Biochemical Pharmacology, Vol. 60, pp. 307–315, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00322-1

Induction of Apoptosis by *Hibiscus* Protocatechuic Acid in Human Leukemia Cells via Reduction of Retinoblastoma (RB) Phosphorylation and Bcl-2 Expression

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ABSTRACT. *Hibiscus* protocatechuic acid (PCA), a phenolic compound isolated from the dried flower of *Hibiscus sabdariffa* L. (Malvaceae), demonstrated antioxidant and antitumor promotion effects in our previous study. In the present study, *Hibiscus* PCA was found to inhibit the survival of human promyelocytic leukemia HL-60 cells in a concentration- and time-dependent manner. The study revealed that HL-60 cells underwent internucleosomal DNA fragmentation and morphological changes characteristic of apoptosis after a 9-hr treatment with *Hibiscus* PCA (2 mM). Flow cytometric analysis of the DNA content of cells treated with PCA for 12 hr showed that the cells were distributed mainly in the hypodiploid phase (apoptotic peak, 46.7%), less in the G_1 (34.2%) and S phase (14.0%), and few in the G_2/M phase (5.1%). Moreover, PCA treatment caused an increase in the level of hypophosphorylated retinoblastoma (RB; 180% of control at the 6-hr time point) and, on the contrary, a decline in hyperphosphorylated RB. A rapid loss of RB was observed when the treatment period was extended. Further studies showed that *Hibiscus* PCA application reduced Bcl-2 protein expression to 47%, and increased Bax protein expression to 181% after 1.5 hr as compared with time 0. Overexpression of Bcl-2 in HL-60 cells delayed the occurrence of *Hibiscus* PCA-induced apoptosis. These data suggest that *Hibiscus* PCA is an apoptosis inducer in human leukemia cells, and that RB phosphorylation and Bcl-2 protein may play a crucial role in the early stage. BIOCHEM PHARMACOL **60**;3:307–315, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; Bcl-2; *Hibiscus* protocatechuic acid; leukemia; RB

Hibiscus PCA§ (Fig. 1), a phenolic compound, is isolated from the dried flower of *Hibiscus sabdariffa* L. (Malvaceae), which is an ingredient of local beverages and a Chinese herbal medicine used to treat hypertension, pyrexia, and liver damage. Recently, PCA has been demonstrated to be an efficacious agent in inhibiting the carcinogenic action of various chemicals in different tissues, such as diethylnitrosamine in the liver [1], 4-nitroquinoline-1-oxide in the oral cavity [2], azoxymethane in the colon [3], *N*-methyl-*N*-nitrosourea in glandular stomach tissue [4], and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine in the bladder [5]. In our previous studies, PCA showed strong antioxidant and antitumor promotion effects [6, 7]. Thus, PCA possesses anticarcinogenic potential and may play a role in chemoprevention.

Recently, considerable attention has focused on the sequence of events referred to as programmed cell death or apoptosis, and the possible role of this process in mediating the lethal effects of diverse antineoplastic agents in leukemia cells [8]. Apoptosis is a highly regulated process that involves activation of a cascade of molecular events leading to cell death as characterized by cell shrinkage, membrane blebbing, chromatin condensation, and formation of a DNA ladder of multiples of 180–200 bp, caused by internucleosomal DNA cleavage [9, 10]. The prototypic regulator of mammalian apoptosis is the proto-oncogene *bcl-2* [11]. Transfection experiments indicated that *bcl-2* could protect many cell types from apoptosis induced by exposure to a wide variety of adverse conditions and stimuli. On the other hand, studies using transgenic mice suggest that loss of RB function is associated with the induction of p53-dependent apoptosis [12]. It also has been reported that anticancer drugs induce RB hypophosphorylation and consequent G_1 arrest and apoptosis in p53-independent cell lines such as human leukemia HL-60 and U937 cells [13].

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§ *Abbreviations*: PCA, protocatechuic acid; BrdU, 5-bromo-2′-deoxyuridine; ICE, interleukin 1β-converting enzyme; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and RB, retinoblastoma (protein).

Received 23 August 1999; accepted 17 December 1999.

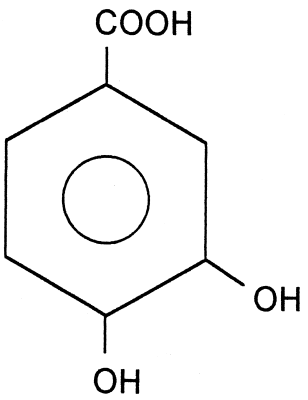

FIG. 1. Structure of PCA.

The phosphorylation of RB protein may mediate cell proliferation [14], differentiation [15], and senescence [16].

Many studies have demonstrated that polyphenols in medicinal and edible plants have various pharmacological activities, such as anti-inflammatory/antioxidant and anti-carcinogenesis activities [17, 18]. Recently, scientists have reported that natural antioxidants such as quercetin [19], gallic acid [20], and tea polyphenols [21] inhibit the growth of cancer cells through the induction of apoptosis [19, 20]. *Hibiscus* PCA, one of the plant phenols well known as natural antioxidants, shows mild cytotoxicity to PC14 and MKN45 human tumor cells [22]. Here, we present findings demonstrating that *Hibiscus* PCA induced apoptosis in human leukemia HL-60 cells, and that this process was associated with the reduction of RB phosphorylation and Bcl-2 expression.

