Regulation of p53-, Bcl-2- and Caspase-dependent Signaling Pathway in Xanthorrhizol-induced Apoptosis of HepG2 Hepatoma Cells

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Abstract. *Xanthorrhizol is a sesquiterpenoid compound extracted from the rhizome of Curcuma xanthorrhiza. This study investigated the antiproliferative effect and the mechanism of action of xanthorrhizol on human hepatoma cells, HepG2, and the mode of cell death. An antiproliferative assay using methylene blue staining revealed that xanthorrhizol inhibited the proliferation of the HepG2 cells with a 50% inhibition of cell growth (IC_{50}) value of 4.17±0.053 μg/ml. The antiproliferative activity of xanthorrhizol was due to apoptosis induced in the HepG2 cells and not necrosis, which was confirmed by the Tdt-mediated dUTP nick end labeling (TUNEL) assay. The xanthorrhizol-treated HepG2 cells showed typical apoptotic morphology such as DNA fragmentation, cell shrinkage and elongated lamellipodia. The apoptosis mediated by xanthorrhizol in the HepG2 cells was associated with the activation of tumor suppressor p53 and down-regulation of antiapoptotic Bcl-2 protein expression, but not Bax. The levels of Bcl-2 protein expression decreased 24-h after treatment with xanthorrhizol and remained lower than controls throughout the experiment, resulting in a shift in the Bax to Bcl-2 ratio thus favouring apoptosis. The processing of the initiator procaspase-9 was detected. Caspase-3 was also found to be activated, but not caspase-7. Xanthorrhizol exerts antiproliferative effects on HepG2 cells by inducing apoptosis via the mitochondrial pathway.*

Liver cancer is the sixth most common cancer worldwide with an average of 626,000 new cases per year (1). It is the third most common cause of death from cancer after lung and stomach cancer. In Malaysia, liver cancer is the tenth most common cancer among males with an incidence of 378 per 100,000 (2). Hepatocellular carcinoma (HCC), or hepatoma is one of the most lethal liver malignancies and there is no effective treatment to date (3).

Over the past few years, research for new anticancer drugs has focused on natural products which may control cell growth (4). In order to search for a natural bioactive compound with potential as a new chemotherapeutic agent, the rhizomes of *Curcuma xanthorrhiza* Roxb. were selected for investigation. *C. xanthorrhiza*, known locally as Temulawak, originates from Indonesia where it still grows wild, as well as being cultivated in Java (5-8). It is a ginger-like plant of the family *Zingiberaceae* with a round tuber (5, 9).

The rhizome of *C. xanthorrhiza* has been used in folk medicine for the treatment of various ailments (10-18). Several classes of compounds have been identified from the rhizomes of *C. xanthorrhiza* including essential oil, curcuminoids, cinnamaldehyde, starch, fat, cellulose and minerals (11, 12, 14, 19-30). The essential oil of *C. xanthorrhiza* is made up mainly of sesquiterpenoids of which xanthorrhizol is the major constituent (31). Previous studies have reported that xanthorrhizol possesses antibacterial activity (30), antioxidant (32, 33), anti-inflamatory (33) and anti-microbial activity (34, 35), hepatoprotective (36) and nephroprotective effects (37). The extract of *C. xanthorrhiza* has been shown to protect the liver from hepatotoxins such as carbon tetrachloride and acetaminophen and to be useful in the treatment of liver injuries (38). The rhizome of *C. xanthorrhiza* has been utilized as a popular remedy for disorders of the liver and digestive organs in Indonesia (25) and may decrease liver cholesterol (39). Xanthorrhizol has also been found to have anti-tumor activity when tested on Sarcoma 180 ascites in mice (23), to suppress tumor promotion in DMBA-stimulated mouse skin cancer (40) and to exhibit anti-metastatic potential by the attenuation of COX-2,

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MMP-9 and ERK in an experimental mouse lung metastasis model (41). In addition, xanthorrhizol has been discovered to have antiproliferative activity by inducing the modulation of the tumor suppressor protein p53 and the pro-apoptotic Bax in HeLa cervical cancer cells (42). Here, the cytotoxic effects of xanthorrhizol on the HepG2 human hepatocellular carcinoma have been studied.

