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The Effect of Alkaloid from *Oxytropis ochrocephala* on Growth Inhibition and Expression of PCNA and p53 in Mice Bearing H_{22} Hepatocellular Carcinoma

Ling LONG[a] and Qingwang LI*[,a,b]

College of Animal Science, Northwest Agriculture and Forestry University, [a]Yangling, Shannxi Province 712100, People's Republic of China and Department of Biological Engineering, College of Environment & Chemical Engineering, Yanshan University, [b]Qinghuangdao, Hebei Province 066004, People's Republic of China

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To study the antitumor activity of alkaloid extracted from *Oxytropis ochrocephala* and its possible mechanism, we observed the effect of alkaloid on tumor weight and expression of PCNA and p53 in mice bearing H_{22} hepatocellular carcinoma by means of immunohistochemistry SP method. After treatment with alkaloid from *Oxytropis ochrocephala*, the results showed that alkaloid administration (25 and 50 mg/kg body weight, p.o.) could inhibit H_{22} hepatocellular carcinoma growth to various extent, and the rates of inhibition were 48.5% and 57.7% respectively ($p<0.01$). The antitumor activity of the alkaloid is in a dose dependent manner, with no signs of toxicity to weight, kidney and liver. The sections of tumor showed the number of tumor cell decreased and nucleus appeared putrescence such as nucleus atrophy, disintegrating and dissolving. Meanwhile, the expression of PCNA and mutant p53 protein positive cell numbers in mice bearing H_{22} hepatocellular carcinoma also suppressed by alkaloid ($p<0.05$). It suggested that Alkaloid from *Oxytropis ochrocephala* showed antitumor effect and its possible mechanism might be associated with the expression inhibition of PCNA and mutant p53 protein. Further studies are needed to explore the antitumor activity of the other compounds of *Oxytropis ochrocephala* and to specify their possible mechanism of action.

Key words——antitumor activity; *Oxytropis ochrocephala*; alkaloid; PCNA; mutant p53 protein; H_{22} hepatocellular carcinoma

INTRODUCTION

It is well established that plants have been a useful source of clinically relevant antitumor compound.[1] Indeed, worldwide efforts were made to discover new anticancer agents from plants which would prevent, slow and/or reverse the cancer induction and its subsequent development.[2] There are many approaches for the selection of plants that may contain biologically active compounds.[3—5] One of the approaches used is the selection of a plant based on the prior information on the folk medicinal use of plant.[5]

Oxytropis ochrocephala Bunge is one of toxic *Oxytropis* plants, which distributes natural grassland in northwestern China , has high nutrient value and toxicity, and seriously threatens the local stockbreading and causes massive economic losing.[6—12] Alkaloid from *Oxytropis ochrocephala* is main antitumor compound, but the research of alkaloid in cancer prevention has only few reports and is not indepth and not systemic.[6,13,14]

e-mail: liqingwangysu@yahoo.com.cn

The purpose of this study was to investigate the in vivo antitumor activity of the alkaloid from *Oxytropis ochrocephala* and lays a foundation for exploiting Oxytropis *ochrocephala* to a new and effective antitumor herbal medicine.

MATERIALS AND METHODS

Chemicals Cyclophosphamide was purchased from Taisheng Pharmacy Inc. (Shanxi province, China). Hematoxylin and eosin were obtained from Sigma Chemical Co. (St. Louis, USA). Mouse antiproliferating cell nuclear antigen (PCNA) and anti-p53 monoclonal antibodies and Streptavidin-Peroxidase immunohistochemical staining kit were obtained from Maixin Biotechnology Inc. (Fujian province, China). All other chemicals used were of analytical reagent grade.

Cell Lines H_{22} hepatocellular carcinoma was obtained from Institute of Materia Medica, Chinese Academy of Medical Sciences.

Animals Male Kunming mice (6 weeks old, 18 ~22 g) are provided by the Experimental Animal Center of the Fourth Military Medical University.

They were kept in groups of five animals per cage in a temperature-controlled room at 20±2℃. Animals were fed with a standard pellet diet and water ad libitum.