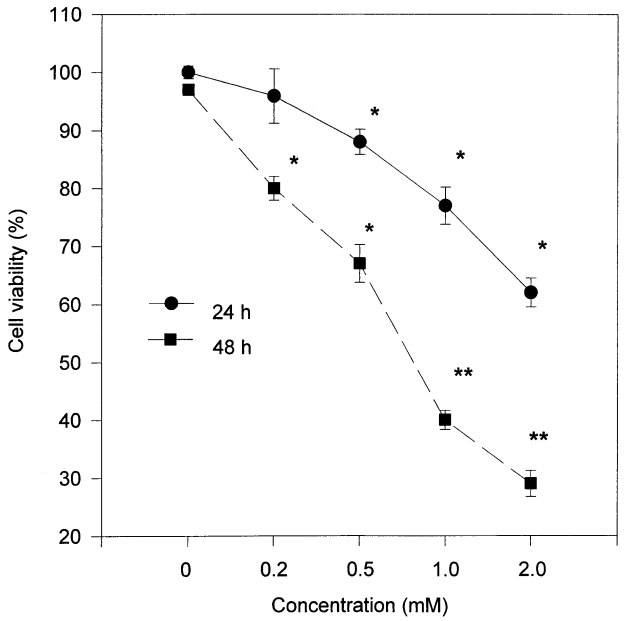

FIG. 2. Effect of *Hibiscus* PCA on HL-60 cell proliferation. Cells were treated as described in the text. The results are presented as means ± SD of three independent experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$, compared with the control group (0.1% DMSO).

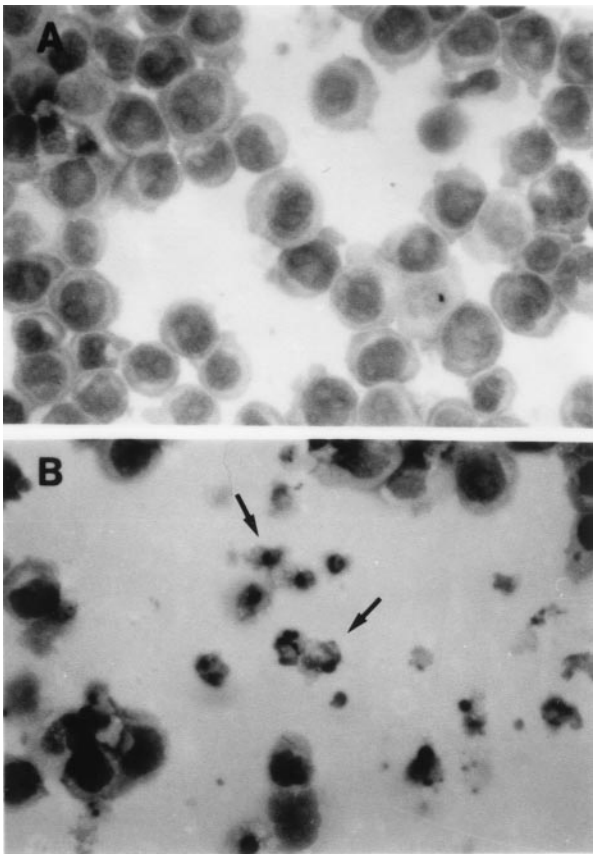

FIG. 3. Microscopic appearance of hematoxylin-stained nuclei of *Hibiscus* PCA-treated HL-60 cells. HL-60 cells were (A) untreated (solvent control) or (B) treated with 2 mM *Hibiscus* PCA for 24 hr, then stained with hematoxylin and examined under a microscope (x 400). Arrows indicate condensed and fragmented nuclei.

MATERIALS AND METHODS

Chemicals

Hibiscus PCA was isolated from *Hibiscus sabdariffa* L., and its structure has been described in one of our previous papers [6]. RPMI and PBS were purchased from GIBCO, Ltd. (BRL Life Technologies, Inc.). Mouse monoclonal antibodies to RB and Bcl-2 were purchased from Transduction Laboratories. A rabbit polyclonal antibody to Bax was obtained from Oncogene Products (CN Bioscience, Inc.). An ECL kit was obtained from Amersham Life Science (Amersham). A cellular DNA fragmentation ELISA kit was purchased from Boehringer Mannheim (Becton Dickinson Co., LTD.). A protein assay kit from Bio-Rad Laboratories was used. All other materials were obtained from the Sigma Chemical Co.

