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Xanthorrhizol Induces Apoptosis *Via* the Up-regulation of Bax and p53 in HeLa Cells

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Abstract. *Xanthorrhizol is a sesquiterpenoid compound extracted from Curcuma xanthorrhiza, which is known locally as Temulawak. Traditionally, C. xanthorrhiza was found to have antibacterial, anticancer and anti-inflammatory activity. The rhizome has also been used to treat inflammation in postpartum uterine bleeding. An antiproliferative assay using methylene blue staining revealed that xanthorrhizol inhibited the proliferation of the cervical cancer cell line HeLa with an EC_{50} value of 6.16 μg/ml. Xanthorrhizol significantly increased apoptosis in HeLa cells, as evaluated by the Tdt-mediated dUTP nick end-labelling (TUNEL) assay and nuclear morphology by Hoechst 33258 staining. Western blot analysis, which was further confirmed by the immunostaining results, implied an up-regulation of tumor suppressor protein p53 and the pro-apoptotic protein Bax, following the treatment with xanthorrhizol. Xanthorrhizol, however, did not affect the expression of the anti-apoptotic protein, Bcl-2 and the viral oncoprotein, E6. Hence, xanthorrhizol is a promising antiproliferative and anticancer agent which induces p53 and Bax-dependent apoptosis in HeLa cervical cancer cells.*

Curcuma xanthorrhiza Roxburgh (Roxb.) is well known as a remedy for various ailments. It can also be taken as a nutritional supplement. The oil of *C. xanthorrhiza* is made up of mainly sesquiterpenoids of which xanthorrhizol (44.5%) is the major constituent (1). According to Yasni *et al.*, the major component (approximately 65%) of the essential oils was identified as α-curcumene by chromatography - mass spectrometry (2). On the contrary, the analysis done by Zwaving and Bos revealed that the essential oil consists of ar-curcumene (41.4%) and xanthorrhizol (21.5%) (3).

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According to Itokawa *et al.*, xanthorrhizol was one of the major antitumor constituents when tested on Sarcoma 180 ascites in mice (4). Xanthorrhizol was also found to inhibit COX-2 or iNOS, which are the important mediators in inflammation and carcinogenesis, respectively (5), showing 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 (6).

Cancer of the uterine cervix is currently the second most common cancer among females in Malaysia after breast cancer, with an incidence of 11.6 per 100,000 population. For the past twenty years, the Annual Reports of the Ministry of Health recorded an average of 2,200 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 female population is about 10.5 million (7).

The cervix is 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 (8). 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 (9). Cervical cancer chemotherapy *in vivo* improved in cases with high p53 expression in the tumor tissue (10, 11). 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 cancer therapy drugs kill tumor cells through apoptosis (12). 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 (13).

Over the past 10 years, research for new drugs to be used in oncology has refocused on natural products, and many potent compounds have been obtained including xanthorrhizol. This research investigated the *in vitro* antiproliferative and anticancer activity of xanthorrhizol

against the 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 the American Type Culture Collection (ATCC) and cultured under standard conditions (95% air, 5% CO_2 at 37˚C) in Dulbecco's modified Eagle Medium (DMEM), containing 5% fetal calf serum (FCS), fungizone and antibiotics. Xanthorrhizol was obtained from the Center for Engineering and Pilot Plant (CEPP), University of Technology, Malaysia.

Antiproliferative assay. Parallel cultures of HeLa, Chang's 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 h with xanthorrhizol, tamoxifen (positive control) and DMSO (negative control) in the final concentration range 0.02 μg/ml to 5.00 μg/ml. To measure cell viability, the methylene blue method was used (14).

Apoptotic index. Staining with Hoechst 33258 was performed as described elsewhere (15). Cells were grown on microscope slides and fixed by immersing the slide in 4% methanol-free paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 4˚C. After washing with PBS, the cells were incubated in Hoechst 33258 (Sigma Chemical Co., St Louis, USA) at a final concentration of 30 μg/ml, at room temperature for 30 min. Nuclear morphology was then observed under a fluorescent microscope. DNA fragmentation, which is the characteristic of apoptotic cells, was evaluated by Tdt-mediated dUTP nick end-labelling (TUNEL) using the Apoptosis Detection Kit, Fluorescein (Promega Inc., USA), 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. Thirty 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 polyvinyl-difluoride (PVDF) membranes (PolyScreen, NEN Life Sciences, USA). 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 labelled to rabbit or mouse IgG. A densitometriy analysis was performed using a GS 670 Imaging Densitometer with software Molecular Analyst (BioRad, Hercules, USA) after exposure on a Kodak OMAT X-ray film. The membranes were reprobed with β-actin antibodies (Sigma) as an internal control and to confirm equal loading.

