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Effects of the anti-tumor composition of the acetoacetate extract of vitex negundo seed on the growth of human cervical carcinoma xenografts in nude mice*

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Abstract ***Objective:*** The aim of our study was to investigate the effects of the anti-tumor composition of the acetoacetate extract of Vitex Negundo Seed (EVn-50) on the growth of human cervical carcinoma HeLa cells xenografts in nude mice and its possible molecular mechanism. ***Methods:*** Models of human cervical cancer HeLa cells xenografts transplanted subcutaneously in nude mice were established and randomly divided into 7 groups (each group including 5 nude mice): saline group, Taxol group, EVn-50 group, comp-6 group, comp-7 group, comp-8 group and comp-10 group. The volume and weight of Xenograts were observed and compared. The alteration of the weight of nude mice, and the change of serum levels of LDH, ALT, Cr and WBC counts were examined and compared. The apoptotic rate of human cervical carcinoma HeLa cells xenografts was analyzed by FCM. The expressions of P53 and Bcl-2 proteins of HeLa cells xenografts were determined by Western blotting. ***Results***: EVn-50 and its fractionated extracts could significantly suppress the increasing volume and weight of human cervical carcinoma HeLa cells xenografts in nude mice models in time-dependent manner, yet had no significant effect on the weight of nude mice, the serum levels of LDH, ALT, Cr and WBC were counted. When the xenografts were treated with EVn-50 and its fractionated extracts for 16 days, the apoptotic rate of xenografts cells were significantly increased, and the expression of P53 protein was up-regulated and protein level of Bcl-2 was decreased. ***Conclusion:*** EVn-50 and its fractionated extracts could suppress the growth of human cervical carcinoma HeLa cells xenografts in nude mice, which may be related to its promotion on xenografts cells apoptosis through down-regulation of Bcl-2 expression and activation of P53 expression.

Key words cervical cancer; EVn-50; xenografts; apoptosis

The acetoacetate extract of Vitex negundo seed (EVN-50) is one of the lignan compounds with extensive pharmacological activity. Recently, some researches reported that EVn-50 could inhibit cancer cell proliferation and induction of apoptosis *in vitro* [1], and the role of anti-tumor attracted many researchers attention, but the anti-tumor effect of EVn-50 on cancer cell *in vivo* has not reported [2]. So in our present study, we established models of human cervical cancer HeLa cells subcutaneous xenografts in nude mice, then observed the effect of EVn-50 and its fractionated extracts on the growth inhibition of xenografts and induction of apoptosis. We also detected the possible mechanism of molecular biology, and provided the experimental basis for clinical treatment of cervical cancer.

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Materials and methods

Major reagents and animals

EVn-50 and its fractionated extracts were extracted, isolated, and purified in Laboratory of Medicine Engineering of Medical College, Hunan Normal University, China. Human cervical cancer HeLa cells were purchased from China Centre for Type Culture Collection (CCTCC).

RPMI-1640 medium was obtained from Gibco Company, China. Fetal calf serum (FCS) was bought from Hangzhou Evergreen Biological Engineering Materials Co., Ltd, China. TAX was purchased from Hengrui Medicine Co., Ltd, China. Rabbit monoclonal Bcl-2 and rabbit polyclonal P53 were obtained from Abzoom, USA. Thirty-five SPF female mice (4–6 weeks old, 14–18 g) with Balb/c-nu mutation were obtained from Vital River Laboratory Animal Technology Co., Ltd, China. The certificate number was 0093102, and the license number was SCXK (Beijing) 2013–0025. Mice were housed in a pathogen-free environment.

Cell culture

Human cervical cancer HeLa cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum at 37 ℃ in a 5% CO_2 humidified atmosphere. Exponentially growing cells were used for the experiments.

Experimental treatment of HeLa cells xenografts in nude mice

Each nude mouse was subcutaneously injected 2×10^6 HeLa cells to make xenografts model. When the volume of xenografts reached 100 mm^3, the 35 nude mice were randomly divided into 7 groups according to xenografts volume and xenografts weight: saline group, Taxol group, EVn-50 group, comp-6 group, comp-7 group, comp-8 group and comp-10 group. Each mouse was subcutaneously injected the experimental medicine every day for 16 days. The longest/shortest diameter of xenografts tumor were measured in vernier caliper every 4 days when the nude mice received the administration, and the volume of xenografts tumor were calculated as $V = L \times W2 \times 0.52$, and then the curve of xenografts growth was drew. The nude mice were weighted every 4 days, and the mice weight curves were generated. After 48 h of the last medicine administrated, the blood of nude mice were collected, and the serum levels of lactate dehydrogenase (LDH), alanine aminotransferase (ALT), creatinine (Cr) and peripheral blood leukocyte count (WBC counts) were valuated. The xenogrfts tumors were cut out and weighted, and the growth inhibition rate of xenogrfts tumor (%) = (1 − Average tumor weight of experimental group / Average tumor weight of control group) × 100% were calculated [3].