Cell Line and Cell Culture

Human leukemia cells (HL-60) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM L-glutamine. Bcl-2-overexpressing HL-60 (HL-60/Bcl-2-350) cells (provided by Dr. Min-Liang Kuo, Institute of

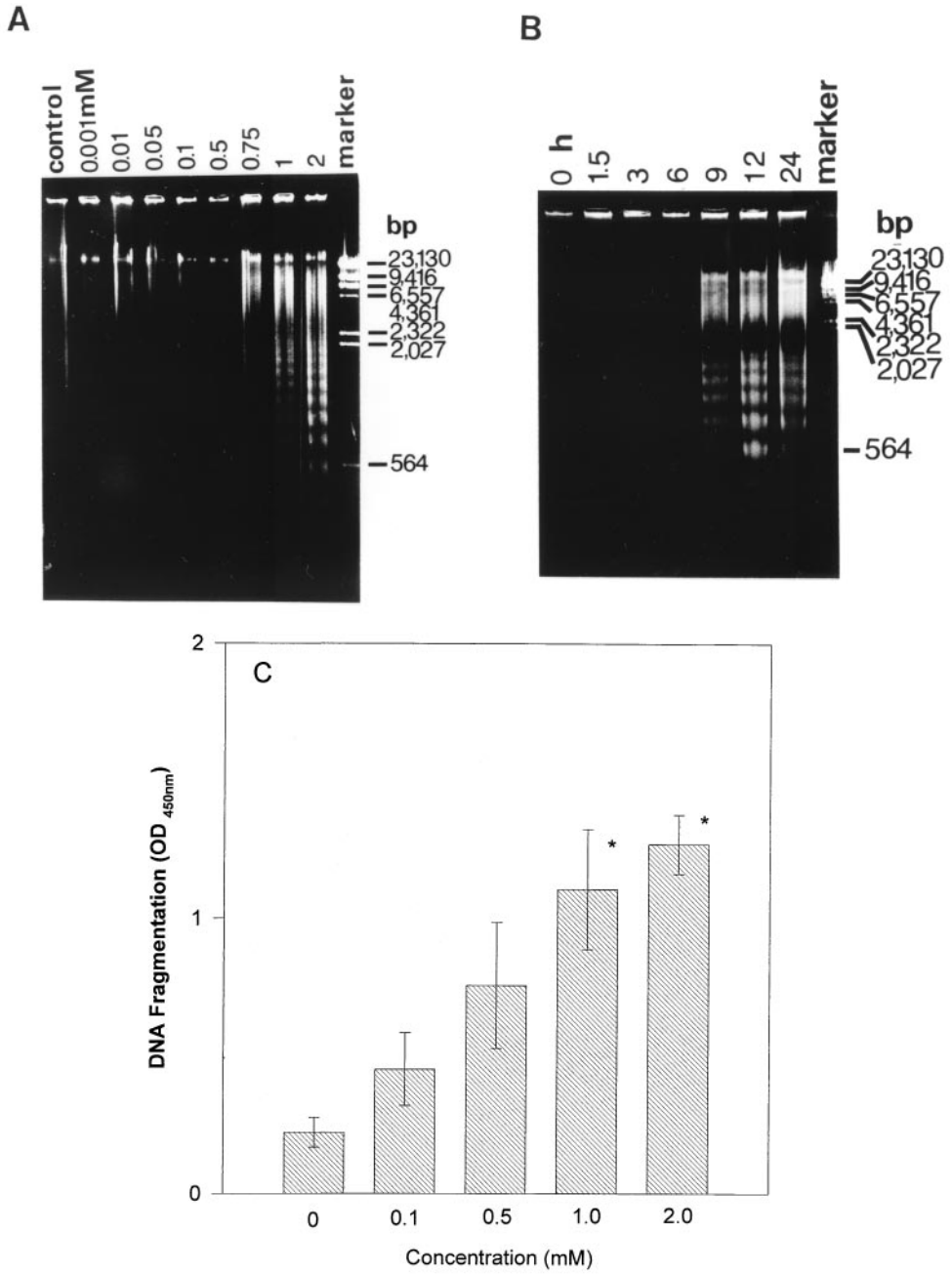

FIG. 4. Apoptotic phenomena induced by *Hibiscus* PCA. Agarose gel electrophoresis of DNA isolated from HL-60 cells treated with (A) various concentrations of *Hibiscus* PCA for 24 hr, and (B) 2 mM *Hibiscus* PCA for the indicated times. After extraction, DNA samples (20 μg) were separated on a 1.5% agarose gel, stained with ethidium bromide, and photographed under UV illumination. (C) DNA fragmentation induced by 2 mM *Hibiscus* PCA for 12 hr was determined photometrically (450 nm) by an ELISA kit. The results are presented as means ± SD of three independent experiments. Key: (*) $P < 0.05$, compared with the control group (0.1% DMSO).

Toxicology, College of Medicine, National Taiwan University) were employed in this study, and the Bcl-2 protein level of these cells was elevated as described by Kuo *et al.* [23]. Bcl-2-overexpressing HL-60 cells were maintained in the same conditions as HL-60 cells with the addition of 1 mg/mL of G418 (Geneticin; Life Technologies, Inc.). Both cell lines were subcultured on day 3 and were diluted 1 day before each experiment. Cell density in culture did not exceed 5×10^5 cells/mL.

Assessment of Cell Viability

Cells were seeded at a density of 2×10^5 cells/mL and incubated with *Hibiscus* PCA at various concentrations (0, 0.2, 0.5, 1, and 2 mM) for 24 and 48 hr. Thereafter, the medium was changed and incubated with MTT (0.1 mg/mL) for 4 hr. The viable cell number was directly proportional to the production of formazan, which was solubilized in isopropanol and measured spectrophotometrically at 563 nm.

