[PHAR04] Xanthorrhizol Induces Apoptosis via the Up-Regulation of Bax and p53 in HeLa Cells

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Introduction

Curcuma xanthorrhiza Roxburgh (Roxb.) or Temulawak is well known by the local people as a remedy for various ailments. It can also be taken as a nutritional supplement. Essential oil is one of the important and beneficial products from *C.xanthorrhiza*. The oil of *C.xanthorrhiza* is made up of mainly sesquiterpenoids of which xanthorrhizol (44.5%) is the major constituent (Ibrahim *et al*., 1999). According to Yasni *et al*. (1994), the major component (approximately 65%) of the essential oils was identified as α-curcumene by chromatography - mass spectrometry (Yasni *et al*., 1994).

According to Itokawa *et al*. (1985), xanthorrhizol is one of the major antitumor constituents when tested on Sarcoma 180 ascites in mice (Itokawa *et al*., 1985). Xanthorrhizol was also found to inhibit the COX-2 or iNOS which are the important mediators in inflammation and carcinogenesis respectively (Lee *et al*., 2002). It shows that xanthorrhizol has both anti-inflammatory and chemopreventive properties. In Thailand, *C. xanthorrhiza* dried rhizome has been used to treat inflammation in postpartum uterine bleeding (Suksamrarn *et al*., 1994).

Cancer of the uterine cervix is currently the second most common cancer among females in Malaysia after breast cancer where the incidence is 11.6 per 100,000 populations. For the past twenty years the Annual Reports of Ministry of Health recorded an average of 2200 new cases per year (Ministry of Health Annual Reports (1980-2000). This number is higher than the speculated 16 cases per 100,000 females in this country where the population of females is about 10.5 million (Nor Hayati, 2003).

Cervix is the lower part of the uterus and very highly threatened by the human papillomavirus (HPV). High risk HPVs, code for at least 3 proteins for growth stimulation and transformation. These are E5, E6 and E7 (Zur Hausen, 2000). The HPV oncoproteins target p53 protein for degradation, leading to abolition of G1 arrest or apoptosis in response to ionizing radiation and DNA damaging agents (Fan *et al.,* 1994). Cervical cancer chemotherapy *in vivo* improved in cases with high p53 expression in the tumor tissue (Garzetti *et al*., 1996). Apoptosis is the ability of a cell to self-destruct by the activation of an intrinsic cellular program when the cells are seriously damaged or no longer needed. There is much evidence to show that most of cancer therapy drugs kill tumor cells through apoptosis (Hannun, 1997). BAX is a protein from the BCL-2 family which has been associated with apoptotic cell death *in vitro* and *in vivo*. The ratio of various BCL-2 family members control apoptosis (Reed *et al*., 1996).

Over the past 10 years, research for new drugs to be used in oncology has refocused on natural products. The rediscovery of natural products has obtained many potent compounds including xanthorrhizol. This research investigated the *in vitro* antiproliferative and anticancer activity of xanthorrhizol against cervical cancer cell line HeLa as well as the mechanism of its action.

Materials and Methods

Cell culture. HeLa (HPV 18-positive), Chang's Liver and MDBK were obtained from American Type Culture Collection (ATCC) and cultured under the standard condition (95% air, 5% CO_2 at 37°C) in the Dulbecco's modified Eagle Medium (DMEM); containing 5% fetal calf serum (FCS), fungizone and antibiotics. Xanthorrhizol was obtained from Center for Engineering and Pilot Plant (CEPP), University of Technology Malaysia.

Antiproliferative assay. Parallel cultures of HeLa, Chang Liver and MDBK cells were conducted in 96 well plates, using triplicate wells for each treatment. Following overnight

incubation to allow attachment and at 70 to 90% confluency, cells were treated for 72 hours with xanthorrhizol, tamoxifen (positive control) and DMSO (negative control) in the range of final concentration 0.02μg/ml to 5.00μg/ml. To measure cell viability, the methylene blue method was used (Lin & Hwang, 1991).

Apoptotic Index. Staining with Hoechst 33258 was performed as described elsewhere (Hishikawa *et al*., 1999). Cells were grown on microscope slides and fixed by immersing slide in 4% methanol-free paraformaldehyde in PBS for 30 minutes at 4^oC. After washing with PBS, cells were incubated in Hoechst 33258 (Sigma) at final concentration of 30 μg/ml at room temperature for 30 minutes. Nuclear morphology then observed under a fluorescent microscope. DNA fragmentation which is the characteristic of apoptotic cells was evaluated by Tdt-mediated dUTP nick end-labeling (TUNEL) using the Apoptosis Detection Kit, Fluorescein (Promega) according to the manufacturer's instruction.The cells from four random microscopic fields were counted to get the percentage of TUNEL positive cells.

