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Induction of Apoptosis by Selected Pakistani Medicinal Plants in Human Lymphoma U937 Cells

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ABSTRACT

Traditional medicines have long been utilized to treat different ailments and their anti-cancer potential has been well developed. In the present study, the apoptosis inducing ability of selected Pakistani medicinal plants was investigated in human lymphoma U937 cell. When the U937 cells were treated with ethanol extracts of medicinal plants for 24 h at a concentration of 100 µg/ml, evidence of apoptotic features, such as increase in the DNA fragmentation % was obtained. Among the four medicinal plants tested, *Polygonumbistota* (PB) showed significant (*p<0.001) increased in the DNA fragmentation % at 50 µg/ml and 100 µg/ml, respectively. Furthermore, PB treatment resulted in the activation of caspase-3. In addition, the activation of Bid was also observed following treatment with PB. Taken together, our findings provide insight into the possible mechanism of action of PB and its anti-cancer potential.**

Keywords: *Apoptosis, Polygonum bistorta, Pakistani medicinal plants.*

INTRODUCTION

Cancer is still the leading cause of deaths worldwide. Despite, all the developments made in the field of radiotherapy, integrate surgery, hyperthermia and chemotherapy, complete remission of cancer has not been achieved due to the unresolved toxicity issues and resistance to available treatments. This clearly demands for further identification of novel agents or treatments with high efficacy and low toxicity. Since, it has been known that the therapeutics effects of many therapies are based on their apoptosis inducing ability, and aberrant regulation of apoptosis can promote carcinogenesis and tumor progression[1]. Therefore, the identification of newer apoptosis inducers is required to allow improved therapeutic effects.

Apoptosis, which is often termed as a cell suicide or genetically programmed-cell death, is characterized by the changes in the cell morphology that include plasma membrane blebbing, shrinkage of cell, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation [2,3]. Apoptosis play crucial roles in the host defense and cancer suppression; dysregulation of apoptosis results in the pathogenesis of several diseases including cancer, autoimmune and degenerative disorders [4, 5]. It is triggered via two main pathways, extrinsic (death receptor

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pathway) due to the engagement of cell surface death receptors such as Fas and tumor necrosis factor (TNFR) with their ligands [6]. The intrinsic (mitochondrial) pathway is activated due to the release of cytochrome-c into the cytoplasm, because of the interactions between Bcl-2 family proteins[7].

In recent decades, traditional or herbal medicines have gained increased attention due to their anti-cancer potential. It has been widely accepted that herbal medicines and dietary supplements improve the organ function and human immunity, and have been found particularly beneficial in the treatment of cancer [8]. Accordingly, the purpose of this study is to determine the possible apoptosis inducing effects of some selected Pakistani medicinal plants on the human lymphoma U937 cells.

EXPERIMENTAL

Cell culture

A human myelomonocytic lymphoma cell line, U937, was obtained from Human Sciences Research Resource Bank (Japan Human Sciences Foundation, Tokyo, Japan). The cells were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in humidified air with 5% CO_2.

Preparation of extracts

Plant extracts were kindly provided by Dr. Faisal Haider Zaidi (Department of Basic Medical Sciences, College of Medicine, King Saud bin Abdulaziz University of Health Sciences Jeddah, Kingdom of Saudi Arabia). All the extracts were prepared as previously described method. Briefly, total four medicinal plants were randomly selected in this study on the basis of their use in GI disorders. All the plants were purchased from a local market of Karachi, Pakistan, authenticated by Dr Iqbal Ahzar, Department of pharmacognosy, University of Karachi, Karachi, Pakistan and authentic voucher specimens have been deposited in the Museum of Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine,University of Toyama, Toyama, Japan [9].

The powdered plant material (5-50g) was soaked twice with 50-100 ml of aqueous ethanol(30:70) for 48h at room temperature[10]. Solvent was evaporated under reduced pressure and each extract was dissolved at 100 mg/ml with dimethylsulfoxide (DMSO) and the final concentration of DMSO was <0.1% in the cell culture medium which has no effect on any of the experiment performed in this study.

Cell treatments

A cell suspension containing 1 x 10^6 cells/ml was treated with or without medicinal plants extract for desired time period. After the treatment, all the cells were incubated at 37°C until analysis.

DNA fragmentation assay

For the detection of apoptosis the percentage of DNA fragmentation was assessed 6, 12 and 24 h post treatment using the method of Sellins and Cohen[11] with minor modifications. Briefly, approximately 3 x 10^6 cells were lysed using 200 µl of lysis buffer (10 mMTris, 1 mM EDTA and 0.2% Triton X- 100, pH 7.5) and centrifuged at 13,000g for 10 min. Subsequently, each DNA sample in the supernatant and the resulting pellet was precipitated in the 25% trichloroacetic acid (TCA) at 4°C overnight and quantified using a diphenylamine reagent after hydrolysis in 5% TCA at 90°C for 20 min. The percentage of fragmented DNA in each sample was calculated as the amount of DNA in the supernatant divided by total DNA for that sample (supernatant plus pellet).

