Chinese-German J Clin Oncol
DOI 10.1007/s10330-014-0026-z
August 2014, Vol. 13, No. 8, P349–P354

The anti-cancer effect of Huaier aqueous extract with rh-Endostatin and DDP*

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Received: 12 June 2014 / Revised: 1 July 2014 / Accepted: 25 July 2014

Abstract ***Objective:*** The aim of our study was to explore the inhibition and apoptosis-inducing effect of the combination of Huaier aqueous extract with recombinant human Endostatin and DDP in human lung adenocarcinoma A549 cells. We also investigated the reversal effect of Huaier aqueous extract in reversing cisplatin resistance in human lung adenocarcinoma A549/DDP cells. ***Methods:*** We treated A549 cells with Huaier aqueous extract and the combination of Huaier aqueous extract and DDP or rh-Endostatin for 24 h, 36 h and 48 h. And then we calculated the inhibition rate through MTT approach and detected the apoptosis rate by flow cytometry. We also treated A549 and A549/DDP cells with DDP, Huaier aqueous extract, DDP and Huaier aqueous extract for 72 h, respectively. ***Results:*** Huaier aqueous extract can inhibit the growth of A549 cells and the inhibition rate improved with the increase of the concentration. The inhibition rate of the combination of rh-Endostatin and 4 mg/mL of Huaier aqueous extract in three time points and the combination of rh-Endostatin and 2 mg/mL of Huaier aqueous extract in the time point of 48 h on the growth of A549 cells all improved ($P < 0.005$). The inhibition rate of the combination of DDP and Huaier aqueous extract with the concentration of 2 mg/mL or 4 mg/mL on the growth of A549 cells all improved ($P < 0.005$). The combination of Huaier aqueous extract and DDP and the combination of Huaier aqueous extract with rh-Endostatin and DDP can improve the inhibition on the growth of A549 cells ($P < 0.005$). ***Conclusion:*** Huaier aqueous extract has the inhibition and apoptosis-inducing effects on the A549 cells. And the combination of Huaier aqueous extract and rh-Endostatin or DDP has the synergistic effects on the inhibition of A549 cells. The combination of Huaier aqueous extract with rh-Endostatin and DDP has the synergistic effects on the inhibition of A549 cells. Huaier aqueous extract can reverse the cisplatin resistance in human lung adenocarcinoma A549/DDP cells.

Key words Huaier aqueous extract; rh-Endostatin; anti-cancer effects; A549 cells; A549/DDP cells

Lung cancer is the major cause resulting in cancer death in the United States both in men and women [1]. There are a lot of therapies such as surgery, chemical therapy, radiotherapy, targeted therapy, Chinese medicine therapy and so on. With the discovery of taxanes, pemetrexed, gemcitabine and other new drugs, the curative effect has improved. However, the drug resistance phenomenon is the main cause for the failure of chemotherapy. When the tumor cells are resistant to one drug, it may be resistant to other drugs with different mechanism and structures. The phenomenon is called multidrug resistance (MDR), which mainly leads to the failure of chemotherapy. The mechanism of MDR is complicated, which may be related to the increase expression of transport proteins, the increase expression of glutathione and its transferase, the decrease expression of topoisomerase II (Topo II) and so on [2]. Therefore, novel anti-cancer drugs must be developed, which may be contained from Chinese herbal medicines. And several herbs have been found to have anti-cancer effect and become the main sources of the anti-cancer drugs [3–5].

Huaier aqueous extract is a kind of hot water extract from fungus Huaier. And the principal active component is proteoglycans (containing 8.72% water, 12.93% amino acids and 41.53% polysaccharides) [6–7], which compose of heteropolysaccharide with 6 kinds of monosaccharide and 18 amino acids [8]. Li [9] *et al* demonstrated that Huaier aqueous extract has been used for the treatment of many diseases such as viral hepatitis in China for many years. Some reports suggested that Huaier aqueous extract has anti-cancer activity via the inhibition of tumor cells growth, induction of apoptosis and anti-angiogenic effects [10–11]. And some experiments *in vitro* indicated that Huaier aqueous extract can suppress the growth of breast cancer cell MCF-7, rectal cancer cell HR8348, esophageal cancer cell Eca-109, lung adenocarcinoma cell H1299, he-

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* Supported by a grant from the Natural Science Foundation of Liaoning Province (No. 201202043).

patic cancer cell HepG-2 and many other tumor cells, and induce the tumor cells apoptosis [12–16]. The rh-Endostatin can inhibit the formation of tumor vessels by inhibiting the migration of vascular endothelial cells, which suppress the proliferation or metastasis of the tumor cells. Some reports have certificated that Huaier aqueous extract can partially reverse the drug resistance in A549/DDP cells.

