http://dx.doi.org/10.4314/ajtcam.v10i5.16

A STUDY ON INHIBITORY EFFECT OF *SPICA PRUNELLAE* EXTRACT ON T LYMPHOMA CELL EL-4 TUMOUR

Xingjiang Mao[1*],Gengfu Wang[2],Wei Zhang[3],Shaoping Li[3]

[1]Department of General Surgery，the First Affiliated Hospital of Xinxiang Medical University, Wei Hui 453100, China, [2]Department of Anesthesiology，the First Affiliated Hospital of Xinxiang Medical University, Wei Hui 453100, China, [3]Department of Obstetrics and Gynecology，the First Affiliated Hospital of Xinxiang Medical University, Wei Hui 453100, China

***Email:** maoxingjiang88@126.com

Abstract

The objective of the study was to investigate the *in-vivo* anti-tumour activity of *Spica prunellae* extract, and to preliminarily explore the possible mechanism of *in-vivo* anti-tumour effect of *Spica prunellae* extract. Tumour inhibition rate and tumour apoptosis-related protein status were determined using the mice model of transplanted T lymphoma cell EL-4 tumour, and by immunohistochemical method. The results revealed that *Spica prunellae* extract showed certain tumour inhibitory effect, and compared with the model group. Tumour weight in *Spica prunellae* high-dose group was highly significantly different ($P<0.01$). Tumour weight in *Spica prunellae* medium-dose group was also significantly different ($P<0.05$) compared with the model group. *Spica prunellae* high-dose group enabled the high expression of Bcl-2 protein (47.54%) and low expression of Bax protein (13.14%). The study concluded that *Spica prunellae* extract has certain *in vivo* anti-tumour effect, which may be achieved through regulation of apoptosis related proteins.

Key words: *Spica prunellae*, EL-4 solid tumour, tumour inhibition, mechanism

Introduction

Spica prunellae is the dried fruit-spike of *Prunella vulgaris L.*, which is in the genus Prunella, family Lamiaceae. It can clear liver fire, improve eyesight, and disperse swelling, and is mainly used in the treatment of swollen eyes, eyeball pain at night, headaches, dizziness, and swollen breasts (China Medical Science Pree, 2010). It gets its name as it "withers soon after the summer solstice" (Chinese Pharmacopoeia Commission, 2010). Its dried mature fruit-spike has been medicinally used in China for many centuries. According to the records of "Shen Nong's Herbal Classic," "Spica prunellae is bitter and pungent in taste, and is mainly used in the treatment of scrofula, head sores, lump pain, goiter, knot..." (Han Wu-xiang, 2006) As early as in 1988, Lee et al. (Lee KH, et al., 1988) found through in vitro study that the rich ursolic acid contained in *Prunella vulgaris* had a significant cytotoxic activity on A549 human lung adenocarcinoma cells and L1210 leukaemia cells. (Wang Ping, et al., 2010). In vitro apoptosis experiments have suggested that it can induce apoptosis of SGC-7901 gastric cancer cells and K562 human erythroleukemia cells (Wang Kun, et al., 2000; Zhang Ke-jie, et al., 2005). Horikawa K, et al. (Horikawa K, et al., 1994) found that the Prunella plants had an obvious antagonistic effect against carcinogenic and mutagenic actions of benzo[a]pyrene. Modern clinical experience has shown that *Spica prunellae*, whether used alone or combined with other medicines, has a good therapeutic effect for the treatment of various malignant tumours such as lung cancer (Lan Hong-qin, et al., 2007; Li Yan, 2008), breast cancer (Gu Yan-fang, et al., 2002), brain glioblastoma (Tu Yun, et

http://dx.doi.org/10.4314/ajtcam.v10i5.16

al., 2007), and lymphoma (Zhou Jian-hua & Xu Ai-hua, 2005). In this paper, mouse model of transplanted T lymphoma cell EL-4 tumour was established to observe the *in-vivo* anti-tumour activity of *Spica prunellae* extract, and immunohistochemical techniques were applied to preliminarily explore the possible mechanism of *in-vivo* anti-tumour effect of *Spica prunellae* extract.

