Xanthorrhizol Exhibits Antiproliferative Activity on MCF-7 Breast Cancer Cells via Apoptosis Induction

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Abstract. Xanthorrhizol is a natural sesquiterpenoid compound isolated from the rhizome of Curcuma xanthorrhiza Roxb (Zingiberaceae). Xanthorrhizol was tested for a variety of important pharmacological activities including antioxidant and anti-inflammatory activities. An antiproliferation assay using the MTT method indicated that xanthorrhizol inhibited the proliferation of the human breast cancer cell line, MCF-7, with an EC₅₀ value of 1.71 µg/ml. Three parameters including annexin-V binding assay, Hoechst 33258 staining and accumulation of sub-G₁ population in DNA histogram confirmed the apoptosis induction in response to xanthorrhizol treatment. Western-blotting revealed down-regulation of the anti-apoptotic bcl-2 protein expression. However, xanthorrhizol did not affect the expression of the pro-apoptotic protein, bax, at a concentration of 1 µg/ml, 2.5 µg/ml and 5 µg/ml. The level of p53 was greatly increased, whilst PARP-1 was cleaved to 85 kDa subunits, following the treatment with xanthorrhizol at a dose-dependent manner. These results, thereby, suggest that xanthorrhizol has antiproliferative effects on MCF-7 cells by inducing apoptosis through the modulation of bcl-2, p53 and PARP-1 protein levels.

Tropical regions host a variety of plants which have provided a substantial number of medicinal products throughout history. Curcuma xanthorrhiza Roxburgh (Roxb.) from the family of Zingiberaceae is one of the most extensively studied medicinal plants, and is recognized as a remedy for various ailments in folklore uses (1). The rhizome of C. xanthorrhiza has been traditionally used for food and medicinal purposes, such as tonic (2, 3), cholagogues, aromatic stomachics, analgesics, rheumatic remedy (4) and liver disorder treatment (2, 4-6).

The fresh rhizome of C. xanthorrhiza has been found to contain large amounts of sesquiterpenoids followed by curcuminoïds and monoterpenoids when analyzed by capillary GC-MS (7). Xanthorrhizol is the major natural sesquiterpenoid in the essential oils extracted from the rhizome and makes up nearly 51% of the essential oils (8). Interestingly, xanthorrhizol has been widely investigated in disease-oriented drug discovery, and has been found to possess antioxidant (9, 10) and anti-inflammatory activity (10, 11), neuroprotective (10), nephroprotective (12) and hepatoprotective effects (2, 5) and antibacterial activity (6).

Xanthorrhizol has been identified as one of the major antitumor constituents when tested on Sarcoma 180 ascites in mice (3). Moreover, it has been discovered to have a suppressive effect on COX-2 and iNOS, which are important mediators in the processes of inflammation and carcinogenesis (5, 11). Thus, xanthorrhizol was considered to exert anti-inflammatory and cancer chemopreventive properties. Xanthorrhizol has been also found to suppress tumor promotion in mouse skin stimulated with TPA (9) and to exhibit anti-metastatic potential by the attenuation of COX-2, MMP-9 and ERK in an in vivo lung metastasis model (13). In an in vitro study, xanthorrhizol has been found to induce p53 and bax-dependent apoptosis in HeLa cervical cancer cells (14).

The tremendous pharmacological activities of xanthorrhizol have prompted us to investigate the biological effects of the compound on human breast cancer cells.

Breast cancer remains the most frequently diagnosed cancer among females in Malaysia. About 3,738 female breast cancer cases have been reported in 2003, which accounted for 31% of the total newly diagnosed female cancer cases (15). To date, no studies have been carried out to determine the anticancer potential of xanthorrhizol towards human breast cancer cells.

Apoptosis is an evolutionary process that leads to programmed cell death. This intrinsic cellular suicide program is triggered to kill and remove excess, unwanted or damaged cells during development and homeostasis of
normal tissue (16). In most cancer types, apoptosis was thwarted so that the cancerous cells were able to acquire survival advantages (17), and to alter the normal apoptotic pathway by regulating the balance between pro-apoptotic protein (bax, bak and bok) and anti-apoptotic protein (bcl-2, bcl-XL and bid) expression (18-20). Bcl-2 and bax are members of the bcl-2 family of proteins that has been associated with apoptotic cell death (19, 21). A direct inverse relationship exists between the levels of bcl-2 and bax in cells during apoptosis process. Decreased bcl-2 levels cause an unbalanced bcl-2/bax ratio, which, in turn, decides the cancer cell’s destiny towards apoptosis (21). It has been found that most of the cytotoxic treatments involving chemotherapy drugs kill cancer cells by inducing the apoptosis pathway (16, 20, 22).

