

Wei-Zheng Kou<sup>1</sup>, Jun Yang<sup>2</sup>, Qing-Hui Yang<sup>1</sup>, Ying Wang<sup>1</sup>,  
Zhi-Fen Wang<sup>3</sup>, Su-Ling Xu<sup>1</sup>, Jing Liu<sup>1\*</sup>

<sup>1</sup>\*Department of Oncology, First Affiliated Hospital of Xinxiang Medical University  
Weihui 453100, Henan, China, <sup>2</sup>Department of Nephrology, First Affiliated Hospital of Xinxiang  
Medical University, <sup>3</sup>Department of Hematology, First Affiliated Hospital of Xinxiang Medical  
University

\*E-mail: liujing6301@163.com

## Abstract

We investigated the anti-tumor effects of *Verbena officinalis* extract on H22 tumor-bearing mice and its effect on immune function. Mice model of H22 solid tumor was established, the mice were divided into five groups and administered the extract, later, tumors were removed and inhibition rates were calculated; spleens were removed and spleen indices were calculated, and the sheep red blood cell-delayed-type hypersensitivity (SRBC-DTH) and the serum hemolysin level were determined. The *Verbena officinalis* extract had anti-tumor effect, with the inhibition rate reaching 38.78%, it also increased the spleen index to a certain extent, in addition, the changes in DTA and HA were not obvious compared with the model group. The *Verbena officinalis* extract had *in vivo* anti-tumor effect, while causing no damage on the immune function.

**Key words:** *Verbena officinalis* extract, H22 mouse, tumor inhibition, immune function

## Introduction

*Verbena officinalis* is the dried aerial parts of *Verbena officinalis* L. and bought from Zaoyang City in Hubei province, which belongs to the Verbenaceae family (Chinese Pharmacopoeia Commission, 2010), it is mainly used for the treatment of abdominal mass, amenorrhea, dysmenorrhea, malaria, pharyngitis, carbuncles, edema, etc. The main chemical constituents of *Verbena officinalis* include iridoid glycosides such as verbenalin and its derivatives (Tian et al., 2005; Zhang et al., 2000), and flavonoid compounds such as luteolin, kaempferol (Chen et al., 2006), ursolic acid (Ma et al., 2005), and volatile oils (Khaled et al., 2010). Studies on its pharmacological activities have mainly focused on the anti-inflammatory (Calvo et al., 2006; Vilalta et al., 1998; M.I. Calvo, N et al., 1998)(not in the references list), antitussive (Gui et al., 1985) effects as well as the effect on uterine smooth muscle (Reproductive Physiology Group i et al., 1974), recent studies have reported the inhibitory effect of *Verbena officinalis* extract on human choriocarcinoma JAR cells and its mechanism of action has been studied (Xu et al., 2001; Zhang et al., 2005; Wang et al., 2004). In this paper, the anti-tumor effect of *Verbena officinalis* extract was studied with the mice H22 hepatoma ascites as the animal model, and its anti-tumor mechanism was initially investigated.

## Materials and Methods

### Drugs and Reagents

*Verbena officinalis* (Baidu Medical Co., Ltd.); cisplatin (Qilu Pharmaceutical Co., Ltd.); SRBC (sterile sheep blood, after defiberization, stored at a 4 °C refrigerator for later use).

### Apparatus

FM-2000A dual-head gamma immune counter (Xi'an Kaipu Mechanical & Electrical Equipment Co., Ltd.); AE31

**Kou et al., Afr J Tradit Complement Altern Med. (2013) 10(3):512a-517**

<http://dx.doi.org/10.4314/ajtcam.v10i3.19>

inverted phase contrast microscope (Motic); SW-CJ-IF Clean Bench (Suzhou Purification Equipment Co., Ltd.); low-temperature refrigerated centrifuge (Eppendorf, Germany); electronic balance (Beijing Sartorius Instrument System Co., Ltd.); blood cell counting chamber (Shanghai Qiujiang Biochemical Instrument Co., Ltd.).

**Animals**

Kunming mice, half male and half female, weight 18~22 g, purchased from the Laboratory Animal Center of the China Medical University. H22 Mice Ascites Hepatoma Cell Lines, purchased from Nanjing KeyGen Biotech. Inc. All experimental animals were approved by the Animal Research Ethics Committee of Xinxiang Medical University, Henan, China (XXMU809).

**Preparation of Verbena Officinalis Extract**

Dried *Verbena officinalis* herb was crushed and adequate (2 kg) was weighed out, which was then added with distilled water 25 times its volume, and extracted for three times using water bath reflux with 2 h each, the extracts were combined together, and after freeze-drying, *Verbena officinalis* extract powder was obtained. The dried powder was diluted to required concentrations when needed.