Preparation of Extracts and Fractions The aerial parts of *Oxytropis ochrocephala* was collected in Gansu province, China in August 2003. Authentication of plant material was carried out by Dr Li at the College of Animal Science, Northwest Agriculture and Forestry University, China, where the herbarium voucher has been kept.

Air-dried (30±2℃) *Oxytropis ochrocephala* (4.5 kg) was minced and exhaustively extracted with 98% ethanol by maceration for 5 times of 7 days in each extraction. Dry ethanolic extracts (A1, 310 g) were obtained after removing the solvent by evaporation under reduced pressure. The residue extracted sequentially with distilled water/ethanol (4 : 6) mixture and distilled water by maceration for 3 times of 3 days in each extraction and got two extracts (A2, 52 g; A3, 41 g), respectively. Then A1, A2 and A3 were dissolved in a little ethanol and evaporated under reduced pressure. The dry mixture was named crude extract of *Oxytropis ochrocephala* (390 g) (Fig. 1). The crude extract was dissolved in 1 mol/l aqueous HCl and filtrated. The supernate was processed with preparative positive ion exchange resin. In brief, the deionized water and 1 mol/l aqueous NH_4OH as eluent was used to sequentially elute the supernate. Then the eluant eluted with aqueous NH_4OH was collected and evaporated to get crude alkaloid extract (71 g).[10,15,16]

The qualitative analysis of alkaloid was carried out with Mayer, Bertrand and Sonnenschein precipitation reagent and the color of precipitation showed light yellow, brown red and brown yellow.[15]

Effect of Alkaloid on Solid Tumor Growth Inhibition Animals were divided into five groups of ten animals in each group. Under the sterile condition, all the animals were injected into the right fore limb (s.c.) with 1.46×10^6 H_{22} hepatocellular carcinoma cells in sterile physiological saline (aspirated from 7～10 day old H_{22} ascites tumor in mice) in 0.2 ml/mouse. After 24 h of tumor inoculation alkaloid was administered orally at a dose of 25 and 50 mg/kg body weight and continued for 10 consecutive days. The group administered with vehicle alone (distilled water, p.o.) was maintained as control. Cyclophosphamide (CTX, 25 mg/kg body weight, i.p.) was used as the standard reference drug. On day 11, all animals were executed and the mice and tumor were weighed. The rate of tumor inhibition was calculated by the formula: (C-T)/C×100, where "T" and "C" are mean average tumor weight of treated group and control group.[17]

Effect of Alkaloid on Liver and Kidney Liver and kidney of executed mice administrated with alkaloid (50 mg/kg body weight, p.o.) were excised and observed with eyes. Then sections of pathology were made and observed with light microscope.

Effect of Alkaloid on Tumor Cell Morphology The tumors of control, alkaloid (50 mg/kg body

