Anticancer effect of two diterpenoid compounds isolated from *Annona glabra* Linn[1]

Yong-hong ZHANG[2], Hai-yan PENG[3], Guo-hao XIA[3], Ming-yan WANG[3], Ying HAN[4]

First Clinical College; [3]Basic Medicine College, Nanjing University of Traditional Chinese Medicine, Nanjing 210029; [4]Jiangsu Institute of Materia Medica, Nanjing 210009, China

KEY WORDS *Annona glabra* Linn; diterpenes; hepatocellular carcinoma; apoptosis; gene expression

ABSTRACT

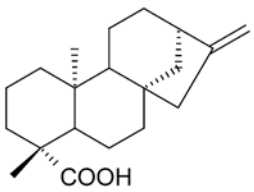
AIM: To study the inhibitory effect of two diterpenoid compounds isolated from *Annona glabra* Linn (Cunabic acid and ent-kauran-19-al-17-oic acid) on the proliferation of Human Liver Cancer (HLC) cell line SMMC-7721 and its mechanism. **METHODS:** Inhibition of cell proliferation was measured by MTT assay. The morphological changes of SMMC-7721 cells were observed under inverted phase-contrast microscope, fluorescent microscope, transmission electron microscope (TEM), and scanning electron microscope (SEM). Flow cytometer (FCM) was used to calculate the cell apoptotic rate, and immunohistochemical staining was used to observe the regulation of gene expression. **RESULTS:** The proliferation of SMMC-7721 cells was obviously inhibited after being treated with Cunabic acid at the concentration >5 μmol/L and ent-kauran-19-al-17-oic acid >10 μmol/L. The biggest inhibitory effect was 81.05 % when treated with Cunabic acid at the concentration of 25 μmol/L. The effect had a linear relationship with concentration. The result indicated that drug-treated cells exhibit typical morphological changes of apoptosis, including condensed chromatin and a reduction in volume. Sub-G_0/G_1 peak was found by FCM analysis and the cell cycle was arrested at G_0/G_1 stage. The apoptotic rates of the cells treated by Cunabic acid and ent-kauran-19-al-17-oic acid were 43.31 % and 24.95 %, respectively. It was visualized by immunohistochemical staining that the drugs down-regulated the gene expression of *bcl*-2 gene and up-regulated that of *bax* gene. **CONCLUSION:** The two diterpenoid compounds isolated from *Annona glabra* Linn, Cunabic acid and ent-kauran-19-al-17-oic acid can obviously inhibit the proliferation of HLC cell line SMMC-7721. The mechanism is correlated with the induction of cell apoptosis by down-regulating the gene expression of *bcl*-2 gene and up-regulating that of *bax* gene.

INTRODUCTION

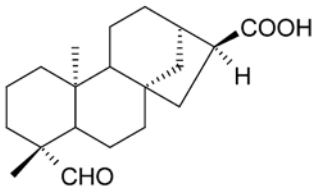
Traditional Chinese medicine afforded a valuable approach in the searching for new anticancer drugs. Studies on the pharmacological mechanism and searching for new chemical structures from herbal extract for new anticancer drugs caught great interest[1]. Taxol, isolated from the stem bark of *Taxus brevifolia*, provided a typical example in this respect. Taxol was ranked as "the most important new drug we have had in cancer for 15 years"[2]. *Annonaceous acetogenius* were a new group of compounds isolated from *Annona* plants exhibiting strong anticancer activity. *In vivo* experiments on the leukemia-bearing mice, Bullatacin, Asinicin,

[1] Project supported by Jiangsu Province Natural Science Foundation (No BK2002201).
[2] Correspondence to Yong-hong ZHANG. Phn 86-25-8679-8128. Fax 86-25-8659-3077. E-mail zyh19511227@sohu.com
Received 2003-09-15 Accepted 2004-02-20

and Bullatacinone (three kinds of extract from *Annona* plants) exhibited 300, 80, 40-times higher anticancer effect than taxol, respectively[3-5]. Liu *et al* found two new extracts from *Annona glabra*, which exhibited anticancer effect 100 000 times higher than adriamycin. *Annona* plants became a new promising resource for new anticancer medicine[6]. We isolated two kinds of diterpenoid compounds from *Annona glabra* Linn, and the following experiment showed that the two new diterpenoid compounds had strong inhibitory effect on the proliferation of HLC cell lines. The two diterpenoid compounds were Cunabic acid (ent-kaur-16-en-19-oic acid) and ent-kauran-19-al-17-oic acid.