The tumor suppressor gene p53 also plays an important role in the etiology of cancer. p53 mediates either apoptosis or cell cycle control in response to DNA damage (23, 24) and has been found to mediate both intrinsic (bax, bcl-2 and bcl-XL) and extrinsic (Fas, DR5 and PERP) pathways in apoptosis (25, 26). Many types of cancers encounter the loss of p53 function (25, 27). Therefore, the restoration or expression of functional p53 induced by cancer chemotherapeutic drugs might be able to re-establish normal cell growth control and restore the appropriate response to DNA-damaging agent, i.e., apoptosis cell death or cell cycle arrest (28).

Another powerful mediator that can influence viability cancer cells is poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1, in response to DNA damage induced by chemotherapy drugs, can initiate either necrosis or apoptosis (29, 30). In the apoptosis process, PARP-1 is cleaved into two fragments of 24 kDa and 89 kDa (31). The process of PARP-1 inactivation is believed to inhibit DNA repair, specific gene transcription, other cellular processes that are not needed for a dying cell, and to prevent cellular depletion of ATP, which is necessary for apoptosis accomplishment (30, 32).

The aim of the present study was to investigate the antiproliferative activity and mode of cell death induced by xanthorrhizol against the human breast cancer cells, MCF-7. The xanthorrhizol mechanism of action was examined by investigating the modulation of the bcl-2/bax, p53 and PARP-1 expression.

**Materials and Methods**

**Cell culture.** MCF-7 and MCF-10A cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Co., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen Co.), 100 U/ml penicillin and 100 mg/ml streptomycin (Flowlab, Sydney, Australia), whereas MCF-10A cells were maintained in Mammary Epithelium Growth Medium (MEGM; Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA) supplemented with 10 ng/ml epidermal growth factor, 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.5 µg/ml gentamisin, 0.5 µg/ml fungizon (Cambrex Bio Science Walkersville Inc.) and 100 ng/ml cholera toxin (Gift from Prof. Azimah, National University of Malaysia, Malaysia) in a humidified atmosphere of 5% CO2 in air at 37°C. Cells were kept in the logarithmic growth phase by routine passage every 2-3 days using 0.025% trypsin-EDTA treatment.

**Cell proliferation assay.** The antiproliferative activity was evaluated by using the MTT method, as previously described (26). Cells were seeded 24 hours prior to treatment in a 96-well plate at 8x103 cells/well in order to obtain semi-confluent cultures. Before addition to the culture medium, xanthorrhizol was dissolved in DMSO (Sigma Chemical Co., St. Louis, MO, USA) and followed by a 2x serial dilution for 10 points ranged from 0.2 µg/ml to 100 µg/ml. The final concentration of DMSO used in the corresponding wells did not exceed 1% (v/v), which affect cell viability. Control cultures received the same concentration of solvent alone and tamoxifen (Sigma Chemical Co.) was used as a positive control. The 96-well plate was incubated for 72 hours. At the end of incubation, 50 µl MTT solution (2 mg/ml MTT in plain culture medium; Sigma Chemical Co.) was added to each well. The plate was then incubated for 4 h, after which the remaining MTT solution was removed and the purple formazan crystal formed at the bottom of the wells was dissolved with 200 µl DMSO for 20 min. The absorbance at 570 nm was read on a spectrophotometric plate reader (Thermo Electron Co., Waltham, MA, USA) and the proportion of surviving cells was calculated as (OD of drug-treated sample – OD of blank) / (OD of control –OD of blank) x 100%. Dose-response curves were constructed to obtain the EC50 values. All data were derived from at least 3 independent experiments.

**Annexin-V binding assay.** The apoptotic event was quantified by the binding of annexin-V to the externalized protein, phosphatidylserine, on the cell membrane during apoptosis with the Apoptest™-FITC Kit (Dako, Glostrup, Denmark), according to the manufacturer’s instructions. Briefly, the floating and trypsinized-adherent of treated and untreated cells were collected and washed with PBS. The cells were then stained with 10 µl Annexin-V-FITC (20 µg/ml) and 5 µl propidium iodide (50 µg/ml) for 15 min at room temperature prior to analysis, using a CyAn™ ADP flow cytometry analyzer (Dako). A total of 20,000 events were gated for the analysis.

