**Dracocephalum palmatum Stephan extract induces apoptosis in human prostate cancer cells via the caspase-8-mediated extrinsic pathway**

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**ABSTRACT** Dracocephalum palmatum Stephan is a medicinal plant traditionally used by nomadic people in Eastern Russia; however, research on this plant is currently limited. Recently, although studies have been conducted on the constituents of this plant and their antioxidant effects, data on its various pharmacological activities are still lacking. Thus, this study examined the anticancer potential of the dried leaves of *D. palmatum* S. (DpL) using human prostate cancer PC-3 cells. The antioxidant potential of DpL was evaluated by estimating the total flavonoid and total phenolic content (TFC and TPC, respectively). Additionally, we investigated the effects of the DpL ethyl acetate fraction (DpLE) on cell proliferation, intracellular reactive oxygen species (ROS) generation, apoptosis, and cell cycle arrest in this cell line. The expression levels of superoxide dismutase (SOD)-1, SOD-2, B-cell lymphoma 2 (Bcl-2) and Bcl-2 associated X (Bax) ratio, phospho-protein kinase B (p-AKT), cleaved caspase-8, poly adenosine diphosphate (ADP) ribose polymerase (PARP), and cleaved-PARP were evaluated by western blotting. The results indicated that DpLE causes apoptosis and exerts intracellular ROS-independent anticancer effects on prostate cancer cells, associated with increased SOD-2, cleaved caspase-8, and cleaved-PARP expression and inhibited p-AKT signaling. Thus, DpLE may be a potential resource for the development of promising chemotherapeutic agents for prostate cancer.

**KEY WORDS** Dracocephalum palmatum Stephan; Prostate cancer; Apoptosis; Caspase-8

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**Introduction**

Prostate cancer, one of the most common cancers occurring globally, is the third leading cause of cancer-related death in the United States. Prostate cancer exhibits a slower multiplication rate than most other cancers at the early stages, showing relatively few or no noticeable symptoms. However, if these cancer cells undergo advanced metastasis, it could spread to the bones or lymph nodes. Prostate cancer treatment includes various therapies, such as surgery as well as hormone and radiotherapy, which have been used worldwide; however, many of these treatments have side effects (1-3). Therefore, it is necessary to develop anticancer agents that are effective but have low side effects.

When normal cells transform into cancerous cells, they lose their inherent properties and show unlimited proliferation and metastasis. Cancer cell growth is caused by an imbalance in proliferation and apoptosis. Deficiency of apoptosis prolongs the life of cancer cells and induces the accumulation of gene abnormalities, thus facilitating cancer progression, including metastasis (4, 9). Therefore, the development of a therapeutic agent to target apoptosis in cancer cells may be a suitable approach to develop a major anticancer drug.

Apoptosis can occur via either the extrinsic (cell death receptor-mediated) or intrinsic (mitochondria-mediated) pathways; these major modes of cell death, along with their underlying molecular mechanisms, have been studied extensively. The extrinsic pathway can be induced via the activation of cell death receptors, including first apoptosis signal (Fas), tumor necrosis factor (TNF)-α, death receptor 4 (DR4, TRAIL-receptor 1), and DR5 (TRAIL-receptor 2), by their respective ligands. The cell death receptor ligands, through receptor oligomerization, initiate characteristic early stage signaling, which involves the recruitment and activation of the caspase cascades. Among these, sufficient recruitment of...
caspase-8 and its effector caspase can lead to apoptosis (Type I extrinsic pathway); this pathway also involves the activation of caspase-3 and DNA fragmentation \([8, 10-13]\). During DNA replication, the poly adenosine diphosphate (ADP) ribose polymerase (PARP) protein recognizes and initiates the repair of damaged DNA in the nucleus, by activating DNA repair-related proteins via a post-translational process \([14, 15]\).

Antioxidants are potent chelators of redox-active metal ions; they inactivate free radical chain reactions by hindering the conversion of hydroperoxides to reactive oxygen radicals. These radicals are well known to initiate molecular reactions in many physical and biological systems \([16, 17]\). Lu et al. reported that antioxidants can cause direct damage to DNA and cells because they are rich in weakly binding electrons \([18]\), leading to speculations that antioxidants could also act to kill cancer cells. However, Akan et al. reported that, although the reduction of superoxides by antioxidants and the resulting cellular protective effects may help prevent cancer, intake of antioxidants during cancer treatment may also protect cancer cells \([19]\). Therefore, whether antioxidant use will help patients with cancer remains controversial.