Immunostaining. This method was 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˚C and blocked with 2% FCS in PBS for 2 h at 37˚C. Next, the cells were washed and incubated overnight with anti-Bax and anti-p53 antibodies at a 1:250 dilution at 4˚C. Then, the slides were incubated with secondary antibodies conjugated with FITC. The slides were visualized under a fluorescence microscope.

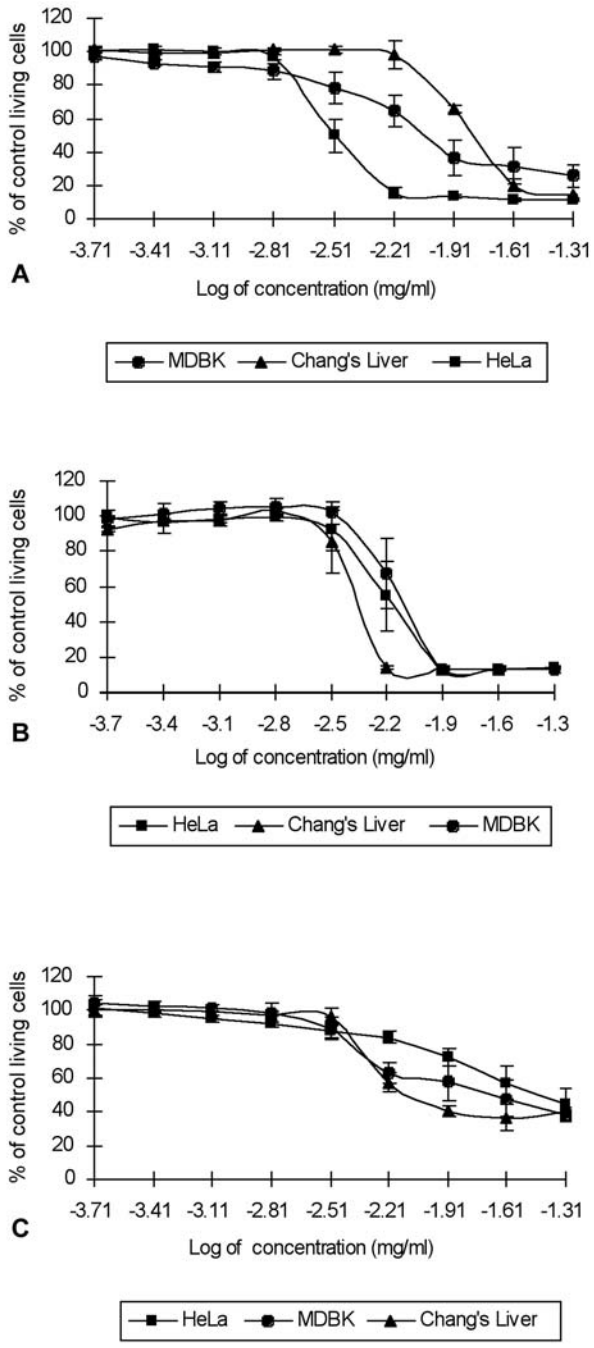


Figure 1. *Cytotoxic effects of xanthorrhizol on cell viability. (A) Treatment of HeLa cells with xanthorrhizol significantly decreased the number of viable cells in a dose-dependent manner. The EC_{50} obtained was 6.16 μg/ml. The EC_{50} for non-malignant Chang's Liver and MDBK cells were 29.17 μg/ml and 17.38 μg/ml, respectively, which were higher than HeLa. (B) Treatment with tamoxifen showed the non-cytoselective effect on HeLa, Chang's Liver and MDBK with EC_{50} values of 6.44 μg/ml, 4.37 μg/ml and 7.65 μg/ml, respectively. (c) Treatment with cisplatin was less effective compared to xanthorrhizol and tamoxifen with EC_{50} values of 74 μg/ml, 15.49 μg/ml and 44.67 μg/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.*