**Hoechst 33258 nuclear staining.** Nuclear staining with Hoechst 33258 (Sigma Chemical Co.) was performed as described elsewhere (26, 33). Briefly, the floating and trypsinized-adherent of treated and untreated cells were collected and washed with PBS. The cells were then fixed with 4% methanol-free paraformaldehyde in PBS for 30 min at 4°C. After washing, the cells were smeared onto microscope slides followed by incubation in Hoechst 33258 at a final concentration of 30 µg/ml at room temperature for 30 min. Nuclear morphology was then examined under a fluorescent microscope (Imaging Source Europe GmbH, Bremen, Germany).

**Flow cytometry analysis.** Accumulation of sub-G1 population and cell cycle arrest were evaluated by the single staining of propidium iodide (Sigma Chemical Co.) as described elsewhere (26). The floating and trypsinized-adherent of treated and untreated cells
were collected and washed with PBS. The pelleted cells were then fixed in 70% ethanol at 4°C overnight. After washing with PBS, the cells were stained with propidium iodide (50 μg/ml) and treated with RNase A (20 μg/ml; Bio Basic Inc., Markham Ontario, Canada) for 30 min at 37°C. The cells were then kept for 3 h at 4°C before flow cytometry analysis. A total of 20,000 events were gated for the analysis.

**Western-blotting.** An equal amount of protein (30 μg) from both treated and untreated cells were loaded and electrophoresed on 12-15% 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 membranes were then dried, preblocked with 5% skimmed milk (Oxoid Ltd., Basingstoke, Hampshire, England) in 0.1% PBS-tween prior to incubation with the primary antibodies (bcl-2, bax, p53 and PARP-1; BD Biosciences Pharmingen., Franklin Lakes, NJ, USA) diluted 1:500, and then probed with secondary antibody conjugated to horseradish peroxidase (1:20,000; 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). Densitometry analysis was conducted using a GS 670 Imaging Densitometer with Molecular Analyst software (BioRad, Hercules, USA). The membranes were reprobed with β-actin antibody (Sigma Chemical Co.) as an internal control and to ensure equal loading.

**Results**

**Effect of xanthorrhizol on cell viability.** The antiproliferative effect of xanthorrhizol on certain cell lines was evaluated by obtaining the EC\textsubscript{50} values as in Figure 1. Xanthorrhizol significantly reduced the viability of MCF-7 cells by 50% at 1.71±0.16 μg/ml. Xanthorrhizol treatment did not exhibit any cytoselectivity towards non-malignant breast cells, MCF-10A (Figure 1A). The non-cytoselectivity effect was similar to the treatment with tamoxifen which is a standard chemotherapeutic drug for breast cancer (Figure 1B). Nevertheless, xanthorrhizol showed nearly 2.5-fold greater cytotoxicity to MCF-7 cells as compared to tamoxifen. Other malignant cell lines including T-47D, HepG2, HM3KO and MG-63 were also killed by the treatment of xanthorrhizol at a very low concentration (EC\textsubscript{50}: 4.92±0.39 μg/ml, 2.10±0.05 μg/ml, 2.26±0.28 μg/ml and 2.72±0.28 μg/ml) (Figure 1C).

**Xanthorrhizol increased Annexin-V-FITC binding.** In flow cytometry analysis, MCF-7 cells treated with xanthorrhizol were stained with Annexin-V-FITC and PI. Early apoptotic cells (Annexin-V-positive/PI-negative) were localized in the lower right (LR) region, late apoptotic, and necrotic cells (Annexin-V-positive/PI-positive) in the upper right (UR) region, necrotic cells (Annexin-V-negative/PI-positive) in the upper left (UL) region and viable cells (double negative) in the lower left region.
From the dot plots (Figure 2A), xanthorrhizol at an increasing concentration, killed nearly 50% of MCF-7 cells without showing apparent changes in necrotic cell regions during 24-h incubation. Although a small population of treated cells was undergoing early apoptosis, most of the cells were observed to localize into the UR region after 24-h incubation. Shifting of treated cells into this region thereby suggests the possibility of apoptosis process being involved in cell death. However, in this particular region, late apoptotic cells cannot be well discriminated from necrotic cells due to an increase in membrane permeability (34). Thus, further investigation including Hoechst 33258 staining (Figure 2B) and sub-G1 peak observation in DNA histogram (Figure 2C) were subsequently conducted to confirm whether apoptotic events took place in xanthorrhizol-treated MCF-7 cells.