Materials and Methods

Materials. Xanthorrhizol was kindly provided by Prof. Dr. Azimahtol Hawariah Lope Pihie (Faculty of Science and Technology, National University of Malaysia). Xanthorrhizol was dissolved in DMSO (dimethyl sulfoxide) (Sigma Chemical Co., USA) to prepare a stock solution of 5 mg/ml. DMEM (Dulbecco's modified Eagle's medium) and penicillin/streptomycin were obtained from Gibco BRL Life Technologies (New York, USA). FBS (fetal bovine serum) was purchased from Mycoplex (Austria). Triton X-100 and propidium iodide (PI) were purchased from Sigma Chemical Co. DeadEnd™ Flurometric TUNEL (Tdt-mediated dUTP nick end labeling assay) System Kit was procured from Promega (USA). The antibodies against p53, Bcl-2, Bax, caspase-9, caspase-7, caspase-3 and β-actin were purchased from Pharmingen (USA).

Cell culture and cytotoxicity assay. HepG2, Chang's Liver and Vero cell lines were obtained from ATCC (American Type Culture Collection) and maintained in DMEM supplemented with 5% FBS and penicillin/streptomycin in a 37˚C, 5% CO_2 incubator. The medium was changed every two days. For the cytotoxicity assay, cells were treated with xanthorrhizol at increasing concentrations to evaluate its *in vitro* antitumor activity. The IC_{50} (50% inhibition of cell growth) values were obtained for these cell lines, as previously described (43).

Apoptotic cell detection. HepG2 cells were untreated or treated with 4 μg/ml xanthorrhizol for 24, 48 and 72 h. DNA fragmentation characteristic of apoptotic cells was quantified by TUNEL with the DeadEnd™ Flurometric TUNEL System Kit according to the manufacturer's instruction, and then investigated using fluorescent microscopy. The Apoptotic Index was calculated for six random microscopic fields at x1000 magnification.

Protein extraction and Western blotting analysis. Protein extracts were prepared as described in detail previously (44). Briefly, cells were lysed with cell lysis buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, 25 mM NaF, 100 μM Na_3VO_4 and 1 mM DTT. The lysates were then sonicated for 10 sec twice and the supernatants were boiled in SDS sample buffer for 5 min. Protein concentration was determined by using a Bradford assay (45). Protein aliquots of 20 μg from both the untreated and treated cells were separated on 15% SDS-polyacrylamide gels and electrophoretically transferred to PVDF membrane (PerkinElmer Life Sciences Inc., Boston, MA, USA). The membranes were dried, preblocked with 5% non-fat milk in phosphate-buffered saline and 0.1% Tween-20, then incubated with a primary antibody for p53, Bcl-2, Bax, caspase-3, -7, -8 and -9 diluted 1:1500 and detected with horseradish peroxidase-labeled antibodies to mouse IgG. Following exposure on a Kodak OMAT X-ray film, a densitometric analysis was performed with a GS 670 Imaging Densitometer with the Molecular Analyst software (Bio Rad, Hercules, USA). Beta-actin was used as internal control to confirm equal loading of protein.

Results

Effect of xanthorrhizol on cell viability. The cytotoxicity of xanthorrhizol on the HepG2, Chang's Liver and Vero cells is shown in Figure 1. HepG2 cells were treated with xanthorrhizol in the concentration range of 0.2 to 50 μg/ml. Control cells received only DMSO at a final concentration of 1% (v/v) or less. Xanthorrhizol significantly reduced HepG2 cell viability in a dose-dependent manner. At a concentration of 4.17±0.03 μg/ml xanthorrhizol, the HepG2 cell viability decreased to 50%. Xanthorrhizol also significantly reduced non-malignant Chang's Liver and Vero cell viability with IC_{50} values of 8.7±0.065 μg/ml and 6.6±0.061 μg/ml, respectively. Despite killing non-malignant cells, xanthorrhizol was found to have a greater propensity to kill malignant cells as indicated by the lower IC_{50} values with the cancer cells.

