EJGO European Journal of Gynaecological Oncology

Plumbagin shows anti-cancer activity in human breast cancer cells by the upregulation of p53 and p21 and suppression of G1 cell cycle regulators

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Summary

Purpose: Plumbagin, a naphthoquinone constituent of *Plumbago zeylanica* L. (Plumbaginaceae), is known to exhibit proapoptotic, antiangiogenic and antimetastatic effects in cancer cells. However, the effect of Plumbagin on breast cancer cells and the underlying molecular mechanism has not yet been elucidated. *Materials and Methods:* MCF-7 (a human breast cancer cell line) was exposed different concentrations of Plumbagin (PG), and the anti-proliferative activity was evaluated by the MTT assay. The mechanism of action for the growth inhibitory activity of Plumbagin on MCF-7 cancer cells was evaluated using flow cytometry for cell cycle distribution, and western blot for assessment of expression of potential target proteins. *Results:* Plumbagin exhibited a significant anti-proliferative activity against human breast cancer cells. Flow cytometric analysis revealed that Plumbagin caused cell cycle arrest at G1 phase. The cell cycle arrest was well correlated with the inhibition of cyclin D1, cyclin E, and upregulation of tumor suppressor protein p53. It further inhibited the expression of anti-apoptotic Bcl-2 family members such as Bcl-xL and Bcl-2, and activated pro-apoptotic proteins like Bax and Bak. *Conclusion:* These findings suggest that the anti-proliferative effect of Plumbagin is due to upregulation of p53 and p21 and suppression of G1 cell cycle regulators.

Key words: Plumbagin; MCF-7; Cyclin D1; Cyclin E; p53; Proliferation.

Introduction

Breast cancer causes significant morbidity and mortality among women [1], and metastasis mainly affects outcome of the disease [2]. Lack of effective therapeutic strategies for control and treatment of breast cancers, and the important financial burden placed on individuals and nations mean urgent action must be taken in the fight against breast cancer. Also, side effects due to conventional pharmacological agents have necessitated the search for newer therapies mostly in the form of natural products. In recent years, interest in natural products has grown, and in light of long-term and safe cancer prevention, current approaches have been focused on the use of food and edible medicinal herbs as sources of products that could effectively control cancers [3-5]. This is evident by the fact that approximately 74% of new anticancer compounds are either natural products or natural product-derived [6-9]. In fact, it has been argued that plants may be sources of multiple bioactive compounds that could provide more benefit than single pharmacological agents against chronic diseases, and this may well be beneficial in managing breast cancer. It has been recognized that a large group of therapeutic agents can stop cancer cells proliferation by inducing apoptosis. The induction of apoptosis has been emphasized in anticancer strategies [10]. Apoptosis is a gene regulated phenomenon which is induced by many chemotherapeutic agents in cancer treatment [11]. It is characterized by a series of typical morphological features, such as nuclear and cellular convolution, chromatin condensation, and the final disintegration of the cell into membrane-bound apoptotic bodies which are phagocytosed by neighboring cells [12]. Most normal cells can die by apoptosis but tumor cells very often have some defects in the apoptotic pathway, leading not only to the increase of tumor mass, but also to tumor resistance to chemotherapy. Since chemotherapy and irradiation act primarily by inducing apoptosis, defects in the apoptotic pathway make the therapy less efficient [13]. Increasing evidences suggest that the related processes of neoplastic transformation involve alteration of the normal apoptotic pathway [14]. The major focus of the research in chemotherapy for cancer in recent times is the use of naturally occurring compounds with the chemopreventive and chemotherapeutic properties in the treatment of cancers [15, 16]. Tumors develop not only from abnormal cell pro-

Revised manuscript accepted for publication October 22, 2014

liferation but also from reduced cell death due to inhibition of apoptosis [17]. The possibility of modulating apoptosis of tumor cells suggests new strategies for improving chemotherapy [18]. Apoptosis is regulated by gene products, which are conserved from nematodes to mammals [19]. Among all apoptosis-related genes, *p53* is of particular importance [20]. *p53* is well known for suppression of cellular proliferation through two mechanisms, each operating in a distinct manner. In normal fibroblasts, p53 induces G1 arrest in response to DNA-damaging agents, presumably allowing the cells to perform critical repair functions before progressing through the cell cycle [21]. In abnormally proliferating cells or irradiated thymocytes, induction of p53 leads to apoptosis [22]. Furthermore, wild-type p53 protein was increased during apoptosis induced by DNA-damaging agents [23].