Several plants belonging to the genus Dracocephalum have been reported to possess anticancer, antioxidant, and cardio-protective effects \([19-24]\). However, no study, to our knowledge, has reported the anticancer activity of Dracocephalum palmatum Stephan. Therefore, here, we comprehensively examined the dried leaves of D. palmatum (DpL) and the effects of various extracts on intracellular reactive oxygen species (ROS) levels, cell cycle distribution, apoptotic cell population, and apoptosis-related protein levels to assess any potential anticancer activity.

**Materials and Methods**

**Reagents**

Methanol was purchased from SK Chemicals (Ulsan, Korea). Dimethyl sulfoxide (DMSO) was obtained from Junsei Chemical Co., Tokyo, Japan. Standards (rosmarinic acid, naringin, tannic acid, and paclitaxel), phosphomolybdic–phosphotungstic acid reagent (Folin–Ciocalteu’s phenol reagent), sodium carbonate (Na₂CO₃), sodium hydroxide (NaOH), diethylene glycol, thiazolyl blue tetrazolium bromide (MTT), 2′, 7′-dichlorodihydro-fluorescein diacetate (DCF-DA), and the remaining reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Welgene Inc., (Gyeongsuk, Korea). Penicillin–streptomycin and fetal bovine serum were purchased from Gibco (Los Angeles, CA, USA). The apoptosis detection kit was obtained from BD Biosciences (San Jose, CA, USA). Tris and glycine were purchased from LPS Solution (Daejeon, Korea). BCA reagent was obtained from Thermo Scientific, (Waltham, MA, USA). Antibodies against superoxide dismutase (SOD)-1, SOD-2, B-cell lymphoma 2 (Bcl-2), Bcl-2 associated X (Bax), cleaved caspase-8, and PARP were purchased from Cell Signaling Technology (Danvers, MA, US).

β-Actin and p-AKT antibodies were obtained from Santa Cruz (Dallas, TX, US). Horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies were purchased from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Enhanced chemiluminescence (ECL) substrate was purchased from GenDEPOT (Katy, TX, USA). All other solvents were of analytical grade.

**Preparation of D. palmatum leaf extracts**

Samples of dried leaves of D. palmatum S. (DpL) were procured from the North-Eastern Federal University located in Yakutsk, Russia, and authenticated by Dr. OKHLOP-KOVA (Department of Biology, North-Eastern Federal University). The drug (606.3 g) was extracted in methanol. In addition to the methanol extract of DpL (DpLM), fractions of five solvents from DpLM were obtained (Fig. S1). The fractionation procedures were performed using a glass pyriform separation funnel. The solvents used for fractionation included hexane (DpLH), chloroform (DpLC), ethyl acetate (DpLE), butanol (DpLB), and water (DpLW). The extract and solvent fractions were concentrated under reduced pressure on an EYELA 1 L rotary evaporator (Tokyo Rikakikai Co., Ltd., Japan). The obtained samples were kept in sterile tubes and stored at 4 °C. The samples were dissolved in DMSO and methanol before each experiment.

To validate the content of rosmarinic acid, one of the main components of DpL, we determined the presence of rosmarinic acid, to obtain the fingerprints of DpLM and DpLE used in this experiment (Fig. 1), by high performance liquid chromatography (HPLC) equipped with a YL9100 system, YL9101 vacuum degasser, YL9110 quaternary pump, and YL9120 ultraviolet/visible (UV/vis) detector. Data from the UV/vis detector were stored and processed using the chromatographic software Autochro 3000 (Young Lin instrument Co., Ltd., Gyeonggi, Korea). Sample separation was performed by using an Agilent Eclipse XDB-C₁₈ HPLC column (250 mm × 4.6 mm, 5 μm). The mobile phase and elution system are shown in Fig. 1B. Quantitative analysis was performed by applying 5-point calibration curves for rosmarinic acid. The regression coefficient value for rosmarinic acid was 0.995.

