Induction and mechanism of HeLa cell apoptosis by 9-oxo-10, 11-dehydroageraphorone from *Eupatorium adenophorum*

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**Abstract.** 9-Oxo-10, 11-dehydroageraphorone (euptox A), a cadenine sesquiterpene, is the main toxin from *Eupatorium adenophorum*. The aim of the present study was to examine the induction and mechanism of HeLa cell apoptosis by euptox A. The apoptosis-inducing effect of the euptox A on HeLa cells was examined by MTT assay. The underlying mechanism was analyzed by flow cytometry and quantitative PCR. Flow cytometry results suggested that euptox A effectively inhibited the proliferation of HeLa cells, arrested the cell cycle transition from S to G2/M phase, did not continue to complete the cell cycle activity (mainly from 4 times and mitosis), and induced cell proliferation. The RT-qPCR detection results showed that euptox A induced apoptosis by improving the gene expression level of apoptotic proteases such as caspase-10 in HeLa cells. Its mechanism of action was associated with the upregulation of apoptotic gene expression and arresting of the cell cycle.

**Introduction**

*Eupatorium adenophorum* (*E. adenophorum*), native to Mexico and Costa Rica of Central America, is a worldwide noxious invasive weed (1). After its introduction as an ornamental plant to the USA in the 1960s, it has spread worldwide (2), as a non-native species to India, New Zealand, and Australia. In China, it first invaded southern regions of Yunnan Province from Burma in the 1940s (3). At present, *E. adenophorum* can be found in Chongqing, Yunnan, Sichuan, Guizhou, Tibet, Guangxi, Taiwan and Hubei provinces. The annual spreading rate of *E. adenophorum* is estimated to be 10-60 km from south to north and from west to east in China (4). It is considered a threat to the local economy and biodiversity. However, as has been reported, several compounds have been separated and characterized from the *E. adenophorum* stem, flowers and leaves, including hemiterpenes, sterides, triterpenes, flavonoid and phenylpropanoids phenol, which have extensive biological activity, such as anti-inflammatory potential (5), acaricidal (6,7) and antioxidant activity (8). *E. adenophorum* can be used as a food (9), medical (10), and chemical material resource (11).

9-Oxo-10, 11-dehydroageraphorone (euptox A), a cadenine sesquiterpene, is the main toxin from *E. adenophorum*. Results of previous studies have shown that the euptox A from *E. adenophorum* exhibited hepatotoxicity (12) and allelopathy (13). Our laboratory has proved that euptox A exhibited highly acaricidal activity for *S. scabiei* and *P. cuniculi* in vitro (14) and euptox A presented significantly antitumor activity against the human lung cancer A549, HeLa and Hep-2 cell lines in vitro in a dose-dependent manner (15).

In the present study, MTT, flow cytometry and RT-PCR were used to detect the change of indices prior to and following drug action, followed by a comparison of the availability of different methods.

**Materials and methods**

**Materials.** Euptox A was provided by the laboratory of bitoxicin and molecular toxicology of Sichuan Agriculture University, China. The purity of the toxin we extracted was >96% (16).

**Cell cultures.** HeLa cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2 in DMEM supplemented with 10% FCS, glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cell cultures were divided every 3 days.

**Colorimetric MTT assay for cell proliferation.** The cytotoxicity of euptox A against HeLa cells was measured by the MTT assay (17). The HeLa cell line was maintained in DMEM supple-
Table I. PCR primers.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-10 (F)</td>
<td>GTATCGGCTACCCAGTCC</td>
<td>151</td>
</tr>
<tr>
<td>Caspase-10 (R)</td>
<td>CAGATCAAGCTCCACCAA</td>
<td>192</td>
</tr>
<tr>
<td>Caspase-3 (F)</td>
<td>GGTTCATCCAGTCGCTTTG</td>
<td>97</td>
</tr>
<tr>
<td>Caspase-3 (R)</td>
<td>CTCCTGTGCCACCTTTTC</td>
<td></td>
</tr>
<tr>
<td>β-actin (F)</td>
<td>GTTCGTTACACCCTTTC</td>
<td>151</td>
</tr>
<tr>
<td>β-actin (R)</td>
<td>CTGCACCTTACCGTTTC</td>
<td></td>
</tr>
</tbody>
</table>