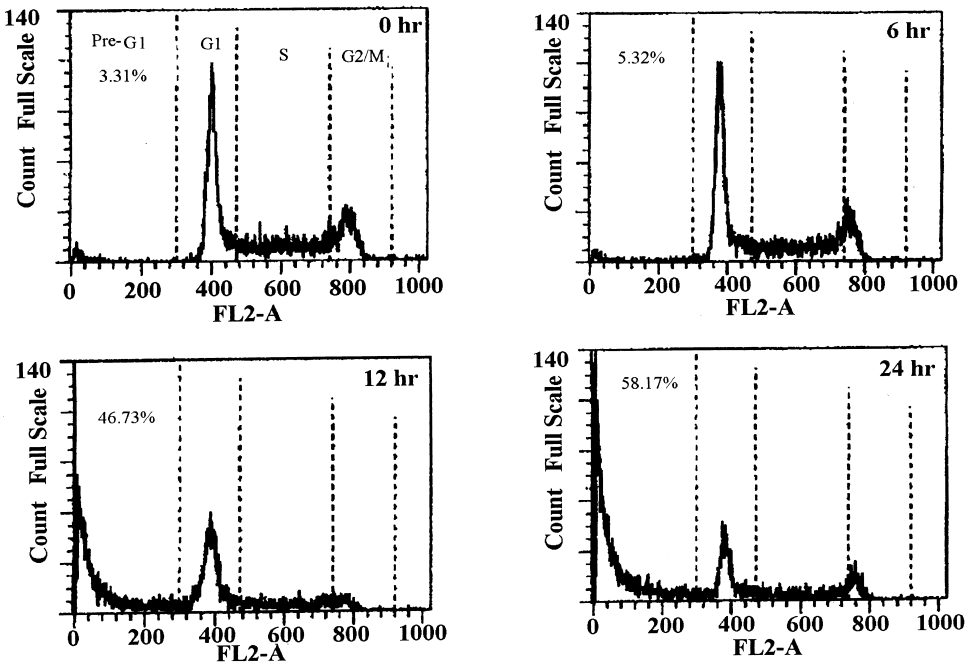

FIG. 5. Effect of *Hibiscus* PCA on cell cycle distribution of HL-60 cells. HL-60 cells were exposed to 2 mM *Hibiscus* PCA for the indicated times, then washed and harvested. The cells were fixed and stained with propidium iodide, and the DNA content in each phase, pre-G_1, G_1, S, and G_2/M, was analyzed by flow cytometry (FACS). The percentage of hypodiploid cell number (pre-G_1 phase) over total cells was calculated and expressed.

Analysis of Morphological Change

Untreated or *Hibiscus* PCA-treated (2 mM, 24 hr) HL-60 cells were examined for morphological change by inverted microscopy. To assay nuclear morphology, cells were harvested, placed on glass slides using a Cytospin apparatus, fixed with 95% ethanol for 1 hr, and stained with 0.1% hematoxylin and 50 mg/mL of propidium iodide.

DNA Gel Electrophoresis

Untreated or *Hibiscus* PCA-treated cells were collected by centrifugation (200 *g*, 10 min), washed in PBS (with 1 mM $ZnCl_2$), resuspended (5×10^6 cells/mL) in 0.5 mL lysis buffer (0.5% SDS, 100 mM EDTA, 10 mM Tris, and 200 mg/mL of RNase A; pH 8.0), and incubated at 37° for 1 hr. Proteinase K was added at a concentration of 0.5 mg/mL and incubated at 50° for 12 hr. DNA was extracted with phenol, and then was precipitated by ethanol at −80° overnight. An equal amount of DNA (20 μg) from each sample was electrophoresed on an agarose gel (1.5%) at 1.5 V/cm for 3 hr. DNA in the gel was visualized under UV light after being stained with ethidium bromide (0.5 mg/mL).

Measurement of DNA Fragmentation

DNA fragmentation of apoptotic cells induced by *Hibiscus* PCA was determined by a sandwich enzyme immunoassay (ELISA) kit. Briefly, HL-60 cells or HL-60/Bcl-2–350 cells grown in flasks were labeled with BrdU (10 μmol/mL) for 18 hr. After labeling, the cells were collected and resuspended at a density of 1×10^5 cells/mL, and then incubated with *Hibiscus* PCA (0–2 mM) for 12 hr. After centrifugation (250 *g*, 10 min), part of the nuclear DNA of the apoptotic cells was released into the supernatant. The BrdU-labeled DNA can be easily detected and quantified photometrically (wavelength at 450 nm) by ELISA using a monoclonal antibody against BrdU.

Analysis of Cell Cycle Distribution

Cell cycle distribution of Hibiscus PCA-treated HL-60, HL-60/neo, or HL-60//Bcl-2–350 cells was determined using a Becton Dickinson Immunocytometer with a Cycle TEST^{TM} PLUS DNA reagent kit. Briefly, cell pellets (5×10^5 cells) were suspended in 0.25 mL solution A (trypsin buffer) at 25° for 10 min. To those suspensions, 200 μL solution B (trypsin inhibitors) was added and incubated at 25° for 10 min, and then 200 μL solution C (propidium iodide) at 4° was added for 10 min. The suspension was filtered through 50-μm nylon mesh, and the DNA content of stained nuclei was analyzed by a flow cytometer (Becton Dickinson). The distribution of DNA content was expressed as pre-G_1, G_1, S, and G_2/M phases. The percentage of hypodiploid cells (pre-G_1) over total cells was calculated and expressed as percent of apoptosis.

