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 biotoxin and molecular toxicology of Sichun 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% CO_2 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-

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Table I. PCR primers.

Primer name	Primer sequence	Size of PCR product (bp)
Caspase-10 (F)	GTATCAGGCTACCCAGTCC	
Caspase-10 (R)	CAGATCAAGCTCCACCAA	192
Caspase-3 (F)	GGTTCATCCAGTCGCTTTG	
Caspase-3 (R)	CTCTGTTGCCACCTTTCG	97
β-actin (F)	GTTGCGTTACACCCTTTC	
β-actin (R)	CTGTCACCTTCACCGTTC	151

mented with 10% fetal bovine serum, 100 mU/l streptomycin, and 100 mU/l penicillin at 37˚C in a humidified atmosphere of 5% CO_2. Cells ($3x10^3$/well) in their exponential growth phase were seeded into each well of a 96-well flat-bottomed culture plate and incubated for 24 h. HeLa cells were incubated with the samples (euptox A) at concentrations of 0, 0.25, 0.5, 1 and 2 μg/ml. After 48 h, 20 μl of 5 μg/ml of MTT was added to each well and incubated for another 4 h. Following removal of the culture media, 150 μl of DMSO were added to each well. Absorbance at 490 nm was detected by a microplate ELISA reader. 5-Fu was treated as a positive control. The inhibition rate was calculated according to the formula:

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

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 for each incubation period. The HeLa cell cycle was assessed by flow cytometry analyzing DNA content in propidium iodide (PI)-stained cells. Briefly, the cells were collected with trypsin/EDTA, washed, incubated for 15 min with PI, and analyzed by flow cytometry.

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, 30 and 50 μg/ml, respectively) and positive control (5-Fu was treated as the positive control). The cells were extracted after 48 h, then used to measure gene expression by RT-qPCR.

Total RNA was isolated from 10^6 to 10^7 cells in a growing phase using TRIzol (Aidlab, China) according to the manual. The RNA was then chloroform-extracted and precipitated with isopropanol. Synthesis of single-stranded cDNA from 5 μg of RNA was performed according to the TUREscript 1st strand cDNA Synthesis Kit from Aidlab. Pairs of 5' and 3' primers used to amplify each type of cDNA are shown in Table I. The reaction conditions used were: 94˚C, 55˚C and 72˚C for 1 min (40 amplification cycles were necessary to achieve exponential amplification in which product formation was proportional to starting cDNA). Relative gene expression was then defined as a ratio of target gene (*caspase-10* and *-3*) expression vs. β-actin gene expression. Each ratio was determined independently three times.

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

Treatment	Concentration (mg/ml)	OD (490 nm) mean ± SE	Inhibition ratio (%)
HeLa			
Control	-	2.790±0.226[a]	0
5-Fu	1	0.487±0.064[b,c]	82.54
Euptox A	0.25	2.201±0.393[d]	21.09
	0.5	0.884±0.226[b]	68.30
	1	0.218±0.024[c]	92.16

[a-c]Difference between data is significant (P<0.01). [d]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).

	G0/G1 phase	S phase	G2/M phase
0 μ/ml	47.533±1.783[b]	31.280±2.779[d]	21.190±1.008[a,e]
10 μ/ml	36.876±2.392[c]	40.936±1.518[c]	22.190±3.419[a]
30 μ/ml	34.093±2.494[c]	48.583±1.519[b]	17.320±0.979[e]
50 μ/ml	24.366±3.170[d]	56.950±1.870[a]	18.683±1.363[e,f]
5-Fu	54.553±2.201[a]	29.603±1.745[d]	15.843±0.813[f]

[a-d]Difference between data is significant (P<0.01). [e,f]Difference between data is significant (P<0.05).

Statistical analysis. Data are presented as mean ± SEM and/or confidence interval. Statistical analyses were performed to compare the treated groups with the respective control group using a one-way analysis of variance (ANOVA) complemented with the Tukey-Kramer multiple comparison test with equal sample size. Computations were performed by employing the statistical software (SPSS, version 20.0) (18).

Results

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 polyg-

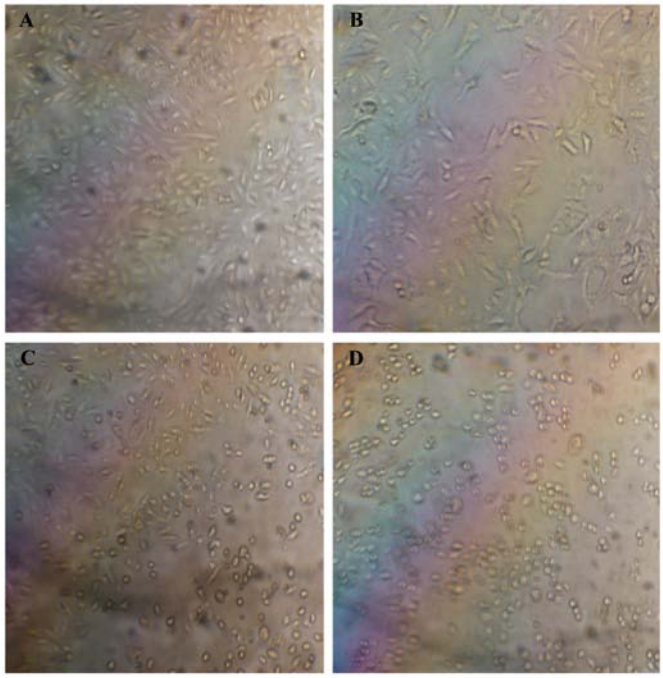

Figure 1. Morphological observation of HeLa cells treated with euptox A for 24 h (10x20). (A) 0 μ/ml, (B) 10 μ/ml, (C) 30 μ/ml, (D) and 50 μ/ml euptox A.