**Hoechst 33258 staining.** The Hoechst 33258 dye was able to diffuse through intact membranes of MCF-7 cells and stain their DNA. As the concentration of xanthorrhizol was increased, multiple strong fluorescence were produced (Figure 2B). The untreated MCF-7 cells remained uniformly stained, whereas the treated cells showed clear apoptotic morphology (arrow). Shrinkage of cells, DNA condensations, nuclear and plasma membrane convolution and nuclear fragmentation were all observed in the treated cells.

**Accumulation of Sub-G1 population.** Another evidence of apoptosis was the appearance of a sub-G1 peak in the DNA histogram from flow cytometry analysis. Apoptotic cells showed a low DNA stainability (hypodiploid DNA) resulting in a distinct, quantifiable region below the G1 peak. Treatments of xanthorrhizol with 5 μg/ml and 10 μg/ml resulted in a marked increased of the sub-G1 peak from 4.40% to 45.65% in the DNA histogram. There was no accumulation of cells in each G1-, S- or G2/M-phase, indicating no cell cycle arrest (Figure 2C).

**Xanthorrhizol modulates bcl-2, p53 and PARP-1 protein expression (Figure 3).** The protein expressions of bax, bcl-2, p53 and PARP-1 were conducted after 24 h incubation with 1 μg/ml, 2.5 μg/ml, 5 μg/ml, 6 μg/ml and 10 μg/ml of xanthorrhizol. Exposure of MCF-7 cells to xanthorrhizol did not alter the expression of bax at low concentrations (1 μg/ml, 2.5 μg/ml and 5 μg/ml) as compared to the control. On the other hand, xanthorrhizol down regulated bcl-2 expression throughout the experiment. Nevertheless, the Bcl-2/Bax ratio overall decreased in a dose-dependent manner (data not shown). The p53 protein was significantly up-regulated by xanthorrhizol treatment. The expression of p53 increased greatly, 50-fold as compared to the control. Cleavage of intact 116 kDa PARP-1 to 85 kDa fragments after xanthorrhizol treatment in a dose-dependent manner is shown in Figure 3. The down-regulation of bcl-2 and increased expression of p53 levels indicated that the cell death was favoring apoptosis. Antagonist expression of PARP-1 also suggested that the cell death of MCF-7 took place via the apoptotic pathway after the treatment of xanthorrhizol.

**Discussion**

Naturally occurring compounds in plants have shown their potential value in combating cancers for many years. Over 50% of anticancer drugs approved by United States Food and Drug Administration since 1960 originate from plants (35, 36). There is still a mega-diversity of unexploited plants from tropical regions, considered to have potential as sources of anticancer agents. Therefore, development of effective new anticancer drugs, drug combinations and chemotherapy strategies, through scientific exploration of the enormous pool of these natural resources is required (37).

Apoptosis has been shown as a significant way of cell death after cytotoxic drug treatment in a variety of cancer types. The induction of apoptosis has been recognized as a strategy for the identification of anti-cancer drugs (20). There is substantial evidence that alteration in the cellular and molecular pathways that control the cell cycle and apoptosis may change the sensitivity and resistance to anticancer agents (18). Therefore, an understanding of apoptosis events and its pathway may allow the development of novel or even better chemotherapeutic agents for the treatment of cancer.

Our findings show that xanthorrhizol, extracted from *C. xanthorrhiza*, demonstrated antiproliferative action in cultured MCF-7 human breast cancer cells. The dying cells exhibit ultrastructural and biochemical features that characterized apoptosis, as shown by loss of cell viability (21), increased annexin-V binding (34), DNA condensation, DNA fragmentation (30, 38), cell shrinkage (39) and sub-G1-phase accumulation (40, 41). Apoptosis-associated proteins, bcl-2, p53 and PARP-1 play a key role in this process.