Plumbagin, a naphthoquinone constituent of *Plumbago zeylanica* L. (Plumbaginaceae) (Figure 1), has been used in traditional medicine as an antifungal, antibacterial, and anti-inflammatory agent [24]. Plumbagin is known to exhibit proapoptotic [25], antiangiogenic [26], and antimetastatic effects in cancer cells [27]. Plumbagin is also known to inhibit NF-κB [28], JNK [29], PKCε, and STAT-3 [30]. This compound activates GSK-3β by inhibiting its inhibitory phosphorylation at Ser9 [31], decreases the expression of cyclin D1 [32], and modulates the acetyltransferase activity of p300 [33]. However, the effect of Plumbagin on breast cancer cells and the underlying molecular mechanism has not been elucidated yet. The anti-proliferative activity was evaluated by the MTT assay. The mechanism of action for the growth inhibitory activity of Plumbagin on MCF-7 cancer cells was evaluated using flow cytometry for cell cycle distribution, and western blot for assessment of expression of potential target proteins. Plumbagin exhibited a significant anti-proliferative activity against human breast cancer cells. Flow cytometric analysis revealed that Plumbagin caused cell cycle arrest at G1 phase. The cell cycle arrest was well correlated with the inhibition of cyclin D1, cyclin E and upregulation of tumor suppressor protein p53. It further inhibited the expression of anti-apoptotic Bcl-2 family members such as Bcl-xL and Bcl-2, and activated pro-apoptotic proteins like Bax and Bak. These findings suggest that the anti-proliferative effect of Plumbagin is due to upregulation of p53 and p21 and suppression of G1 cell cycle regulators.

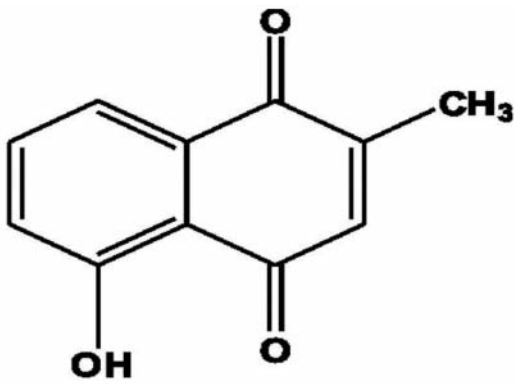

Figure 1. — Chemical structure of Plumbagin (molecular formula: 5-hydroxy-2-methyl-1,4-naphthoquinone, molecular weight: 188.18).

Materials and Methods

Cell lines

MCF-7, a human breast cancer cell line, was cultured as monolayers in RPMI 1640 supplemnted with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic (ABAM) at 37°C in a humidified incubator of 95% air and 5% CO2.

Cell proliferation analysis

The anti-proliferative effect of Plumbagin on MCF-7 cells was examined using MTT colorimetric assay. Cells were seeded in 96-well plates at a density of $5x10^3$ cells per well for 24 hours before exposure to the indicated concentrations (0.1, 1, 10 μg/ml) of PG for 24, 48 and 72 hours, respectively. 0.5% DMSO was used as a negative control (0 mg/ml). MTT reagent was dissolved at a concentration of five mg/ml in sterile PBS at room temperature. After removal of the medium, 20 ml was added to each well and followed by four hours incubation. The MTT solution was carefully aspirated and the purple formazan crystals produced by the mitochondrial dehydrogenase enzymes were dissolved in DMSO. The optical density of each well was measured at 570 nm on a scanning multiwall spectrophotometer.

Flow cytometric cell cycle analysis

The effect of PG on MCF-7 cell cycle distribution was determined by flow cytometric analysis. Cells were seeded in six-well plates at $1.4X10^5$ cells per well, cultured for 24 h prior to the indicated concentrations of PG exposure for 24, 48, and 72 hours, respectively. The cells were detached from the plates by trypsinization and fixed in 70% cold ethanol (added in a dropwise manner) for at least two hours at 4°C. Prior to flow cytometric analysis, the cell solutions were centrifuged at 300g for five minutes and the pellet was re-suspended in one ml of PBS/1% FBS. After centrifuging at 300 g for five minutes, the fixed cells were then treated with 0.5 ml of RNase A (200 mg/ml) and incubated for ten minutes at room temperature. The DNA content per cell was analyzed using flow cytometry after being stained with PI staining solution (two mg/ml) for 20 minutes at room temperature in darkness. At least 10,000 cells were analyzed for each experimental condition. Data analysis was performed using CellQuest cell cycle analysis software.

