Co.), to represent a measurement of the cell population for each cell line at the time of drug addition (T). Test agents were dissolved in dimethyl sulfoxide (DMSO) (Sigma Chemical Co.) and followed by a 2x serial dilution for 6 points ranged from 0.8 μg/ml to 25 μg/ml. The final concentration of DMSO used in the corresponding wells did not exceed 1% (v/v), which affects cell viability. Control cultures received the same concentration of solvent alone. Tamoxifen and curcumin were used as positive controls. Following drug addition, the plates were incubated for an additional 48 hours.

At the end of incubation, cells were fixed in situ with 50 μl of cold 50% (w/v) TCA and incubated for 1 hour at 4°C. The plates were then washed with tap water and air dried. SRB (Sigma Chemical Co.) solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to the wells and the plates were further incubated for 10 minutes at room temperature. After staining, unbound dye was washed out by 1% acetic acid and the bound stain was subsequently solubilized with 10 mM trizma base (Sigma Chemical Co.) solution (100 μl) at 0.4% (w/v) in 1% acetic acid.

In Table I, Antiproliferative and cytotoxic activity of xanthorrhizol, curcumin and tamoxifen towards 5 human breast cancer cell lines.

<table>
<thead>
<tr>
<th>Breast cancer cell lines</th>
<th>Antiproliferative activity (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xanthorrhizol</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td></td>
</tr>
<tr>
<td>GI₅₀</td>
<td>8.67±0.79</td>
</tr>
<tr>
<td>TGI</td>
<td>11.00±0.54</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>21.43±0.93</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
</tr>
<tr>
<td>GI₅₀</td>
<td>10.86±0.74</td>
</tr>
<tr>
<td>TGI</td>
<td>17.92±0.94</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>&gt;25</td>
</tr>
<tr>
<td>T-47D</td>
<td></td>
</tr>
<tr>
<td>GI₅₀</td>
<td>10.90±0.63</td>
</tr>
<tr>
<td>TGI</td>
<td>15.63±0.69</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>21.52±0.83</td>
</tr>
<tr>
<td>MDA-MB-453</td>
<td></td>
</tr>
<tr>
<td>GI₅₀</td>
<td>7.51±0.33</td>
</tr>
<tr>
<td>TGI</td>
<td>9.87±0.69</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>19.52±2.83</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td></td>
</tr>
<tr>
<td>GI₅₀</td>
<td>11.54±1.02</td>
</tr>
<tr>
<td>TGI</td>
<td>16.21±0.14</td>
</tr>
<tr>
<td>LC₅₀</td>
<td>20.45±0.97</td>
</tr>
</tbody>
</table>

GI₅₀, growth inhibition activity (inhibition activity); TGI, total growth inhibition (cytostatic activity); LC₅₀, lethal concentration (cytotoxic activity).

At the end of incubation, cells were fixed in situ with 50 μl of cold 50% (w/v) TCA and incubated for 1 hour at 4°C. The plates were then washed with tap water and air dried. SRB (Sigma Chemical Co.) solution (100 μl) at 0.4% (w/v) in 1% acetic acid was added to the wells and the plates were further incubated for 10 minutes at room temperature. After staining, unbound dye was washed out by 1% acetic acid and the bound stain was subsequently solubilized with 10 mM trizma base (Sigma Chemical Co.). The absorbance at 505 nm was read on a spectrophotometric plate reader.