For quality assurance of DpLM and its five solvent fractions, additional high-performance thin layer liquid chromatography (HPTLC) analysis was performed (Fig. S2).

**Measurement of total phenolic content**

The total phenolic content (TPC) of the extracts was determined using the Folin–Ciocalteu method \([25]\), with modifications. To 1 mL of each sample, 1 mL of Folin–Ciocalteu’s reagent (1 mol) and 20 mL of sodium carbonate solution (2%) were added. The mixture was left standing in the dark at room temperature for 30 min. Subsequently, optical density was measured at 725 nm. The results were calculated based on a calibration curve of tannic acid equivalent (TAE) per gram of dried sample.

**Measurement of total flavonoid content**

The total flavonoid content (TFC) of the extracts was determined by the application of a diethylene glycol colorimet-
The rate of apoptosis was measured by annexin V–FITC/PI double staining. In brief, following treatment with DpLE, cells were harvested by digestion with 0.05% trypsin, washed thrice with ice-cold PBS, and resuspended in binding buffer (500 μL). Subsequently, annexin V–FITC and propidium iodide (PI) were added to the cell suspension, which was then incubated for 15 min in the dark at room temperature. The apoptotic percentage of $1 \times 10^6$ cells was determined. After staining, single-cell suspensions were analyzed using flow cytometry (BD FACSCanto II, USA).

**Cell cycle analysis**

PI staining was used to examine the cell cycle progression. Briefly, DpLE-treated PC-3 cells ($1 \times 10^6$ cells/well) were fixed overnight with 70% ethanol at −20 °C. At the end of the incubation, the fixed samples were resuspended in PI staining solution (0.01 mg mL$^{-1}$ PI and 0.1 mg mL$^{-1}$ RNase A). After staining, single-cell suspensions were analyzed using flow cytometry (BD FACSCanto II, USA).

**Western blot analysis**

PC-3 cells were treated with various concentrations of DpLE for 24 h. Total cell lysates were extracted using a prep protein extraction solution purchased from iNtRON (Gyeonggi, Korea). Total protein extracts were cleared by centrifugation at $13,250 \times g$ for 10 min at 4 °C and assayed for protein content using the BCA method. Equal amounts of protein were separated by 6 to 15% sodium-dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF (Millipore, Darmstadt, Germany) membranes. After transfer, the membranes were blocked using 5% skim milk in Tris-buffered saline with Tween 20 (TBST) buffer for 1 h at room temperature; subsequently, the membranes were incubated overnight with primary antibodies at 4 °C. After three washes with TBST buffer, the HRP-conjugated secondary antibody was applied and the membranes were incubated for 1 h at room temperature. The membranes were then treated with ECL solution, and the expression level of each protein was detected using a photosensitive luminescent analyzer system (Amersham™ Imager 600, Little Chalfont, UK). Band quanti-
fication was performed using ImageJ software (NIH, Bethesda, MD, USA).

Data analysis
All data are expressed as the mean ± standard deviation (SD) and were analyzed using one-way analysis of variance (ANOVA). Dunnett’s test was used to assess the variance between experimental groups. SigmaPlot (version 12.0) was used for statistical analysis. Statistical significance was set at \( P < 0.05 \).

Results

Quantification of rosmarinic acid in DpLM and DpLE
The DpLM and DpLE samples were analyzed by HPLC. The separation patterns of the samples are shown at the top of Fig. 1A. Among the peaks, rosmarinic acid was shown at about 14 min. The rosmarinic acid quantitation values in DpLE (22.61 ± 4.87 mg g\(^{-1}\)) were higher than those in DpLM (10.85 ± 1.63 mg g\(^{-1}\)). The quantitation values are shown at the bottom of Fig. 1A.

Determination of TPC and TFC
Olennikov et al. investigated the bio-availability of \( D. \) palmatum S., an arctic plant, and reported its antioxidant effects, attributable to the presence of high levels of phenolic compounds \([23]\). They isolated 23 compounds from the aerial parts of the plant, including phenylpropanoids, coumarins, and flavonoids. However, to our knowledge, there is no report on the anticancer effect of this plant. Therefore, we confirmed, through a preliminary study, that the methanol extract of DpLM has antioxidant and anticancer activity. In this study, we attempted to confirm the most potent fraction of DpLM and its related mechanism of action.