Western blot analysis

Cells were seeded in six-well plates at 2 x 10^5 cells per well and followed by overnight incubation before treating with PG at the indicated concentrations and time intervals. Proteins from PG treated MCF-7 and untreated cells were extracted with ice-cold cell lysis buffer containing protease inhibitor cocktail. Protein lysates were boiled in Laemmli sample buffer (1:1 dilution) at 100°C for five minutes and resolved by electrophoresis on 10%

or 12% SDS polyacrylamide gels. After gel electrophoresis, the proteins were electro-transferred to a nitrocellulose membrane (0.45 mm) at 15 V for 28 minutes. Membranes were then blocked with 5% milk in TBS-Tween 20 for 30 minutes at 70 rpm, room temperature, followed by washing for three times, for ten minutes each at 110 rpm. Thereafter, the membranes were probed with corresponding primary antibodies (p53, p21, cyclin D1, cyclin E, Bcl2 and Bcl-xl, Bax and Bak) for proteins of interest overnight at 4°C, 70 rpm. After overnight incubation, the membranes were washed three times, for ten minutes each at 110 rpm before probing with corresponding secondary antibodies. The immunoblots were examined with the enhanced chemiluminescence kit. Antibodies were diluted as recommended by the manufacturers for Western blotting.

Statistical analysis

Results were analysed by one-way analysis of variance (ANOVA) or Student's t-test, and differences were considered statistically significant at the level of p-values < 0.05.

Results

Effect on cell proliferation

PG was first examined for its ability to inhibit the proliferation of MCF-7 cells using MTT assay. As shown in Figure 2, PG inhibited the proliferation of MCF-7 cells in a time- and dose-dependent manner. Interestingly, at the highest dose of PG (ten µg/ml), cell viability was more significant at all the three indicated time points (Figure 2).

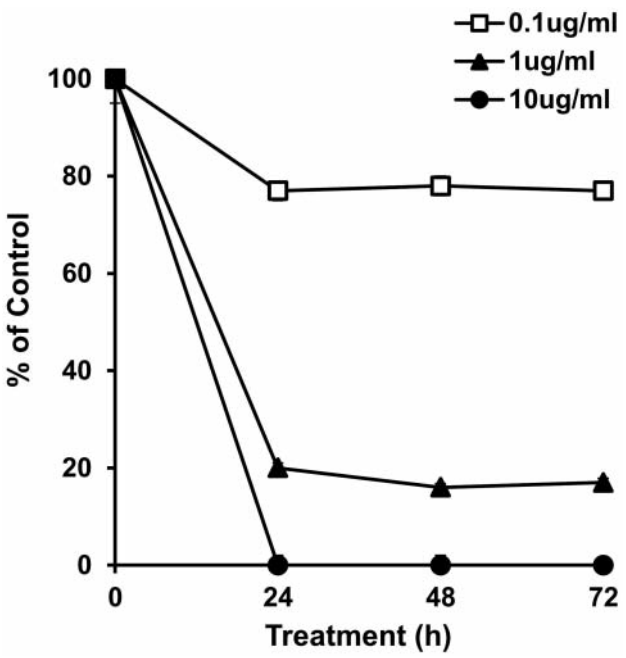

Figure 2. — Effect of Plumbagin on proliferation of MCF-7 cells. MCF-7 cells were plated in a 96-well plate in RPMI supplemented with 10% FBS, and incubated with different concentrations of the test compound for 24, 48, and 72 hours. Anti-proliferative effect was determined using MTT assay. Absorbancies were measured at 570 nm and expressed in relation to absorbancies of control (untreated) cells. Values are expressed as means ± SD of three experiments.

Effect on cell cycle distribution

Considering the fact that PG inhibited cell proliferation, flow cytometric analysis on cell cycle progression was performed to determine the mechanism for this anti-proliferative effect of PG on the human breast cancer cells. In these cells, PG induced time- and dose-dependent growth arrest in the G1 phase of the cell cycle. PG showed a marked increase in proportion of cells at all its concentrations with a maximum at ten µg/ml (58.9%) as compared to control (15.4%). The percentage of cells accumulating in G1 phase of cell cycle for other two doses was 43.4% for 0.1 µg/ml and 50.7% for one µg/ml of PG (Figures 3A, 3B).

