Studies on the Apoptosis-Inducing Effect of *Ulmi Pumilae Cortex* on Human Leukemia HL-60 Cells

Jun Ki Rhyu, Bong Seon Yu, Jae Eun Jeong, Jin Yeong Bak, In Hwan Son, Ju Seok Lee, Byeong Hun Jeon[1], Byung Soon Mun*

Department of internal medicine, College of Oriental medicine, Wonkwang university,
1: Professional Graduate School of Oriental Medicine, Wonkwang university

The antiproliferative effect of the water extract of the branch and root bark of *Ulmi Pumilae Cortex*(WEUPC) was investigated on the p53-negative human leukemia cell line (HL-60). A dose- and time-dependent inhibition of cell growth was observed; this effect appears to be due to induction of apoptosis. Involvement of oxidative stress is indicated by a dose-dependent increase in intracellular reactive oxygen species levels. In addition, anti-apoptic effect was observed in the cells simultaneously treated with WEUPC and the anti-oxidant N-acetylcysteine. WEUPC did not affect the anti-apoptotic Bcl-2 and the pro-apoptotic Bax, whereas $p21^{WAF1/CIP1}$ was enhanced in a dose- and time-dependent fashion; this effect was partially inhibited by N-acetylcysteine. The increase in $p21^{WAF1/CIP1}$ was accompanied by a parallel accumulation of cells in the G1 phase of the cycle. These results suggest that the p53-independent induction of $p21^{WAF1/CIP1}$ and the induction of apoptosis may mediate the antiproliferative effect of WEUPC at least in this study; on the basis of this observation, WEUPC could be proposed as an useful adjunct to the treatment of p53-deficient tumors, which are often refractory to standard chemotherapy.

Key words : *Ulmi Pumilae Cortex*, Human Leukemia HL-60 Cells, Apoptosis

Introduction

Apoptosis is a selective process of physiological cell deletion that plays an important role in the balance between cell replication and cell death. Since it has recently been suggested that cancer chemotherapeutic as well as chemopreventive agents exert their pharmacological effects by triggering apoptotic cell death or cell cycle transition, the induction of apoptosis in tumor cells has become a predictor of tumor treatment response[1-12]. Conversely, several tumor promoters have also been shown to inhibit apoptosis[1-3,9].

Whereas various insults can initiate apoptosis[13-18], a consistent picture that emerges from studies in various laboratories points to the pivotal role of redox homeostasis in apoptotic process. Oxidative stress and reactive oxygen species (ROS) can be either the initiating stimulus or mediator of apoptosis[19-23]. Factors that regulate the levels and responses to oxidative stress are believed to play a decisive role in determining the "point of no return" in cell death[19-23]. In that context, the mitochondria are now well established as critical for processing and integrating pro-apoptotic signals. Diverse apoptotic stimuli can cause mitochondrial dysfunction leading to pro-oxidative changes in redox homeostasis. Pro-oxidants *per se* cause the efflux of mitochondrial components, further increasing the oxidative stress[13-23]. The cross-talk between cellular signaling and the redox status of the cell is presently believed to dictate, more than any other factors, whether or not a cell will undergo apoptosis[19-23]. A balance of pro- and anti-apoptotic factors allows cells to survive despite various insults for prolonged times. However, apoptosis seems to proceed like a chain reaction and relatively small perturbations in the equilibrium may either rapidly amplify or successfully attenuate the process. The amplification or attenuation of pro-apoptotic responses is tightly coupled to the redox status of the cell[19-23].

Lesions in DNA are generally considered as the primary apoptotic stimulus for DNA-damaging agents. DNA damage elicits complex responses which are mediated by various intracellular and extracellular factors such as p53, abl, c-myc, Rb, E2F, growth factors, and which often involve multiple and redundant pathways[24-25]. If these responses are not

* To whom correspondence should be addressed at : Byung Soon Mun, Professional Graduate School of Oriental Medicine, Wonkwang university, Iksan 570-749, Korea.
· E-mail : mbs@wonkwang.ac.kr, · Tel : 063-850-2102.
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extinguished by anti-apoptotic signals (such as Bcl-2), Bax and related death proteins decrease mitochondrial membrane potential[26-29], which, in turn, leads to cytochrome c release and oxidative stress[26-29], followed by the activation of cysteine proteases of the caspase family (among 11 groups of caspase, caspase-3 has been shown to play an important role in executing apoptosis) and subsequent apoptotic DNA fragmentation[26-29].This general route has been implicated in the action of currently used standard anticancer drugs, such as a cisplatin[10,12]. Additionally, the induction of apoptosis in cancer cells is recognized as a valuable tool for cancer treatment[10].