Experimental Materials

Drugs and reagents

Spica prunellae was purchased from Xinxiang Pharmaceutical Co., Ltd. DMEM medium (Beijing Borunlaite Science & Technology Co., Ltd.), which was identified by Professor Wang Kun, and placed at experiment centre (JM-2314-KL). Other reagents included foetal horse serum (Beijing Biodee Biotechnology Co., Ltd.), trypsin (Jiangsu Yake Chemical Co., Ltd.), MTT (Sigma-Aldrich), cyclophosphamide (Jiangsu Hengrui Medicine Co., Ltd.), mouse anti-mouse Bcl-2 monoclonal antibody concentrate (Beijing ZhongShan Golden Bridge Biotechnology Co., Ltd.), mouse anti-mouse Bax monoclonal antibody concentrate (Beijing ZhongShan Golden Bridge Biotechnology Co., Ltd.), Streptavidin-Peroxidase Histostain TM-Plus Kit (Beijing ZhongShan Golden Bridge Biotechnology Co., Ltd.), and DAB chromogenic kit (Beijing ZhongShan Golden Bridge Biotechnology Co., Ltd.). Other reagents were of domestic analytical grade.

Main instruments

The main instruments used for the study included the following: NUAIRE US AUTOFLOW CO2 incubator (NUAIRE, Germany); SW-CJ-IF clean bench (Suzhou Purification Equipment Factory); AE31 inverted phase contrast microscope (Motic Group); Mod550 fully automated microplate reader (BioRad, USA); stainless steel positive pressure filter (Haining Yatai Pharmaceutical Machinery Co., Ltd.); sterile 96-well culture plate (Costar); RE-52AA rotary evaporator (Shanghai Ya Rong Biochemical Instrument Factory); HH-8 digital thermostat water bath (Changzhou Guohua Electric Appliance Co., Ltd.); electronic balance (Beijing Sartorius Instrument System Co., Ltd.); DZF-6020 vacuum oven (Shanghai Yiheng Instruments Co., Ltd.); hemacytometer (Shanghai Qiujing Biochemical Instrument Factory).

Experimental animals

C57BL/6 inbred mice, weighing 18~22 g, were purchased from the Laboratory Animal Center of Xinxiang Medical University. All experimental procedures were approved by the Animal Research Ethics Committee of Yunnan Medical College University

Cell lines

Mouse T lymphoma leukaemia cell lines EL-4 were purchased from the Cell Center, Institute of Basic Medical College, Peking Union Medical College.

Preparation of *Spica prunellae* extract

Dried *Spica prunellae* was crushed and an appropriate amount was weighed. It was then added with a 25-fold volume of 60% ethanol and ultrasonically extracted three times with 30 min each. The extracted solutions

http://dx.doi.org/10.4314/ajtcam.v10i5.16

were combined, cryoconcentrated, and then purified by polyamide resin. After the effluent was cryoconcentrated and freeze-dried, *Spica prunellae* extract powder was obtained.

Cell cultivation

EL-4 cells were grown in suspension in DMEM high glucose medium containing 10% foetal horse serum, and cultured routinely under the conditions of a temperature of 37 ℃, CO_2 concentration of 5%, and saturated humidity. Cells were passaged once every three days, and cells after thawing were regarded as the first generation cells. In this experiment, the third generation cells after thawing were used.

Model establishment (Li Yi-kui, et al., 2006)

Cells in the logarithmic growth phase were collected, prepared as a 1×10^7 cells/mL single-cell suspension with sterile saline. They were subcutaneously inoculated in the right axilla of C57BL/6 mice, with an inoculum concentration of 0.2 ml cell suspension/mouse. After EL-4 cells were stably passaged in mice, EL-4 tumour-bearing mice on the 14th day after passaging were sacrificed by cervical dislocation. Tumour tissues were harvested from the axilla in a clean bench. Under sterile conditions, fibrous capsules and necrotic tissues were removed, and well-grown tumour tissues were picked out, cut into pieces in a sterile dish, gently ground in a tissue grinder by adding appropriate amount of normal saline, and then filtered with a 200 mesh nylon filter to prepare the tumour cell suspension. After staining with 0.4% trypan blue, cells were counted on the haemocytometer. Cell viability was greater than 95%, and cell concentration was adjusted to 4×10^5 cells/mL with sterile saline. 0.2 mL of the above tumour cell suspension was taken and inoculated subcutaneously into the right axilla of C57BL/6 mice.