Materials and methods

Materials

Huaier aqueous extract was obtained from Gaitianli pharmacy Co. Ltd., China. Human lung adenocarcinoma A549 cells and A549/DDP cells were purchased from Science cell Co. (USA). The PRMI 1640 culture was supplied by Hyclone Co. (USA). The MTT was obtained from Ameresco Co. (USA). And DMSO and trypsin were purchased from Sigma Co. (USA). The rh-Endostatin was obtained by Maideji Co. (China). DDP was purchased from Haosen pharmacy Co. (Jiangsu, China). Fetal bovine serum (FBS) was supplied by TBD biological Co. (China). AnnexinV-FITC apoptosis kit was purchased from Biouniquer Co. (USA). DMSO and trypsin were purchased from Sigma Co. (USA).

Tumor cell preparation

The human lung adenocarcinoma A549 cells and A549/DDP cells were intensively cultured in the RPMI 1640. After the cryopreservation and resuscitation, the cancer cells were then used for culturing in RPMI 1640 until they reached 80% confluence. Finally, the cancer cells were washed in PBS solution. And when the shape of cells turned round and there was gap between cells, the reaction was terminated by adding complete medium. Single cell suspension was prepared and then passaged as a ratio of 1 : 2 or 1 : 3.

Detection of DDP, rh-Endostatin, Huaier aqueous extract on the inhibition of A549 cells proliferation

The cancer cells were plated in 96-chamber culture plates at 20,000 cells per well, allowed to adhere and incubate with the different concentrations of DDP, rh-Endostatin and Huaier aqueous extract respectively for 24 h, 36 h and 48 h. And then the MTT liquid was added to each plate and culture for 4 h. Absorbance (*A* value) at 592 nm was measured with Model 550 Type microplate reader after dissolving with DMSO. And the inhibition rate was calculated to determine the inhibition effects of antitumor drugs on tumor cells. Absorbance was detected repeatedly 3 times on different days and expressed as mean ± standard deviation. The apoptosis rate was detected by MTT approach with the excitation wavelength of 488 nm.

Detection of DDP, Huaier aqueous extract and the combination of DDP and Huaier aqueous extract on the inhibition of A549/DDP cells proliferation

The cancer cells were plated in 96-chamber culture plates at 20,000 cells per well, allowed to adhere and incubate with the different concentration of DDP, Huaier aqueous extract and the combination of DDP and Huaier aqueous extract respectively for 72 h. Absorbance (*A* value) at 570 nm was measured with Model 550 Type microplate reader after dissolving with DMSO. And the inhibition rate was calculated to determine the inhibitory effects of antitumor drugs on tumor cells. Absorbance was detected repeatedly 3 times on different days and expressed as mean ± standard deviation.

Statistical analysis

The statistical analysis was performed with SPSS 13.0 software and statistical differences were determined by one-way ANOVA. The data were expressed as the mean ± standard deviation and all experiments were performed in twice. The value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

The influence of different concentration of Huaier aqueous extract on the growth of human lung adenocarcinoma A549 cells by MTT approach (Table 1)

The results showed that the Huaier aqueous extract can inhibit the growth of human lung adenocarcinoma

Table 1 The influence of Huaier aqueous extract on the growth of human lung adenocarcinoma A549 cells