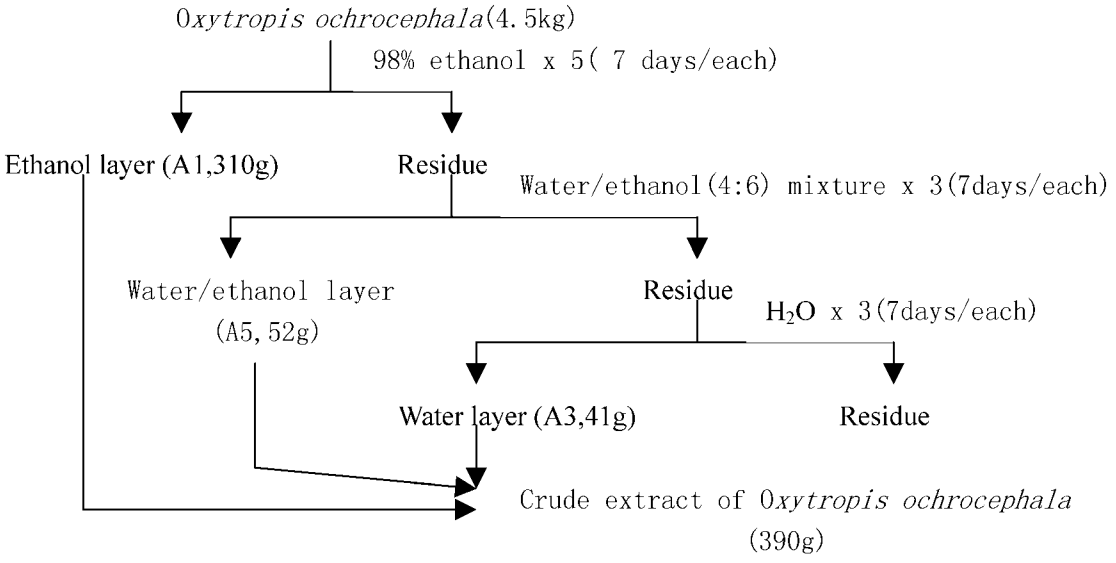


Fig. 1. Flow Chart of Extraction of Crude Extract of *Oxytropis ochrocephala*

weight, p.o.) and CTX (25 mg/kg body weight, i.p.) administration extirpated were fixed in 12% acidic formalin at 4℃, embeded in paraffin and sectioned. Sections were stained with HE staining method and then observed with light microscope.[18—21)]

Expression of Proliferating Cell Nuclear Antigen (PCNA) and Mutant p53 Protein Sections were stained with standard immunohistochemical SP (streptavidin peroxidase conjunction) method and observed with light microscope. The distinctly brown nucleus expressed positive cell and blue nucleus expressed negative cells counterstained by hematoxylin. The numbers of positive cells were counted with a hemacytometer and the mean was calculated.[18—22)]

Statistical Analysis Statistical differences were evaluated using the Student's *t*-test.

RESULTS

Effect of Alkaloid on Solid Tumors The alkaloid from *Oxytropis ochrocephala* administration and standard reference drug (CTX) reduced the tumor weight in a dose dependent way. The tumor weight of control group animals after tumor inoculation was found to be 1.30±0.53 g. The tumor weight was reduced to 0.67±0.19, 0.55±0.16 and 0.43±0.13 g and the tumor inhibition rate was found to be 48.5, 57.7 and 66.9% by administration of alkaloid (25 and 50 mg/kg body weight, p.o.) and CTX (25 mg/kg body weight, i.p.), respectively (Table 1).

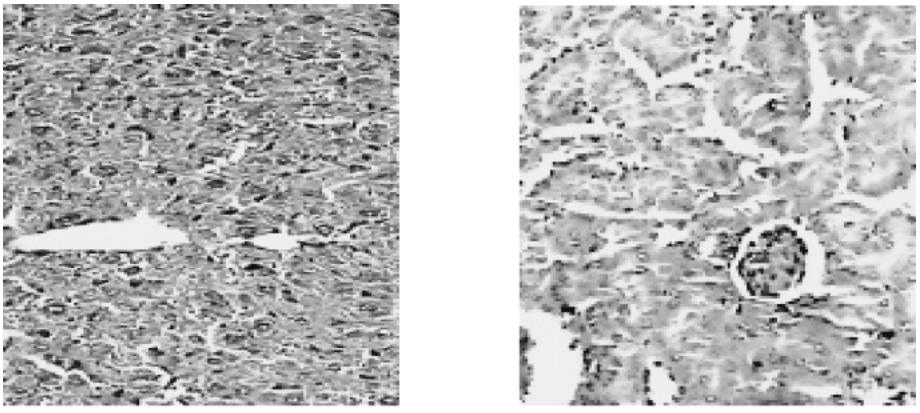
Toxicity Observation on Liver and Kidney The color, luster and texture of liver and kidney observed with eyes were all normality and no pathological changes in alkaloid administration (50 mg/kg body weight, p.o.). The sections of liver and kidney showed that central vein and hepatic lobule were distinct, hepatocellular disposed compact and orderly and glomerular and renal tubular were also evident (Fig. 2).