Preparation of Cell Extracts and Immunoblot Analysis

Cells were plated on dishes (15 cm^2) at a density of 2×10^5 cells/mL with (2 mM) or without (0.1% DMSO as solvent control) *Hibiscus* PCA for the indicated times and then harvested. To prepare whole-cell extracts, cells were washed with PBS containing zinc ion (1 mM) and suspended in a lysis buffer (50 mM Tris, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 81 μg/mL of aprotinin, 170 μg/mL of leupeptin, 100 μg/mL of phenylmethylsulfonyl fluoride; pH 7.5). After 30 min of mixing at 4° the mixtures were centrifuged (10,000 *g*) for 10 min, and the supernatants were collected as whole-cell extracts. Then, protein content was determined with the Bio-Rad protein assay reagent using BSA as a standard. To perform western blotting, whole cell lysates (50 μg protein) from control and *Hibiscus* PCA (2 mM)-treated samples were resolved on 12% SDS–PAGE gels along with prestained protein molecular weight standards (Bio-Rad). Proteins were then blotted onto NC membrane (Sartorius) and reacted with primary antibodies (anti-RB and anti-Bcl-2 from Transduction Laboratories; anti-Bax from Oncogene Products; anti-β-tubulin from Sigma as internal control). The secondary antibody was a peroxidase-conjugated goat anti-mouse antibody. After binding, the bands were revealed by enhanced chemiluminescence using an ECL commercial kit.

Statistical Analysis

Data were reported as means ± SD of three independent experiments and evaluated by one-way ANOVA. Significant differences were established at $P < 0.05$.

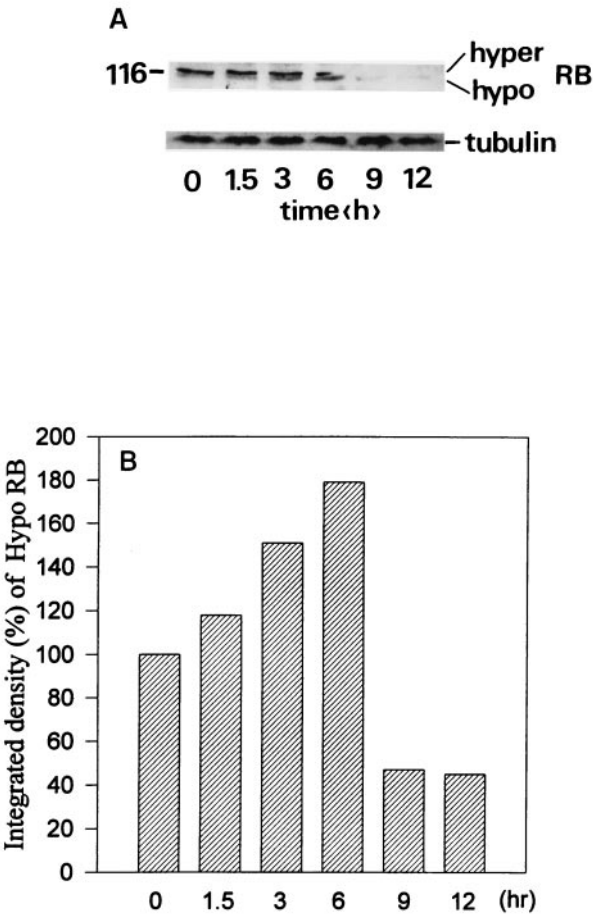

FIG. 6. Effect of *Hibiscus* PCA on the RB protein level of HL-60 cells. (A) Total cell lysates (50 μg protein) of HL-60 cells treated with 2 mM *Hibiscus* PCA for the indicated times were analyzed by 8% SDS–PAGE, and subsequently immunoblotted with antisera against RB and β-tubulin, which served as an internal control. (B) Densitometric quantitation of the autoradiogram in arbitrary units. The result shown is one data set representative of two independent experiments.

RESULTS

Cytotoxicity of Hibiscus *PCA to HL-60 Cells*

Human leukemia HL-60 cells were treated with different amounts of *Hibiscus* PCA (0.2 to2 mM) for 24 and 48 hr. Figure 2 shows that *Hibiscus* PCA presented a concentration-dependent inhibitory effect on the growth of HL-60 cells. Morphological examination showed that *Hibiscus* PCA-treated cells expressed typical characteristics of apoptosis, including cell shrinkage, membrane blebbing, and apoptotic bodies (data not shown). Treatment with *Hibiscus* PCA (2 mM) for 24 hr also induced the appearance of condensed and fragmented nuclei as stained by hematoxylin (Fig. 3).