Concentration	24 h		36 h		48 h	
(mg/mL)	OD value	Inhibition rate (%)	OD value	Inhibition rate (%)	OD value	Inhibition rate (%)
0	0.09	0	0.091	0	0.091	0
2	0.750 ± 0.007	9.5	0.699 ± 0.007	16.9	0.706 ± 0.003	17.53
4	0.689 ± 0.012	17.1	0.603 ± 0.008	30.1	0.521 ± 0.008	31.21
8	0.622 ± 0.008	26.3	0.519 ± 0.005	41.5	0.458 ± 0.003	50.78
12	0.535 ± 0.003	38.4	0.430 ± 0.004	53.7	0.388 ± 0.004	60.15
16	0.471 ± 0.008	47.2	0.367 ± 0.002	59.6	0.298 ± 0.005	72.30

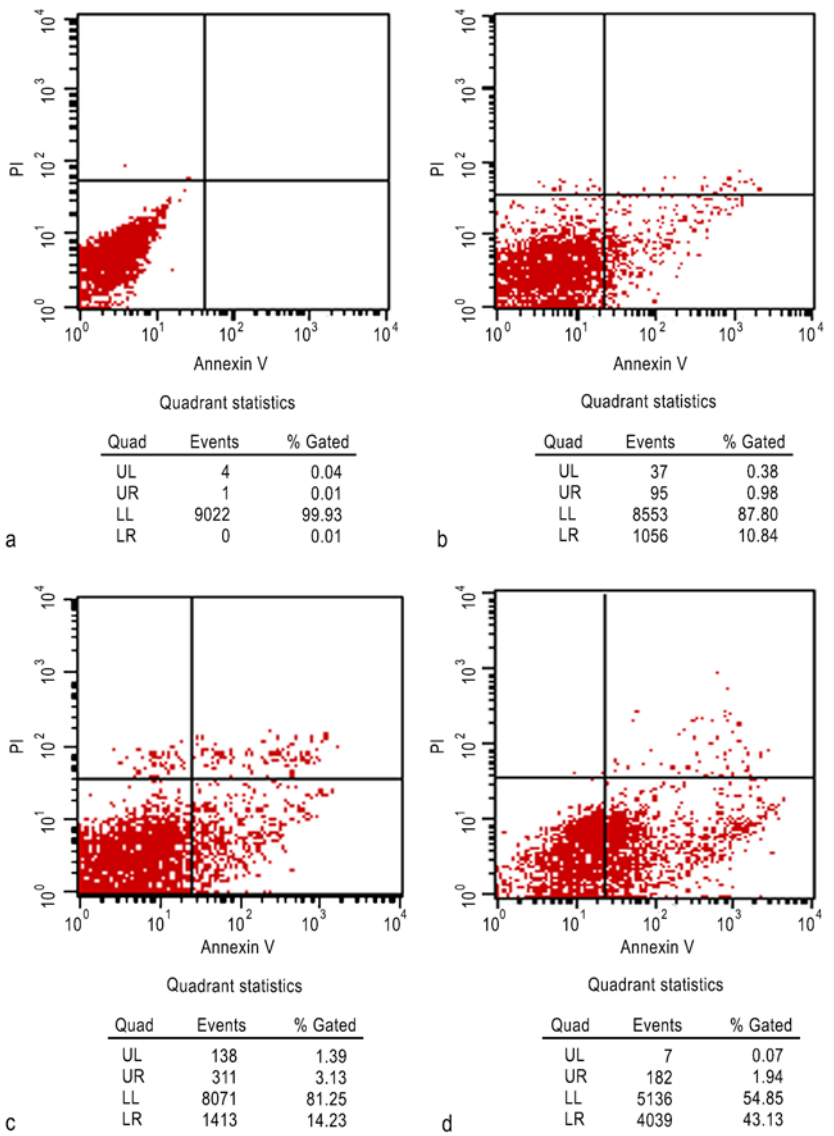

Fig. 1 Effect of Huaier aqueous extract on apopotosis of A549 cells. (a) control group; (b) 4 mg/mL Huaier aqueous extract; (c) 8 mg/mL Huaier aqueous extract; (d) 16 mg/mL Huaier aqueous extract

A549 cells and the inhibition rate improved with the increase of the concentration, which may be dose-time dependent. And the time point of 48 h after the administration has the best inhibition effect.

The apoptosis-inducing effect of Huaier aqueous extract on the human lung adenocarcinoma A549 cells (Fig. 1)

The results showed that the time point of 48 h after the administration of Huaier aqueous extract has the best inhibition effect on the human lung adenocarcinoma A549 cells. So the apoptosis rate was detected at the time point of 48 h after the administration by flow cytometry.