Effect of Alkaloid on Tumor Cell Morphology The sections of tumor showed that, in contrast to control group, the administration of alkaloid (50 mg/kg body weight, p.o.) and CTX (25 mg/kg body weight, i.p.) significantly reduced the numbers of tumor cells and inhibited the malignant phenotype of

Table 1. Effect of Alkaloid Treatment on the Tumor Inhibition

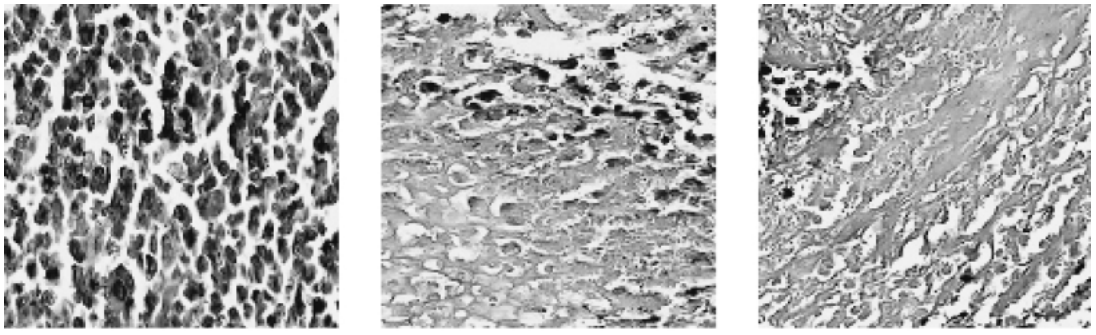
Groups	Treatment (mg/kg)	Animal number		Body weight (g)		Tumor weight (g)	Inhibition (%)
		Beginning	End	Beginning	End		
Control	Vehicle	10	10	20.2±0.93	25.1±1.15	1.30±0.53	
CTX	25	10	10	20.9±0.97	24.4±1.01	0.43±0.13*	66.9
Alkaloid	25	10	10	21.3±0.89	25.6±1.20	0.67±0.19*	48.5
	50	10	10	20.7±1.12	24.6±1.11	0.55±0.16*	57.7

* $p<0.01$ as compared with control group. Values are mean±S.D.



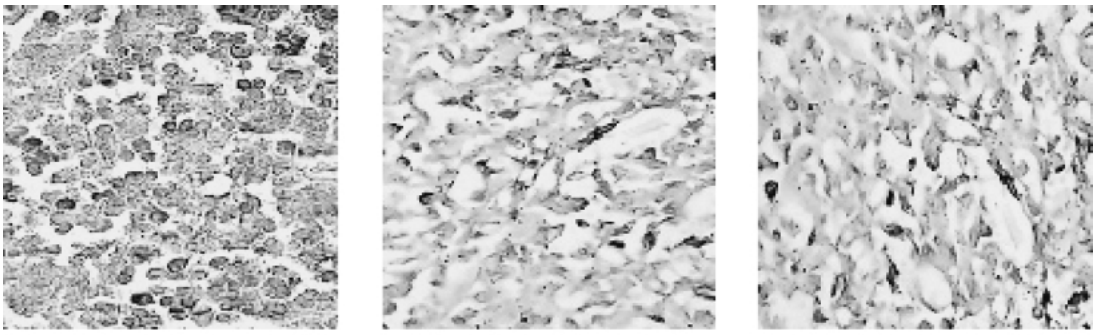
Left: liver; Right: kidney

Fig. 2. Pathology Sections of Liver and Kidney (H.E×200)



Left: control group; Middle: CTX group; Right: alkaloid group

Fig. 3. Sections of H_{22} Hepatocellular Carcinoma Cell (H.E×200)



Left: control group; Middle: CTX group; Right: alkaloid group

Fig. 4. Expression of PCNA in Nucleus (H.E×200)

tumor cells such as heteromorphism, heteropyknosis and decrease of nucleus/plasma ratio in comparison with control group. In groups of alkaloid and CTX administration, the tumor cells appeared scattered monolayer distribution and putrescence such as nucleus atrophy, disintegrating and dissolving and structureless red staining region (Fig. 3).