The TPC and TFC results were significantly different according to the parts of \( D. \) palmatum S. (Table 1). Thus, the extracts, in terms of their TPC and TFC, can be categorized in the order: DpLE > DpLM > DpLB > DpLH > DpLC > DpLW.

Effect of DpLM and DpLE on cell viability and effect of DpLE on apoptotic cell death
We measured the viability of PC-3 prostate cancer cells using two extracts, the methanol extract and its ethyl acetate fraction, which are expected to have high antioxidant activity and bio-availability. The cell viability of PC-3 in the presence of 0–100 μg mL\(^{-1}\) DpLM and DpLE for 24 h was measured using the MTT assay. PC-3 cell viability was significantly decreased upon treatment with 25, 50, and 100 μg mL\(^{-1}\) DpLM (85.11% ± 3.09%, 77.59% ± 2.24%, and 65.24% ± 6.02%; respectively, compared to the control; Fig. 2A). DpLE treatment showed greater cytotoxicity compared to the control (81.20% ± 9.89%, 45.46% ± 5.77%, and 15.56% ± 1.04%; respectively, compared to the control; Fig. 2A). DpLE treatment showed greater cytotoxicity towards PC-3 cells at the same concentration as DpLM (81.20% ± 9.89%, 45.46% ± 5.77%, and 15.56% ± 1.04%; respectively, compared to the control). This cytotoxicity was dependent on the concentration of DpLE (Fig. 2A). The viability of paclitaxel-treated cells was significantly decreased at concentrations of 0.6 and 2.5 μmol L\(^{-1}\) (55.16% ± 5.61% and 46.71% ± 2.03%; respectively, compared to the control, Fig. 2B).

Anneixin V-FITC and PI double staining was performed to investigate the effects of DpLE on PC-3 cells. Fig. 2C shows that the distribution of annexin V-FITC and PI-double positive cells was increased after 24 h incubation of PC-3 cells with various concentrations of DpLE. Apoptotic cells were significantly increased after treatment with 50 and 100 μg mL\(^{-1}\) DpLE (28.20% ± 0.30% and 66.03% ± 0.15%; respectively, compared to the control; Fig. 2D); these effects were dependent on the DpLE dose.

Effect of DpLE on intracellular ROS and sub-G1 phase apoptosis in PC-3 cells
Flow cytometry analysis of PI staining was performed to investigate the apoptotic effect of DpLE during the sub-G1 phase in PC-3 cells. Cell cycle distribution enhancing Sub-G1 phase was observed at 100 μg mL\(^{-1}\); however, this was not seen at the other concentrations (\( P < 0.01 \)). This result was compared to the control (Fig. 3A). Fig. 3D shows that the PI-positive PC-3 cells in the sub-G1 phase increased upon treatment with 100 μg mL\(^{-1}\) DpLE (46.59% ± 5.45%; \( P < 0.001 \), compared to the control).

Effect of DpLE on protein expression in PC-3 cells
To investigate the presence of an association between the apoptotic effect of DpLE (0, 25, 50, and 100 μg mL\(^{-1}\)) on PC-3 cells and the extrinsic signaling pathway of apoptosis, protein expression of SOD-1, SOD-2, Bcl-2/Bax ratio, p-AKT, cleaved caspase-8, PARP, and cleaved-PARP in PC-3 cells was determined by western blot analysis.

Mitochondrial SOD-2 protein activation was observed in DpLE-treated cells at 100 μg mL\(^{-1}\) (\( P < 0.001 \), Figs. 4A and 4C). However, under the same conditions, the expression level of SOD-1 was not affected by DpLE treatment (Figs. 4A and 4B). As seen in Fig. 5A, the Bcl-2/Bax ratio was markedly increased following DpLE treatment (100 μg mL\(^{-1}\)) for 24 h (\( P < 0.01 \)). DpLE-treated cells at 100 μg mL\(^{-1}\) showed altered phosphorylation of AKT, indicating that DpLE inflicts a potent inhibitory effect on p-AKT signaling.