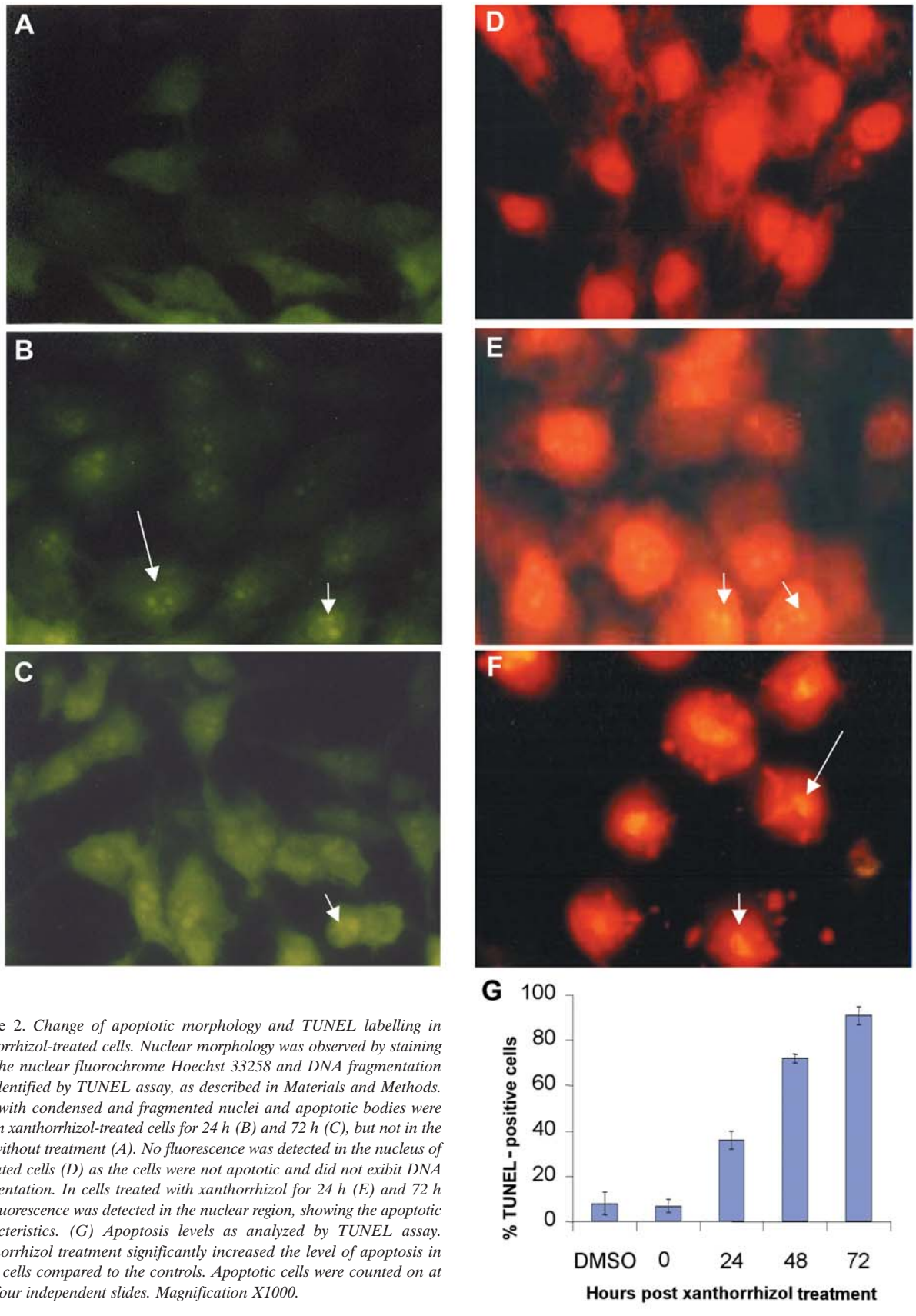


Figure 2. *Change of apoptotic morphology and TUNEL labelling in xanthorrhizol-treated cells. Nuclear morphology was observed by staining with the nuclear fluorochrome Hoechst 33258 and DNA fragmentation was identified by TUNEL assay, as described in Materials and Methods. Cells with condensed and fragmented nuclei and apoptotic bodies were seen in xanthorrhizol-treated cells for 24 h (B) and 72 h (C), but not in the cells without treatment (A). No fluorescence was detected in the nucleus of untreated cells (D) as the cells were not apototic and did not exibit DNA fragmentation. In cells treated with xanthorrhizol for 24 h (E) and 72 h (F), fluorescence was detected in the nuclear region, showing the apoptotic characteristics. (G) Apoptosis levels as analyzed by TUNEL assay. Xanthorrhizol treatment significantly increased the level of apoptosis in HeLa cells compared to the controls. Apoptotic cells were counted on at least four independent slides. Magnification X1000.*