Western blot analysis

The cells were collected and washed with cold PBS. They were lysed at a density of 2.5 x 10^6 cells / 70 µl of RIPA buffer (50 mMTris-HCl, 150 mMNaCl, 1% Nonidet P-40 (v / v), 1% sodium deoxycholate, 0.05% SDS, 1 µg of each aprotinin, pepstatin and leupeptin and 1mm phenylmethylsulfonyl fluoride) for 20 min. Following brief sonification, the lysates were centrifuged at 12,000 g for 10 min at 4°C, and the protein content in the supernatant was measured using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Protein lysates were denatured at 96°C for 5 min after mixing with2 µl SDS-loading buffers, applied on an SDS-polyacrylamidegel for electrophoresis, and transferred to nitrocellulose membrane. Western blot analysis was performed to detect Caspase-3 and Bid expression using specific antibodies. Antibodies against Caspase-3, and Bid were obtained from Cell Signalling technology (Danvers M.A). Blots were then probed with either secondary horseradish peroxide (HRP)-conjugated anti-rabbit or anti-mouse IgG antibodies obtained from Cell Signaling. Band signals were visualized on a luminescent image analyzer (LAS 4000, Fujifilm Co., Tokyo, Japan) by using chemi-luminescence ECL detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Statistical analysis

All values are expressed as the means ± S.D. of the respective test or control group. Statistical significance between the control group and test groups was evaluated by Student's t-test. Values of $p < 0.05$ were considered to be significant.

Table. 1 Effects of medicinal plants on U937 cells. **p<0.01 vs control.

	Con	12 h	24 h
Polygonum bistorta L	9.6	19.5**	41.1**
Alpinia galaugal Wild	8.8	14	13
Ruta graveolens L	9.5	12.9	16.1
Rosa damascene Miller	9.7	12	10

RESULTS AND DISCUSSION

Herbal or traditional medicines such as Indian, Chinese, and Kampo (Japanese herbal) medicines are widely accepted as complementary and alternative cancer treatments [12,13]. In this study, human lymphoma U937 cells were treated with extracts of Polygonumbistorta, Alpinia galangal Wild, Rutagraveolens L, Rosa damascene Miller at a concentration of 100 µg/ml for 12 and 24 h, followed by the colorometric DNA fragmentation assay, a hallmark of apoptosis. As illustrated in Table. 1, treatment with Alpinia galangal Wild, Rutagraveolens

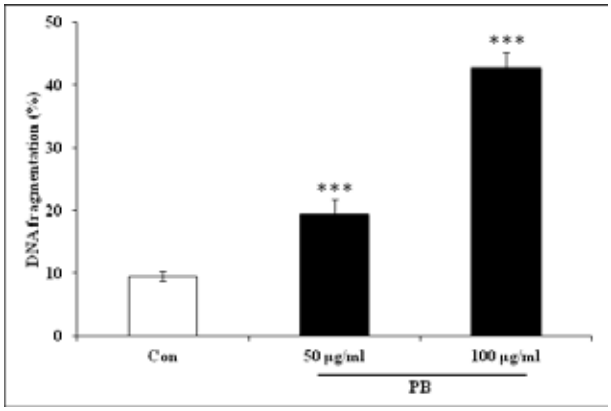

Fig. 1. DNA fragmentation induced by PB. U937 cells were treated with PB at different concentrations (50 and 100 µg/ml) for 24 h, followed by DNA fragmentation assay. Where indicated, values for the treated cells are significantly different to the untreated cells at the same time point at ***$p < 0.001$. The results are presented as the mean ± S.D. (n = 3).

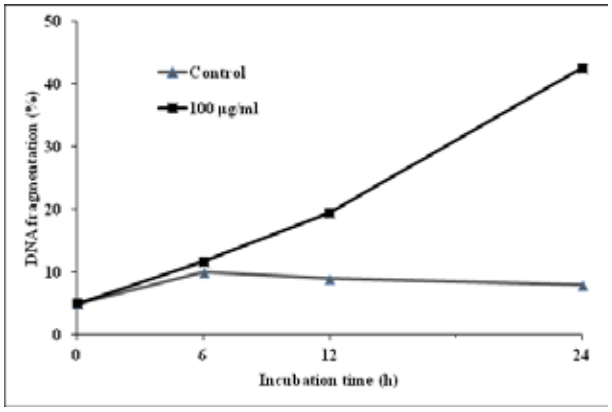

Fig. 2. Time-dependent induction of DNA fragmentation by PB 100 µg/ml in U937 cells. U937 cells were treated with PB 100 µg/ml for 6, 12 and 24h followed by DNA fragmentation assay. The results are presented as the mean ± S.D. (n = 3).

L and Rosa damascene Miller showed no marked increased in the DNA fragmentation (%) as compared to control. However, the DNA fragmentation (%) was significantly increased following treatment with extract of Polygonumbistorta (**p<0.01, **p<0.01 at 12 and 24h, respectively), as compared to control.