Ulmi pumila L.(native of China) is a deciduous tree with uneven pinnate leaves and classified in the subfamily of Ulmuceae, which contains many pharmacologically active constituents such as β-sitosterol, phytosterol, stigmasterol and tanin[30-34]. The wood, root and stem barks of the plant have all been reported to contain these compounds, the β-sitosterol being the major constituents[31]. As one of Koran traditional herbal medicines, the root bark of *Ulmi pumila L.*(榆白皮) has been used to settle hematuria, some kinds of fungal disease, and tumor, and also as an insecticide. However, other pharmacological activities of this drug, such as anticancer activity, remain to be uncovered.

At present, no academic information is available on the therapeutic potential of the root bark of *Ulmi pumila L.* on human tumors. But *Ulmi pumila L.* has been used for the therapy of tumor as a folk remedy. Therefore, the anticancer effects of the water extract of the root bark of *Ulmi pumila L.* (WEUPC) was examined in HL-60 cells, a human myeloid leukemic cell line. Herein I have reported that WEUPC exerts an antiproliferative effect on HL-60 cells by inducing cell cycle arrest in G1 phase and apoptosis, which are pharmacological targets of various standard anticancer drugs.

Materials and Methods

1. Chemicals

The anti-oxidant N-acetylcysteine(NAC), propidium iodide(PI), DAPI and 3-(4,5-dimethylthiozol-2-yl)-2 ,5-diphenyltetrazolium bromide(MTT) was obtained from Sigma(St. Louis, MO).

2. WEUPC

The air-dried materials of the branch and root bark of Ulmi Pumilae Cortex was obtained from the Medicinal Resources Research Center of Wonkwang University. One hundred gram of the materials was extracted with 1,000 ㎖ distilled water for 2 hours at 100 ℃, and then the extracted water filtered with 3M paper and was centrifuged at 3,000 rpm for 20 min. The supernatant was filtered again, and condensed using rotary evaporator and dried using freeze- dryer, and stored at -70 ℃ until used. When used, the sample was filtered with 0.22 ㎛ pore filter paper for sterilizatiopn.

3. Cell culture

The HL-60 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10 % heat-inactivated fetal bovine serum(GIBCO-RBL, Grand Island, NY), 100 U/mL of penicillin(GIBCO-RBL), and 100 μg/ml of streptomycin (GIBCO-RBL). The cells were grown in a humidified incubator at 37 ℃ under a 5 %CO_2/95 %O_2 atmosphere and used for assays during the exponential phase of growth. Cell counts were performed routinely to maintain a low-density population and cell viability was assayed by the Trypan blue exclusion method. HL-60 cells (5×10^5 or 1×10^6) generally were treated in 1mL of RPMI 1640 medium containing 10 % heat-inactivated fetal bovine serum, and then were incubated in an O_2/CO_2 culture incubator. Before adding the reagents, pre-incubation was normally performed for at least 1h.

4. Treatment of HL-60 cells with WEUPC

WEUPC was dissolved in RPMI 1640 medium. Various doses of WEUPC were used to treat the cells for 0h, 6h, 12h, 18h and 24h.

5. Cell viability assay

MTT is converted in living cells into insoluble formazan, and the amount of formazan produced is proportional to the number of viable cells. Cells (5×10^5) were incubated for 2h at 37 ℃ with 0.5mg/mL of MTT. The cells were lysed with an equal volume of isopropanol/1M HCl (24:1). Reduced MTT was measured at 550nm in a 96-microtiter plate by using a ELISA reader.

6. Nuclear staining

Cells (5×10^5) were labeled with 2 μg/mL of the DNA dye DAPI (Sigma) for 30min at 37 ℃ and visualized on a fluorescence microscope. DAPI permeates the plasma membrane and yields blue chromatin. Viable cells display normal nuclear size and blue fluorescence, whereas apoptotic cells show condensed chromatin and fragmented nuclei.