Xanthorrhizol inhibited the proliferation of HepG2 cells through the induction of apoptosis. The xanthorrhizol-treated HepG2 cells were labeled to visualize the extent of DNA fragmentation in a time-course manner. Fragmented DNA produces 3'-OH DNA ends that can be labeled with fluorescein-12-dUTP using the principle of the TUNEL assay. The labeled DNA was then visualized directly by fluorescence microscopy (Figure 2) and the percentage of apoptotic cells was quantified from the average of at least six separate experiments (Figure 3). The TUNEL assay demonstrated the typical appearance of apoptosis fragmented DNA, elongated lamellipodia and many detached cells following xanthorrhizol treatment. Xanthorrhizol treatment increased the number of apoptotic cells up to >70% by 24 h in contrast to less than 5% in untreated cells.

Effects of xanthorrhizol on the levels of Bcl-2 and Bax. The Bcl-2 and Bax proteins have been shown to be associated with anti-apoptotic and pro-apoptotic functions. Bcl-2, which is an anti-apoptotic protein, binds to Bax, thereby blocking Bax-induced apoptosis (46-48). To explore the possible role of Bcl-2 family members in the xanthorrhizol-induced apoptosis, the effects of xanthorrhizol on the expression of Bcl-2 and Bax protein was examined by Western blot analysis. As shown in Figure 4, 4 μg/ml of xanthorrhizol decreased the Bcl-2 protein level in contrast to the levels of the control cells at 24 h. Bax protein expression, however, remained unchanged in HepG2 cells after treatment with xanthorrhizol. This indicates that Bax is not involved in xanthorrhizol-mediated apoptosis in HepG2 cells.