Dose-response curves were constructed to obtain the response parameters which were the GI₅₀, TGI and LC₅₀. Percentage growth is calculated as: [(T (C-Tₜ))/(C-Tₜ)] ×100 where (Tₜ) is measurement of cell population at the time of drug addition, (C) is control growth and (T) is test growth in the presence of drug at the six concentration levels. The GI₅₀ value corresponds to the concentration of test agents causing 50% decrease in net cell growth which was calculated from ([T (C-Tₜ)]/(C-Tₜ)) ×100=50, the TGI value is the concentration of test agents resulting in total growth inhibition which was calculated from Tₜ-Tₜ, and the LC₅₀ value is the concentration of the test agents causing net 50% loss of initial cells at the end of the incubation period which was calculated from ([T (Tₜ-Tₜ)]Tₚ) ×100=(−50). Applying the same experimental study, the antiproliferative activity of xanthorrhizol on normal cell lines, Vero and MDBK, was also determined by using SRB assay as described above. All data were derived from 3 independent experiments.
Determination of selectivity ratio. The sensitivity of a particular breast cancer cell line towards a test agent was determined as described elsewhere (30). The selectivity ratio of each test agent at the GI50 and LC50 was calculated by comparing the full panel mean-graph mid-point values (MG-MID) which is the average sensitivity of all cell lines toward test agent to individual GI50 and LC50.

Hoechst 33258 nuclear staining. Nuclear staining with Hoechst 33258 (Sigma Chemical Co.) was performed as described elsewhere (18, 31). Briefly, the floating and trypsinized-adherent of treated and untreated cells were collected and washed with phosphate-buffered saline (PBS). After washing, the cells were incubated in Hoechst 33258 at a final concentration of 10 μM at room temperature for 20 min. At the end of incubation, cells were washed once with PBS before being immediately smeared onto microscope slides. Nuclear morphology was then examined under a fluorescence microscope (Imaging Source Europe GmbH, Bremen, Germany). To quantify the apoptotic index, the percentage of single and multi intense-fluoresced cells (apoptotic morphology) were calculated from five random microscopic fields at ×40 magnification.

Analysis of alteration of mitochondrial membrane potential. Changes in the mitochondrial membrane potential of treated MDA-MB-231 cells were examined using a fluorescence microscope (Imaging Source Europe GmbH) and MitoCapture™ Mitochondrial Apoptosis Detection Kit (BioVision Research, Moutain View, CA, USA), according to the manufacturer’s instructions. Briefly, the floating and trypsinized-adherent of treated and untreated cells were collected and washed with PBS. One μl of MitoCapture dye was diluted to 1 ml pre-warmed incubation buffer immediately prior to use. A pellet of MDA-MB-231 cells was resuspended in 500 μl of the diluted MitoCapture solution then incubated for 20 minutes at 37˚C in a CO2 incubator. Cells were then centrifuged at 900 ×g for 5 min. The pellet was then resuspended in 100 μl pre-warm incubation buffer, immediately smeared onto microscope slides and observed under a fluorescence microscope.

Immunofluorescence microscopic evaluation of cytochrome c release. MDA-MB-231 cells were seeded 24 hours prior to treatment in a 6-well plate at 2×10^5 cells/well. The floating and trypsinized-adherent of treated and untreated cells were collected and washed with PBS. Cells were centrifuged at 900 xg for 5 minutes followed by the process of fixation and permeabilization, according to the manufacturer’s instructions (Dako, Glostrup, Denmark). Briefly, 100 μl of fixative solution was added to the cell pellet and incubated at RT for 15 min. Cells were then washed in 2 ml of PBS and centrifuge at 900 xg for 5 min. The remaining pellet was then permeabilized with 100 μl of permeabilization solution. Meanwhile, FITC-labelled monoclonal antibody against cytochrome c (clone 6H2.B4; BioLegend, San Diego).

Table II. Median growth inhibitory concentrations (GI50, μg/ml) of in vitro panel of breast cancer cell linesa.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MDA-MB-231</th>
<th>MCF-7</th>
<th>T-47D</th>
<th>MDA-MB-453</th>
<th>SK-BR-3</th>
<th>MG-MIDb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthorrhizol</td>
<td>8.67</td>
<td>10.86</td>
<td>10.90</td>
<td>7.51</td>
<td>11.54</td>
<td>9.90</td>
</tr>
<tr>
<td></td>
<td>(1.14)</td>
<td>(0.91)</td>
<td>(0.91)</td>
<td>(1.32)</td>
<td>(0.86)</td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>8.74</td>
<td>4.11</td>
<td>4.70</td>
<td>5.16</td>
<td>3.06</td>
<td>5.15</td>
</tr>
<tr>
<td></td>
<td>(0.59)</td>
<td>(1.25)</td>
<td>(1.10)</td>
<td>(1.00)</td>
<td>(1.68)</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>4.59</td>
<td>8.29</td>
<td>3.26</td>
<td>3.70</td>
<td>2.90</td>
<td>4.55</td>
</tr>
<tr>
<td></td>
<td>(0.99)</td>
<td>(0.55)</td>
<td>(1.40)</td>
<td>(1.23)</td>
<td>(1.57)</td>
<td></td>
</tr>
</tbody>
</table>