Induction of Apoptosis by Hibiscus *PCA*

Agarose gel electrophoresis of the chromosomal DNA extracted from *Hibiscus* PCA-treated cells revealed a ladder pattern of DNA fragments consisting of multiples of approximately 180–200 bp, using a PCA concentration above 0.75 mM for a 24-hr duration or 2 mM for a duration longer than 9 hr (Fig. 4, A and B). Quantitative analysis of the DNA fragmentation induced by *Hibiscus* PCA was performed using an ELISA kit with BrdU DNA labeling. A 12-hr exposure of HL-60 cells to *Hibiscus* PCA produced a concentration-dependent increase in the degradation of cellular DNA into small double-stranded fragments (Fig. 4C), being significantly higher than the basal level at concentrations of 1 and 2 mM *Hibiscus* PCA. To further examine the degree of apoptosis, we employed flow cytometry to quantify the apoptotic state. Figure 5 shows that HL-60 cells exposed to 2 mM *Hibiscus* PCA for 0, 6, 12, and 24 hr demonstrated 3.31, 5.32, 46.73, and 58.17% apoptosis, respectively. In addition to the hypodiploid phase (apoptosis peak, 46.7%), DNA content analysis exhibited 34.2% of cells in the G_1 phase, 14.0% in the S phase, and 5.1% in the G_2/M phase after a 12-hr treatment with *Hibiscus* PCA (Fig. 5).

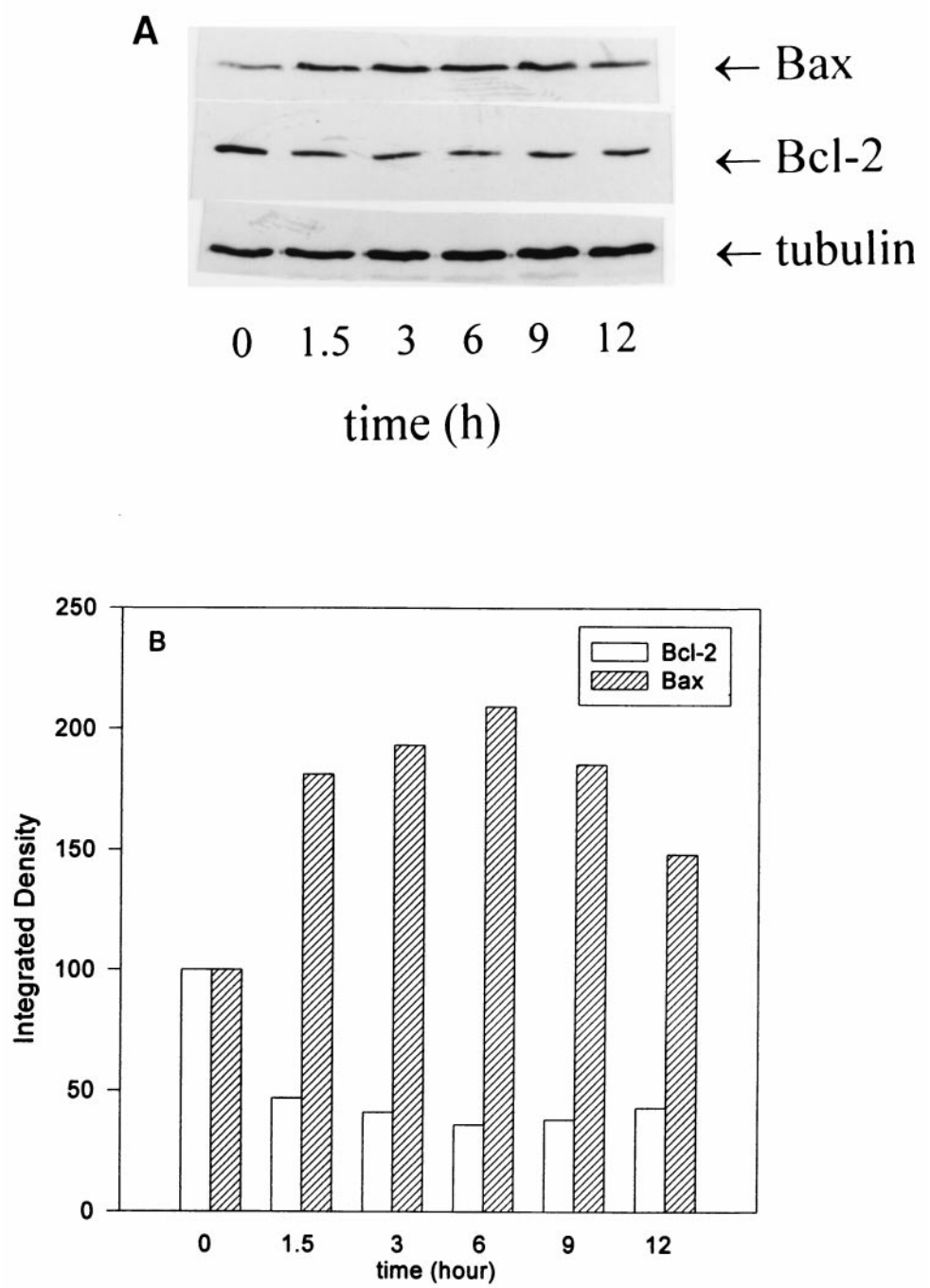

FIG. 7. Effect of *Hibiscus* PCA on Bcl-2 and Bax protein levels of HL-60 cells. (A) Total cell lysates (50 μg protein) of HL-60 cells treated with 2 mM *Hibiscus* PCA for the indicated times were analyzed by 12% SDS–PAGE and subsequently immunoblotted with antisera against Bcl-2, Bax, and β-tubulin, which served as an internal control. (B) Densitometric quantitation of the autoradiogram in arbitrary units. The result shown is one data set representative of two independent experiments.