Effect on the expression of cell cycle regulators and other signalling proteins

In order to understand the possible molecular events associated with PG -induced growth arrest in MCF-7 cells, various cell cycle regulatory proteins were examined by western blot analysis. Since PG was shown to induce cell specific G1 cell cycle arrest, and it is known that MCF-7 cells express wild type p53, it was hypothesized that PG can induce activation of p53, a tumour suppressor gene that plays a vital role in the cell cycle. p53 activation is the most commonly implicated mechanism of G1-phase arrest following drug exposure [1]. Hence, p53, p21Waf1/Kip1 (a downstream transcriptional target gene of p53 and a cyclin-dependent kinase inhibitor) and other cell cycle proteins regulating cell cycle progression at the G1 boundary, such as cyclin D1 and cyclin E were detected by western blotting. As depicted in Figure 4A and 4B, PG treatment remarkably increased the p53 expression level in MCF-7 cells. Expectedly, the increased p53 level was correlated with an upregulation of its transcriptional target gene, p21. The expression levels of cyclin D1 and cyclin E also decreased significantly. Together, these results suggest that PG arrested MCF-7 cells in the G1 phase of the cell cycle through the upregulation of p53 and p21 genes and the suppression of G1 cell cycle regulators. PG also inhibited the expression of anti-apoptotic Bcl-2 family members such as Bcl-xL and Bcl-2, and activated pro-apoptotic proteins like Bax and Bak (Figures 4C, 4D).

Discussion

The aim of this study was to determine the anti-cancer effects of PG against human breast cancer cells and its possible mechanisms of action. The present report thus delineates the underlying mechanisms by which PG induces cell