Cunabid acid



Ent-kauran-19-al-17-oic acid

MATERIALS AND METHODS

Drugs Cunabid acid and ent-kauran-19-al-17-oic acid were isolated from *Annona glabra* Linn and both white powdery. Drugs were dissolved with absolute ethanol, and diluted with culture medium till the final concentration of ethanol was less than 1 %. Molecular formulas of Cunabid acid and ent-kauran-19-al-17-oic acid were $C_{20}H_{30}O_2$ and $C_{20}H_{30}O_3$, respectively.

Reagents RPMI-1640, produced by Gibco Co in USA, dissolved in double distill water and the pH value was adjusted to 7.0 with $NaHCO_3$, disinfected, stored at -20 ºC. Fetal bovine serum was purchased from Sijiqing Biological Engineering Co in Hangzhou, China, sterilized, stored at -20 ºC. PBS (phosphate-buffered saline), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], PI (propidium iodide), and Triton X-100 were purchased from Sigma Co, USA. AO (acridine orange) was purchased from Molecular Probe Inc Co, USA, and EB (ethidium bromide) was purchased from Fluka Co, diluted with PBS (pH 7.2, 1:1) into the concentration of 0.1 μg/L, stored at 4 ºC, protected from light. RNase A (ribonuclease A), was purchased from Germany. The polyclonal rabbit anti-human antibodies of bcl-2 and bax were purchased from Santa Cruz Co, USA. SP (streptomyces anti-biotin protein-peroxidase) immunohistochemical staining kit was produced by Maixin Biotechnique Co, Fuzhou, China.

Cell culture SMMC-7721 cell lines (provided by Microbiology Department, Nanjing University of Traditional Chinese Medicine) were cultured in the RPMI-1640 medium containing 10 % fetal bovine serum, penicillin 1×10^5 IU/L, streptomycin 0.1 g/L, at 37 ºC in a humidified atmosphere of 5 % CO_2. The single layer adherent cells generated every 3-5 days, were trypsinized for 3 min, and then made into supernatant for seeding.

Drug treatment Cells at the exponential growth stage were implanted on the culture bottles at the density of 2×10^3/L. After 4 h, culture medium containing drugs was added and then incubated for required time.

MTT assay Living cells 2×10^3/L were plated in 96-well culture dishes at the density of 90 μL per well. After the cells adhered to the walls, 10 μL medium containing one of the six different concentrations of Cunabic acid or ent-kauran-19-al-17-oic acid was added, respectively, and PBS alone as the control. After being incubated at 37 ºC in a humidified atmosphere of 5 % CO_2 for required time, then MTT (concentration of 1 g/L) was added (50 μL per well). Four hours later, supernatant was discarded, and Me_2SO (100 μL per well) was added to dissolve the formazan. Optical density (*OD*) was measured on an EL340 ELISA reader at 490 nm. The growth inhibitory rate (%) (IR) was calculated according to the following formula: IR= (1– mean *OD* of tested cells/mean *OD* of control cells) ×100 %. IC_{50} was calculated according to modified Cou's method[7].

Morphological assessment of apoptosis Inverted phase-contrast microscope (Olympus, Melville, NY) was used to observe the morphological changes of the cells and photos were taken. Cells treated with Cunabic acid and ent-kauran-19-al-17-oic acid at the concentration of 0.1, 0.25, and 0.5 μmol/L for 24, 48, and 72 h, respectively, then harvested, centrifuged (20PR-520, Hitachi Co, Japan), and stained with AO/ EB solution (4 μL) for 2 min, and observed under the fluorescent microscope. The cells were also observed under scanning electron microscope (SEM)(JSM-6300, Electron Co, Japan) and transmission electron microscope (TEM) (H-300, Hitachi Co, Japan) [8,9]. Briefly, after incubation for 72 h, cells were fixed with glutaraldehyde, air-dried, and gilded by Pt irons, then observed under SEM. The cells were fixed with glutaraldehyde, washed by PBS (0.1 mmol/L), dehydrated, embedded, thin-sectioned and double-stained, then observed under TEM.

FCM analysis All the samples were measured with flow cytometer (FACS Calibur, produced by Becton

Dickinson Co, USA)[8]. Briefly, the drug-treated cells were trypsinized for 2 min, rinsed by PBS (0.01 mol/L). Single cell suspension was centrifuged at 1000 r/min for 3 min, and supernatant was discarded. Pellets were dissolved with PBS, fixed with 75 % ethanol at room temperature for 30 min, and then averted to -20 ºC. Cells were digested by 1 % RNase A (ribonuclease A, Sigma) at 37 ºC for 30 min, stained by PI fluorescent staining at 4 ºC, protected from light, filtered twice, then measured by FCM. The red fluorescence at 490 nm (ELISA reader DJ3200) was recorded, and the results were analyzed by CELLQUEST MODFIT LT computer systems (Becton Cickinson Ltd).