7. Cell cycle analysis

Control and WEUPC-treated cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS) (pH 7.4) and fixed in methanol/PBS (9/1, by volume) at -20 ℃

for at least 30 min. The fixed cells were then washed twice with ice-cold PBS and stained with 50 μg/mL of PI in the presence 25 μg/mL of RNase A (Sigma). Cell cycle phase distribution was analyzed in three different experiments using FACS flow cytometer(FACS vantage, Becton Dickinson Immunocytometry Systems, San Jose, USA). Data from 10000 events/sample were collected and analyzed using the Cell Fit cell analysis program (Becton Dickinson Immunocytometry Systems, San Jose, USA).

8. Discrimination of apoptotic cells by flow cytometry

The numbers of cells in each phase of the cell cycle and in apoptosis were determined by use of a FACS vantage flow cytometer and CELLQuest software (Becton Dickinson) after staining the DNA with PI. The cells (1×10^6) treated with WEUPC were fixed in 200 μL of 70 % ethanol at 4 ℃ for 4 h, and then incubated in 40 μL of a running buffer (containing 0.2 M Na_2HPO_4 and 0.1 M citrate) at room temperature for 30 min.

Following treatment with 0.1mg/mL of RNase A in 100 μL of phosphate-buffered saline (PBS) at 37 ℃ for 30 min, the cells were stained with 50 mg/mL of PI in 1mL of PBS at 37 ℃ for 30 min in the dark. Analytic flow cytometric measurements were performed using a FACS vantage flow cytometer (Becton Dickinson), and the fluorescence was detected through a 564-606 nm band-pass filter. Twenty thousand cells were analyzed in each sample using CELLQuest software (Becton Dickinson).

9. DNA fragmentation assay

Control and WEUPC-treated cells were harvested, washed twice with ice-cold PBS (pH 7.2), and lysed for 1 h in a lysis solution (0.5 % SDS, 10 mM EDTA, 0.5 mg/mL proteinase K, and 50 mM Tris-HCl, pH 8.0). DNA was extracted with a standard phenol-chloroform method. 30 μg of DNA of each sample was subjected to electrophoresis on 1.5 % agarose gel at 5 V/cm. The DNA was visualized and photographed under UV illumination after staining with ethidium bromide (Sigma).

10. Detection of caspase-3 activation

Activation of caspase-3 was determined by using a fluorometric assay kit (R&D Systems Inc., Mineapolis, MN) according to the manufacturer's instructions. Briefly, 5×10^6 cells were collected and lysed in 50 μL of lysis buffer and incubated with fluorochromic caspase substrate. After incubation at 37 ℃ for 1 h, the fluorescence was measured by a spectrofluorometer (RF540, Shimadzu, Tokyo) with excitation at 400 nm and emission at 550 nm.

11. Western blot analysis for $p21^{WAF1/CIP1}$, Bcl-2, and Bax

Protein concentration was determined by the Bradford assay kit (Sigma). Equal amounts of protein were loaded on each lane and electrophoresed on sodium dodecylsulfate-polyacrylamide gels with Tris-glycine running buffer. They were then transferred to nitrocellulose membranes by using a semi-dry electrotransfer for 50 min at 40 V. Membranes were incubated with antibodies (anti-$p21^{WAF1/CIP1}$, anti-Bcl-2, anti-Bax and anti-tubulin; Santa Cruz Biotechnology, Santa Cruz, CA).

The antibodies were used at a dilution of 1:100. After washing, the membranes were incubated with secondary antibody labeled with horseradish peroxidase (Amersham, Arlington Heights, IL; diluted 1:1000), for 1h at room temperature, washed again, and developed with the chemiluminescence detection kit (Amersham), followed by exposure of the membranes to autoradiographic films (Amersham).

12. Measurement of Intracellular ROS level

2′,7′-Dichlorofluorescein diacetate (DCFH-DA; Sigma) is a molecular probe for ROS. The principle of this assay is that DCFH-DA diffuses through the cell membrane and is enzymatically hydrolzed by intracellular esterases to nonfluorescent dichlorofluorescein (DCFH). In the presence of ROS, this compound is rapidly oxidized to highly fluorescent dichlorofluorescein (DCF). For the assay, cells (1×10^6 cells per 3 mL in 6-well plates) were incubated with WEUPC, rinsed three times with PBS, and added with 10 μM of DCFH-DA. After 30 min incubation at 37 ℃, the cells were harvested and washed with PBS twice. The samples were analyzed using a flow cytometer (Becton Dickinson).

13. Statistical analysis

Each experiment was performed at least in triplicate. Results are expressed as the mean±S.D. Statistical analysis was performed using a Student's *t*-test programmed by Microcal Origin Software Co (USA). P values < 0.05 were considered significantly different from control group.