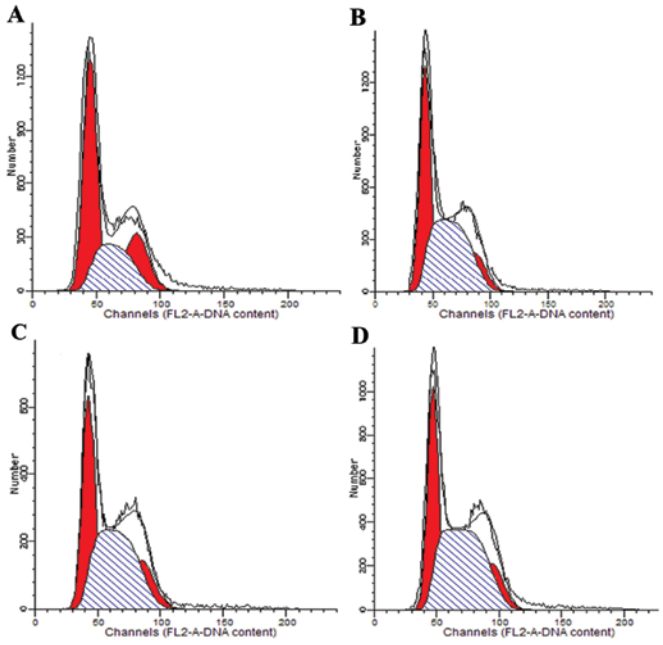

Figure 2. Effect of euptox A on the cell cycle of HeLa cells. (A) 0 μ/ml, (B) 10 μ/ml, (C) 30 μ/ml and (D) 50 μ/ml euptox A.

onal, 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 apoptotoc changes were observed (Fig. 1B-D).

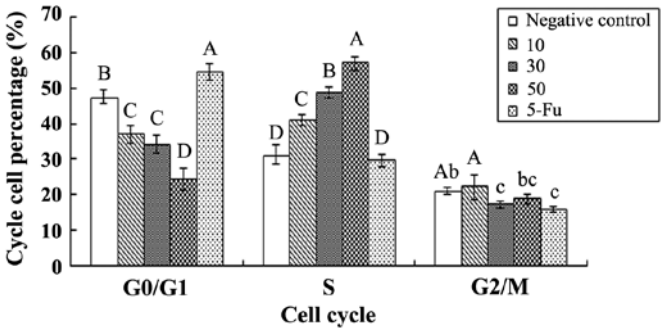

Figure 3. Effect of euptox A on the cell cycle of HeLa cells (mean ± SD, n=3) 5-Fu: positive control group; negative control group: 0 μ/ml euptox A; 10: 10 μg/ml of euptox A group; 30: 30 μg/ml of euptox A group group; 50: 50 μg/ml of euptox A group.

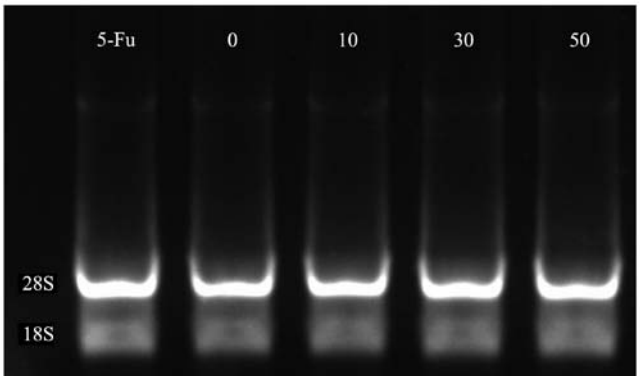

Figure 4. Total RNA was extracted from HeLa cells. 5-Fu: positive control group; 0: negative control group; 10: 10 μg/ml of euptox A group; 30: 30 μg/ml of euptox A group group and 50: 50 μg/ml of euptox A group.

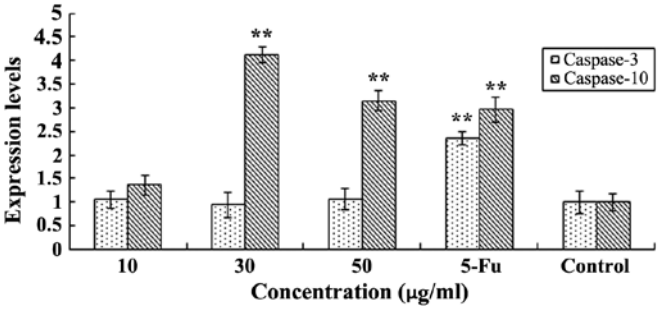

Figure 5. Effect of euptox A blend on mRNA expression of caspase-10 and -3 in HeLa cells. 5-Fu: positive control group; control: 0 μ/ml euptox A; 10: 10 μg/ml of euptox A group, 30: 30 μg/ml of euptox A group group; 50: 50μg/ml of euptox A group.

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 in the experimental control 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).

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 antimetabolite 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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