Cell apoptosis analysis

Tissues of HeLa cells xenografts (10 mm^3) were washed in cold PBS (4 ℃) twice, and cut into small pieces, then the xenografts tissues were filtered with 200 mesh nylon mesh filter twice, and made into single cell suspension. Then 1×10^6 cells were washed by cold PBS (4 ℃) twice and fixed in ethanol 70%, and stained with PI in dark. Cell apoptosis rate was analyzed by using flow cytometry [4]. The experiment was repeated three times.

Western blotting

Thirty μg of proteins coming from HeLa cells xenograft administrated to NS, TAX, and EVn-50 and its fractionated extracts for 48 h were separated by 10% SDS-PAGE gel electrophoresis, and then electro-transferred to the PVDF membranes. Membranes were blocked with TBST containing 5% non-flat dry milk and incubated with the indicated primary antibodies overnight at 4 ℃, then membranes were incubated with HRP-conjugated second antibody. Protein-antibodies complexes were detected by enhanced chemiluminescene (ELC) according to the manufacturer's recommendations. Band densities in Western blotting measured using Imaging J for Windows software (NIH) [5]. The experiment was repeated three times.

Statistical analysis

All the experimental data were expressed as $\bar{\chi} \pm s$, and statistical analysis was performed by using SPSS 16.0 software. Differences between groups were examined with One-Way ANOVA, and a probability level of 0.05 was chosen for statistical significance.

Results

Effects of EVn-50 and its fractionated extracts on the volume of human cervical cancer HeLa cells xenografts

When the xenogrfts had treated with EVn-50 and its fractionated extracts for 16 d, the volume of xenografts significantly reduced ($V_{EVn\text{-}50}$ = 201.28 mm^3, $V_{comp\text{-}6}$ = 395.58 mm^3, $V_{comp\text{-}7}$ = 418.62 mm^3, $V_{comp\text{-}8}$ = 125.83 mm^3, $V_{comp\text{-}10}$ = 405.21 mm^3) compared with the volume of NS group (V_{NS} = 751.26 mm^3) ($P < 0.05$), while there was no difference among comp-6 group, comp-7 group and comp-10 group ($P > 0.05$). The volume of EVn-50 group/TAX group was dramatically smaller than the comp-6 group, comp-7 group and comp-10 ($P < 0.05$), and there was no statistical significance between TAX group and EVn-50 group ($P > 0.05$; Fig. 1). Our research suggested that EVn-50 and its fractionated extracts could inhibit the growth of HeLa cells xenografts, and the effect of 10 mg/kg EVn-50 was similar to 1 mg/kg TAX.

Effects of EVn-50 and its fractionated extracts on the weight of human cervical cancer HeLa cells xenografts

The mice were killed after the xenogrfts were treated with EVn-50 and its fractionated extracts for 16 d. Then the xenograts were cut out and weighted, and their growth inhibition rates were counted. The growth inhibition rates of comp-6 group, comp-7 group and comp-

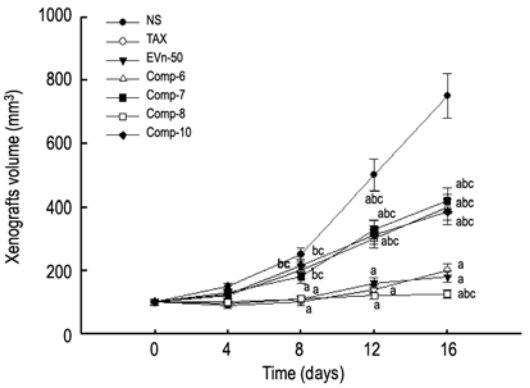

Fig. 1 The effects of EVn-50 and its fractionated extracts on the volume of human cervical carcinoma HeLa cells xenografts in nude mice. Compared with NS group, [a] $P < 0.01$; compared with TAX group, [b] $P < 0.05$; compared with EVn-50 group, [c] $P < 0.05$