aData obtained from Table I in vitro human breast cancer cell lines; bGI50 (μg/ml) full panel mean-graph mid point (MG-MID) = the average sensitivity of all breast cancer cell lines toward the test agent. Note: Values in brackets indicate the selectivity ratios of test agents on different types of breast cancer cell lines.

Table III. Median cytotoxic concentrations (LC50, μg/ml) of in vitro panel of breast cancer cell linesa.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MDA-MB-231</th>
<th>MCF-7</th>
<th>T-47D</th>
<th>MDA-MB-453</th>
<th>SK-BR-3</th>
<th>MG-MIDb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthorrhizol</td>
<td>21.43</td>
<td>N/A</td>
<td>21.52</td>
<td>19.52</td>
<td>20.45</td>
<td>20.73</td>
</tr>
<tr>
<td></td>
<td>(0.97)</td>
<td>(N/A)</td>
<td>(0.96)</td>
<td>(1.06)</td>
<td>(1.01)</td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td>N/A</td>
<td>N/A</td>
<td>21.35</td>
<td>16.14</td>
<td>24.59</td>
<td>20.69</td>
</tr>
<tr>
<td></td>
<td>(N/A)</td>
<td>(N/A)</td>
<td>(0.97)</td>
<td>(1.28)</td>
<td>(0.84)</td>
<td></td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>11.20</td>
<td>N/A</td>
<td>12.01</td>
<td>5.93</td>
<td>7.25</td>
<td>9.10</td>
</tr>
<tr>
<td></td>
<td>(0.81)</td>
<td>(N/A)</td>
<td>(0.76)</td>
<td>(1.53)</td>
<td>(1.26)</td>
<td></td>
</tr>
</tbody>
</table>

aData obtained from Table I in vitro human breast cancer cell lines; bLC50 (μg/ml) panel mean-graph midpoint (MG-MID) = the average sensitivity of all breast cancer cell lines toward the test agent. NA, not available.
USA) at a dilution of 1:500 and final concentration of 10 μM Hoechst 33258 was added to the suspension and incubated in the dark at room temperature for 30 minutes. After extensive washing, cells were immediately smeared onto microscope slides and observed under a fluorescence microscope (Imaging Source Europe GmbH) to evaluate the release of cytochrome c from the mitochondrial intermembrane space as described elsewhere (32, 33).

Western-blotting. An equal amount of protein (30 μg) from both treated and untreated cells was loaded and electrophoresed on 15% SDS-polyacrylamide gels. At the end of electrophoresis, the proteins were blotted onto polyvinyl-difluoride (PVDF) membranes (PerkinElmer Life Sciences Inc., Boston, MA, USA). The membrane was then dried, preblocked with 5% skimmed milk (Oxoid Ltd., Basingstoke, Hampshire, UK) in 0.1% TBS-tween prior to incubation with the primary antibodies (Cytochrome c and PARP; BD Biosciences Pharmingen, Franklin Lakes, NJ, USA) diluted 1:500. The membrane was then probed with secondary antibody conjugated to horseradish peroxidase (1:10,000; BD Biosciences Pharmingen). The immunoreactions between the antibodies were detected by an enhanced chemiluminescence system (PerkinElmer Life Sciences Inc.) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY, USA). Integrated density value (IDV) of each band was determined using a AlphaImager HP with spot densitometry software (Alpha Innotech, San Leandro, CA, USA). The membrane was re probed with β-actin antibody (Sigma Chemical Co.) as an internal control and to ensure equal loading.