Expression of Proliferating Cell Nuclear Antigen (PCNA) and Mutant p53 Protein In contrast to the control group, administration of the alkaloid from *Oxytropis ochrocephala* reduced the expression of PCNA and mutant p53 protein in a dose dependent way. The percent of PCNA positive cells in control group was 78.2±5.67%. But alkaloid (50 mg/kg body weight, p.o.) and CTX (25 mg/kg body weight, i.p.) administration significantly reduced the percent of positive cells to 45.2±5.46 and 34.2±4.54 (Table 2, Fig. 4). Similarly, the percent of mutant p53 protein positive cells was found to be 70.5±6.48%, which was reduced to 46.1±5.84 and 36.5±4.96% in the group of animals treated with alkaloid and CTX (Table 2, Fig. 5).

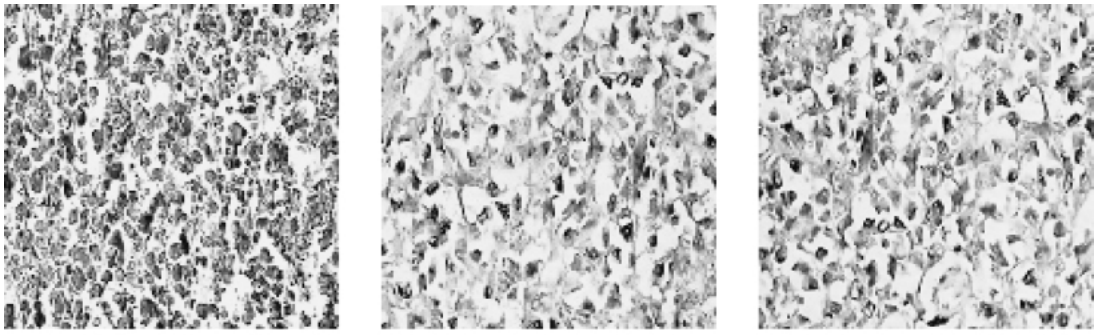
Table 2. Effect of Alkaloid Treatment on the Expression of PCNA and Mutant p53 Protein

Groups	Treatment (mg/kg)	Animal number	PCNA (%)	Mutant p53 protein (%)
Control	Vehicle	10	78.2±5.67	70.5±6.48
CTX	25	10	34.2±4.54*	36.5±4.96*
Alkaloid	50	10	45.2±5.46*	46.1±5.84*

* $p<0.01$ as compared with control group. Values are mean±S.D.

DISCUSSION

The results of the present investigation demonstrate the significant inhibition activity of alkaloid from *Oxytropis ochrocephala* on tumor growth in mice bearing H_{22} hepatocellular carcinoma. The dose (25 and 50 mg/kg body weight, p.o.) were selected based on the preliminary studies carried out. The antitumor activity of the alkaloid is in a dose dependent manner, with no signs of toxicity to weight, kidney and liver. The sections of tumor showed the number of tumor cell decreased and nucleus appeared putrescence such



Left: control group; Middle: CTX group; Right: alkaloid group

Fig. 5. Expression of Mutant p53 Protein in Nucleus (H.E×200)

as nucleus atrophy, disintegrating and dissolving. It indicated that alkaloid from *Oxytropis ochrocephala* possessed direct effect of killing and wounding tumor cell.

PCNA is a cell nuclear protein and is close correlative with DNA replication. PCNA regulates the transition from G1 phase to S phase and is connected with the proliferation of tumor cells.[20] The wild p53 protein is tumor-suppressor gene, but mutant p53 protein loses the activity of tumor suppression and induces tumor proliferation abnormally.[22—24] The expression of PCNA and mutant p53 protein in alkaloid administration was lower than those of control administration in our study. The result showed that alkaloid from *Oxytropis ochrocephala* could inhibit proliferation of tumor cell in our study. It showed the antitumor mechanism of alkaloid from *Oxytropis ochrocephala* might be related with the low expression of PCNA and mutant p53 protein.

There are close positive correlation between PCNA and mutant p53 protein. Mutant p53 protein makes PCNA high expression due to increasing cell proliferation by activating genes related with proliferation. So there are positive correlation between PCNA and mutant p53 protein.[20] The close and synchro relation between PCNA and mutant p53 protein was observed in our study.