10 group (42.86%, 45.86% and 43.54% respectively) were significantly increased compared with that in the NS group ($P < 0.05$). The growth inhibition rates of EVn-50 group, comp-8 group and TAX group were 62.76%, 77.75% and 63.47% respectively, and also significantly increased compared with that in the NS group ($P < 0.01$). There was no difference in the growth inhibition rates among comp-6 group, comp-7 group and comp-10 group ($P > 0.05$). There existed statistical significance between EVn-50 group/TAX group and comp-6, comp-7, comp-10 groups in the growth inhibition rates ($P < 0.05$). The growth inhibition rate of comp-8 group was higher than EVn-50 group/TAX group ($P < 0.05$). All the results were showed in Table 1. Our research suggested that EVn-50 and its fractionated extracts could inhibit the growth of HeLa cells xenografts, and the effect of 10 mg/kg EVn-50 was equivalent to 1 mg/kg TAX.

Effects of EVn-50 and its fractionated extracts on the weight of the nude mice

After treatment with EVn-50 and its fractionated extracts for 16 days, the nude mice were killed and the body weigh were measured. The weigh of the nude mice had no statistical significance among NS group, EVn-50 group and its fractionated extracts groups ($P > 0.05$), while the body weigh of TAX group markedly decreased compared with NS group, EVn-50 and its fractionated extracts groups ($P > 0.05$) (Table 2). Our results indicated that EVn-50 and its fractionated extracts have no significant influence on the weigh of nude mice.

Table 1 The effects of EVn-50 and its fractionated extracts on the weight of human cervical carcinoma HeLa cells xenografts in nude mice ($\bar{\chi} \pm s$)

Groups	Xenogrfts weight (mg)	Inhibition rate (%)
NS	392 ± 118	–
TAX	105 ± 27	73.47[b]
EVn-50	228 ± 52	43.12[a]
Comp-6	214 ± 46	40.46[a]
Comp-7	231 ± 54	45.27[a]
Comp-8	88 ± 16	76.46[b]
Comp-10	226 ± 60	46.75[a]

Compared with NS group, [a] $P < 0.05$, [b] $P < 0.01$

Effects of EVn-50 and its fractionated extracts on the serum level of WBC, ALT, LDH and Cr of the nude mice

The nude mice received EVn-50 and its fractionated extracts 16 days, then the nude mice blood were collected and measured. The serum level of WBC, ALT, LDH and Cr of the nude mice had no statistical significance among NS group, EVn-50 and its fractionated extracts groups ($P > 0.05$), while the serum level of WBC, ALT, LDH and Cr of TAX group markedly decreased compared with NS group, EVn-50 and its fractionated extracts groups ($P > 0.05$). Results were shown in Table 3. Our finding suggested that EVn-50 and its fractionated extracts have no significant influence on the liver and kidney function of nude mice.

Effects of EVn-50 and its fractionated extracts on the apoptotic rate of HeLa xenografts

The xenogrfts received EVn-50 and its fractionated extracts treatment for 16 days, and the cell apoptosis rate dramatically increased. The apoptotic rates of EVn-50

Table 2 The effects of EVn-50 and its fractionated extracts on the weight of nude mice after human cervical carcinoma HeLa cells xenograft ($\bar{\chi} \pm s$)

Groups	0 d	4 d	8 d	12 d	16 d
NS	21.6 ± 0.6	22.4 ± 0.5	23.8 ± 0.6	24.6 ± 1.0	26.2 ± 0.5
TAX	21.1 ± 0.8	19.8 ± 0.4	18.2 ± 1.1[a]	18.1 ± 1.3[a]	17.5 ± 2.6[b]
EVn-50	19.8 ± 0.8	21.7 ± 0.6	22.6 ± 0.5	23.4 ± 0.4	24.2 ± 0.8
Comp-6	20.2 ± 1.0	21.3 ± 1.2	22.6 ± 0.7	24.5 ± 0.4	25.6 ± 0.3
Comp-7	20.0 ± 0.6	20.8 ± 0.5	22.4 ± 0.7	23.2 ± 0.3	23.5 ± 2.8
Comp-8	20.3 ± 1.2	21.4 ± 1.0	23.5 ± 0.4	24.3 ± 0.6	25.4 ± 0.7
Comp-10	19.9 ± 0.8	20.9 ± 0.5	22.8 ± 0.6	24.1 ± 0.5	24.4 ± 3.2