Results

1. Antiproliferative effect of WEUPC

The inhibitory effect of WEUPC on the growth of human myeloid leukemia HL-60 cells was assessed by measuring the number of cells surviving after extract treatment for 24h by a colorimetric MTT assay. The absorbance of the WEUPC-treated

cells was expressed as percentage of the control untreated cells in order to show the extent of inhibition on cell growth (Fig. 1). WEUPC showed levels of cell growth inhibition by 50 % compared with control cells at doses of 61 μg/mL (ED_{50}) and above (Fig. 1A). At a dose of 50 μg/mL, WEUPC was able to reduce the cell growth of HL-60 cells up to about 45 % in a time-dependent manner (Fig. 1B).

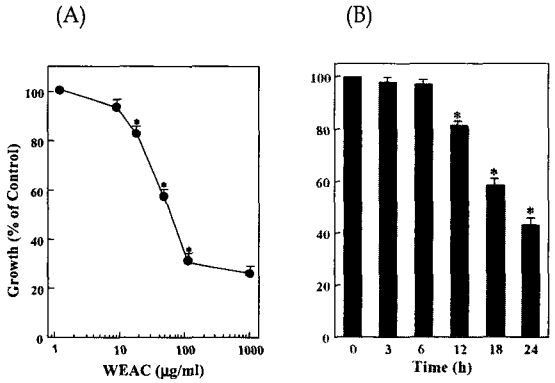

Fig. 1. The effect of WEUPC on the growth of HL-60 cells. The cells seeded at 4×10^3 cells in 96-well plates were incubated with WEUPC: (A), 1-1000 μg/mL for 24h in the dose-response study; or (B), with 50 μg/mL in the time-course study. Values represent means±S.D. ($n=8$). *P < 0.05 compared with WEUPC-untreated group.

2. Effect of WEUPC on Cell cycle changes

The results of cell cycle analysis of WEUPC-treated HL-60 cells showed a significant dose-dependent increase in G1 cells (Table 1). A parallel decrease was observed in the percentage of cells in the G2/M phases (Table I). At a dose of 50 μg/mL, WEUPC was able to increase the percentage of cells in the G1 phase up to about 80% in a time-dependent manner (Fig. 2). DNA profiles also showed a dose-dependent increase in the percentage of cells with sub-G1 DNA contents (Fig. 3). Such increase was also time-dependent, thus supporting the hypothesis of an apoptotic mode of cell death.

Table I. Cell cycle analysis of HL-60 cells exposed to different doses of WEUPC for 18h. Each value is the mean±S.D. of three independent experiments

Group	Quantities of cell types			
	G0/G1	S	G2/M	Sub-G1
Control	54 ± 1.7	27 ± 2.1	17 ± 4.3	< 2
WEUPC, 5 μg/mL	59 ± 1.3	24 ± 2.2	14 ± 3.1	< 3
WEUPC, 10 μg/mL	65 ± 1.2	21 ± 2.2	10 ± 3.4	4 ± 1.1
WEUPC, 25 μg/mL	73 ± 1.5	10 ± 2.5	7 ± 2.2	10 ± 1.3
WEUPC, 50 μg/mL	80 ± 1.7	4 ± 2.3	4 ± 2.3	12 ± 1.5
WEUPC, 100 μg/mL	70 ± 1.4	2 ± 1.9	3 ± 2.5	25 ± 1.6

3. Induction of apoptosis by WEUPC

The apoptotic cell death induced by WEUPC in HL-60 cells was verified by fluorescent staining of fragmented nuclei by DAPI, DNA fragmentation and caspase-3 activity. After treatment with 50 μg/mL of WEUPC for 24h, the characteristic fragmented and condensed nuclei, or apoptotic bodies, were clearly shown in WEUPC-treated cells after staining with DAPI (Fig. 4A). Another distinct feature of apoptosis, DNA fragmentation pattern, was also seen in HL-60 cells after treatment with 50 μg/mL for 24h (Fig. 4B). At a dose of 50 μg/mL, WEUPC was able to induce a time-dependent activation of caspase-3 (Fig. 4C), which is thought to be the main effector of apoptosis.