Immunohistochemistry analysis SABC-AP was used for immunohistochemistry analysis[10]. Briefly, cells with and without drug-treatment were seeded on the 6-well culture dishes(10 mm×10 mm) at the density of 1×10^6 per well. After incubation for required time, cells were fixed with acetone for 30 min, air dried, washed with PBS (pH 7.4) for three times (3 min each time), then the activity of endogenous peroxidase was checked. After cells were re-washed with PBS for three times, the serum of non-immunized animal was added and incubated at room temperature for 10 min, then anti-bcl-2 and anti-bax antibodies were added [1 drop (50 μL) per slide]. After being incubated at room temperature for 60 min, the cells were washed with PBS, and biotinylated second antibody was added. Then PBS was discarded and SP (streptomyces anti-biotin protein-peroxidase) solution was added. The cells were again incubated for 10 min and washed with PBS, stained with fresh DBA staining solution and re-stained with haematoxylin back to blue, sealed with gel for microscopy.

The results were analyzed according to the following standards: The cell containing brown homogenous cytolymph was regarded as positive cell. The grading standard was: "-", positive cells<5 %; "+", 5 %<positive cells<25 % (light brown); "++", 25 %<positive cells <50 % (brown); "+++", positive cells>50 % (dark brown).

Statistical methods All data were presented as mean±SD. Statistical significance was determined by *t*-test and χ^2-test. $P<0.05$ was considered significant.

RESULTS

Effects on cell proliferation The inhibitory effects of Cunabid acid and ent-kauran-19-al-17-oic acid were concentration and time-dependent. The biggest IR of Cunabid acid was 81.05 % at the concentration of 25 μmol/L, 72 h (Tab 1). It was found that when the concentration of Cunabid acid was lower than 10 μmol/L, the inhibitory effect was weak; when the concentration ranged from 10 μmol/L to 25 μmol/L, the inhibitory effect became obvious, the higher concentration, the better effect; while the concentration was higher than 25 μmol/L, the higher concentration, the less effect. The best IR of ent-kauran-19-al-17-oic acid was 56.86 % at the concentration of 75 μmol/L, 72 h, and the effect was time- and concentration-dependent (Tab 1).

Effects on cell apoptosis The analysis of cellular DNA content by FCM showed that there was a sub-G_0/G_1 peak in the graph of drug-treated groups. That was a typical apoptotic peak, which was not shown in the graph of control groups (Fig 1). After treatment with Cunabic acid or ent-kauran-19-al-17-oic acid at the concentration of 25 μmol/L for 48 h, most SMMC-7721 cells were blocked at G_0-G_1 stage. Compared with the control group, cells at G_0-G_1 stage of Cunabic acid-treated group increased by 15.21 %, and cells at S stage decreased by 11.68 %. The apoptotic rates of Cunabic acid and ent-kauran-19-al-17-oic acid were

Tab 1. Inhibitory effect on SMMC-7721 cells by the drugs. A: treated with Cunabic acid; B: treated with ent-kauran-19-al-17-oic acid.

	Inhibitory rate/%										
	1 μmol·L^{-1}	5 μmol·L^{-1}		10 μmol·L^{-1}		25 μmol·L^{-1}		50 μmol·L^{-1}		75 μmol·L^{-1}	
Time/h	A	A	B	A	B	A	B	A	B	A	B
24	4.82	15.66	3.61	30.12	12.05	46.98	26.51	40.96	40.96	36.14	46.99
48	4.92	18.85	4.10	39.34	16.36	62.30	33.61	56.56	47.54	50.82	53.28
72	5.23	22.22	4.58	52.94	18.95	81.05	37.91	69.28	51.63	59.48	56.86

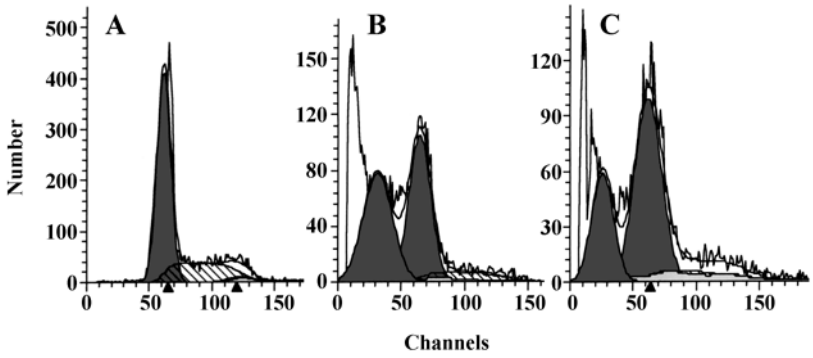


Fig 1. The analysis of cellular DNA content by FCM. A) control cells. B) cells treated with Cunabic acid for 48 h. Sub-diploid peak was found before the G_0/G_1 stage, which was a typical apoptotic cell peak. C) cells treated with ent-kauran-19-al-17-oic acid for 48 h. Sub-diploid peak was found before the G_0/G_1 stage, which was a typical apoptotic cell peak.