In summary, our study suggests that the alkaloid from *Oxytropis ochrocephala* is valuable antitumor herb. Further investigations are in progress to clarify antitumor activity of the other compounds of *Oxytropis ochrocephala* and to specify their possible mechanism of action.

REFERENCES

1) Cragg G. M., Boyd M. R., Cardellina J. H., Newman D. J., Snader K. M., McCloud T. G., "Ethnobotany and Search for New Drugs," the US National Cancer Institute, 1994 p. 3.
2) Chuang S. E., Kuo M. L., Hsu C. H., Chen C. R., Lin J. K., Lai G. M., Hsieh, C.Y., Cheng, A. L., *Carcinogenesis*, **21**, 331–335 (2000).
3) Cordell G. A., Beecher C. W. Pezzuto J. M., *J. Ethnopharmacol.*, **32**, 117–133 (1991).
4) Schwartsmann G., Workman, P., *Eur. J. Cancer*, **29**, 3–14 (1993).
5) Appolinary K., Charles N., Peter D. W., *J. Ethnopharmacol.*, **70**, 143–149 (2000).
6) Meng X. Z., Hu X. Q., Zhang R. M., You Y. T., *J. Ningxia Univ.* (Natural Science Edition), **15**(2), 67–70 (1994).
7) Wu Y. K., Tian H. Y., Chang G. Q., Dan L., Li J. K., *Anim. Toxicol.*, **13**(1), 31–34 (1998).
8) Wu Y. K., Tian H. Y., Dan L., Yang Y. S., Li D. J., Chang G. Q., Li J. K. *Chinese J. Vet. Sci.*, **19**(1), 55–60 (1999).
9) Zhang J., Liu X. C., *Chinese J. Vet. Med.*, (1), 46–48 (1994).
10) Cao G. R., Li S. J., Duan D. X., *J. Northwest Agr. Univ.*, **17**(3), 1–6 (1989).
11) Cao G. R., Li S. J., Duan D. X., *Chinese J. Anim. Vet. Sci.*, **23**, 135–140 (1992).
12) Wang K., Cao G. R., Duan D. X., *Chinese J. Anim. Vet. Sci.*, **21**, 80–86 (1999).
13) Zhang S. F., He B. X., Li X. M., *J. Qinghai Anim. Vet.*, **91**(1), 15–16 (1991).

14) Li S. Q., *Cancer, Aberrance and Mutation*, **2** (2), 53–59 (1990).
15) Li Y. K., ''Chinese Medicine Experimental Methods in Pharmacology,'' Shanghai Science Technology Press, Shanghai, 1991, pp. 43–44.
16) Zhang J. T., ''Modern Experimental Methods in Pharmacology,'' Beijing Medical University & Peking Union Medical College United Press, Beijing, 1998, pp. 75–76.
17) Chihara G., Hamuro J., Maeda Y. Y., Arai Y., Fukuoka F., *Cancer Res.*, **30**, 2776–2781 (1970).
18) Li Q. M., Yu Q., Min C. Y., *World Chin. J. Digestol.*, **11**(7), 997–1000 (2003).
19) Sasano H., Date F., Imatania A., Asaki S., Nagura H., *Hum. Pathol.*, **24**, 584–589 (1994).
20) Chen Q. Y., Wu Y. Q., He Y. S., Zhang C. H., Zhu Y. F., *Med. J. Chin.*, **26**(11), 835–836 (2001).
21) Wasseen X., Lane D., *J. Cell Sci.*, **96**(1), 121–123 (1990).
22) Chen Q. Y., Zhu Y. F., He Y. S., Wu Y. Q., Wang Z. Y., Cai Y., *J. Tumor*, **22**(1), 68–69 (2002).
23) Hall P., Lane D., *J. Pathol.*, **172**(1), 1–5 (1994).
24) Rodrigus N., Rowan A., Smith M., *Proc. Nat. Acad. Sci. U.S.A.*, **87**(19), 7553–7554 (1990).