In this study, both bcl-2 and bax were expressed in MCF-7 cells. Xanthorrhizol was found to potently downregulate bcl-2 levels. However, there were no apparent changes in bax levels at lower doses of xanthorrhizol, while at higher doses the decrease in bax level was less than the reduction of bcl-2. The resulting net effect throughout the whole study could, thus, lead to a significantly lowered ratio of bcl-2/bax (data not shown), which might be responsible for the xanthorrhizol-induced apoptosis in MCF-7 cells. It has been reported that even when the expression of Bax is conserved but the bcl-2 expression level is lowered, homodimers of bax will still always be formed to stimulate apoptosis (21). Bcl-2 was highly expressed in human malignant estrogen receptor-positive breast tissue to facilitate tumor progression (42).
Figure 2. Determination of apoptosis events in MCF-7 cells after treatment of xanthorrhizol at 5 µg/ml and 10 µg/ml as described in Materials and Methods. (A) The early apoptosis event was detected using the double staining of Annexin-V-FITC and PI. The cell population which underwent early apoptosis (LR) was slightly increased compared to the untreated control cells. On the other hand, the cells in the late apoptosis quadrant (UR) were significantly greater with xanthorrhizol treatment. (B) Nuclear morphology of xanthorrhizol-treated MCF-7 cells was evaluated using Hoechst 33258 staining. No fluorescence was observed in the nucleus of cells without treatment. On the contrary, fluorescence (arrow) was clearly detected in the nuclear region of the treated cells, indicating the apoptotic morphology. (C) Apoptosis was further confirmed by the appearance of a sub-G1 peak in flow cytometry analysis. Treatment with xanthorrhizol was substantially increased the sub-G1 peak in each DNA histogram as compared to the untreated control cells, suggesting apoptosis. UR: Upper right area, LR: Lower right area, UL: Upper left area, LL: Lower left area.
Therefore, the reduction of this protein by xanthorrhizol can serve as a promising tool in restoring sensitivity of these breast cancer cells to apoptotic stimuli.

Under normal conditions, the level of wild-type p53 in MCF-7 cells is very low but will always be expressed in response to DNA damage (27). Xanthorrhizol treatment in this study caused serious DNA damage to MCF-7 cells. The up-regulation of functional p53 resulted in apoptosis instead of cell cycle arrest due to the higher content of p53 throughout the whole experiment (43, 44). From our results, the expression of p53 is not in line with the up-regulation of bax levels. Hence, the highly expressed p53 may initiate apoptosis through other pro-apoptotic proteins, such as Fas (CD95/APO-1) (45, 46), bel-2, bak and cytochrome c (26, 46, 47). Besides, p53 may tilt the balance of bel-2/bax ratio towards apoptosis in the mitochondrial pathway by down-regulating the anti apoptotic bel-2 (48). Interestingly, our observations were similar to a previous study, where xanthorrhizol have been found to initiate apoptosis in HeLa cells by decreasing the level of bel-2 and up-regulating p53 (14).

PARP has been shown as a potential target of novel compounds to counteract PARP-mediated cell death or to enhance the efficacy of cancer chemotherapeutic agents, since the suppression of PARP can improve cell susceptibility to those agents (29, 32, 49). PARP-1 cleavage observed in MCF-7 cells upon xanthorrhizol treatment provided conclusive and definitive evidence for the induction of apoptosis (20, 29, 32). PARP-1 is essential in maintaining genome integrity by its poly(ADP-ribosyl)ation process. 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 (20, 50). However, in our study, PARP-1 (116 kDa) was cleaved into fragments of 85 kDa, which has been reported to possess automodification and a catalytic domain incapable of activation by DNA nicks (31, 32). Thus, they might be involved in certain mechanisms to prevent energy depletion or DNA-damaging signaling. This preserved energy is believed vital in starting an energy-dependent form of programmed cell death, i.e., apoptosis (29, 30, 32).

In this present study, xanthorrhizol showed an enormous potential in combating malignancy by inducing breast cancer cell death via the modulation of bel-2, p53 and PARP-1. Taken together with the organ protective effects and anti-metastatic activity that have been reported (2, 5, 10, 12, 13), we believe that xanthorrhizol may have a great advantage as an antiproliferative agent. However, further studies will be required to delineate the involvement of xanthorrhizol as a potential chemotherapeutic agent. Thus, in-depth understanding of the mechanism of action of xanthorrhizol should lead to a greater ability to evaluate its capacity in the treatment of breast cancer in the future.

Acknowledgements

We thank the Director of the Institute for Medical Research, Kuala Lumpur, Malaysia, for the encouragement and permission to publish this paper. This work received funding from the Malaysia Government Research and Development Fund and the Institute for Medical Research Fund.
References


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Received June 19, 2006
Revised October 13, 2006
Accepted October 17, 2006