Compared with NS group, [a] $P < 0.05$, [b] $P < 0.01$

Table 3 The effects of EVn-50 and its fractionated extracts on peripheral blood leukocytes, heart, liver and kidney function of human cervical carcinoma HeLa cells xenografts in nude mice ($\overline{\chi} \pm s$)

Groups	LDH (U/L)	ALT (U/L)	Cr (μmol/L)	WBC (1×10^9/L)
NS	1986 ± 305	66 ± 13	65 ± 7	7.92 ± 1.58
TAX	2395 ± 426[a]	102 ± 16[b]	82 ± 10[a]	4.64 ± 1.22[b]
EVn-50	1886 ± 321	75 ± 14	58 ± 6	7.28 ± 1.62
Comp-6	1906 ± 285	74 ± 13	59 ± 12	7.89 ± 2.28
Comp-7	2006 ± 195	68 ± 24	61 ± 5	8.23 ±1.14
Comp-8	1934 ± 208	70 ± 21	57 ± 6	8.27 ± 1.16
Comp-10	2015 ± 156	73 ± 18	68 ± 8	7.84 ± 1.08

Compared with NS group, [a] $P < 0.05$, [b] $P < 0.01$

group, comp-6, comp-7, comp-8 and comp-10 group were 40.5%, 22.8%, 23.4%, 55.2% and 25.0% respectively, and there was statistical significance compared with NS group (apoptosis rate was 2.64%) ($P < 0.05$). The apoptotic rate of EVn-50 group/TAX group was significant increased than that in comp-6, comp-7 and comp-10 group ($P < 0.05$), but it was significant decreased than that in comp-8 group ($P < 0.05$). There was no difference of the apoptotic rate between EVn-50 group and TAX group ($P > 0.05$). Data were showed in Fig. 2. Our study suggested that the effect of 10 mg/kg EVn-50 on the apoptosis of HeLa xenografts cell was equivalent to 1 mg/kg TAX.

Effects of EVn-50 and its fractionated extracts on the apoptotic related proteins of HeLa xenografts

The xenogrfts received EVn-50 and its fractionated extracts treatment for 16 days, and the expression of P53 protein dramatically improved, while Bcl-2 protein decreased, and there was significant difference compared with NS group ($P < 0.05$). The expression of P53 protein of EVn-50 group was significantly higher than that in com-6, com-7 and com-8 groups ($P < 0.05$). The mean relative density of Bcl-2 protein was significantly lower than that in com-6, com-7 and com-8 groups ($P < 0.05$). The up-regulating effect of EVn-50 on P53 protein and the down-regulating effect of EVn-50 on Bcl-2 protein were equivalent to TAX ($P > 0.05$). The P53/Bcl-2 protein regulating effect of comp-8 was superior to EVn-50/TAX ($P < 0.05$). There were no statistical significance between comp-6 and comp-7 groups, comp-6 and com-10 groups, comp-7 and comp-10 groups in the mean relative density of P53/Bcl-2 proteins ($P > 0.05$), as shown in Fig. 3.

Discussion

Cervical cancer is one of the most common gynecological malignancy. With the research progress of tumor and the improvement of treating methods, chemotherapy has already controlled the primary tumor, killed the subclinical metastases, sensitized radiotherapy, treated recurrence, improved the survival rate of cervical can-

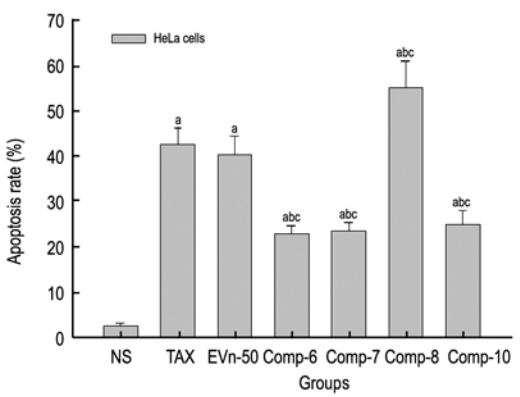

Fig. 2 The effects of EVn-50 and its fractionated extracts on apoptotic rate of human cervical carcinoma HeLa cells xenografts in nude mice ($\overline{\chi} \pm s$; $n = 3$). Compared with NS group, [a] $P < 0.01$; compared with TAX group; [b] $P < 0.05$; compared with EVn-50 group, [c] $P < 0.05$