43.31 % and 24.95 %, respectively, while that of the control group was 0.05 % (Tab 2).

Tab 2. Inhibitory rate of SMMC-7721cells at different stages of cell cycle. A: treated with Cunabic acid at the concentration of 25 μmol/L for 48 h; B: treated with ent-kauran-19-al-17-oic acid at the concentration of 25 μmol/L for 48 h.

	G_0-G_1/%	G_2-M/%	S/%	Apoptosis/%
Control	68.47	3.53	28.00	0.05
A	83.68	0.00	16.32	43.31
B	80.88	6.47	12.65	24.95

Effects on morphological changes Through inverted phase-contrast microscope (400-time), it was found that the growth of drug-treated SMMC-7721 cells was obviously inhibited. The drug-treated cells (T-groups) became round and lost cell membrane, the inter-cellular connection became loose, the granules in the cytoplasm increased, and the proliferation slowed. The untreated cells exhibited normal form with clear outline. The inter-cellular connection was firm, and the proliferation was rapid. Under fluorescent microscopy, normal nucleus DNA was stained yellow or yellow-green homogeneously, cytoplasm and nucleolus RNA stained orange or orange-red. In the nucleus or cytoplasm of apoptotic cells, there were dense dark yellow-green stained fragments. SMMC-7721 cells treated with Cunabic acid or ent-kauran-19-al-17-oic acid for 72 h showed typical apoptotic features under fluorescent microscopy. Under TEM, the drug-treated cells were wrinkled with cytolymph condensation and the concentrated chromatin into mass inside the nuclear membrane. The apoptotic bodies were observed in the cytoplasm. While the control cells were found integrate cell membrane, normal organelles, big several nucleolus, homogeneous chromatin, and abundant normal chromatin (Fig 2). Under SEM, the drug-treated cells were found wrinkle and smaller, irregular outline, broken surface, and loss of villus, while the control

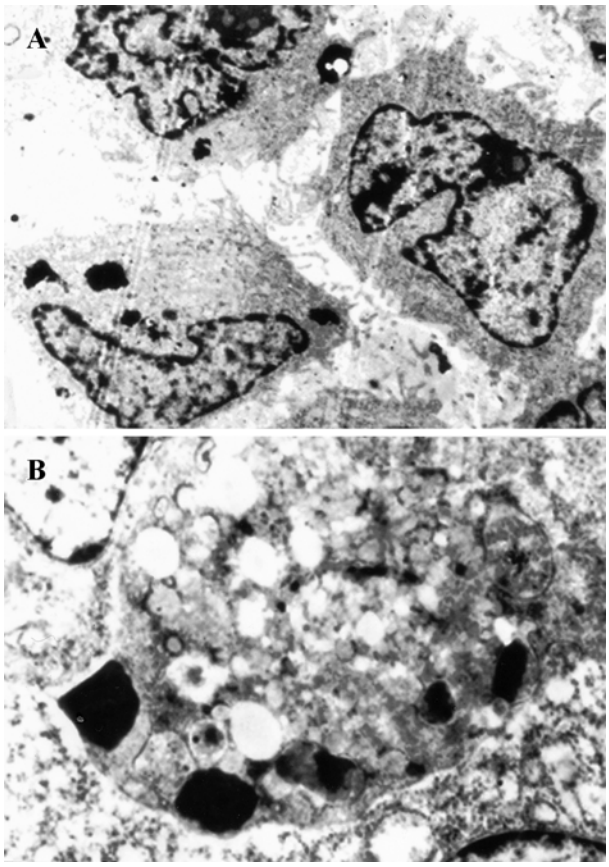


Fig 2. Morphological observation by transmission electron microscopy. A) control cells (×7500); B) Cunabic acid-treated cells (0.25 mmol/L, 72 h) (×10500).

cells were found global form, integrate surface, wealthy villus (Fig 3).