Determination of caspase activity. Caspase-3, -6, -8 and -9 activity was determined using Caspase Colorimetric Assay Kit (BioVision Research, Mountain View, CA, USA), according to the manufacturer’s instructions. Briefly, the floating and trypsinized-adherent treated and untreated cells were collected and washed with PBS. Cells were then lysed with cell lysis buffer. The suspension was centrifuged for 1 min at 10,000 ×g and the supernatant (cytosolic extract) was transferred to a fresh tube. The protein concentration assay was then performed by using Bradford assay. Two hundred μg cytosolic protein was diluted to 50 μl cell lysis buffer for each assay in a 96-well plate. Each reaction buffer containing 10 mM dithiothreitol (DTT) and 5 μl of 4 mM Asp-Glu-Val-Asp (DEVD)-pNA substrate (caspase-3), 4 mM Val-Glu-Ile-Asp (VEID)-pNA substrate (caspase-6), 4 mM Ile-Glu-Thr-Asp (IETD)-pNA substrate (caspase-8), or 4 mM Leu-Glu-His-Asp (LEHD)-pNA substrate (caspase-9) was added into each well of the designated enzyme assay. The plate was then incubated at 37°C for 2 hours. At the end of incubation, the plate was read in a microtiter plate reader at 412 nm.

Figure 1. Effect of xanthorrhizol on growth of human breast cancer cells, MDA-MB-231 as compared to normal mammalian cell lines, VERO and MDBK. The cell lines were exposed to graded concentration of xanthorrhizol for 48 h. The GI<sub>50</sub>, TGI, and LC<sub>50</sub> values obtained for VERO cells were 13.58±0.81 μg/ml, 20.36±0.53 μg/ml, and >25 μg/ml respectively. Meanwhile, the GI<sub>50</sub>, TGI, and LC<sub>50</sub> values obtained for MDBK cells were 13.26±0.74 μg/ml, 19.42±0.87 μg/ml and > 25 μg/ml respectively. The growth inhibition and cytotoxic values obtained for both VERO and MDBK cells were higher than MDA-MB-231 for which GI<sub>50</sub>, TGI and LC<sub>50</sub> values were 8.67±0.79 μg/ml, 11.00±0.54 μg/ml and 21.43±0.93 μg/ml respectively. Values were expressed as mean±SD of at least three separate experiments.
Fold-increase in caspase activity was determined by comparing the results of treated samples with the level of the uninduced control.

**Results**

**Effect of xanthorrhizol on human breast cancer cells proliferation.** The antiproliferative activity of xanthorrhizol on human breast cancer cell lines was evaluated by obtaining GI₅₀, TGI, and LC₅₀ values as shown in Table I. Tamoxifen which

Figure 2. Xanthorrhizol induces apoptosis, alters the mitochondrial transmembrane potential and modulates the release of cytochrome c. A, Nuclear morphology of xanthorrhizol-treated MDA-MB-231 cells was evaluated using Hoechst 33258 staining. No fluorescence was observed in most of the nuclei of untreated cells. In contrast, fluorescence (arrow) was clearly detected in the nuclear region of the treated cells, indicating their apoptotic morphology. B, MitoCapture™ dye was found localized and fluoresced red in the mitochondria of untreated cells (arrow). However, the number of cells fluorescing red decreased after the treatment with xanthorrhizol and tamoxifen in a dose-dependent manner, suggesting the disruption of the mitochondrial transmembrane potential. C, The distribution of cellular cytochrome c was determined by using the FITC-labelled antibody cytochrome c (green fluorescence) counterstained with Hoechst 33258 stain (blue fluorescence). As xanthorrhizol- and tamoxifen-treated cells underwent apoptotic cell death, a more diffused stain of cytochrome c was observed as compared to that of the control. This implies the released of cytochrome c occurred in parallel with apoptosis. Results are representative of three independent experiments.