Since, increased DNA fragmentation (%) was observed particularly with Polygonumbistorta (PB) compared to other medicinal plants. Therefore, PB was selected for further analysis. U937 cells were treated with PB in a concentration-dependent manner at 50 µg/ml and 100 µg/ml for 24 h. As shown in Fig.1, PB can induce significant apoptosis in a concentration-dependent manner. PB at 50 µg/ml and 100 µg/ml caused significant increase in the DNA fragmentation (%) compared to control (control 9.5 ± 0.8, PB 50 µg/ml 19.2± 2.5 %, PB 100 µg/ml 42.6 ± 2.6%). Based on the data, 100 µg/ml of PB was selected to evaluate the time-dependent effects on U937 cells. Treatment with PB 100 µg/ml showed no DNA fragmentation at initial 6 h. However, DNA fragmentation was apparent at 12 and markedly increased at 24 h with 100 µg/ml of PB (Fig. 2). Taken together these findings indicate that PB induces apoptosis in human lymphoma U937 cells in a concentration- and -time-dependent manner, as evidenced by DNA fragmentation (%).

Caspases are considered the main executor of apoptosis and play an important role in the morphological changes associated with apoptosis. In all the caspases, caspase-3 is the common executor of apoptosis and involved in both the apoptotic pathways. However, caspase-8 is located at upstream in the caspase cascade; which is capable of inducing the release of cytochrome-c in the cytosol from the

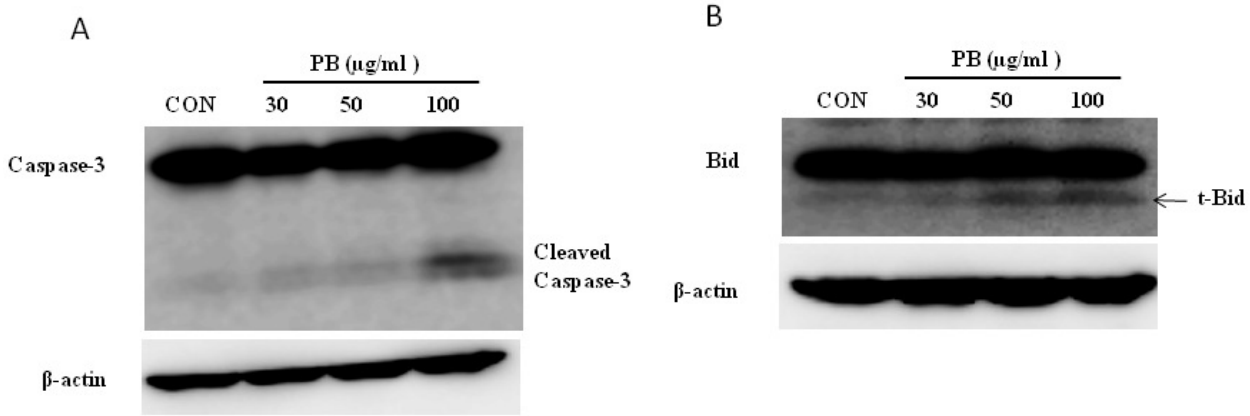

Fig. 3. Assessment of apoptosis related proteins. U937 cells were treated with increasing concentration of PB at 30 µg/ml, 50 µg/ml and 100 µg/ml for 24 h. Changes in the expression of apoptosis related proteins were detected by western blot. (A) Expression of caspase-3. (B) Expression of Bid. One representative image of western blot is shown here.

damaged mitochondria[14,15]. To determine the role of caspase-3 in the apoptosis induced by PB, cells were treated with PB; activation of caspase cascade was determined by western blot using caspase 3 specific antibody. An increased in the cleaved form of caspase-3 was observed following treatment with PB at 24 h (Fig. 3A) .In addition, Bcl-2 family proteins are involved in pro or anti apoptotic process by interacting with the mitochondria [16]. Bid a pro-apoptotic Bcl-2 family member containing BH3 domain, can be cleaved by caspase-8, and the cleaved Bid, the carboxyl-terminal fragment, translocates to the mitochondria to induce the release of cytochrome-c [17, 18]. The two apoptotic pathways ultimately trigger the effector caspase, and could be interconnected by the caspase-8-mediated cleavage of Bid, which triggers the activation of the mitochondrial pathway. In this study, increased Bid activation was observed following PB treatment at 24 h (Fig.3B). Although, the effects of PB were not determined on MMP loss and caspase-8 activation, we speculate that caspase-8 mediated activation of Bid might contribute in the mitochondrial pathway. Taken together; these findings suggest the involvement of the caspase-dependent mitochondrial pathway in PB-induced apoptosis.

CONCLUSION

In conclusion, the present study provides some preliminary insights into the anti-cancer potential of selected Pakistani medicinal plants. Especially, PB can induce apoptosis in human lymphoma U937 cells. However, future experiments are necessary to determine the detail molecular mechanism involved in the PB- induced apoptosis.

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DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest.

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