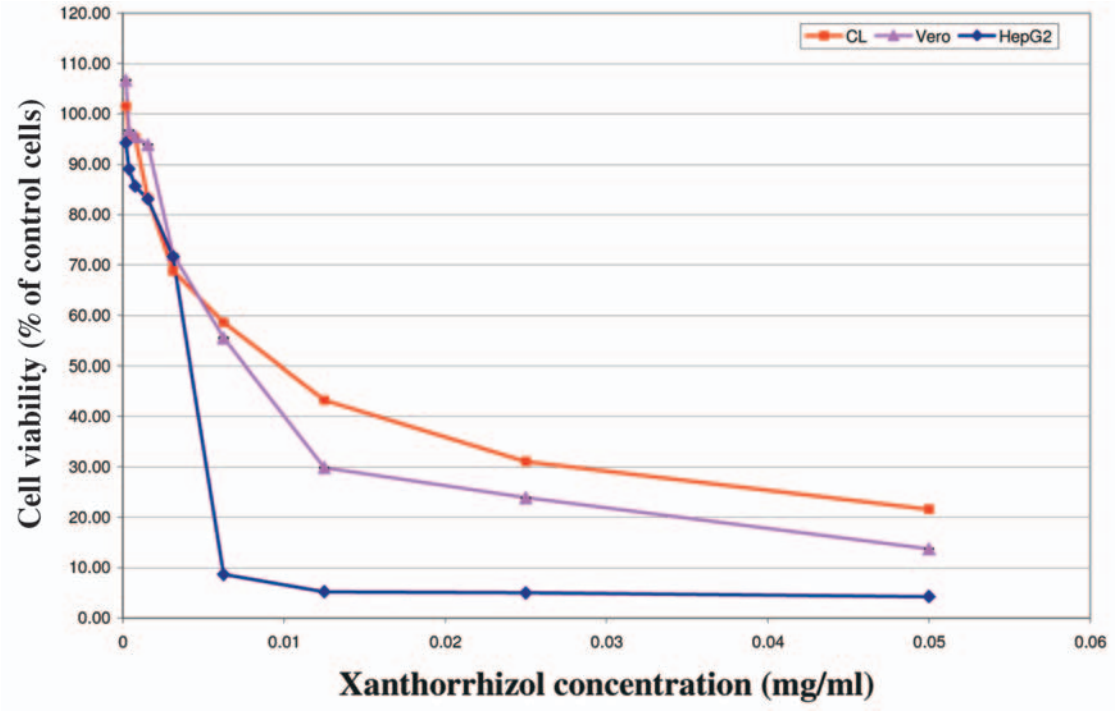

Figure 1. *Effect of xanthorrhizol on cell viability. Treatment by xanthorrhizol of HepG2, Chang's Liver and Vero cells significantly decreased the number of viable cells in a dose-dependent manner. The IC_{50} obtained for HepG2 cells was 4.17±0.03 μg/ml. The IC_{50} for non-malignant Chang's Liver and Vero cells was 8.7±0.065 μg/ml and 6.6±0.061 μg/ml, respectively. Cytotoxicity was determined by methylene blue assay and was expressed as mean±S.E.M. of three separate experiments.*

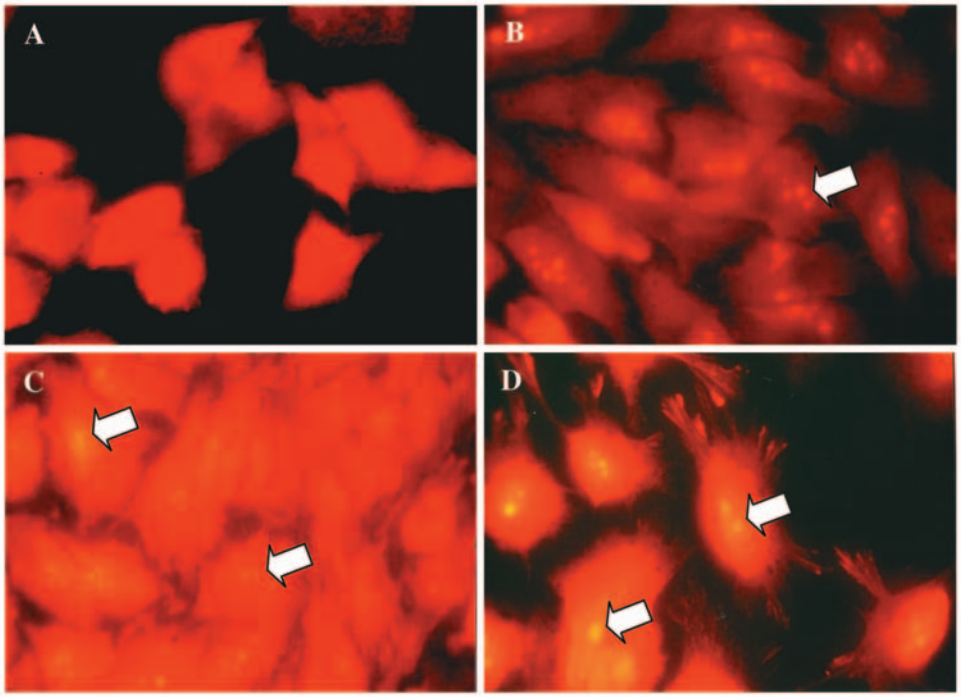

Figure 2. *TUNEL labeling of xanthorrhizol-treated HepG2 cells. Untreated and treated cells were identified for DNA fragmentation by TUNEL assay. No fluorescence was detected in the nucleus of untreated cells (A), as the cells were not apoptotic and did not exhibit DNA fragmentation. In cells treated with 4 μg/ml xanthorrhizol, fluorescence was detected in the nuclear region, indicating DNA fragmentation and nuclear condensation, characteristic of apoptosis. At 48 h (C) and 72 h (D) post xanthorrhizol treatment, the intensity of fluorescence was more apparent when compared to 24 h (B), indicating more DNA fragmentation.*