The results showed that Huaier aqueous extracts has the apoptosis-inducing effect on the A549 cells and the apoptosis rate improved with the increase concentration of Huaier aqueous extract.

The inhibition rate of the combination of Huaier aqueous extract and IC50 concentration of rh-Endostatin on the growth of A549 cells by MTT approach (Table 2)

The rh-Endostatin has the inhibition effect on the growth of A549 cells by MTT approach and the IC50 concentration was 1.6 mg/mL.

The results showed that the inhibition rate increased as the time prolonged after the administration, which may be time-dependent. Through the statistical analysis, we found that the inhibition rate of the combination of rh-Endostatin and 4 mg/mL of Huaier aqueous extract in three time points and the combination of rh-Endostatin and 2 mg/mL of Huaier aqueous extract at the time point of 48 h on the growth of A549 cells all improved, which was significant ($P < 0.005$). The inhibition rate of other

Table 2 The influence of the combination of Huaier aqueous extracts and IC50 concentration of rh-Endostatin on the growth of A549 cells

Group	24 h		36 h		48 h	
	Inhibition rate (%)	*P*	Inhibition rate (%)	*P*	Inhibition rate (%)	*P*
IC50 rh-Endostatin + Huaier aqueous extract (2 mg/mL)	45.7	0.139	50.6	0.236	54.3	0.0295
IC50 rh-Endostatin + Huaier aqueous extract (4 mg/mL)	49.3	< 0.005	55.2	< 0.005	57.6	< 0.005

Table 3 The influence of the combination of Huaier aqueous extract and IC50 concentration of DDP on the growth of A549 cells

Group	24 h		36 h		48 h	
	Inhibition rate (%)	*P*	Inhibition rate (%)	*P*	Inhibition rate (%)	*P*
IC50 DDP+Huaier aqueous extracts (2 mg/mL)	47.2	< 0.005	59.7	< 0.005	69.73	< 0.005
IC50 DDP+Huaier aqueous extracts (4 mg/mL)	54.3	< 0.005	65.9	< 0.005	79.81	< 0.005

Table 4 The influence of the combination of Huaier aqueous extract and DDP or rh-Endostatin on the growth of A549 cells

Group	Inhibition rate (%)		
	24 h	36 h	48 h
1/2IC50 rh-Endostatin + 1/2IC50 Huaier aqueous extracts	39.4	47.1	52.3
1/2IC50 DDP + 1/2IC50 Huaier aqueous extracts	42.3	53.6	60.1
1/3IC50 rh-Endostatin+1/3IC50 Huaier aqueous extracts + 1/3IC50 DDP	48.7	56.9	63.2

Table 5 The difference between the single drug and the combination

Group	IC50 Huaier aqueous extracts		IC50 rh-Endostatin		IC50 DDP	
	χ^2	*P*	χ^2	*P*	χ^2	*P*
1/2IC50 rh-Endostatin + 1/2IC50 Huaier aqueous extracts	0.45	0.5	1.68	0.211		
1/2IC50 DDP + 1/2IC50 Huaier aqueous extracts	17.5	< 0.005			16.4	< 0.005
1/3IC50 rh-Endostatin + 1/3IC50 Huaier aqueous extracts + 1/3IC50 DDP	31.36	< 0.005	38.7	< 0.005	29.89	< 0.005

combination of two drugs also improved, though it was not significant.

The inhibition rate of the combination of Huaier aqueous extract and IC50 concentration of DDP on the growth of A549 cells by MTT approach (Table 3)

The DDP has the inhibition effect on the growth of A549 cells by MTT approach and the IC50 concentration was 25 μg/mL.

The results showed that the inhibition rate increased as the time prolonged after the administration, which may be time-dependent. Through the statistical analysis, we found that the inhibition rate of the combination of DDP and Huaier aqueous extract with the concentration of 2 mg/mL or 4 mg/mL on the growth of A549 cells all improved, which was significant ($P < 0.005$).