Grouping and treatment

24 hours after inoculation, 50 mice were randomly divided into model groups – cyclophosphamide group and *Spica prunellae* extract high-, medium-, and low-dose groups, – a total of 5 groups with 10 mice each. All mice were given enough water and food, and mice in each group were weighed and recorded. Method of administration and dose were determined referring to Yao Zhi-hua (2006) as well as the method provided in the “Experimental Methodology of Pharmacology” (Xu Shu-yun, et al., 1991). *Spica prunellae* high-, medium-, and low-doses were 600 mg crude drug/Kg, 400 mg crude drug/Kg, and 200 mg crude drug/Kg respectively. The mental state, activities and feeding status of mice were observed on a daily basis.

Determination of tumour inhibition rate

On the 11th day after administration, mice were weighed and sacrificed. Tumours were harvested and weighed. The tumour inhibition rate was calculated according to the following formula:

Tumour inhibition rate = (average tumour weight of the control group - average tumour weight of the experimental group) / average tumour weight of the control group × 100%

Determination of apoptosis-related gene protein expression by immunohistochemical method

(1) Paraffin sections were dewaxed, and placed in water.

http://dx.doi.org/10.4314/ajtcam.v10i5.16

(2) The sections were rinsed with distilled water, soaked in PBS for 5 min, and then antigen heat-retrieved for 1 h.

(3) Sections were added dropwise with 3% hydrogen peroxide, incubated at 37 ℃ for 10 min to eliminate endogenous peroxidase activity, and then washed in PBS three times with 5 min each.

(4) Sections were added dropwise with 5% normal goat serum to block non-specific binding sites, so as to eliminate non-specific staining, incubated at 37 ℃ for 30 min, and decanted instead of washing.

(5) Sections were added dropwise with primary antibody working solution (mouse anti-mouse BCI-2 monoclonal antibody, mouse anti-mouse Bax monoclonal antibody), and incubated at 37℃ in wet box overnight.

(6) Sections were washed in PBS three times, with 5 min each.

(7) Sections were added dropwise with biotin-labelled secondary antibody working solution, and incubated at 37℃ for 30 min.

(8) Sections were washed in PBS three times, with 5 min each.

(9) Sections were added dropwise with horseradish peroxidase-labelled streptavidin, and incubated at 37℃ for 30 min.

(10) Sections were washed in PBS three times, with 5 min each.

(11) Sections were developed with DAB.

(12) Sections were thoroughly rinsed with tap water, counterstained, dehydrated and cleared in xylene, and mounted in neutral balsam.

Judgement of immunohistochemical staining results for expression of bcl-2 and bax proteins in EL-4 transplanted tumour tissues in mice

Appearance of yellowish-brown or tan granules in the cytoplasm was regarded as positive. 10 high power fields were randomly selected for counting; 100 cells were counted per high power field, which totalled 1000 cells, and the bcl-2 or bax positive cell ratio (%) was calculated. EL-4 transplanted tumour tissues of mice where drug was not administered were served as the blank control. Counting was performed by at least two individuals, and averaged.

Statistical methods

Experimental data were analysed using SPSS 13.0 software. Comparison between two groups was performed using t-test, and pairwise comparisons among groups were performed using one-way analysis of variance.