**Cell Cultivation**

Single cell suspension was prepared, the number of cells was adjusted to  $1 \times 10^6$ , which was seeded to the peritoneal cavity of mice under sterile conditions (0.2 mL/mouse), and abdominal swelling of the mice was observed on a daily basis. 6 days later, peritoneal effusions in mice were extracted.

**Model Building (Li et al., 2006)**

The mice, whose abdominal circumferences were increased to maximum on the 6th day of inoculation, were sacrificed by cervical dislocation, after disinfection of the abdomen, abdominal cavity was cut open, and ascitic fluid was extracted with 1-mL sterile syringes, the ascitic fluid was diluted with PBS and centrifuged at 1000 rpm/min for 10 min, the supernatant was discarded, and viable cells were counted using the trypan blue dye exclusion assay, the number of tumor cells were adjusted to  $1 \times 10^6$  cells/mL. 50 mice were selected and their right armpit skins were disinfected, tumor cell suspension was injected subcutaneously to the right forelimb armpit of each mouse using 1-mL sterile syringes (0.2 mL/mouse) to create a solid tumor model.

**Grouping and Administration**

24 hours after inoculation, the 50 mice were randomly divided into 5 groups, namely the model group, cisplatin group, high-, medium- and low-dose verbena officinalis extract groups (n=10). All mice were provided with enough water and food, while mice in each group were weighed and their body weights were recorded. The administration and dosage were determined in accordance with the "Experimental Methodology of Pharmacology" (Xu et al., 1991), high-, medium- and low-dosages of verbena officinalis were 40, 20, and 10 g crude drug/Kg respectively. The mental state, activities and dietary intake of mice were observed on a daily basis.

### **Determination of Body Weight**

Before and after the experiment, mice in each group were weighed and their body weights were recorded.

### **Determination of Tumor Inhibition Rate**

24 h after the last administration, mice were sacrificed, tumors were peeled off and weighed, and the tumor inhibition rate was calculated according to the following formula. Tumor inhibition rate = (average tumor weight of control group - average tumor weight of experimental group) / average tumor weight of control group  $\times$  100%

### **Determination of Spleen Index**

After the mice were sacrificed, their spleens were removed and weighed. The ratio between spleen weight (mg) and body weight (g) was taken as the spleen index.

### **Determination of Footpad Swelling (DTH) and Serum Hemolysin Level**

Footpad swelling (DTH) was determined by the footpad swelling method (Cao et al., 2009): Fresh defibred sheep blood was washed with sterile normal saline for three times, and prepared as 2% SRBC, on the 3rd day of the experiment, 0.2 ml of the 2% SRBC was injected intraperitoneally to each mouse to cause immunization, on the 4th day after immunization, the thicknesses of left and right footpads were measured with a vernier caliper, and 20% SRBC (20  $\mu$ l/mouse) was injected subcutaneously at the measured site to induce the attack, 24 h after the attack, thicknesses of left and right footpads were measured, each site was measured for three times and the average value was obtained. DTH degree was represented by difference in thickness of front and rear footpads (footpad swelling) before and after the attack.

Serum hemolysin level was determined by the micro-hemagglutination assay (Xue et al., 1995). On the 3rd day of administration, each mouse was injected intraperitoneally with 0.2 ml of 2% SRBC, 24 h after the last administration, mice eyeballs were enucleated and blood was extracted, serum was obtained by centrifugation, and serum hemolysin level was determined on the U-shaped 96-well microplate, the highest serum dilution factor showing (++) agglutination was taken as the hemolysin titer.

## **Statistical Methods**

The experimental data was analyzed using the SPSS 13.0 software. T-test was used for the comparison between two groups, and pairwise comparisons among groups were performed using the One-way analysis of variance (ANOVA).

## **Results**

### **Effect of Verbena Officinalis Extract on Body Weight of Mice**

The changes in body weights of mice before and after the experiment were as shown in Table 1, body weights increased markedly in all of the experimental groups except for the cisplatin group, but the increases were all smaller compared with the model group, and the amounts of increase were generally negatively correlated with the dosage.