Flow cytometry. HeLa cells were plated at a density of 75x10^3/well in 24-well plates and grown with DMEM containing 10% FBS. The following day, euptox A was added at a concentration of 0, 10, 30 and 50 µg/ml, respectively, and the cells were incubated for 24 h. 5-Fu was treated as a positive control. The inhibition rate was calculated according to the formula:

\[
\text{Growth inhibition rate(%) = } \left( \frac{\text{Absorbance of control group}}{\text{Absorbance of experimental group}} - 1 \right) \times 100\%
\]

RT-qPCR detection HeLa apoptotic gene expression. Logarithmic phase cells were divided into 3 groups, i.e., vehicle, experimental control (the concentration of euptox A was 0, 10, and 30 µg/ml, respectively) and positive control (5-Fu was treated as the positive control). The cells were harvested after 48 h, then used to measure gene expression by RT-qPCR.

Table II. Activation of HeLa cell lines exposed to different concentrations of euptox A.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/ml)</th>
<th>OD (490 nm) mean ± SE</th>
<th>Inhibition ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>2.790±0.226^c</td>
<td>0</td>
</tr>
<tr>
<td>5-Fu</td>
<td>1</td>
<td>0.487±0.064^bc</td>
<td>82.54</td>
</tr>
<tr>
<td>Euptox A</td>
<td>0.25</td>
<td>2.201±0.393^bc</td>
<td>21.09</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.884±0.226^d</td>
<td>68.30</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.218±0.024^d</td>
<td>92.16</td>
</tr>
</tbody>
</table>

*Difference between data is significant (P<0.01). †Difference between data is significant (P<0.05).

Table III. Effect of euptox A on the cell cycle of HeLa cells (mean ± SD, n=3).

<table>
<thead>
<tr>
<th>G0/G1 phase</th>
<th>S phase</th>
<th>G2/M phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µ/ml</td>
<td>47.533±1.783^b</td>
<td>31.280±2.779^b</td>
</tr>
<tr>
<td>10 µ/ml</td>
<td>36.876±2.392^c</td>
<td>40.936±1.519^b</td>
</tr>
<tr>
<td>30 µ/ml</td>
<td>34.093±2.494^c</td>
<td>48.583±1.519^b</td>
</tr>
<tr>
<td>50 µ/ml</td>
<td>24.366±3.170^d</td>
<td>56.950±1.870^c</td>
</tr>
<tr>
<td>5-Fu</td>
<td>54.553±2.201^e</td>
<td>29.603±1.745^d</td>
</tr>
</tbody>
</table>

*Difference between data is significant (P<0.01). †Difference between data is significant (P<0.05).

In vitro cytotoxicity assay. To investigate the effects of euptox A treatment on cell viability, the endpoint of cytotoxicity (MTT) assays in the HeLa cell line was investigated and CR results are shown in Table II. The results showed euptox A had significant antitumor activity against the HeLa cell line in vitro in a dose-dependent manner. When the concentration of euptox A was at 500 µg/ml, the percentage inhibition of HeLa cells was 68.30%.

Flow cytometry. Morphological observation of HeLa cells treated with euptox A for 24 h is shown in Fig. 1. Euptox A effectively inhibited the proliferation of HeLa cells. The shape of Hela cells in the negative control group appeared as poly-
oral, were densely arranged, and firmly adherent (Fig. 1A). Compared with the negative control group, the cells were treated with euptox A at concentrations of 10, 30 and 50 µg/ml. The increase of the concentration of euptox A administration resulted in a gradual increase in cell suspension, a significant reduction in the number of adherent cells, which were sparsely arranged, fusiform, appeared long and had irregular protrusions. Membrane integrity was maintained, although characteristic apoptotic changes were observed (Fig. 1B-D).

The cell cycle was analysed by flow cytometry at various concentrations of euptox A. Significant differences in the distribution of the cell cycle after various concentration of euptox A were observed following treatment for 24 h (Figs. 2 and 3) in a dose-dependent manner. Compared with the negative control group, the amount of S-phase cells increased from 31% to 40.936% and 48.583% to 56.950% at concentrations of 10, 30 and 50 µg/ml of euptox A group, respectively. However, for the positive control group, the amount of G1/M phase cells increased from 47.533% to 54.553% (Tables II and III).