Western blotting. 30 μg of protein from both xanthorrhizol treated and untreated cells were separated on 12 -15% SDS-polyacrylamide gels. After electrophoresis, the proteins were blotted onto polvynyl-difluoride (PVDF) membranes (PolyScreen, New Life Science). The membranes were dried, preblocked with 6% non-fat milk in PBS-Tween (0.1%), then incubated with the primary antibodies (p53, E6, bcl-2 and bax) diluted 1: 2000. The p53 antibody used can detect both wild type and mutant p53 protein. The secondary antibody used was horseradish peroxidase-labeled to rabbit or mouse IgG. A densitometriy analysis was performed using a GS 670 Imaging Densitometer with software Molecular Analyst (BioRad, Hercules, USA) after the exposure on a Kodak OMAT x-ray film. The membranes were reprobed with β-actin antibodies (Sigma) as an internal control and to confirm the equal loading.

Immunostaining. This method is used to detect the expression of Bax and p53. The cells were fixed on slides and permeabilized with 0.2% Triton X-100 for 20 min at 4^oC and blocked with 2% FCS in PBS for 2 h at 37^oC. Next, the cells were washed and incubated overnight with anti-bax and anti-p53 antibodies at a 1:250 dilution at 4^oC. Then, the slides were incubated with secondary antibodies conjugated with FITC. The slides were visualized under a fluorescence microscope.

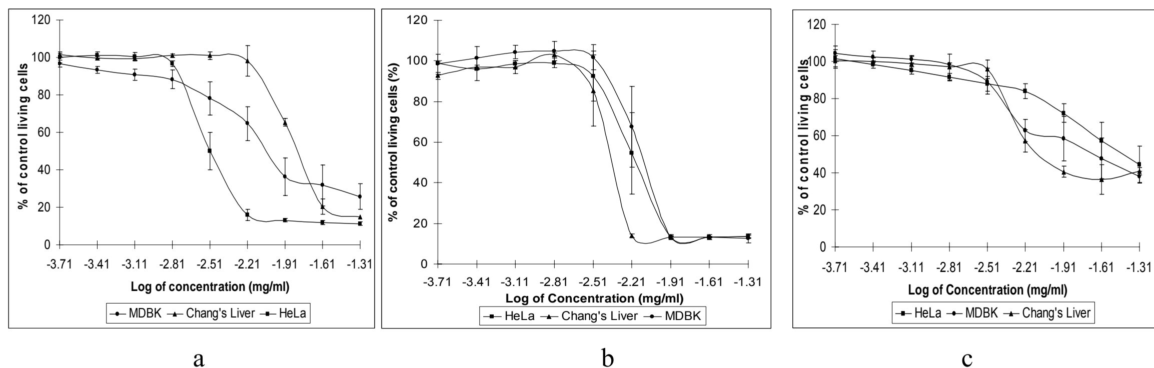

FIGURE 1 Cytotoxic effects of xanthorrhizol on cell viability. (a) Treatment of xanthorrhizol on HeLa cells significantly decreased the number of viable cells in a dose-dependent manner. The EC_{50} obtained was 6.16μg/ml. The EC_{50} for non-malignant Chang's Liver and MDBK cells were 29.17μg/ml and 17.38μg/ml respectively; which were higher than HeLa. (b) Treatment of tamoxifen showed the non-cytoselective effect on HeLa, Chang's Liver and MDBK with EC_{50} values, 6.44μg/ml, 4.37μg/ml and 7.65μg/ml respectively. (c) Treatment of cisplatin was less effective compared to xanthorrhizol and tamoxifen with EC_{50} values, 74μg/ml, 15.49μg/ml and 44.67μg/ml for HeLa, Chang's Liver and MDBK respectively. Cytotoxicity was determined by methylene blue assay and was expressed as mean ± S.E.M of three separate experiments.