The inhibition rate of the combination of 1/2IC50 Huaier aqueous extract and DDP or Endostatin on the growth of A549 cells (Table 4, 5)

The results showed that compared with the single drug Huaier aqueous extract with the concentration of IC50 or rh-Endostatin of IC50 in the time point of 24 h, 36 h and 48h, the inhibition rate has not improved obviously in the group of 1/2IC50 Huaier aqueous extract and rh-Endostatin on the growth of A549 cells, which is not significant ($P > 0.05$). It demonstrated that the inhibition on the growth of A549 cells in the group of Huaier aqueous extract and rh-Endostatin was the same as the single drug, which mean the two drugs do not have synergistic effect. However, the results showed that the combination of Huaier aqueous extract and DDP and the combination of Huaier aqueous extract and rh-Endostatin and DDP can improve the inhibition on the growth of A549 cells, which is significant ($P < 0.005$). It suggested that they had synergistic effect.

The inhibition rate of different concentration of DDP on human lung adenocarcinoma A549 and A549/DDP cells detected by MTT assay (Table 6)

The results showed that DDP can inhibit the human lung adenocarcinoma A549 and A549/DDP cells, and can strengthen with the increasing concentration of DDP. According to the formula, the IC50 of DDP on A549 and A549/DDP cells were (0.79 ± 0.06) μg/mL and (13.3 ± 0.9)

Table 6 The 72 h inhibition rate (%) of different concentrations of DDP on A549 and A549/DDP

Concentration of DDP (μg/mL)	A549 (C/O)	Concentration of DDP (μg/mL)	A549/DDP (C/O)
0.19	22.1	1.5	0.092
0.38	33.2	3.0	0.180
0.75	46.6	6.0	0.383
1.50	69.0	12.0	0.498
3.00	73.3	24.0	0.597
6.00	80.6	30.0	0.625
12.00	91.0	60.0	0.814
24.00	94.3	120.0	0.870

Table 7 The 72h inhibition rate (%) of different concentration of Huaier aqueous extract on A549 and A549/DDP cells

Concentration of Huaier aqueous extract (μg/mL)	Inhibition rate for 72 h (%)	
	A549/DDP cells	A549 cells
0.4	3.2 ± 1.3	4.1 ± 0.3
0.6	5.7 ± 2.1	6.3 ± 1.9
0.8	9.2 ± 2.9	11.7 ± 3.7
1.0	13.7 ± 4.1	15.5 ± 6.3
2.0	19.3 ± 3.4	17.5 ± 4.2
5.0	27.3 ± 5.9	28.2± 8.1
10.0	43.7 ± 0.7	57.7 ± 0.8

The difference between A549 and A549/DDP was analyzed by SPSS 11.5 software, $P = 0.738$, no significant difference

μg/mL respectively. Thus, the resistance index of A549/DDP to DDP was 16.8.

The inhibition rate of different concentration of Huaier aqueous extract on human lung adenocarcinoma A549 and A549/DDP cells detected by MTT assay (Table 7)

The results showed that Huaier aqueous extract can inhibit the proliferation of the human lung adenocarcinoma A549 and A549/DDP cells, and can strengthen with the increase of the concentration. It was found in this experiment that Huaier aqueous extract of 0.4 mg/mL had no significant cell cytotoxicity. Therefore, this study used 0.4 mg/mL as a reversal concentration.

The inhibition rate of different concentration of DDP with Huaier aqueous extract on human lung adenocarcinoma A549 and A549/DDP cells detected by MTT assay (Table 8)

Huaier aqueous extract of 0.4 mg/mL had no significant cytotoxicity. Therefore, in this study A549 and A549/DDP cells were treated with Huaier aqueous extract of 0.4 mg/mL combined with different concentrations of DDP for 72 h. IC50 was detected by MTT assay and the change of IC50 was compared. Reversal fold (RF), relative reverse rate (RRR%) were calculated in order to assess the reversal effects of Huaier aqueous extract on cisplatin resistance of A549/DDP cells.