Figure 1. Morphological observation of HeLa cells treated with euptox A for 24 h (10x20). (A) 0 µ/ml, (B) 10 µ/ml, (C) 30 µ/ml, (D) and 50 µ/ml euptox A.

Figure 2. Effect of euptox A on the cell cycle of HeLa cells. (A) 0 µ/ml, (B) 10 µ/ml, (C) 30 µ/ml and (D) 50 µ/ml euptox A.

Figure 3. Effect of euptox A on the cell cycle of HeLa cells (mean ± SD, n=3) 5-Fu: positive control group; negative control group: 0 µ/ml euptox A; 10: 10 µg/ml of euptox A group; 30: 30 µg/ml of euptox A group; 50: 50 µg/ml of euptox A group.

Figure 4. Total RNA was extracted from HeLa cells. 5-Fu: positive control group; 0: negative control group; 10: 10 µg/ml of euptox A group; 30: 30 µg/ml of euptox A group; 50: 50 µg/ml of euptox A group.

Figure 5. Effect of euptox A blend on mRNA expression of caspase-10 and -3 in HeLa cells. 5-Fu: positive control group; control: 0 µ/ml euptox A; 10: 10 µg/ml of euptox A group, 30: 30 µg/ml of euptox A group and 50: 50 µg/ml of euptox A group.
Expression of the caspase-10 and -3 genes. Total RNA was extracted from HeLa cell. The total RNA was measured for integrity, purity and yield of RNA met the necessary requirement (Fig. 4). The expression levels of caspase-10 and -3 genes in each group are shown in Fig. 5. The expression levels of caspase-10 gene in HeLa cells were increased following incubation with euptox A for 24 h, but were not dose-dependent. An initial increase followed by a decrease with an increase of the euptox A dose was observed. In addition, higher expression levels of caspase-10 gene following treatment with 30 µg/ml of euptox A were observed. However, in HeLa cells the caspase-3 gene showed only a slight decrease following incubation with euptox A for 24 h. However, in the 5-Fu positive control group, an increase of caspase-10 and -3 gene expression levels following incubation for 24 h was observed.

Discussion
Cancer is a common and frequently occurring disease that is a serious threat to human and animal life, its mortality rate is second only to cardiovascular disease. At present, natural anti-neoplastic drugs have become the subject of much investigation. Natural products such as paclitaxel (19,20), camptothecine (21,22), podophyllotoxin (23), matrine (24) and vincristine (25) have been shown to have anticancer activity. As recently demonstrated, euptox A extracted from *E. adenophorum* markedly inhibited the growth of cancer cells directly. Euptox A was found to be highly active against the rapidly growing HeLa cells, and its activity was concentration-dependent. A direct comparison with 5-Fu in the cell lines showed a clear superiority of euptox A, as 5-Fu is an anti-metabolite that has been used as a chemotherapeutic agent for a various types of cancer for over 40 years (26). However, the antitumor activity of euptox A was markedly stronger than that of 5-Fu at the same concentration. The reason is most likely that the test tumors were resistant to the 5-Fu. Our findings are consistent with previous studies which have shown that the *E. adenophorum* had antitumor activity (27).

In the present study, we found that euptox A effectively inhibited the proliferation of HeLa cells, arrest the cell cycle transition from S to G2/M phase, did not continue completion of the cell cycle activity (mainly from 4 times and mitosis), and induced cell proliferation. The findings are consistent with those of previous studies, where 10-hydroxycamptothecine (HCPT) showed marked cell cycle specificity in inducing death and apoptosis in the G1 phase, blocking the S phase (28). Caspase-10 is believed to be crucial in initiation of apoptosis by death receptors (29). The caspase-10 gene expression levels in HeLa cells were increased following treatment with euptox A. It is suggested that euptox A resulting in apoptosis may be regulated by influencing caspase-10 gene expression in HeLa cells.

The present study provides a new approach for the utilization of *E. adenophorum*. Euptox A has the potential to be developed as an antitumor drug. However, future studies are necessary for clinical trials, animal acute toxicity test and safety evaluation.

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References