Statistical analysis. All data were expressed as mean±standard deviation. The statistical differences were analyzed using one-way ANOVA followed by a Tukey Honestly Significantly Different (HSD) test. Values of p<0.05 were considered significant.
is a standard chemotherapeutic drug for breast cancer showed greater antiproliferative activity to the panel of breast cancer cells as compared to xanthorrhizol and curcumin. Curcumin, which is derived from the same family as xanthorrhizol (Zingerberaceae) and currently used for testing in clinical trials (34-36), showed higher antiproliferative effect in all breast cancer cell lines except for MDA-MB-231 cells as compared to the treatment with xanthorrhizol. Xanthorrhizol showed slight advantage over curcumin in growth inhibition and cytotoxic activity in the MDA-MB-231 cell line with GI₅₀: 8.67±0.79 μg/ml, TGI: 11.00±0.54 μg/ml and LC₅₀: 21.43±0.93 μg/ml. Sensitivity of human breast cancer cells toward xanthorrhizol, curcumin and tamoxifen. From Table II and Table III, xanthorrhizol, curcumin, and tamoxifen are rated as being non-selective towards the breast cancer cell lines as they were found to have a low selectivity ratio ranging between 0.55-1.68 and 0.76-1.53 at the GI₅₀ and LC₅₀ levels respectively. These data suggest that xanthorrhizol does not possess cytotoxic preferences toward the breast cancer cell lines tested.

Effect of xanthorrhizol on normal cell line (MDBK and Vero) proliferation and viability as compared to breast cancer cell line (MDA-MB-231). Xanthorrhizol treatment exhibited a low extent of cytoselectivity towards nonmalignant cells, MDBK and Vero. Xanthorrhizol showed 1.5-, 1.8-, and at least 1.2-fold lower growth inhibition and cytotoxicity as shown by GI₅₀, TGI, and LC₅₀ values respectively in both MDBK and Vero cells as compared to MDA-MB-231 cancer cells (Figure 1).

Hoechst 33258 nuclear staining. The Hoechst 33258 dye was able to diffuse through intact membranes of MDA-MB-231 cells and stain their DNA. As the concentration of xanthorrhizol increased, single intense fluorescence and multiple strong fluorescence signals were produced in the cell nuclei (Figure 2A). The control culture (untreated MDA-MB-231) was uniformly stained, whereas the treated cells showed clear apoptotic morphology. Shrinkage of cells and plasma membrane convolution were all observed in the treated cells. Similar apoptotic morphology was also observed in the MDA-MB-231 cells treated with 5 μg/ml tamoxifen.

Treatment with xanthorrhizol increased number of apoptotic cells in a dose-dependent manner. The number of apoptotic cells was determined by Hoechst 33258 staining assay and were expressed as percentage of apoptotic index. Treatment with xanthorrhizol resulted in a dose dependent increase in apoptotic cells (Figure 3).
Mitochondrial transmembrane potential was altered after treatment with xanthorrhizol. The cationic dye MitoCapture™ was able to enter the cell cytoplasm, accumulate and aggregate in mitochondria, thus producing a bright red fluorescence. This red fluorescence was easily distinguished in untreated MDA-MB-231 cells. Remarkably, increasing doses of xanthorrhizol treatment resulted in significant reduction of this red fluorescence (Figure 2B). In contrast, there was an increasing number of treated cells which only fluoresced green as the apoptotic index increased exponentially. This is due to the inability of MitoCapture™ to accumulate in mitochondria as the mitochondrial transmembrane potential alters during apoptosis events. It remains as green monomers in the cytoplasm of apoptotic cells (33, 37, 38). A similar trend of fluorescence was also seen in MDA-MB-231 cells when treated with 5 μg/ml and 10 μg/ml tamoxifen respectively.