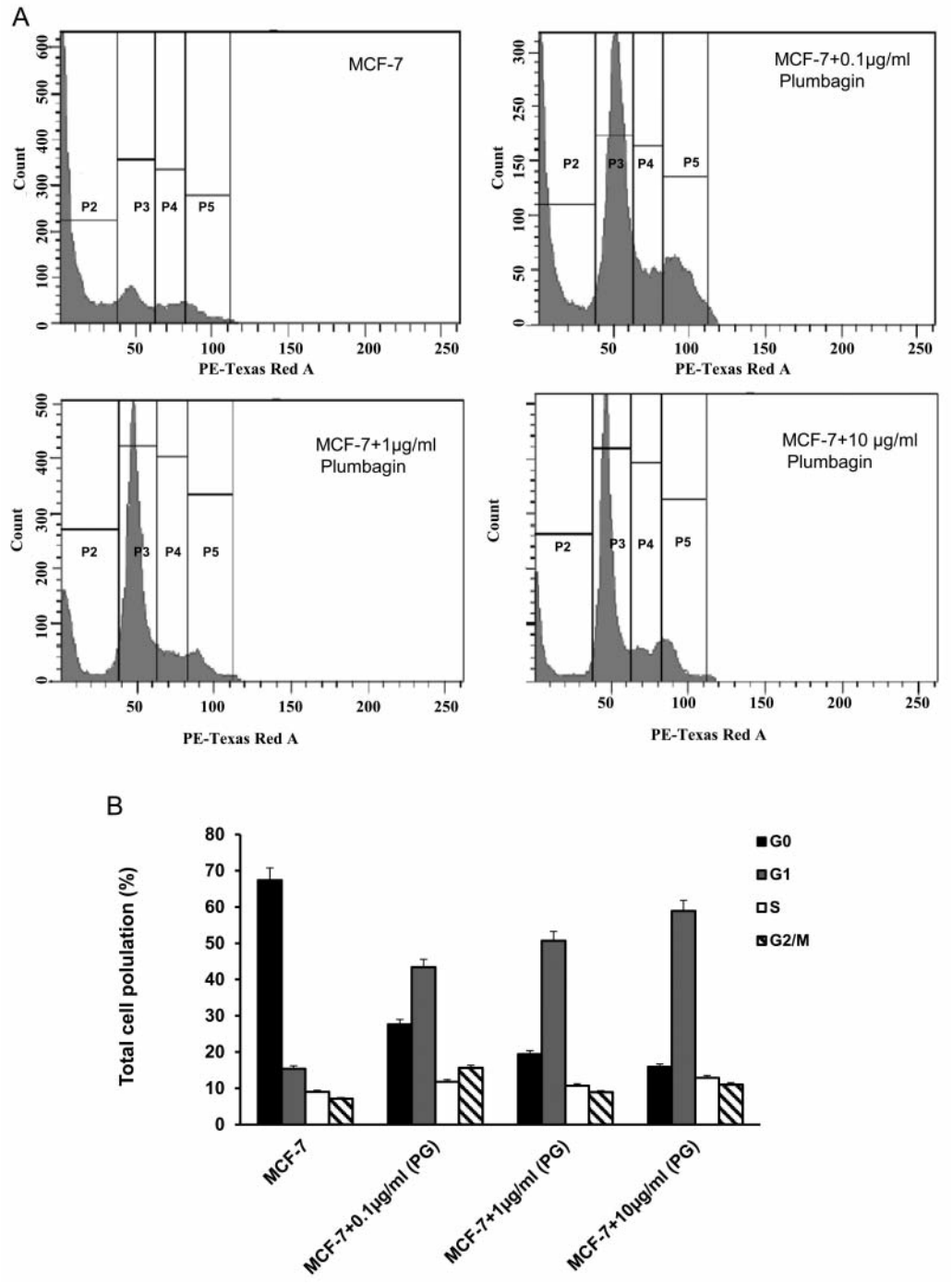

Figure 3. — (A, B) Effect of Plumbagin on regulation of cell cycle distribution in MCF-7 cells. Cells were seeded in six-well plates at 1.4 x 10^5 cells per well, in RPMI supplemented with 10% FBS, and then treated with various concentrations of Plumbagin A for 24 hours. The cell cycle distribution was analyzed by flow cytometry as described in Materials and Methods. Plumbagin arrested the growth of breast cancer cells in G1 phase in a dose dependent manner.

cycle arrest. PG inhibited the proliferation of MCF-7 cells in a time- and dose-dependent manner. In addition, it caused cell-type specific G1 growth arrest in MCF-7 cells through the suppression of the expression of cyclin D1 and cyclin E, but via a p53-independent pathway. It further inhibited the expression of anti-apoptotic Bcl-2 family members such as Bcl-xL and Bcl-2, and activated pro-apoptotic proteins like Bax and Bak.

PG showed a significant anti-proliferative activity, measured by MTT assay. It inhibited the proliferation of MCF-7 cells to a significant extent at its higher doses. PG caused the arrest of MCF-7 cells in G1 phase of cell cycle. Since p53 activation is the most commonly implicated mechanism of G1-phase arrest following drug exposure [34], the present authors evaluated the expression of p53 and p21, a downstream transcriptional target gene