Effect of Hibiscus PCA *on RB, Bcl-2, and Bax Protein*

Since many experiments suggested the involvement of RB phosphorylation in apoptosis [12, 13, 24, 25], we monitored the time–course of expression of RB protein in HL-60 cells exposed to *Hibiscus* PCA. Treatment with *Hibiscus* PCA for 6 hr induced a decline in hyperphosphorylated RB that became undetectable after 9 hr of treatment (Fig. 6). However, the level of hypophosphorylated RB increased after 3 hr of treatment with *Hibiscus* PCA (<9 hr), which then also declined after a 9-hr treatment with *Hibiscus* PCA.

Recent investigations of the *bcl-2* gene family show a complex network regulating apoptosis. It has been found that whereas Bcl-2 protects cells against apoptosis caused by a variety of physiological and pathological stimuli, Bax counters the effect and accelerates apoptotic cell death [11,

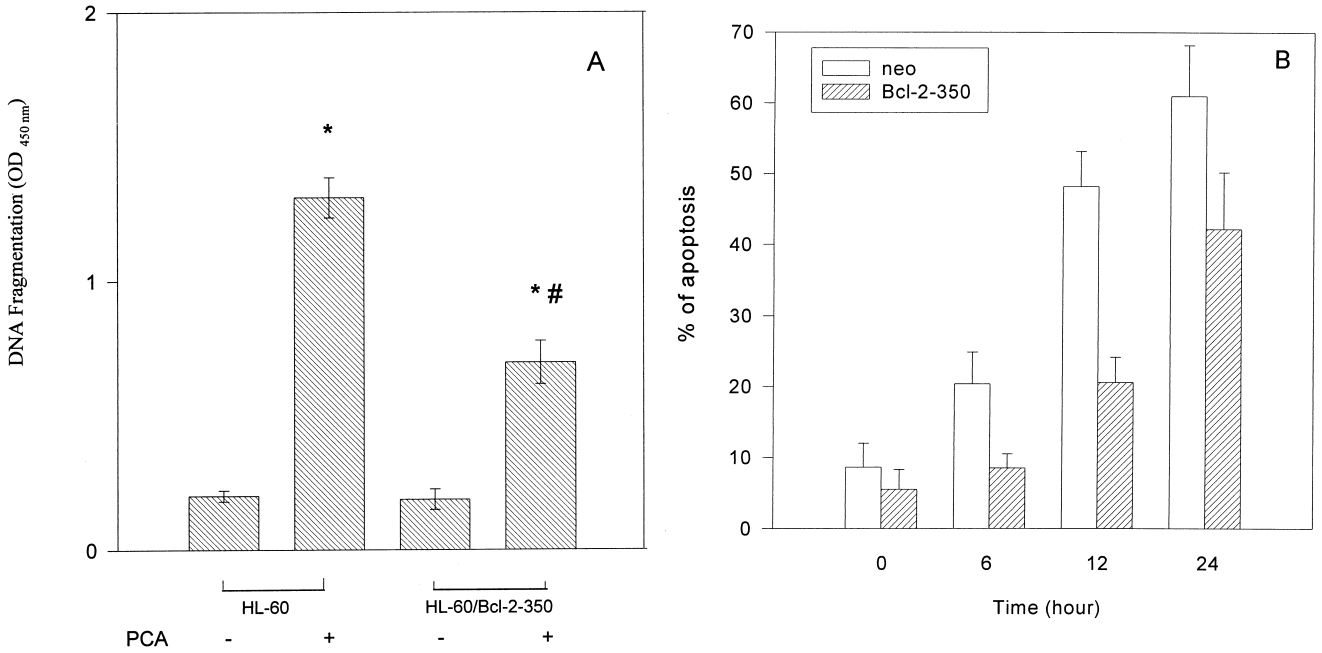

FIG. 8. Resistance of Bcl-2-overexpressing cells to apoptosis mediated by *Hibiscus* PCA. (A) HL-60 and HL-60/Bcl-2–350 cells with prelabeled DNA were incubated in the absence (0.1% DMSO as solvent control) or presence of 2 mM PCA for 12 hr. Then the cells were lysed directly in the wells, and the cytoplasmic DNA fragments were removed and determined photometrically (450 nm) by an ELISA kit. The results are presented as means ± SD of three independent experiments. Key: (*) $P < 0.05$, compared with the control group; and (#) $P < 0.05$, compared with the 2 mM *Hibiscus* PCA-treated HL-60 cell group. (B) Cells transfected with a Bcl-2-expressing vector (HL-60/Bcl-2–350) or with the control neo gene plasmid (HL-60/neo) were exposed to 2 mM PCA for the indicated times. Cells with apoptotic DNA were quantitated by propidium iodide staining and flow cytometry analysis. The results represent means ± SD of three independent experiments.

26]. Therefore, we examined the cellular levels of Bcl-2 and Bax proteins after treatment of HL-60 cells with *Hibiscus* PCA. Bcl-2 was decreased to 47% after a 1.5-hr treatment and subsequently to 36% after a 6-hr treatment, as compared with 0 hr (Fig. 7). On the other hand, Bax protein was increased to 2-fold of the control level after a 6-hr treatment.