Table 1  Total phenolic content (TPC) and total flavonoid content (TFC) of extract and fractions. Tannic acid and naringin were used as standard for measuring contents. Results are expressed as milligram standard equivalents per gram of dry weight (means ± SD, n = 3)

<table>
<thead>
<tr>
<th>Samples</th>
<th>TPC (mg TAE/g)</th>
<th>TFC (mg NE/g)</th>
</tr>
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<tbody>
<tr>
<td>DpLM</td>
<td>138.21 ± 6.44</td>
<td>129.46 ± 3.83</td>
</tr>
<tr>
<td>DpLH</td>
<td>30.58 ± 3.40</td>
<td>17.69 ± 0.48</td>
</tr>
<tr>
<td>DpLC</td>
<td>20.53 ± 1.24</td>
<td>13.21 ± 0.77</td>
</tr>
<tr>
<td>DpLE</td>
<td>297.59 ± 8.82</td>
<td>310.62 ± 0.70</td>
</tr>
<tr>
<td>DpLB</td>
<td>114.46 ± 11.29</td>
<td>97.06 ± 1.41</td>
</tr>
<tr>
<td>DpLW</td>
<td>16.51 ± 3.40</td>
<td>7.30 ± 0.84</td>
</tr>
</tbody>
</table>

Figure 1A shows the separation patterns of the samples. The quantification values of rosmarinic acid in DpLE were 22.61 ± 4.87 mg g\(^{-1}\) and in DpLM were 10.85 ± 1.63 mg g\(^{-1}\). The TPC and TFC results are shown in Table 1.
Fig. 2 Effects of ethyl acetate fraction of *Dracocephalum palmatum* S. methanol extract (DpLE) on apoptosis of PC-3 cells. PC-3 cells were treated with various concentrations of DpLM, DpLE (0–100 μg·mL⁻¹) and paclitaxel (0–2.5 μmol·L⁻¹) for 24 h (A, B). Cell death induced by DpLE was determined using annexin V and propidium iodide (PI) double staining analysis. (a) Control, (b) 0, (c) 25, (d) 50, and (e) 100 μg·mL⁻¹ DpLE treatment. Percentage of cell located areas is shown (C, D). Data presented as means ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs the control group.

(P < 0.05; Fig. 5B). Fig. 5C shows that cleaved caspase-8 was increased by DpLE treatment (100 μg·mL⁻¹). PARP cleavage is an important signal that appears when DNA is fragmented; this was observed after treatment with DpLE at a concentration of 100 μg·mL⁻¹ (***P < 0.001; Fig. 5D).

Discussion

In recent studies, numerous herbal resources from the genus *Dracocephalum* have been reported to have beneficial effects, such as anti-oxidative and anticancer effects [19-23]. Olennikov et al. [24] isolated 23 compounds from the aerial parts of this plant, including phenylpropanoids, coumarins, and flavonoids; however, to our knowledge, there is no report on the anticancer effect of this plant.

Taxol, one of the most commonly used chemotherapeutic agents for prostate cancer, has been widely used for the treatment of prostate, breast, liver, lung, and other cancers [25-29]. In our study, paclitaxel, a medication used in the management of cancers, showed a powerful effect on PC-3 cells (Fig. 2B). However, paclitaxel is reported to have several side effects, such as reduction of white and red blood cell counts as well as accompanying pains, neuropathy, and hypersensitivity. Therefore, studies on natural products as potential cancer treatments are needed to minimize the use of anticancer drugs with such side effects.

In our preliminary studies, DpLM showed anticancer activity; thus, in this study, we attempted to determine the most potent fraction of DpLM and its related mechanism. Of the fractions investigated, DpLE showed the highest anticancer activity. This anticancer activity of DpLE against PC-3 cells, which was found to be stronger than that of DpLM, was confirmed to be related to apoptosis and sub-G₁ phase cell cycle arrest (Figs. 2, 3C and 3D).

SOD defends against the pathogenesis of numerous cardiovascular diseases and cancers; its isofrom, SOD-1, is a major soluble cytoplasmic antioxidant enzyme, whereas SOD-2 is a mitochondrial matrix enzyme that scavenges the ROS produced in the mitochondria [30, 31]. Antioxidants are defense chemicals against oxidative stress; our results showed the highest TPC and TFC in the DpLE. However, in the cells, ROS generation was reduced and SOD-2 protein signaling was activated by DpLE treatment. Therefore, we assumed that the inhibition of intracellular ROS production by DpLE and the increased SOD-2 expression were correlated (Figs. 3 and 4).