Xanthorrhizol induced cytochrome c release-mediated apoptosis. Immunofluorescence microscopic analysis of distribution of cytochrome c in MDA-MB-231 cells after treatment with xanthorrhizol showed that cytochrome c was released from mitochondria during xanthorrhizol-induced cell death (Figure 2C). In control culture, cells exhibited a very sharp and intense green fluorescence which indicates cytochrome c accumulated in mitochondria. On the contrary, a more diffused stain of cytochrome c antibody was observed in both the xanthorrhizol- and tamoxifen-treated cells. These observations were in line with the increase of apoptotic cells and the disruption of mitochondria membrane potential following treatment of xanthorrhizol at increasing doses. This result was then confirmed by western blotting where the expression of cytochrome c was down-regulated in the mitochondrial fraction of the treated cells. In contrast, the expression of cytochrome c was up-regulated in the cytosolic fraction of the treated cells as compared to untreated cells (Figure 4). This expression trend suggests cytochrome c migrated from the mitochondria to the cytosol.

Xanthorrhizol modulates caspase-3 and caspase-9 activity but not caspase-6 or caspase-8 activity. To test whether caspases are involved in xanthorrhizol-induced apoptosis,
the enzymatic activity of initiator caspases (caspase-8 and caspase-9), and effector caspases (caspase-3 and caspase-6) was analyzed. There was a marked increased of caspase-3 and -9 activity after treatment with xanthorrhizol in a concentration-dependent manner. Their activity started to rise gradually at 10 μg/ml. At 20 μg/ml, caspase-9 achieved highest activity (~12-fold), followed by caspase-3 (~7-fold) as compared to the control. In contrast, caspase-6 and caspase-8 activity did not change throughout the experiment (Figure 5).

**Figure 5. Modulation of caspase-3, -6, -8, and -9 activity after treatment with xanthorrhizol.** Quantification of the caspase activity was performed and normalized with respect to untreated cells, shown as fold change. Here, the caspase activity analysis revealed that caspase-3 and -9 activity increased, whereas the caspase-6 and -8 activity were not elevated by the treatment with xanthorrhizol as compared to the control. Results are presented as means±SD of 3 independent experiments. *p<0.05, **p<0.005 Statistically significant values relative to untreated control.

**Discussion**

There are tremendous precedents for looking to nature for drug discovery as naturally occurring compounds, especially in plants have shown potential value in combating cancer for many years. According to the United States Food and Drug Administration, over 50% of anticancer drugs approved since 1960 were derived from plants (39, 40). The efficacious drug, Taxol, which is used to treat breast and ovarian cancer, originated from the bark of the Pacific yew tree (41). Nowadays, most cancer in advanced stages is not curable by chemotherapy (42) and breast cancer arises due to series of frequent recurrent alterations in the genome of normal epithelial cell (43). Therefore, new drug discovery and development from natural products still continue to play a vital role in the fight against cancer.

Our findings show that xanthorrhizol, extracted from *C. xanthorrhiza*, demonstrated potent antiproliferative action in highly proliferative human breast cancer cell lines. Most of the chemotherapeutic agents achieved excellent...
cytotoxicity when tested in human cancer cells with high rates of cell proliferation (44). In our study, the ability of xanthorrhizol to inhibit the growth of various human breast cancer cell lines was compared to that of tamoxifen, which is recognized as a 'gold standard' agent for treatment of breast cancer (45), and to curcumin, which is a well established natural product for potential breast and ovarian cancer treatment (34-36). Tamoxifen was the most active test agent against breast cancer cell lines in our experiment. Although curcumin exhibited higher growth inhibition in most of the cell lines as compared to xanthorrhizol, xanthorrhizol was able to achieve competitive cytotoxic activity with curcumin in the same panel of cell lines, particularly against MDA-MB-231 cells. Since tamoxifen was found to have a number of adverse effects (45-47), while xanthorrhizol was found to have organ-protective properties and antitumorigenic and antiproliferative effect, we believe xanthorrhizol may have greater advantage as an antiproliferative agent as compared to tamoxifen and curcumin. In addition, natural products which are reported to have potential therapeutic value for treatment of breast cancer have demonstrated higher GI50 value as compared to xanthorrhizol on MDA-MB-231 cells (48, 49). Hence, xanthorrhizol may have a role in a new potential therapeutic approach for breast cancer.