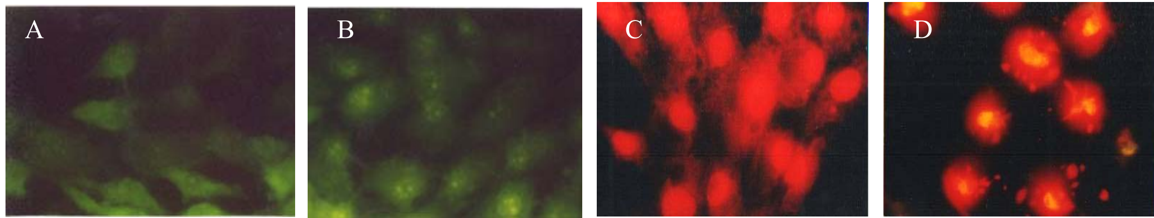

FIGURE 2 Change of apoptotic morphology and TUNEL labeling in xanthorrhizol-treated cells. Nuclear morphology was observed by staining with the nuclear fluorochrome Hoechst 33258 and DNA fragmentation were identified by TUNEL assay as described in Materials and Methods. Cells with condensed and fragmented nuclei and apoptotic bodies are seen in xanthorrhizol-treated cells for 48 h (B), but not in the cells without treatment (A). No fluorescence was detected in the nucleus of untreated cells (C) as the cells were not apototic and did not exibit DNA fragmentation. In cells treated with xanthorrhizol for 48 h (D), fluorescence was detected in the nuclear region, showing the apoptotic characteristics. Xanthorrhizol treatment significantly increased the level of apoptosis in HeLa cells compared to the controls. Apoptosis cells were counted on at least four independet slides. Magnification X1000.

Results

Effect of xanthorrhizol on cell viability

Using methylene blue assay, the cytotoxicity of xanthorrhizol on HeLa, Chang's Liver and MDBK cells are shown in FIGURE 1. HeLa cells were treated with xanthorrhizol in the range of 0.2 to 5µg/ml.Tamoxifen and cisplatin were used as positive controls while DMSO as negative control. Xanthorrhizol significantly reduced HeLa cell viability in a dose-dependent manner. Xanthorrhizol decreased 50% of the HeLa cells viability at 6.16µg/ml. Although it also affected the normal cells, the EC_{50} values for normal cells were much higher than HeLa.

Xanthorrhizol-induced apoptotic cell death

Staining with Hoechst 33258 showed fragmentation and condensation of chromatin in HeLa cells treated with 6µg/ml xanthorrhizol for 24 h and 72 h (Fig. 2B & 2C), compared to untreated control (Fig. 2A). Control cells exibited a normal nuclear morphology characterized by a diffuse chromatin structure concentration and therefore light staining. The fragmented DNA produces 3-OH DNA ends that can be labeled with fluorescein-12-dUTP using the principle of TUNEL assay. The extent of DNA fragmentation was visualized by labeling the xanthorrhizol-treated cells in a time-course manner. Apoptotic cells were counted on at least four independent slides. Each experiment was repeated at least three times.

Xanthorrhizol up-regulated the bax and p53 protein expression

To determine which apoptosis-related proteins are regulated by xanthorrhizol, the expression of p53, bax, bcl-2 and E6 protein was measured after 6µg/ml xanthorrhizol treatment for 2, 6, 12 and 24 h in HeLa cells using western blot analysis. Exposure of HeLa cells to xanthorrhizol increased the pro-apoptotic protein p53 and bax. However, the expression of anti-apoptotic bcl-2 and E6 did not show any change in neither the control nor the treated cells throughout the treatment period (Fig. 3A). The up-regulation of p53 and bax protein by xanthorrhizol was confirmed by the immunostaining of both proteins (Fig. 3B), as well as bcl-2 and E6 (data not shown). This result implies that apoptosis induced by xanthorrhizol may be mediated by the p53 and bax pathway in cervical cancer cells.

Discussion

The normal epithelium of the cervix is constantly undergoing regeneration which is a maintenance program for tissue-specific homeostasis by establishing the equilibrium between cellular proliferation and apoptosis. Interruption in these processes can let the viral invaders to impair normal cells to neoplastic transformation. The cause for poor responsiveness to chemotherapy lies in the etiopathogenesis of cervical cancer such as HPV infection and loss of tumor suppressor gene function due to inactivation of p53 and Rb by HPV encoded viral oncoproteins. The

restoration of p53 levels could be a potential strategy to increase responsiveness. However, different authors had differring reports regarding the role of p53 and chemosensitivity (Fan *et al.,* 1994; Ceraline *et al,*. 1998; Pestell *et al*., 2000).