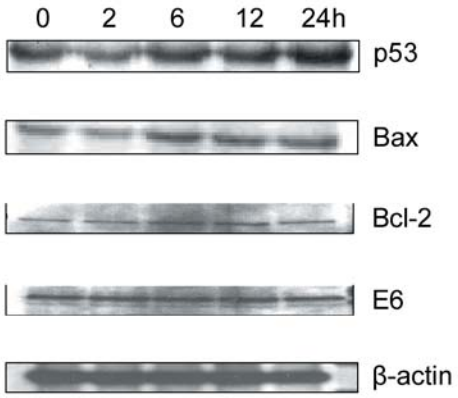
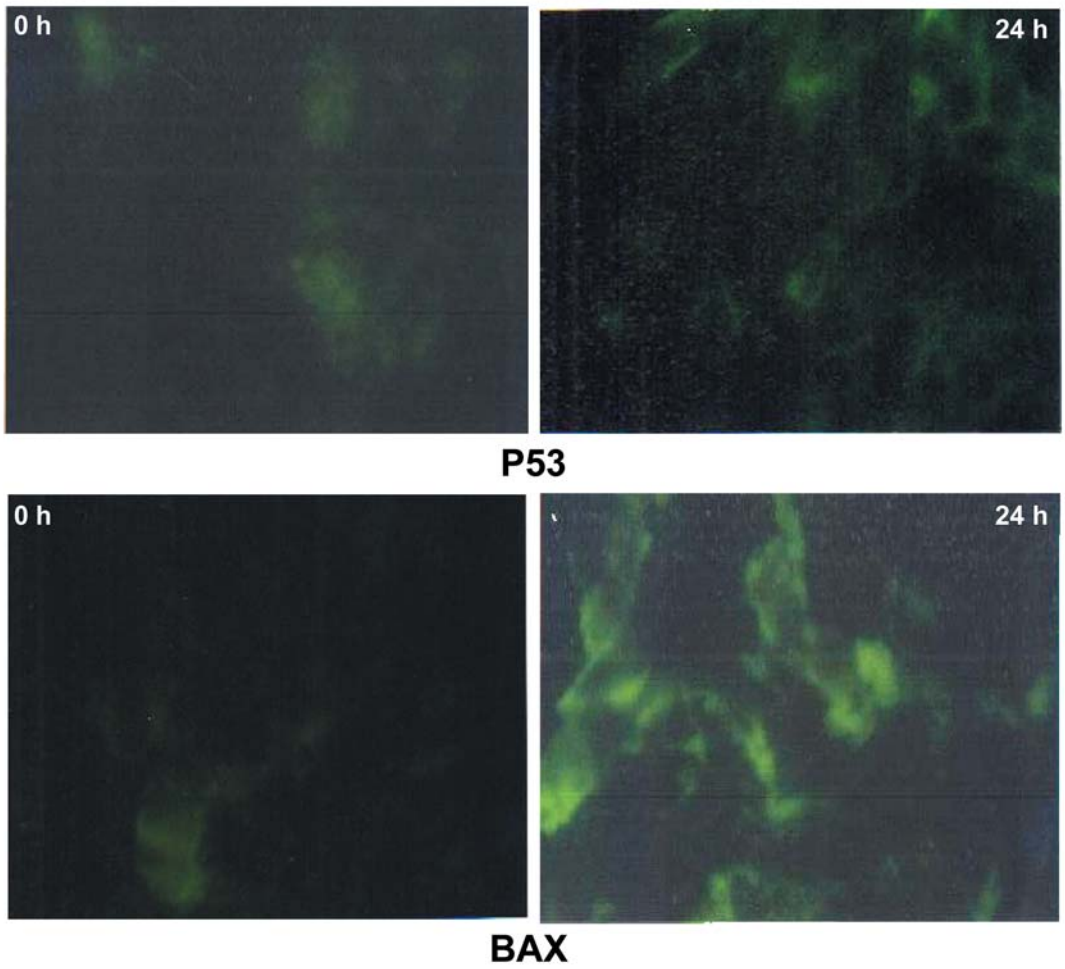