Experimental Results

Effect of *Spica prunellae* extract on body weight of mice

The changes in body weight of mice before and after the experiment are as shown in Table 3-1. Except for the cyclophosphamide group where weight gain was not obvious, body weights were significantly increased in all of the experimental groups, although the increases were less when compared with the model group. Basically, the amount of increase was negatively correlated with the dose.

http://dx.doi.org/10.4314/ajtcam.v10i5.16

Effect of *Spica prunellae* extract on tumour weight and tumour inhibition rate

The results for the effect of *Spica prunellae* extract on tumour weight and tumour inhibition rate are shown in Table 3-2. Tumour weight in *Spica prunellae* high-dose group was highly significantly different ($P<0.01$) compared with the model group. Tumour weight in *Spica prunellae* medium-dose group was also significantly different ($P<0.05$) compared with the model group, and the tumour inhibition rates of the two groups were both greater than 30%, suggesting that the *Spica prunellae* extract can significantly inhibit solid tumour development in EL-4 tumour-bearing mice. Tumour weight in *Spica prunellae* low-dose group was also reduced compared with the model group, but revealed no significant difference.

Table 1: Effect of *Spica prunellae* extract on changes in body weight of EL-4 tumour-bearing mice before and after experiment ($\overline{X}$±S, n=10)

Group	Dose (mg crude drug/Kg)	Number of animals	Average body weight (g) of mice before experiment	Average body weight (g) of mice after experiment
Model group		10	21.98±2.51	29.46±4.13
Cyclophosphamide group	0.02g	10	21.42±2.78	24.24±3.77
Spica prunellae high-dose group	600	10	21.74±2.29	27.69±4.45
Spica prunellae medium-dose group	400	10	21.36±2.78	28.88±4.75
Spica prunellae low-dose group	200	10	21.53±2.19	29.23±5.22

Table 2: Effect of *Spica prunellae* extract on tumour weight and tumour inhibition rate of EL-4 tumour-bearing mice

Group	Dose (mg crude drug/Kg)	Number of animals	Tumour weight (g)	Tumour inhibition rate (%)
Model group		10	2.32±0.57	
Cyclophosphamide group	0.02g	10	0.68±0.34**	70.69
Spica prunellae high-dose group	600	10	1.15±0.39**	50.43
Spica prunellae medium-dose group	400	10	1.38±0.34*	40.52
Spica prunellae low-dose group	200	10	1.75±0.44	24.57

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

http://dx.doi.org/10.4314/ajtcam.v10i5.16

Effect of *Spica prunellae* extract on expression of apoptosis-related genes Bcl-2 and Bax proteins

The effect of *Spica prunellae* extract on expression of apoptosis-related genes Bcl-2 and Bax proteins is shown in Table 3-3. Compared with the model group, Bcl-2 protein expression decreased while Bax protein expression increased in the transplanted tumour tissues of the *Spica prunellae* high-dose group, which were both statistically significant (P<0.01) compared with the model group.

Table 3: Effect of *Spica prunellae* extract on expression of apoptosis-related genes Bcl-2 and Bax proteins

Group	Bcl-2 protein expression ratio (%)	Bax protein expression ratio (%)
Model group	47.54±2.34	13.14±1.17
Spica prunellae high-dose group	5.68±0.25**	34.56±0.74**

Comparison with the model group, ** P<0.01

Discussion

Over the years, although researches on the pathogenesis and control of lymphoid and haematopoietic tumours have made great progress, there still remain many difficulties to be resolved. The search for efficient and low-toxic antineoplastic drugs from different sources is still a top priority. In recent years, due to the advantages of low toxicity, little side effects, and easy acceptance by patients of the traditional Chinese medicines, the anti-tumour active ingredients extracted from them are gaining increasing attention (Liu Xiao, et al., 2011). This study also proved that the prepared *Spica prunellae* extract did have anti-tumour effects. The induction of tumour cell apoptosis is one of the recognised anti-tumour mechanisms. The apoptosis is the self-destructive process of cells regulated by genes, which involves a series of gene expression cascades. The genes identified to be associated with apoptosis so far can be categorised into three: pro-apoptotic genes, anti-apoptotic genes, and the ones that assist in the process of apoptosis. BCl-2 and its family can prevent the occurrence of apoptosis (Yang, et al., 1997), and are associated with tumour cell proliferation and carcinogenesis, while Bcl-2-related x protein (bax) can promote the apoptosis (Cheng EH, et al., 1997). In this study, Bcl-2 protein is highly expressed (47.54%) in the EL-4 tumour tissues of mice in the drug groups, while Bax protein is lowly expressed (13.14%), suggesting that this may be one of the causes for mouse EL-4 tumour cell apoptosis.