**Table 1:** Effect of *Verbena Officinalis* Extract on Body Weight Changes of H22 Tumor-Bearing Mice Before and

After Experiment ( $\bar{X} \pm S$ , n=10)

Group	Dosage (g crude drug/Kg)	Number of animals	Average body weight of mice before the experiment (g)	Average body weight of mice after the experiment (g)
Model	-	10	22.58±2.63	28.46±4.24
Cisplatin	1mg/kg	10	22.52±2.38	25.24±3.67
High-dose	40	10	22.57±2.49	27.65±4.45
Medium-dose	20	10	22.76±2.73	27.85±4.85
Low-dose	10	10	22.13±2.43	29.23±5.34

#### Effect of *Verbena Officinalis* Extract on Tumor Weight and Tumor Inhibition Rate

The results for the effect of *Verbena officinalis* extract on tumor weight and tumor inhibition rate were as shown in Table 2, tumor weights between the model group and high-dose *Verbena officinalis* group had a very significant difference ( $P < 0.01$ ), tumor weights between the model group and medium-dose *Verbena officinalis* group also had a significant difference ( $P < 0.05$ ), moreover, the tumor inhibition rate for the high-dose group was greater than 30%, suggesting that the *Verbena officinalis* extract had an obvious inhibitory effect on H22 solid tumor in mice. Compared with the model group, tumor weight was also reduced in the low-dose *Verbena officinalis* group, but there was no significant difference.

**Table 2:** Effect of *Verbena Officinalis* Extract on Tumor Weight and Tumor Inhibition Rate

Group	Dosage (g crude drug/Kg)	Number of animals	Tumor weight (g)	Inhibition rate (%)
Model	-	10	3.12±0.98	-
Cisplatin	1mg/kg	10	1.78±0.74**	42.94
High-dose	40	10	1.91±0.38**	38.78
Medium-dose	20	10	2.24±0.84*	28.20
Low-dose	10	10	2.63±1.24	15.71

Comparison with the model group, \*  $P < 0.05$ ; \*\*  $P < 0.01$

#### Effect of *Verbena Officinalis* Extract on Spleen Index of H22 Tumor-Bearing Mice

The effect of *Verbena officinalis* extract on spleen index of H22 tumor-bearing mice was as shown in Table 3-3, compared with the model group, spleen indices of the high- and medium-dose *Verbena officinalis* groups were both increased, but the differences were not significant, suggesting that the *Verbena officinalis* extract had no significant effect on spleen index of tumor-bearing mice.

**Table 3:** Effect of *Verbena Officinalis* Extract on Spleen Index of H22 Tumor-Bearing Mice

Group	Spleen index (mg/g)
Model	5.21±1.65
Cisplatin	5.56±1.82
High-dose	5.79±1.13
Medium-dose	5.23±1.06
Low-dose	5.17±1.54

### Results of Determination of Footpad Swelling (DTH) and Serum Hemolysin Level

The effects of *Verbena officinalis* extract on footpad swelling (DTH) and serum hemolysin level of mice were as shown in Table 4, compared with the model group, DTH in each group were increased, but the increase was not significant. HA content in the cisplatin group showed significant difference compared with the model group, while HA content in treatment groups showed no significant differences, indicating that the *Verbena officinalis* extract had no significant suppressive effects on delayed-type hypersensitivity response to SRBC and on antibody production against SRBC, *Verbena officinalis* could inhibit the tumor growth, while causing no apparent damage to the immune organs, cellular immunity and humoral immune functions.

**Table 4:** Effect of *Verbena Officinalis* Extract on Footpad Swelling (DTH) and Serum Hemolysin Level of Mice ( $\bar{X} \pm S$ )

Group	Dosage (g crude drug/Kg)	Number of animals	DTH (0.1 mm)	HA (log)
Model	-	10	3.54±1.52	3.24±0.21
Cisplatin	1mg/kg	10	3.89±1.59	2.15±0.52*
High dose	40	10	3.61±1.49	2.58±0.28
Medium dose	20	10	3.72±1.82	2.74±0.32
Low-dose	10	10	3.85±1.58	2.96±0.49

Comparison with the model group, \* $P < 0.05$

### Discussion

Although conventional tumor therapies can kill the tumor cells, they may also cause damage to the body or even further suppress the immune function. Herbal medicine is China's traditional medicine, which has become a research focus with its advantages such as low toxic and side effects, and acceptable tumor inhibitory effect. As a traditional Chinese herbal medicine, *Verbena officinalis* is abundant in resources, which is widely distributed in China, the study on its anti-tumor activity has a broad prospect. There was also a new triterpenoid compound extracted from *Verbena officinalis* L. which exhibits significantly higher antitumor activity against human hepatoma cell line Bel 7402 *in vitro* (Shu et al., 2012). At the same time, verbenoside A (1) and verbenoside B (2), have been isolated from the ethanol extract of the aerial parts of *Verbena officinalis* L (Xu et al., 2010).

This study demonstrated that the *Verbena officinalis* extract could inhibit the tumor growth in H22 tumor-bearing mice, and on the basis of its *in vivo* anti-tumor effect, its effect on immune function was further explored, and was initially

confirmed that the extract will not damage the immune function. Further in-depth studies should be conducted to clarify its exact mechanisms of action.