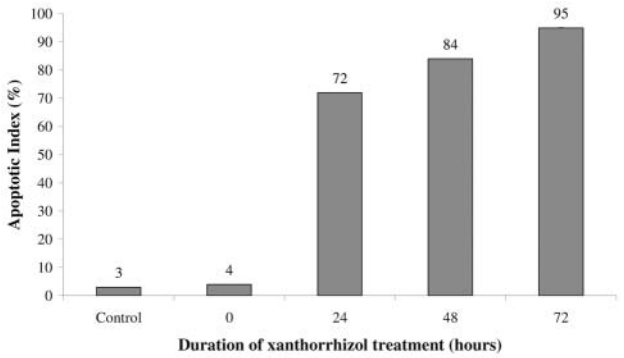

Figure 3. *Apoptosis levels as analyzed by TUNEL assay. Xanthorrhizol treatment significantly increased the level of apoptosis in HepG2 cells compared to the control. Apoptotic cells were counted on at least six independent slides.*

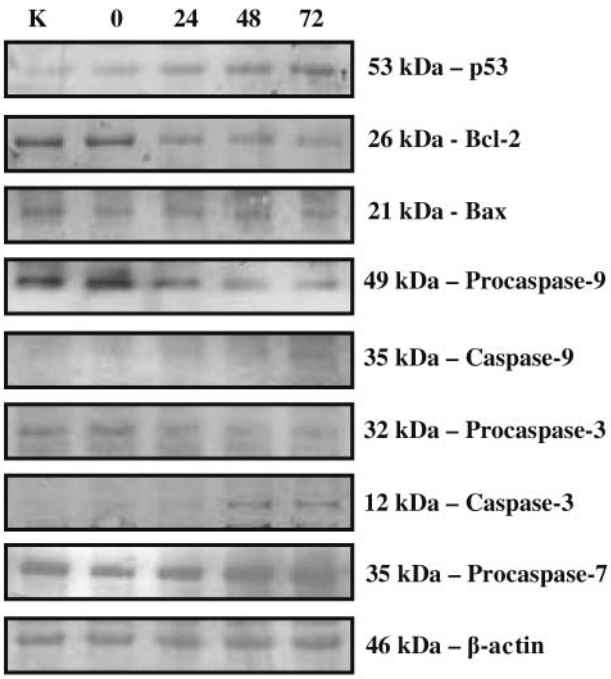

Figure 4. *Western blot analysis of p53, Bcl-2, Bax, caspase-3, -7 and -9 proteins in HepG2 cells. HepG2 cells treated with 4 μg/ml xanthorrhizol for indicated times were resolved on a 15% PAGE and submitted to Western blotting. p53, caspase-3 and -9 protein expression increased after 24 h following xanthorrhizol treatment, while Bcl-2, procaspase-3 and -9 levels decreased. Bax and procaspase-7 levels were not altered and remained low throughout the experiment. β-actin did not show any changes indicating equal loading (internal control).*

Xanthorrhizol increased p53 protein expression. To determine whether Bcl-2 down-regulation was caused *via* a p53-dependent pathway, the effect of xanthorrhizol on the expression of p53 protein in HepG2 cells (expressing wild-type p53) was examined. A marked induction of p53 protein was observed (Figure 4) in a time-dependent manner and the p53 protein level in xanthorrhizol-treated cells increased within 24 h of the commencement of treatment. This indicates that xanthorrhizol-induced apoptosis was mediated *via* a p53-dependent pathway, which is consistent with studies elsewhere (42).

Xanthorrhizol induced caspase activation. Accumulated results indicate that the caspases play a pivotal role in the terminal, execution phase of apoptosis induced by diverse stimuli (48, 49). To see whether, caspases were involved in xanthorrhizol-induced cell death, or not, the expression and activation of caspases were examined. As shown in Figure 4, in the HepG2 cells, xanthorrhizol treatment caused a time-dependent increase in caspase-3 and -9 activities which was evident at 24 h and gradually increased over 72 h. The activity of caspase-7, however, was unaffected by xanthorrhizol treatment. These result demonstrated that caspase-3 and -9 were involved in the xanthorrhizol-induced death pathway.