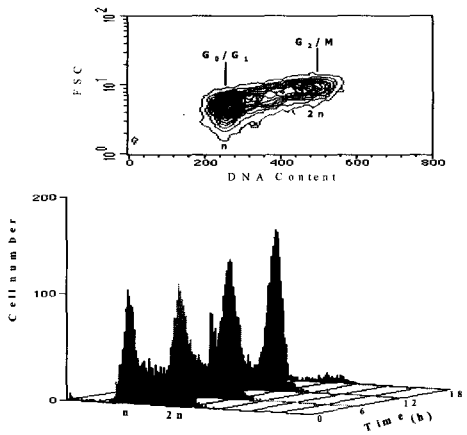

Fig. 2. The effect of WEUPC on HL-60 cell cycle phase distribution. HL-60 cells were treated with 50 μg/mL of WEUPC for indicated times. The cells were harvested and analyzed by a flow cytometer in three separate experiments. The figure shows the results of one of these experiments. The cells treated with WEUPC are accumulated at G_0/G_1 phase of cell cycle (See the inserted Figure for cell cycle phase and DNA content).

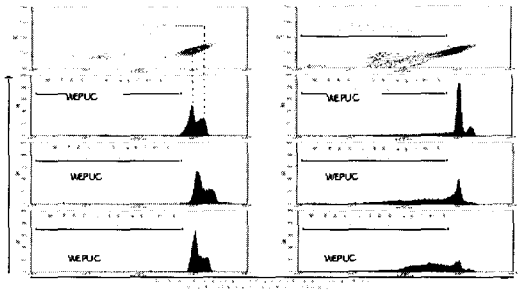

Fig. 3. Determination of sub-G1 cells (apoptotic cells) by a flow cytometry. HL-60 cells were cultured with indicated doses of WEUPC for 24h. The method of flow cytometry used here is described in Materials and Methods. Bar represents % of sub-G1 cells. Similar results were obtained in three separate experiments.

4. Generation of intracellular ROS by WEUPC

Studies in a variety of cell types have suggested that cancer chemotherapy drugs induce apoptosis of tumor cells, in part, by inducing the formation of intracellular ROS[35-37]. I examined whether the intracellular ROS was generated by 3-h treatment of HL-60 cells with WEUPC. The intracellular ROS level was determined by using DCFH-DA and detected by a flow cytometry. WEUPC increased the intracellular ROS levels

in a dose-dependent manner (Fig. 5). A significant difference in peroxide levels, as compared with the control cells, was detected for 50 μg/mL of WEUPC, thus supporting the hypothesis of WEUPC-induced oxidative damage. It should be pointed out that the intracellular ROS formation by WEUPC had been detected before G1 arrest and apoptosis were observed. Suggesting that the formation of intracellular ROS by WEUPC may play an important role in triggering apoptosis.5. Effects of WEUPC on the expressions of $p21^{WAF1/CIP1}$, Bcl-2, and Bax

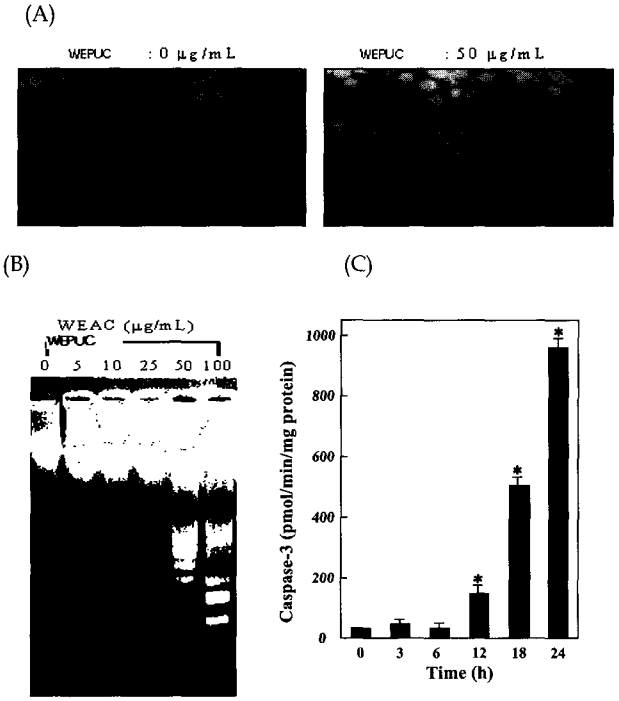

Fig. 4. Induction of apoptosis by WEUPC in HL-60 cells. (A) Nuclear staining confirmation. The cells were incubated with medium or with 50μg/mL of WEUPC for 24h. Fragmented nuclei are illustrated in WEUPC-treated cells. (B) DNA fragmentation analysis. The cells were incubated with medium or with indicated doses of WEUPC for 24h. (C) Effects of WEUPC on the activations of caspase-3. The cells were treated with 50μg/mL of WEUPC for indicated periods, and the caspase-3 activity was determined as described in Materials and Methods. Data represent means±S.D. for three determinations.