## References

1. Calvo, M.I. (2006). Anti-inflammatory and analgesic activity of the topical preparation of *Verbena officinalis* L. J. Ethnopharmacol., 107(3):380-382.
2. Cao, Z.R., Wang, B., Rong, R.X., et al. (2009). Effect of water extract of *Verbena Officinalis* on tumor inhibition and immune function of tumor-bearing mice. J. Chin. Postgraduate. Med. School, 30 (4): 545-546.
3. Chinese Pharmacopoeia Commission. (2010). Pharmacopoeia of the People's Republic of China (Volume 1). China. Press. Med. Sci. Technol., 49.
4. Chen, G.M., Zhang, J.Y., Zhang, X.P., et al. (2006). Study on chemical composition of flavonoids in *Verbena Officinalis*. J. Chin. Med. Mater., 29 (7): 677-678.
5. Gui, C.H., Tang, R.J. (1985). Study on antitussive active ingredients of *Verbena Officinalis*. Bull. Chin. Mater. Med, 10 (10): 35.
6. Khaled A.A., Adnan A.E., Aziza H.Z., Mohamed A.A. (2010). Abdelaaty A Shahat. Investigation of the volatile oils, lipid constituents and biological activity of *Ballota andreuziana* *Teucrium zanonii* and *Verbena tenuisecta* in Libya. Asian.Pacific. J. Tropical. Med., 3(8):594-601.
7. Li, Y.K., Jin, R.M., Wang, Q.M. (2006). Experimental methodology of TCM pharmacology, 2<sup>nd</sup> Ed., Shanghai. Sci. Tech. Publishers, shanghai, pp. 777- 779.
8. Ma, X.P., Jiang, Z.H., Ding, N., et al. (2005). HPLC Determination of ursolic acid content in *Verbena Officinalis*. Chin. Traditi. Patent.Med. , 27 (1): 88-90.
9. M.I. Calvo, N. Vilalta, A. San Julián, M. Fernández. (1998). Anti-inflammatory activity of leaf extract of *Verbena officinalis* L. Phytomedicine, 5(6): 465-467.
10. Reproductive Physiology Group, Physiological Teaching & Research Group, Peking University Health Science Center. (1974). Study on effect of *verbena officinalis* on uterine activity-effect of *verbena officinalis* on human and rat uterine muscle. Acta. Zoologica. Sinica, 20 (4): 335-339.
11. Shu., J.C., Liu, J.Q., Chou, G.X.(2012). A new triterpenoid from *Verbena officinalis* L. Nat Prod Res. 77 :275-9
12. Tian, J., Zhao, Y.M., Luan, X.H. (2005). Study on Chemical composition of *Verbena Officinalis* L. . China. J. Chin. Mater. Med., 30 (4): 268-269.
13. Vilalta, N., Calvo, M.I., Julián, A.S., Fernández, M. (1998). Anti-inflammatory activity of leaf extract of *Verbena officinalis* L. Phytomedicine, 5(6):465-467.
14. Wang, J.J., Luo, L., Zhang, L.P., et al. (2004). Arrest of human choriocarcinoma jar cells at G2/M phase as well as induction of apoptosis by part c of *Verbena Officinalis*. Acta Universitatis Medicinalis Nanjing, 24 (6): 598-601.
15. Xu, S., Jiao, Z.X., Xu, X.J., et al. (2001). Effect of alcohol extract of *Verbena Officinalis* on choriocarcinoma jar cell proliferation and on epidermal growth factor receptor expression. J. China. Pharm. University, 31 (4): 281. 284.
16. Xu, S.Y., Bian, R.L., Chen, X. (1991). Experimental methodology of pharmacology, 2<sup>nd</sup> Ed., People's Medical Publishing House, Beijing, p.202.
17. Xu, W., Xin, F., Sha, Y., Fang, J., Li, Y.S.(2010). Two new secoiridoid glycosides from *Verbena officinalis*. J Asian Nat Prod Res. 12(8):649-53.
18. Xue, B. (1995). Experimental technique of immunotoxicology. Beijing: Beijing Medical University and Peking Union Medical College United Publishing House, 10: 42-43.
19. Zhang, L.P., Xia, B.L., Luo, L., et al. (2005). Study on molecular mechanism of part c of *Verbena Officinalis*-induced apoptosis of human choriocarcinoma JAR cells. Chin. J. Clin. Oncol. , 32 (19): 1089-1092.
20. Zhang, T., Ruan, J.L., Lü, Z.M. (2000). Study on iridoid glycosides of *Verbena Officinalis*. Chin. Traditi. Herb. Drugs., 31 (10): 721-723.