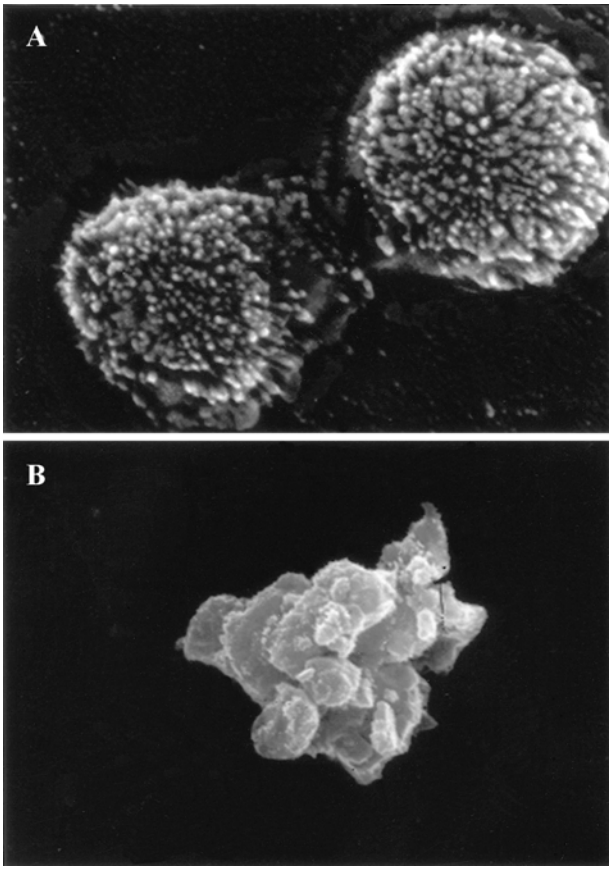


Fig 3. Morphological observation by scanning electron microscopy. A) control cells (×19000); B) Cunabic acid-treated cells (0.25 mmol/L, 72 h) (×7500).

Effects on the regulation of gene expression By immunohistochemistry staining, it was found that the gene expression of *bax* gene of SMMC-7721 cells treated with drugs at the concentration of 25 μmol/L for 72 h increased from + to +++, and that of *bcl*-2 gene decreased from +++ to +.

DISCUSSION

Annona L has long been recorded in classic books of traditional Chinese medicine. It was used to clear away heat, detoxify, kill pest, and stop bleeding[11]. Recently, studies on *Annona* plant are performing widely, a series of reports showed strong anticancer activity of the extract of this plant[6,12,13]. However, study on the anticancer effect and its mechanism of diterpenoid compounds isolated from *Annona glabra Linn* is still few. In our research, experiments were done to study the inhibitory effect of two diterpenoid compounds isolated from *Annona glabra* Linn (Cunabic acid and ent-kauran-19-al-17-oic acid) on the proliferation of human liver cancer (HLC) cell line SMMC-7721 and its possible mechanism. The results showed that the proliferation of SMMC-7721 cells was significantly inhibited by the drugs, and the biggest inhibitory effect was 81.05 %. The inhibitory effect were concentration and time dependent. It was found that Cunabic acid at the concentration of 25 μmol/L showed the best inhibitory effect, no better effect was achieved by only increasing the concentration, so more experiments should be done to find the cause. The drug-treated cells exhibited typical morphological change of cell apoptosis while the control cells showed normal morphology. Cell cycle analysis revealed that the drugs blocked the progression of SMMC-7721 cells through the S-phase and caused arrest at the G_0/G_1 stage. The immunohistochemistry analysis revealed that the drugs induced cell apoptosis by down-regulating the gene expression of *bcl*-2 gene and up-regulating that of *bax* gene.

Apoptosis represents a major protective mechanism against cancer, maintaining normal cell numbers in tissue and deleting cells with severe DNA damage. Apoptosis is an energy-requiring process, characterized by morphological changes, nuclear condensation, plasma membrane blebbing, and the action of an endonuclease that digests DNA into small fragments. It is confirmed that cell factors have close relationship with apoptosis, such as *bcl*-2 gene acts to inhibit apoptosis, while bax gene induces apoptosis. The effect of an anticancer drug was determined in part by how readily the tumor cells undergo apoptosis[14]. The results in this study demonstrated that the *Annona* compounds induced cancer cells apoptosis by regulating gene expression, so they exhibited remarkable inhibitory effect on the proliferation of SMMC-7721 cells. In summary, the two diterpenoid compounds isolated from *Annona glabra Linn* (Cunabic acid and ent-kauran-19-al-17-oic acid) can induce the apoptosis of SMMC-7721 cells at an appropriate concentration, and inhibit the proliferation of cancer cells. The finding of new chemical structures from *Annona* plants provides a fascinating novel resource for anticancer drugs.

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