Apoptosis involves a series of cellular biochemical events leading to various characteristic morphological changes, including cell shrinkage, DNA fragmentation, and chromatin condensation [6, 32]. Bcl-2 proteins are critical regulators of the apoptotic pathway, which includes the major pro-apoptotic protein, Bax [9]. Involvement of the intrinsic (mitochondrial) pathway in DpLE-induced apoptosis would be indicated by a decrease in the Bcl-2/Bax ratio. However, in this study, the Bcl-2/Bax ratio remained unchanged at low con-
Fig. 3  Effects of ethyl acetate fraction of D. palmatum S. methanol extract (DpLE) on intracellular reactive oxygen species (ROS) inhibition and cell cycle arrest in PC-3 cells. PC-3 cells were treated with various concentrations of DpLE (0–100 μg·mL$^{-1}$). Proportion of intracellular ROS inhibited by DpLE was determined using 2', 7'-dichlorodihydro-fluorescein diacetate (DCF-DA) dye (A, B). Proportion of cell cycle distribution affected by DpLE was determined using propidium iodide (PI) staining (C, D). (a) Control, (b) 0, (c) 25, (d) 50, and (e) 100 μg·mL$^{-1}$ DpLE-treated cells. Percentage of cell located area and distribution are shown. Data presented are means ± SD of three independent experiments; *P < 0.05, **P < 0.01, and ***P < 0.001 vs the control group.

Fig. 4  A, effects of ethyl acetate fraction of D. palmatum S. methanol extract (DpLE) on SOD-1 and SOD-2 protein expression in PC-3 cells. PC-3 cells were treated with various concentrations of DpLE (0–100 μg·mL$^{-1}$) for 24 h. Assessment of SOD-1 and SOD-2 protein expression levels in PC-3 cells using western blotting. B, effect of DpLE on ratio of SOD-1/β-actin. C, effect of DpLE on ratio of SOD-2/β-actin. Data are presented as means ± SD of three independent experiments; ***P < 0.001 vs the control group.
centrations of DpLE but was increased at high concentrations (Fig. 5A), suggesting that the intrinsic pathway was not involved in DpLE-induced PC-3 cell apoptosis.

Activation of the AKT signaling pathway promotes cell survival and growth [33]; in this study, we confirmed the decreased protein expression of p-AKT in DpLE-treated PC-3 cells (Fig. 5B). Caspase-8 protein is involved in the extrinsic (death receptor-mediated) apoptotic pathway [34, 35]; here, the expression level of its cleaved form was increased after treatment with a high concentration of DpLE. Subsequently, we assumed that the function of caspase-8 was affected and observed that PARP protein was cleaved in the DpLE-treated PC-3 cells (Figs. 5C and 5D). These results indicate that DpLE shows strong potential as an antioxidant agent and exhibits anticancer effects on PC-3 cells via the apoptotic pathway. Furthermore, the apoptotic effects were likely mediated by the death receptor-related extrinsic pathway.

Collectively, the results indicate that DpLE exerts anticancer effects against human PC-3 prostate cancer cells. Although studies on other Dracophalum species have been ongoing and their anticancer activity is well recognized, findings similar to our results have not been published previously. We demonstrated that the elicited disturbance in the cell cycle and induced apoptosis was mediated by the activation of the extrinsic pathway. In addition, the extract of D. palmatum S. exhibited antioxidant effects. Thus, DpLE could be regarded as a suitable resource for the development of potential novel anticancer agents.

Fig. 5 Effects of ethyl acetate fraction of D. palmatum S. methanol extract (DpLE) on protein expression in PC-3 cells. PC-3 cells were treated with various concentrations of DpLE (0–100 μg·mL⁻¹) for 24 h. Assessment of protein expression levels in PC-3 cells using western blotting. A, effect of DpLE on Bcl-2/Bax values; B, effect of DpLE on p-AKT/β-actin ratio; C, effect of DpLE on cleaved caspase-8/β-actin ratio values; D, effects of DpLE on PARP or cleaved-PARP/β-actin ratio values. Data presented are means ± SD of three independent experiments; *P < 0.05, **P < 0.01, and ***P < 0.001 vs the control group.