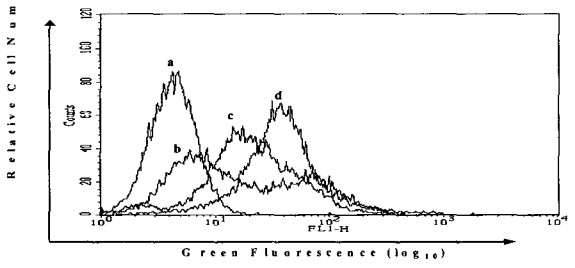

Fig. 5. Effects of WEUPC on intracellular ROS formation. A clear increase in the cells emitting green fluorescence was observed after 3-h treatment of HL-60 cells with 0(a), 25(b), 50(c) and 100(d)μg/mL of WEUPC. The methods are described in Materials and Methods. Similar results were obtained in three separate experiments.

Western blot analysis with both anti-Bcl-2 and anti-Bax antibodies showed a minimal decrease or increase in the intensity of the immunoreactive bands of WEUPC-treated cells (Fig. 6B). In contrast, $p21^{WAF1/CIP1}$ protein levels increased in a dose- and time-dependent manner (Fig. 6A). This effect was partially, but not completely, inhibited by simultaneous treatment with 1 or 5 mM NAC (Fig. 6C). However, treatment with NAC was found to completely block WEUPC-induced apoptotic cell death (Fig. 6D).

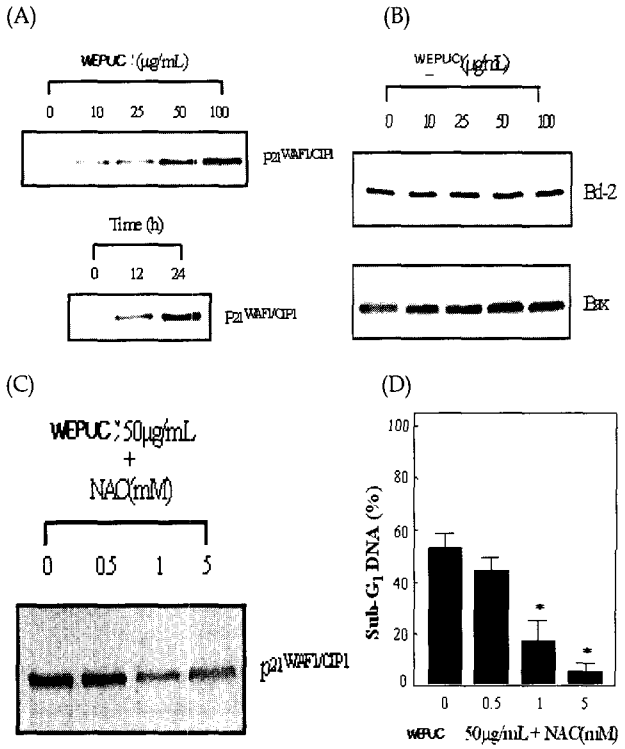

Fig. 6. Representative Western blot analysis demonstrating the effects of WEUPC on $p21^{WAF1/CIP1}$, Bcl-2, and Bax expressions(A, B and C). HL-60 cells were incubated for indicated times or 18 h with indicated doses or 50 μg/mL of WEUPC in the absence or presence of indicated doses of NAC. NAC almost completely blocked WEUPC-induced apoptosis(D).

Discussion

The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs. The Industrial Revolution and the development of organic chemistry resulted in a preference for synthetic products for pharmacological treatment. The reasons for this were that pure compounds were easily obtained, structural modifications to produce potentially more active and safer drugs could be easily performed and the economic power of the pharmaceutical companies was increasing.