Figure 3. *Western blot analysis of p53, Bax, Bcl-2, E6 and immunostaining of p53 and Bax proteins in HeLa cells. (A) HeLa cells treated with 6 μg/ml xanthorrhizol for indicated times were resolved on a 15% PAGE and submitted to Western blotting. p53 and Bax protein expression increased after 6 h following 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). (B) Immunostaining of p53 and Bax proteins showed a low level of p53 and Bax in the untreated HeLa cells. However, a marked increase in immunofluorescence of both proteins can be seen after 24-h treatment with xanthorrhizol.*



Results

Effect of xanthorrhizol on cell viability. Using the 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 concentration range 0.2 to 50 μg/ml. Tamoxifen and cisplatin were used as positive controls, and DMSO as negative control. Xanthorrhizol significantly reduced HeLa cell viability in a dose-dependent manner. Xanthorrhizol decreased 50% of the HeLa cells' viability at 6.16 μg/ml. Although it also affected the normal cells, the EC_{50} values for normal cells were much higher than for HeLa cells.

Xanthorrhizol-induced apoptotic cell death. Staining with Hoechst 33258 showed fragmentation and condensation of chromatin in HeLa cells treated with 6 μg/ml xanthorrhizol for 24 h and 72 h (Figure 2B & 2C), compared to the untreated control (Figure 2A). Control cells exibited a normal nuclear morphology, characterized by a diffuse chromatin structure and, therefore, light staining. The fragmented DNA produces 3-OH DNA ends that can be labelled with fluorescein-12-dUTP using the principle of the TUNEL assay. The extent of DNA fragmentation was visualized by labelling 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 expressions of p53, Bax, Bcl-2 and E6 protein were measured after 6 μg/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 proteins p53 and Bax. However, the expression of the anti-apoptotic Bcl-2 and E6 did not show any change in either the control or the treated cells throughout the treatment period (Figure 3A). The up-regulation of p53 and Bax by xanthorrhizol was confirmed by the immunostaining of both proteins (Figure 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 pathways 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 impair normal cells causing 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, various authors have differing views regarding the role of p53 and chemosensitivity (9, 16, 17).

There is an obvious association between the accumulation of p53 and both HPV 16/18 infection and expression of the E6 protein (18). It has been demonstrated that the p53 protein complexes with the HPV 16/18 E6 protein in the cytoplasm and leads to p53 degradation *via* ubiquitination (19, 20). E6 can interfere with the normal function of p53 by its ability to abrogate both the transcriptional activation and transcriptional repression functions of the gene (21, 22). 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 was increased 6 h after the xanthorrhizol treatment in a time-dependent manner. The Bax expression, however, was increased after 6 h and maintained at a higher level than the controls throughout the experiment. The Bcl-2 and E6 levels were unchanged and maintained at low levels throughout the experiment. P53 protein expression was slightly higher in the cells without treatment compared to 2 h after treatment.

The half-life of the mutant form is 4-6 h, while the wild-type p53 has a short half-life (6-20 min). Thus, detection of p53 by immunostaining is often considered to reflect the mutant form (23, 24). However, this might not be true in all cases, as the immunocytochemical analysis of the p53 protein varies according to the antibody used. Also, in cervical cancer, previous studies showed that p53 gene mutation was infrequent (25, 26). In a mutant-specific p53 analysis of cervical cancer tissues, only 12 out of 230 samples studied were found to be positive (18). 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 cells by up-regulating p53 protein levels and therefore increased the expressions of both p53 and Bax proteins, while suppressing Bcl-2 protein. Several lines of evidence demonstrate the mechanism by which p53 induces apoptosis. P53 may regulate the gene product of the pro-apoptotic protein Bax *via* transcriptional activation (27). A previous study also showed that p53 can transcriptionally repress the expression of anti-apoptotic Bcl-2 (28). Utilizing a temperature-sensitive version of p53, conditional restoration of p53 activity in a p53-deficient leukemia cell line resulted in decreased Bcl-2, increased Bax and stimulated apoptosis (28, 29). The product of the *Bax* gene has antagonistic effects over the Bcl-2 protein, and Bax expression accelerates apoptosis (30).

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 (27). 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 apoptosis still occurred (31).

Since anticancer agents can kill tumor cells through apoptosis, the increase in p53 and Bax protein expression may restore sensitivity to apoptotic stimuli in cervical cancer cells. The results of this study provided evidence that xanthorrhizol from *C. xanthorrhiza* was able to inhibit the proliferation of HeLa cervical cancer cells 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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