Table 8 The 72 h inhibition rate (%) of different concentration of DDP with Huaier aqueous extract on A549 and A549/DDP cells

Concentration of DDP with Huaier aqueous extract (μg/mL)	Inhibition rate for 72 h (%)	
	A549/DDP cells	A549 cells
0.19	8.5 ± 1.2	23.3 ± 1.0
0.38	17.3 ± 1.6	38.0 ± 1.1
0.75	29.1 ± 0.3	67.7 ± 0.2
1.5	39.0 ± 0.6	72.3 ± 0.6
3.0	48.8 ± 0.7	85.6 ± 1.4
6.0	63.0 ± 1.0	87.9 ± 0.3
12.0	79.2 ± 0.5	90.3 ± 0.8
24.0	82.3 ± 1.1	94.3 ± 0.5

IC50 of DDP combined with Huaier aqueous extract on A549 cells was (0.48 ± 0.02) μg/mL. IC50 of DDP combined with Huaier aqueous extract on A549/DDP cells was (4.21± 0.02) μg/mL.

Reversal fold (RF) = $IC50_{(A549/DDP + DDP)}$ / $IC50_{(A549/DDP + DDP + Huaier\ aqueous\ extract)}$ = 13.3 / 4.2 = 3.15

Relative reversal rate (RRR%) = ($IC50_{(A549/DDP + DDP)}$ – $IC50_{(A549/DDP + DDP + Huaier\ aqueous\ extract)}$) / ($IC50_{(A549/DDP + DDP)}$ – $IC50_{(A549 + DDP)}$) = (13.3 – 4.21) / (13.3 – 0.79)% = 72.7%

Discussion

Lung cancer is one of the highly prevalent malignant diseases worldwide which has the highest morbidity and mortality [17]. The chemotherapy has the important position. However, the drug resistance is the major cause for the failure of chemotherapy. Huaier aqueous extract, a traditional Chinese medicine obtained from the extract of the fungi, has been detected that has anti-tumor effects in various types of cancer. For instance, treatment with Huaier has been shown to inhibit the proliferation of breast cancer cells by inducing apoptosis [18]. Also, Huaier aqueous extract acts as an effective agent for eradicating colorectal cancer stem cells (CSCs) and identifies the Wnt/β-catenin pathway as its potential target [19]. Huaier aqueous extract can inhibit the invasion of ovarian cancer cells via the AKT/GSK 3β/β-catenin pathway [20]. Additionally, it has been suggested that Huaier has various biological activities related to metastasis inhibition, immune system activation and drug resistance reversal [21].

Recombinant human Endostatin is a new biological product with anti-cancer effects which is composed of 20-kDa internal fragment of the C-terminal of collagen XVIII. Some reports demonstrated that Endostatin is one of the potent endothelial cell inhibitors of angiogenesis and tumor growth without toxicity and drug resistance. And Endostatin could suppress pathological angiogenesis by downregulating some angiogenc factors such as VEGF-A and FGF-2 [22–24]. A large number of clinical trials demonstrated that rh-Endostatin combined with

chemotherapy has synergistic effects and improves chemotherapeutic efficacy and quality of patients' life. It has been showed that rh-Endostatin significantly improved overall and progression-free survival in combination with the first-line chemotherapy regime in patients with advanved non-small cell lung cancer in a phase III clinical study [25].

In this study, we found that the combination of Huaier aqueous extract and rh-Endostatin or DDP has the synergistic effects on the inhibition of A549 cells. The combination of Huaier aqueous extract and rh-Endostatin and DDP has the synergistic effects on the inhibition of A549 cells. The combination of Huaier aqueous extract and DDP can improve the apoptosis rate, which may be the basis of synergistic effect. And the combination of rh-Endostatin and DDP have the anti-cancer effect, which may inhibit the tumor growth effectively [26]. And we found that Huaier aqueous extract value of 0.4 mg/mL had no significant cell cytotoxicity on A549 and A549/DDP cells for 72 h. Huaier aqueous extract can reverse the cisplatin resistance in human lung adenocarcinoma A549/DDP cells *in vitro*, with the IC50 value of 4.21 ± 0.02 μg. Also the combination of Huaier aqueous extract and DDP can strengthen the reversal effect. Further research on the animal experiments and clinical trials is suggested to be needed.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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DOI 10.1007/s10330-014-0026-z
Cite this article as: Che YX, Zhou MX, Zhan P, *et al*. The anti-cancer effect of Huaier aqueous extract with rh-Endostatin and DDP. Chinese-German J Clin Oncol, 2014, 13: 349–354.