References

1. Cheng EH, Kirsch DG,Clem RJ, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwick JM. (1997) Conversion of Bcl-2 to a Bax-like death dffector by caspases.Science, **278**:1966-1968.
2. Chinese Pharmacopoeia Commission. Pharmacopoeia of the People's Republic of China, 2010 edition Volume 1. Beijing: China Medical Science Press, **2010: 263**.
3. Gu Yan-fang, Zhang Tian-wen, Niu Yu, Gu Yu (2002) Experience in cancer treatment by Gu Mingsan. Shanghai: Shanghai Scientific and Technical Publishers, **2002: 65-69.**
4. Horikawa K, Mohri T, Tanaka Y, Tokiwa (1994) Moderate inhibition of mutagenicity and carcinogenicity of benzo [a] Pyrene,l,6-dinitropyrene and 3,9-dinitrofluoran thene by Chinese medicinal herbs.Mutagenesis, **9(6)**:523-528.
5. Lan Hong-qin, Li Shu-jun, Zhang Zhi-fang. (2007) Clinical observation on treatment of 32 cases of middle-advanced lung cancer by combination of TCM and Western Medicine. Journal of New Chinese Medicine, 39 **(6)**: 88-89.

http://dx.doi.org/10.4314/ajtcam.v10i5.16

6. Lee KH, Lin YM, Wu TS, Zhang DC, Yamagishi T, Hayashi T, Hall IH, Chang JJ, Wu RY, Yang TH. (1988). The cytotoxic principles of Prunella vulgaris, Psychotria serpens, and Hyptia serpens, and Hyptis capitata: ursolic acid and related derivatives. Planta Med, 54 (4): 308-311..

7. Liu Xiao, Wen Rui-xing. (2011) Research progress on anti-tumor effect of traditional Chinese medicine. Progress in Modern Biomedicine, **11 (24)**: 4978-4983.

8. Li Yan. (2008). The Effect on the sCD44v6 of NSCLC Patient by the Therapy of Combination of TCM with Western Medicine. Chinese Archives of Traditional Chinese Medicine, **26 (4)**: 704-707.

9. Li Yi-kui, Jin Ruo-min, Wang Qin-mao. (2006). Experimental Methodology of TCM Pharmacology (Second Edition). Shanghai Scientific and Technical Publishers, 777-779.

10. Tu Yun, Han Pei-hai, Li Chun-an, Fan Tuo-ying. (2007) Observation on curative effect of brain glioblastoma treated by combination of TCM and Western Medicine. Acta Academiae Medicinae Jiangxi, 47 (2): 74.

11. Wang Kun, Dong Hui-fang, Zhang Xiao-ying, Ravi R, Kastan MB, Bedi A, Ueno K, Hardwich JM. (2000). The effect of Prunella vulgaris L. on SGC-7901 Cells. Shanghai Journal of Medical Laboratory Sciences, 15 **(5)**: 305-30.

12. Xu Shu-yun, Bian Ru-lian, Chen Xiu. (1991). Experimental Methodology of Pharmacology (second edition). Beijing: People's Medical Publishing House, 202.

13. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. (1997) Prevention of apoptosis by Bcl-2: release of cytochromec from mitochondria blocked.Science, 275:1129-1132.

14. Yao Zhi-hua. (2006). The Experiment Research of In Vivo Anti-Lymphoma EL-4 Effect of the Extract of Prunella Vulgaris L. Zhengzhou: Zhengzhou University.

15. Zhang Ke-jie, Zhang Ming-zhi, Wang Qing-duan. (2005). Inductive effect of Prunella vulgaris Injection on K562 cells apoptosis. Chinese Traditional and Herbal Drugs, 36 **(7):** 1031-1035.

16. Zhou Jian-hua, Xu Ai-hua. (2005). 30 cases of malignant tumors treated by combination of TCM and Western Medicine. Jiangxi Journal of Traditional Chinese Medicine, **36 (10)**: 415.