Discussion

In cancer therapy, tumor growth can be suppressed by activating the apoptotic machinery in the cell (50-52). Many malignant cells, however, have been shown to be unable to regulate the genes that control apoptosis, rendering them resistant to the induction of apoptosis by a variety of stimuli, including chemotherapeutic drugs and radiotherapy (53). In the present study, xanthorrhizol exerted antiproliferative activity on the growth of HepG2 cells by apoptosis, which was confirmed by DNA fragmentation employing the TUNEL assay.

Apoptosis is a tightly regulated process under the control of several signaling pathways, such as the mitochondrial pathway and caspase cascade (49, 54, 55). In the present study, xanthorrhizol treatment induced the activation of the tumor suppressor protein p53 and caused the anti-apoptotic protein Bcl-2 to decrease in a time-response manner without affecting the expression of the pro-apoptotic protein Bax. Moreover, a time-dependent activation of caspase-3 and -9 was also observed in the xanthorrhizol-treated HepG2 cells.

In particular, the anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax have been reported to regulate the induction of apoptosis at least through the control of mitochondrial function (56-58). The capacity of Bcl-2 and Bax to compete for one another *via* heterodimers suggests a reciprocal relationship in which Bcl-2 monomers or homodimers favour survival and Bax homodimers favour death (47). The ion channel perforation activity of Bcl-2 and Bax may control apoptosis by influencing the permeability

of the membranes and cytochrome *c* release from mitochondria. Overexpression of Bcl-2 blocks cytochrome c release in response to a variety of apoptotic stimuli (56, 57, 59, 60). Down-regulation of Bcl-2 thus contributes to cytochrome *c* release from mitochondria. Moreover, Bcl-2 heterodimerization with Bax exerts dominant negative inhibition of pro-apoptotic Bax activity (47). Therefore, when the Bcl-2 expression level is low and the Bax expression level is maintained, homodimers of Bax will always be formed and apoptosis will be stimulated (61). Previous reports have documented that the ratio between pro- and anti-apoptotic proteins determines, in part, the susceptibility of cells to a death signal (62-64). From this study, it is suggested that down-regulation of Bcl-2 in xanthorrhizol-treated HepG2 cells was induced by p53. p53 has been shown to down-regulate Bcl-2 *via* binding to a negative regulatory element outside of the Bcl-2 gene promoter (65).

Once released from the mitochondria, cytochrome *c* interacts with Apaf-1 favoring the activation of procaspase-9 (48, 49, 66). Caspase-9 can modulate the activation of the executioner caspases, caspase-3 and -7, by proteolysis, thereby transmitting the apoptotic signal to the execution phase (67). When the HepG2 cells were treated with xanthorrhizol, the 49 kDa procaspase-9 slowly diminished in the course of experiment. The xanthorrhizol treatment did not lead to the activation of caspase-7 since the active p17 subunit could not be detected. As processing of this caspase did not occur, it is possible that the other executioner caspase, caspase-3, may be involved in xanthorrhizol-induced apoptosis. Here, it was confirmed that treatment with xanthorrhizol resulted in the activation of procaspase-3, seen as the formation of a 12 kDa protein which corresponds to the catalytically active subunit (48). Caspase-7 is highly related to caspase-3 and shows the same synthetic substrate specificity (68), suggesting that caspase-3 and -7 may have overlapping roles in apoptosis (69).

According to the present data, xanthorrhizol led to p53 activation and Bcl-2 reduction which then activated mitochondria-mediated downstream molecular events including activation of caspase-9 and caspase-3. In conclusion, xanthorrhizol can effectively induce apoptosis of HepG2 cells. The induction of apoptosis by xanthorrhizol involved the activation of a mitochondria-mediated caspase cascade and the inhibition of the anti-apoptotic protein Bcl-2, which was consistent with several studies which have suggested that mitochondria may play a major role in drug-induced apoptosis (70, 71). However, Bax and caspase-7 are not involved in xanthorrhizol-mediated apoptosis. Given that hepatocellular carcinoma is one of the most lethal malignancies with as yet no effective therapy, our findings may suggest a potential use of xanthorrhizol for hepatocellular carcinoma treatment.

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