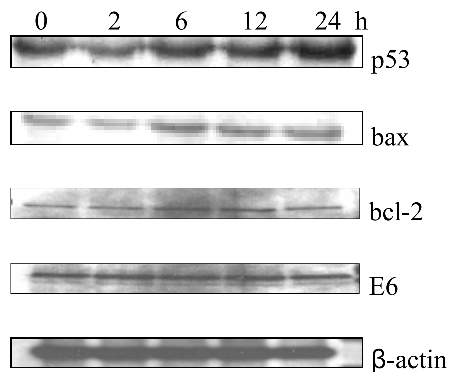

FIGURE 3 Western blot analysis of p53, bax, bcl-2, E6. HeLa cells treated with 6μg/ml xanthorrhizol for indicated times were resolved on a 15% PAGE and submitted to Western blotting. p53 and bax protein expression increased after 6 h following xanthorrhizol treatment, while bcl-2 and E6 levels were not altered and remain low throughout the experiment. β-actin did not show any changes indicating equal loading (internal control).

There was an obvious association between the accumulation of p53 and both HPV 16/18 infection and expression of the E6 protein (Nair *et al*., 1999). It has been demonstrated that p53 protein complexes with the HPV 16/18 E6 protein in the cytoplasm and lead to p53 degradation via ubiquitination (Liang *et al*., 1993; Picksley & Lane, 1996). E6 can interfere with the normal function of p53 by its ability to abrogate both transcriptional activation and transcriptional repression function of the gene (Lechner *et al*., 1992; Miyake *et al*., 2000). In this respect E6 shows similarity to certain mutant forms of p53 which also fail to function as transcriptional regulators themselves and may inactivate the wild type p53 after oligomerization.

The present study showed that the p53 protein level in the xanthorrrhizol-treated cells were increased 6 h after the xanthorrhizol treatment in a time-dependent manner. The BAX expression however, was increased after 6 h and maintained at higher level than the controls throughout the experiment. The bcl-2 and E6 levels were unchanged and maintained a low level throughout the experiment. P53 protein expression was slightly higher in the cells without treatment compared to 2 h after treatment.

Half life of the mutant form is 4-6 hours while the wild type p53 has a short half life (6-20 minutes). Thus detection of p53 by immunostaining is often considered to reflect the mutant form (Hassapoglidou *et al*., 1993; Reihsaus *et al*., 1990). However this could not be true in all cases, as the immunocytochemical analysis of p53 protein vary according to the antibody used. Also, in cervical cancer, previous studies showed that p53 gene mutation was infrequent (Kurvinen *et al*., 1994; Lakshmi *et al*., 1997). In a mutant specific p53 analysis of cervical cancer tissues, only 12 of 230 samples studied were found to be positive (Nair *et al*., 1999). The function of normal p53 can be attenuated by E6 of high risk HPVs or possibly by other endogenous proteins such as MDM-2; resulting in a functionally inactive form.

From this study, it is suggested that xanthorrhizol induced apoptosis in HeLa by upregulating p53 protein levels and therefore increased the expression of both p53 and BAX proteins while suppressing BCL-2 protein. Several lines of evidence demonstrate the mechanisme by which p53 induces apoptosis. P53 may regulate the gene product of the pro-apoptotic protein bax via transcriptional activation (Miyashita *et al*., 1994). Previous study also showed that p53 can transcriptionally repress expression of anti-apoptotic bcl-2 (Miyashita *et al*., 1994). Utilizing a temperature sensitive version of p53, conditional restoration of p53 activity in a p53-deficient leukemia cell line resulted in decrease bcl-2; increased bax and stimulated apoptosis (Miyashita *et al*., 1993; Selvakumaran *et al*., 1994). The product of the *BAX* gene has antagonistic effects over the BCL-2 protein, and BAX expression accelerates apoptosis (Oltvai *et al*., 1993).

The promoter of the human *BAX* gene has been shown to contain several consensus sequences for p53 binding and is strongly transactivated by p53. Thus, the effects of p53 on apoptosis may be mediated in part through its effect on the expression of bcl-2 and bax (Miyashita & Reed, 1995). However, the transcriptional activation of p53 target genes and *de novo* synthesis of their products are not obligatory for p53 to induce apoptosis in

certain experimental models. In the presence of actinomycin D or cycloheximides, which block RNA synthesis, p53-mediated apoptos is still occurred (Caelles *et al*., 1994). Since anticancer agents can kill tumor cells through apoptosis thus the increase in p53 and bax protein expression may restore sensitivity to apoptotic stimuli in cervical cancer cells. The results in this study provided evidence that xanthorrhizol from *C. xanthorrhiza* was able to inhibit the proliferation of cervical cancer cells, HeLa by inducing apoptotic cell death. Additional studies are necessary to determine the downstream effector molecules in the apoptotic pathway. Understanding the functional role of apoptotic events and their regulation by interfering pathways, will provide new insights into mechanisms involved in malignant cell proliferation and avenues to combat malignancy.

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