Although the potent antiproliferative and cytotoxic activity after treatment of xanthorrhizol did not occur selectively towards any of the breast cancer cell line, the highly proliferative and invasive MDA-MB-231 cell line was selected for further investigations on the potential mechanism of action of xanthorrhizol. The MDA-MB-231 cell line has lost its normal breast cell phenotype. The MDA-MB-231 cell line expresses mutant tumor suppressor gene, p53, and invasion factors such as MMP-1, MMP-2, MMP-9 and vimentin but they do not expressed factors such as E-cadherin or steroid receptors such as estrogens and progesterone as compared to the other breast cancer cell lines (21, 50-52). Furthermore, the MDA-MB-231 cell line achieves high tumorigenicity in nu/SCID mice and exhibits invasive properties in vitro (22). Therefore, the altered phenotype in MDA-MB-231 cell line is associated with tumor progression, metastasis formation and resistance to programmed cell death (23, 24). Hence, the ability of xanthorrhizol to show antiproliferative activity at low treatment doses towards the MDA-MB-231 cell line may help to halt its proliferation and thus, prevent progression.

It is generally believed that the balance between proliferation and apoptosis influences the response of tumors to cytotoxic treatment (53). Apoptosis is considered as a significant form of cancer cell death after treatment with cytotoxic drugs and has been recognized as a standard strategy for the selection of anticancer drugs (54, 55). Most chemotherapeutic drugs induce apoptosis in cancer cells (56). The inability of MDA-MB-231 cells to undergo apoptosis in response to anticancer stimuli has been highlighted in recent study (57). However, our investigations showed that xanthorrhizol was able to induce apoptotic cell death in the MDA-MB-231 cell line. The dying cells exhibit ultrastructural and biochemical features that characterized apoptosis, as shown by the loss of cell viability, DNA condensation, DNA fragmentation, cell shrinkage, and altered mitochondrial transmembrane potential (18, 21, 58-60). Recently, xanthorrhizol treatment has been reported to cause apoptotic cell death in MCF-7 (18), HeLa (19) and HepG2 (20) cancer cell lines as well.

<table>
<thead>
<tr>
<th>Control</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative expression</td>
<td>(1.00)</td>
<td>(1.32)</td>
<td>(1.27)</td>
<td>(0.90)</td>
<td>(0.81)</td>
<td>(0.49)</td>
</tr>
<tr>
<td>PARP-1</td>
<td>~116 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>~46 kDa</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Figure 6. Western-blot analysis of PARP-1 protein (~116 kDa) in MDA-MB-231 cells treated with xanthorrhizol at the indicated concentration. Total proteins extracted from treated cells were resolved in 10% SDS-PAGE and subsequently subjected to Western blotting. Quantitation of the bands was performed and normalized changes with respect to untreated cells are shown in brackets, using β-actin as a loading control. Results are representative of three independent experiments. The level of PARP-1 protein (~116 kDa) was found to decrease gradually in a dose-dependent manner and was substantially reduced at concentration of xanthorrhizol 25 μg/ml.
In this present study, apoptosis associated proteins, cytochrome c, caspase-3, caspase-9 and PARP-1 played a key role in MDA-MB-231 cell death.

In line with the marked increase of apoptotic index after treatment with xanthorrhizol, mitochondrial depolarization occurred in a dose-dependent manner. This facilitates the release of cytochrome c from the mitochondria intermembrane to the cytosol which can be seen in the immunofluorescence images. Tamoxifen has also been reported to induce apoptosis in MCF-7 and MDA-MB-231 breast cancer cells via the mitochondrial pathway involving the release of cytochrome c (61). The increased apoptotic index, altered mitochondrial transmembrane potential and the release of cytochrome c after treatment with xanthorrhizol occurred in good correlation. These data suggest mitochondria-mediated apoptosis was triggered upon treatment with xanthorrhizol. The exact mechanism of the mitochondrial membrane permeabilization still remains to be characterized. Cytochrome c is a vital protein in leading the cancer cells to mitochondria-dependent apoptosis (62). In normal apoptotic events, cytochrome c released from mitochondria mediates the formation of the apoptosome complex which consists of scaffold protein Apaf-1, procaspase-9 and ATP. This leads to the activation of caspase-9. Activated caspase-9 initiates downstream caspase-3 activation (63-66). This executioner caspase is then involved in the formation of apoptotic features. Interestingly, xanthorrhizol was also reported to induce apoptosis via the mitochondrial pathway in a human liver cancer cell line (20).