However, even if we only consider the impact of the discovery of the penicillin, obtained from micro-organisms, on the development of anti-infection therapy, the importance of natural products is clearly enormous. About 25 % of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. Of the 252 drugs considered as basic and essential by the World Health Organization (WHO), 11 % are exclusively of plant origin and a significant number of synthetic drugs are obtained from natural precursors. Examples of important drugs obtained from plants are digoxin from *Digitalis spp.*, quinine and quinidine from *Cinchona spp.*, vincristrine and vinblastine from *Catharanthus roseus*, atropine from Atropa belladona and morphine and codeine from *Papaver somniferum*. It is estimated that 60 % of anti-tumors and anti-infectious drugs already on the market or under clinical trial are of natural origin. The vast majority of these cannot yet be synthesised economically and are still obtained from wild or cultivated plants. Natural compounds can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development and the discovery of new therapeutic properties not yet attributed to known compounds. In addition, compounds such as muscarine, physostigmine, cannabinoids, yohimbine, forskolin, colchicine and phorbol esters, all obtained from plants, are important tools used in pharmacological, physiological and biochemical studies.

Furthermore, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. This interest in drugs of plant origin is due to several reasons; namely, conventional medicine can be inefficient (*e.g.* side effects and ineffective therapy), abusive and/or incorrect use of synthetic drugs results in side effects and other problems, a large percentage of the world's population does not have access to conventional pharmacological treatment, and folk medicine and ecological awareness suggest that natural products are harmless. However, the use of these substances is not always authorized by legal authorities dealing with efficacy and safety procedures, and many published papers point to the lack of quality in the production.

Nevertheless, the potential use of medicinal plants as a source of new drugs is still poorly explored. Of the estimated 500,000 plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties; in most cases, only pharmacological screening or preliminary studies have been carried out. It is estimated that 5000 species have been studied for medical use. Between the years 1957 and 1981, the NCI screened around 20,000 plant species from Latin America and Asia for anti-tumour activity, but even these were not screened for other pharmacological activities.

The observed increase in $p21^{WAF1/CIP1}$ levels in WEUPC-treated cells indicates that WEUPC is able to activate a p53-independent pathway for $p21^{WAF1/CIP1}$ induction in HL-60 cells, as reported for other agents[38]. In my data, flow-cytometric analysis revealed that WEUPC could arrest HL-60 cells in a G1 phase. This inhibition of cell-cycle progression might be associated with an altered expression of cell cycle relevant regulator, including $p21^{WAF1/CIP1}$ and its upstream molecule[39].

The mechanism(s) by which WEUPC is able to increase $p21^{WAF1/CIP1}$ levels in HL-60 cells has been shown to be very complex. The partial inhibition of $p21^{WAF1/CIP1}$ expression by the anti-oxidant NAC would suggest that WEUPC-induced oxidative stress is one of inducing factors leading to $p21^{WAF1/CIP1}$ induction in HL-60 cells.

The role of $p21^{WAF1/CIP1}$ in the control of apoptosis is controversial. In some studies,40 authors have reported that increased $p21^{WAF1/CIP1}$ induction promoted apoptosis, whereas in other studies, authors have demonstrated that $p21^{WAF1/CIP1}$ is responsible for cell cycle arrest but not for apoptosis. That the increased expression of $p21^{WAF1/CIP1}$ contributes directly to the observed effect of WEUPC on apoptosis in my study is unlikely because the anti-oxidant NAC partially inhibited $p21^{WAF1/CIP1}$ induction but almost completely blocked apoptotic cell death. Further experiments are in progress to ascertain whether $p21^{WAF1/CIP1}$ induction is merely concidental with apoptosis or whether a causal relationship exists between the two events.

Conclusion

A number of interesting conclusions can be drawn from the present data. WEUPC exerts an antiproliferative effect on human myeloid leukemia HL-60 cells by inducing cell cycle arrest in G1 phase (cytostatic effect) and apoptotic cell death (cytotoxic effect). Oxidative stress seems to play a relevant role in the apoptotic pathway, which is the major mode of cell death in WEUPC-treated HL-60 cells, and to be involved partially in $p21^{WAF1/CIP1}$ induction, which induces growth arrest in G1 phase. The apoptotic effects of WEUPC do not require p53 expression and two of the down-stream mediators of p53-driven responses to cell damages, Bcl-2 and Bax. It is most likely that $p21^{WAF1/CIP1}$ is responsible for cell cycle arrest but not for apoptosis, at least, in WEUPC-treated cells. On the

basis of the results of the present study, WEUPC could be proposed as an useful adjunct to the treatment of p53-deficient tumors, which are often refractory to standard chemotherapy.

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