Hibiscus *PCA-Induced Apoptosis in Bcl-2-Transfected HL-60 Cells*

To determine if the down-regulation of Bcl-2 modifies the susceptibility of cells to death after *Hibiscus* PCA exposure, we compared *Hibiscus* PCA-induced apoptosis in HL-60 cells versus Bcl-2-overexpressing HL-60/Bcl-2–350 cells [26] by using an ELISA kit. The results showed a significant reduction in the *Hibiscus* PCA-induced DNA fragmentation in HL-60/Bcl-2–350 cells as compared with the parental HL-60 cells (Fig. 8A). In addition, FACS analysis of hypodiploid cells was performed on HL-60/Bcl-2–350 and HL-60/neo cells. In Fig. 8B, flow cytometric analysis demonstrated that exposing HL-60/neo cells to 2 mM *Hibiscus* PCA for 12 or 24 hr resulted in 48.2 and 61.0% apoptosis, respectively; however, Bcl-2-overexpressing HL-60 cells exhibited 20.6 and 42.2% apoptosis.

DISCUSSION

Scientific interest in phenolic compounds in food has been provoked in recent years due to their anti-inflammatory, antimutagenic, and anticarcinogenic properties [17, 18]. It has been suggested that plant phenols have multifunctional biochemical activities including (i) trapping of electrophilic radicals, (ii) inhibition of nitrosation, (iii) modulation of arachidonic acid metabolism, and (iv) alteration of carcinogen metabolism [27]. In our previous study, *Hibiscus* PCA, a phenolic compound isolated from the dried flower of *Hibiscus sabdariffa* L., showed antioxidant [6] and antitumor promotion activity [7]. In this study, we first noted that *Hibiscus* PCA (over 0.2 mM in concentration) showed a cytotoxic effect that was accompanied by an induction of apoptosis in HL-60 human leukemic cells. An exposure to *Hibiscus* PCA for 9 hr resulted in easily detectable chromatin DNA fragmentation. Flow cytometric analysis also demonstrated that hypodiploidy appeared conspicuously after 12 hr of treatment with *Hibiscus* PCA, and the surviving cells were distributed mainly in G_1 phase.

Evidence to date has suggested that apoptosis may be a consequence of functional inactivation or loss of RB [28]. The phosphorylated state of the RB protein varies with cell cycle and proliferation events. RB protein becomes transiently hyperphorylated in early S phase. The hyperphosphorylated form of RB leads to release of the E2F transcrip-

tion factor, resulting in the transcription of numerous genes involved in cell cycle progression. In contrast, hypophosphorylated RB binds to and inactivates E2F, contributing to G_1 arrest. Our present data showed that after 6 hr of treatment with *Hibiscus* PCA, the level of hyperphosphorylated RB declined and that of hypophosphorylated RB increased. The observation implies that *Hibiscus* PCA prevented HL-60 cells from entering S phase. Recent investigations have reported that during apoptosis RB protein undergoes rapid dephosphorylation, which is followed by a cleavage (N-terminus or C-terminus cleavage of RB) event catalyzed by the ICE-like proteases [24, 25]. The hypophosphorylated form of RB might serve as a substrate of the activated mammalian ICE-like proteases (caspases) [29]. To date, at least ten different caspases in mammalian cells have been identified. Many of those may play a key role in the initiation and execution of apoptosis induced by various stimuli [30–33]. The dramatic reduction of hyperphosphorylated and hypophosphorylated RB after a 9-hr treatment with *Hibiscus* PCA suggests that while the *Hibiscus* PCA-treated HL-60 cells underwent G_1-arrest, the cleavage of cellular RBs led to the subsequent apoptosis. However, the mechanism by which *Hibiscus* PCA mediates the phosphorylation change of RB protein and the activation of caspase needs further investigation.

Considerable attention has been focused on the proto-oncogene *bcl-2*, which encodes an inner mitochondrial protein that reportedly antagonizes apoptosis in many tumor cells [34, 35]. Decreased expression of this protein might contribute to drug-mediated lethality. Recently, the induction of apoptosis in HL-60 cells by taxol [36, 37], curcumin [23], and the retinoid *N*-(4-hydroxyphenyl) retinamide [38] has been associated temporally with the down-regulation of Bcl-2. Consistent with these reports, our study also showed that *Hibiscus* PCA-induced apoptosis was associated with the down-regulation of Bcl-2 after a 1.5-hr treatment (Fig. 7). Furthermore, a gene transfection study provided convincing evidence that elevation of Bcl-2 protein indeed delayed *Hibiscus* PCA-induced apoptosis (Fig. 8). Taken together, these findings suggest that Bcl-2 protein plays a prominent role in *Hibiscus* PCA-induced apoptotic cell death.

In conclusion, *Hibiscus* PCA exhibited an antiproliferative effect on HL-60 cells by inducing apoptosis, which was associated with the phosphorylation and degradation of RB and the suppression of Bcl-2 protein. Therefore, we suggest that the anti-tumor effects of *Hibiscus* PCA may be, at least in part, attributed to its apoptosis-inducing activity.

This work was supported by a National Science Council Grant (NSC 88–2314-B-040–017) and the Chung Shan Medical and Dental College Research Fund (CSMC 87-OM-A-013).

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