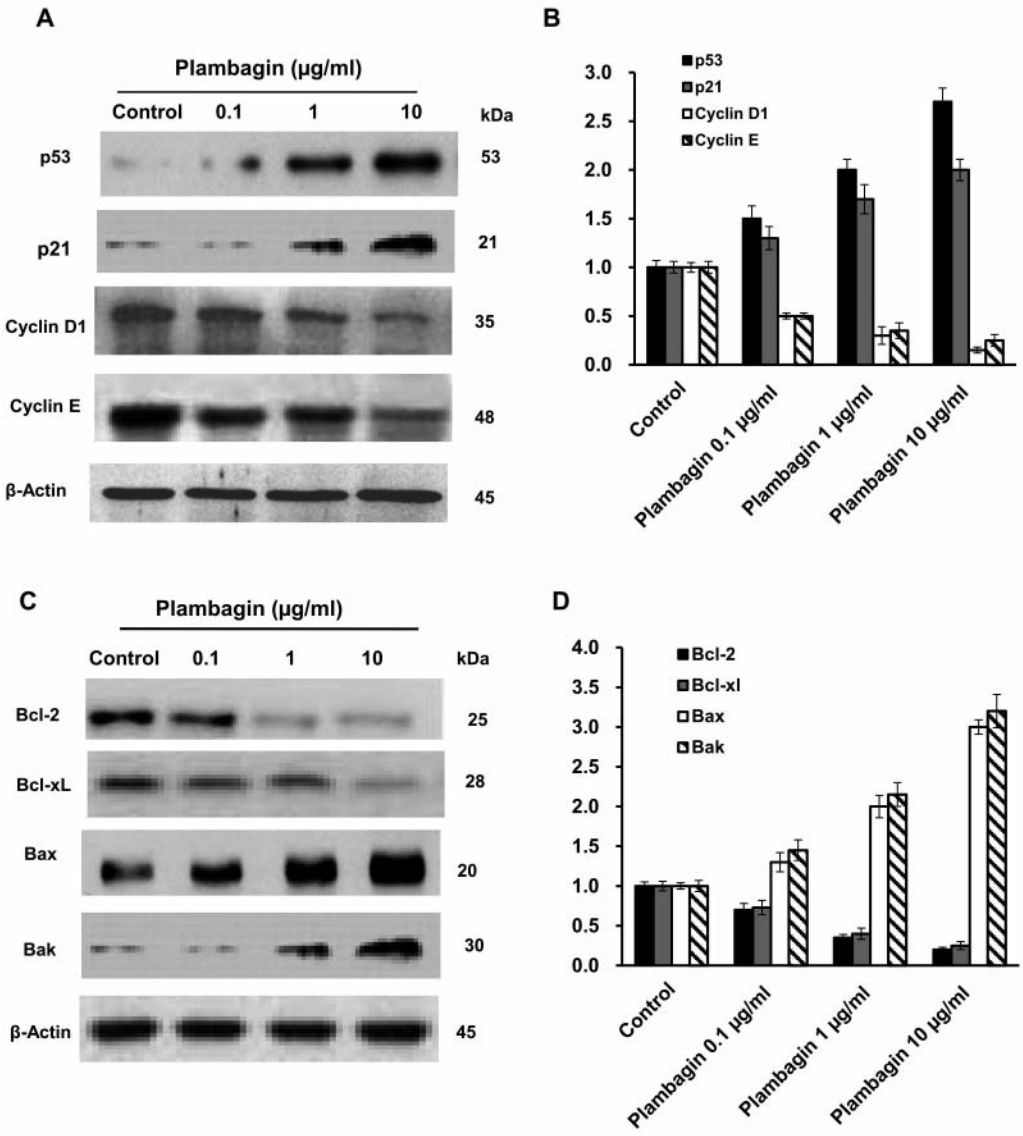

Figure 4. — Western blot analysis (A, B) Effect of Plumbagin on the expression of cell cycle regulator proteins and tumor suppressor proteins including p53, p21, cyclin D1, and cyclin E associated with arrest of G1 phase of cell cycle. (C, D) Effect of Plumbagin on the expression of various anti-apoptotic and pro-apoptotic proteins. It inhibited the expression of anti-apoptotic Bcl-2 family members such as Bcl-xL and Bcl-2 and activated pro-apoptotic proteins like Bax and Bak.

of p53 through western blot. It was found that PG suppressed the expression of both p53 and p21 in a dose dependent manner with highest inhibition at ten µg/ml. It was also observed that PG attenuated the expression of cyclin D1 and cyclin E in these cells, dose dependently. This provides strong evidence that PG arrested MCF-7 cells in the G1 phase, thereby inhibiting their progression to the S phase.

The p53 tumour suppressor has been termed 'the guardian of the genome' because of its pivotal role in safeguarding the integrity of genetic information in response to various genotoxic injuries [35, 36]. Besides the previously mentioned role of suppressing growth arrest, the induction of apoptosis is one of the central activities by which p53 exerts its tumour suppressing function. It has been widely known that p53, as a transcription factor, promotes apoptosis through the transcription of its target genes such as Bcl-2 family members. However, an increasing number of studies has shown the existence of a transcription-independent mechanism – i.e. a direct localization of p53 to the mitochondria, such that p53 can interact directly with Bcl-2 or Bcl-xL to promote apoptosis [36, 37]. Here the results showed that PG treatment downregulated the expression of Bcl-2 and Bcl-xL and upregulated the expression of Bax and Bak in a dose dependent manner.

In conclusion, PG shows anti-cancer effects in MCF-7 mediated through the inhibition of cell proliferation of these cells. This study demonstrates the potential applications of PG as an anti-cancer agent and thus paving the way for further research on PG in the field of anti-cancer drug discovery.

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