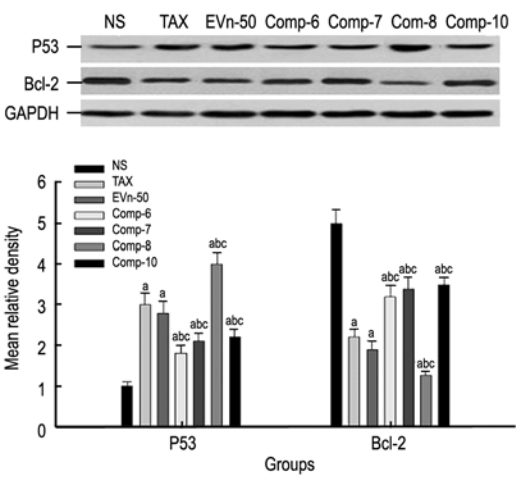

Fig. 3 The effects of EVn-50 and its fractionated extract on the expression levels of p53 protein and Bcl-2 protein of human cervical carcinoma HeLa cells xenografts in nude mice ($\overline{\chi} \pm s$; $n = 3$). Compared with NS group, [a] $P < 0.01$; compared with TAX group; [b] $P < 0.05$; compared with EVn-50 group, [c] $P < 0.05$

cer patients. Therefore, development of new drugs for chemotherapy treatment for cervical cancer has positive means [6]. EVn-50 is one of the lignan compounds with extensive pharmacological activity. Recently, some researches reported that EVn-50 could inhibit cancer cell proliferation and induction of apoptosis *in vitro*, and the role of anti-tumor attracted many researchers attention, but the anti-tumor effect of EVn-50 on cancer cell *in vivo* has not reported [1, 2]. In our study, we established a xebografts model of human HeLa cells in nude mice and administrated different experimental medicines to the mice. We found EVn-50 and its fractionated extracts could dramatically inhibited the growth of HeLa cells xenografts through inducing the cells apoptosis without side effects.

P53 regulates the cell cycle through induction of apoptosis and inhibition of proliferation, and its main function checkpoint lies in G1/S phase. When chromosomal DNA damaged was determined by P53, it will induce cell cycle G1 phase arrest through stimulating CDKI and initiating DNA repair. The cell apoptosis death path will be initiated if DNA repair failed [7]. P53 is one kind of transcription-activating factors, which can initiate mitochondrial apoptotic pathway and death receptor apoptotic pathway [7]. In our study, EVn-50 and its fractionated extracts were administrated to human cervical cancer HeLa cell xenografts. The growth of xenografts was significantly inhibited and the apoptosis of the xenografts cells dramatically induced accompaniment with p53 protein up-regulated. Our results was similar to that reported by Xin *et al* [8].

Bcl-2 gene posses the role of inhibiting apoptosis, and its coding protein extensively exist in mitochondria membrane, cell membrane, endoplasmic reticulum membrane and nucleus membrane of many tumor cell lines. Bcl-2 protein is a direct antioxidant, which can inhibit mitochondria releasing pro-apoptotic proteins, such as cyto-c, AFI, etc., and distress the action of the function of Bax and Bak proteins, un-activate caspase enzyme, and sustain the stability of calcium [9]. Bcl-2 protein may inhibit apoptosis induced by a variety of factors, and the tumor cells had pre-transfected with Bcl-2 expression plasmid would resist apoptosis induced by antitumor medicine [9]. Our study found that the growth of xenografts significantly inhibited and the apoptosis of the xenografts cells dramatically induced accompaniment with Bcl-2 protein down-regulated when EVn-50 and its fractionated extracts had administrated to human cervical cancer HeLa cell xenografts. The results was similar to that reported by Zhou *et al* [10].

Our study indicated that EVn-50 and its fractionated extracts posse were significant inhibited HeLa cells xenografts, while had no influence to the serum levels of LDH, ALT, Cr,WBC counts, and the weight of nude mice self, induced xenografts cell apoptosis, accompanied by P53 protein up-regulation and Bcl-2 protein down- regulation, which suggested EVn-50 and its fractionated extracts inhibited the growth of HeLa cells xenografts related to up-regulation of P53 protein and down-regulation of Bcl-2 protein. In summary, EVn-50 and its fractionated extracts are new kinds of anti-tumor lignan compounds with safe, valid anti-tumor effects and slight side effect, and it posses a broad application prospects and clinical developing value in cancer treatment, but the mechanism related to induction of apoptosis *in vivo* needs further studies.

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