Treatment with xanthorrhizol was found to markedly induce activity of caspase-3 and -9 but not of caspase-6 and caspase-8. Caspase-8 is activated when procaspase-8 is cleaved subsequently from the recruitment of Fas associated protein with death domain (FADD) to the death-effector domain (DED) site of procaspase-8 during the oligo-merization of death receptor and its ligand (66-68). The possibility of caspase-3 activation and cytochrome c release due to the activation of caspase-8 was ruled out since the caspase-8 activity level remained at a basal level throughout the experiment (69-71). These results suggest the treated cells underwent an intrinsic apoptosis pathway, but not the extrinsic pathway (72). Meanwhile, caspase-6 activity which is vital in executing the apoptosis event, was also not induced as compared to the untreated culture. This may also be due to the absence of caspase-8 activity (71).

In an anticancer study on a human liver cancer cell line, xanthorrhizol was reported to modulate the expression of caspase-3 protein and the activation of caspase-9 through the enzymatic processing of initiator procaspase-9 (20). The results showed the involvement of caspase-3 and caspase-9 in the induction of apoptosis in cancer cells, which is parallel with our present study. Caspase-3 activity is an important mediator in apoptosis cell death as it is responsible for the cleavage of the key cellular proteins that leads to the typical morphological changes observed in cells undergoing apoptosis (73, 74). Caspase-3 deficiency and down-regulation have been reported to be associated with breast carcinogenesis (75), this indicates that the increased activity of caspase-3 after treatment with xanthorrhizol may play an important role in cancer prevention and treatment. In addition, we found that the cytotoxic effect of xanthorrhizol on MDA-MB-231 cells was more potent than on MCF-7 cells. This may be explained by the deficiency of caspase-3 in MCF-7 cells (76, 77) resulting in the lower cytotoxic response to xanthorrhizol as described in a report (78).

PARP-1 is essential in maintaining genome integrity by its poly(ADP-ribosyl)ation process (79). Highly activated PARP-1 in response to DNA-damaging agents can lead to the depletion of cellular energy, which would then lead to the loss of all energy-dependent cellular function, thereby initiating necrosis (80-82). However, in our study, the expression of uncleaved PARP-1 protein (116 kDa) was decreased at higher doses of xanthorrhizol. The down-regulation of PARP-1 protein is believed is an inactivation process to prevent cellular depletion of ATP that is necessary for apoptosis accomplishment as suggested in other reports (60, 83). In addition, the disappearance of the 116 kDa PARP-1 band may reflects the processing of the zymogen to generate the cleaved 89 kDa fragment, as has been interpreted in previous reports (84, 85) and it may caused by the enzymatic process of activated caspase-3 as reported elsewhere (86, 87).

The experimental evidence from this study suggests that xanthorrhizol may induce apoptosis via the intrinsic pathway, involving the release of cytochrome c followed by the modulation of caspase-3 and -9 activity, and thereby regulates the inactivation of PARP-1 protein. Taken together with its organ-protective properties and antimetastatic activity, we believe xanthorrhizol could be a potential antiproliferative agent for breast cancer cells. Therefore, additional in vitro and in vivo investigation into its mechanism of action (apoptosis pathway, angiogenesis pathway, and signal transduction pathway), potential drug interactions and adverse effects that may result from its consumption should lead to a greater ability to evaluate its therapeutic capacity in the treatment of breast cancer in the future.

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