Experimental study on the inhibitory effect of total flavonoids of Trifolium repens L. on MCF-7 breast cancer mice

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Abstract: 'Objective' to observe the inhibitory effect of total flavonoids of Trifolium repens L. on tumors in MCF-7 breast cancer mice, and to explore its mechanism and toxicity. 'Methods' Breast cancer MCF-7 cells were subcutaneously inoculated into the right axilla to observe the effects of low, medium and high doses of total flavonoids of Trifolium repens L. on the tumor growth curve and tumor inhibition rate of tumor-bearing mice. The expression of Caspase-3 in tumor tissues was measured, and the serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (BUN), creatinine (CRE) and other biochemical indicators were detected. 'Results' 2.5 and 5.0g/kg total flavonoids of Trifolium repens L. had significant inhibitory effects on the tumor volume and weight of McF-7 breast cancer mice. The inhibition rate of total flavonoids of Trifolium repens L. of 5.0g/kg clover reached 32.18%. The expression of Caspase-3 in subcutaneous transplanted tumor in the three dose groups was higher than that in the control group ($P < 0.05$). The total flavonoids of Trifolium repens L. had no significant effect on serum ALT, AST, BUN and CRE. 'Conclusion' the flavonoids can inhibit the growth of MCF-7 transplanted tumors. This effect may be related to the enhancement of Caspase-3 expression and promote the apoptosis of tumor cells. No flavonoids of Trifolium repens L. have liver and kidney toxicity in experimental mice.

1. Introduction

Breast cancer is one of the most common malignant tumors, and its incidence rate ranks first in women's cancer incidence, which seriously endangers women's health. [1] At present, the treatment of breast cancer is mainly based on surgical treatment, combined with radiation therapy and systemic chemotherapy. [2] Comprehensive treatment can achieve a certain therapeutic effect, but has a certain degree of damage to the autoimmune system, especially traditional chemotherapy drugs, and serious adverse reactions.

Trifolium repens L. is a cliff climbing vine plant of grape family. It is medicated with root tubers or whole herbs. It has the functions of clearing heat and detoxifying toxins, removing wind and phlegm, promoting blood circulation and analgesia. In recent years, the anti-tumor effect of Trifolium repens L. has been extensively studied. Researchers found that the anticancer activity of Trifolium repens L. is related to its flavonoids, which can induce apoptosis of SGC-7901 human gastric cancer cells, inhibit the proliferation of hepatoma H-22 cells in tumor-bearing mice, and inhibit the proliferation and migration of breast cancer cells MCF-7. In this study, the inhibitory effect of total flavonoids of Trifolium repens L. on subcutaneous transplantation of MCF-7 cells was studied, which provided some experimental basis for the treatment of breast cancer with Trifolium repens L.

2. Material

2.1 Drug

Trifolium repens L. decoction: purchased from Jiangxi Academy of Traditional Chinese Medicine; total flavonoids of Trifolium repens L.: total flavonoids of Trifolium repens L. were extracted by

condensing reflux method, roots of trifoliate greens were washed, and the juicer was processed into a homogenate, taking 25g in a 1L three-necked flask, 68% ethanol was added at a ratio of 1:25, and condensed at 69°C for 100min, and the extraction was repeated twice. The extracts were combined, vacuum filtered, and the filtrate was taken to determine the total flavonoids concentration.

2.2 Animals

Balb/c nude mice, 4 to 6 weeks old, 32, weighing 18 to 22g, purchased from Ningbo Ruyao Biotechnology Co., Ltd., in a specific pathogen free (SPF) animal house, temperature 21-25°C, humidity 45% to 55%.

3. Method

3.1 Establishment of tumor-bearing model

The breast cancer MCF-7 cells were collected and the liver cancer MCF-7 cells were made into a tumor cell suspension with a density of 1×10^7 cells/mL by subcutaneous implantation; under sterile conditions, each mouse 0.2mL was injected subcutaneously into the right axilla. The growth of tumors in mice was observed and all mice were successfully modeled.

3.2 Animal grouping and administration

After 48 hours of subcutaneous inoculation in mice, the mice were randomly divided into 4 groups: control group, low-dose group of Trifolium repens L., medium-dose group of Trifolium repens L., and high-dose group of Trifolium repens L., with 8 rats in each group. The low, middle and high dose groups of Trifolium repens L. were given by intragastric administration of total flavonoids extracted from Trifolium repens L. at doses of 1.25, 2.5 and 5.0g/kg, respectively, each of which was 0.2mL, once a day, continuously administered 21 day. The model control group was administered the same volume of physiological saline daily for 28 consecutive days.

3.3 Observation indicators

3.3.1 Tumor growth curves

The long and short diameters of the tumor were measured once a day using a Vernier caliper to calculate the tumor volume and draw a growth curves.

3.3.2 Tumor weight and tumor inhibition rate

After 24 hours of the last administration, blood was taken from the eye frame, and the cervical spine was dislocated and sacrificed. The tumor tissue was completely stripped and weighed with 10% neutral formaldehyde. The tumor inhibition rate was calculated as (C-T)/C×100%, T was the average tumor weight of each drug group, and C was the average tumor weight of the model control group.

3.3.3 Determination of Caspase-3 expression in tumor tissue

Immunohistochemistry was performed using the EnVision method. The tumor tissue was routinely embedded in paraffin, 4μm thick section, dewaxed with xylene, dehydrated with gradient alcohol, and subjected to high temperature autoclave antigen retrieval (pH 6.0 citrate buffer), and then added with primary antibody at 4°C overnight. The box instructions were carried out, DAB coloring, neutral gum sealing, observation and counting under an optical microscope. Caspase-3 was positively localized to the cytoplasm, and positive staining was observed with yellow or brownish yellow particles in the cytoplasm, which was determined by the Fromowitz comprehensive scoring method [7].

3.3.4 Determination of serum biochemical indicators

Serum was taken to measure liver function and renal function biochemical indicators: alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea nitrogen (BUN), and creatinine (CRE), according to the kit instructions.

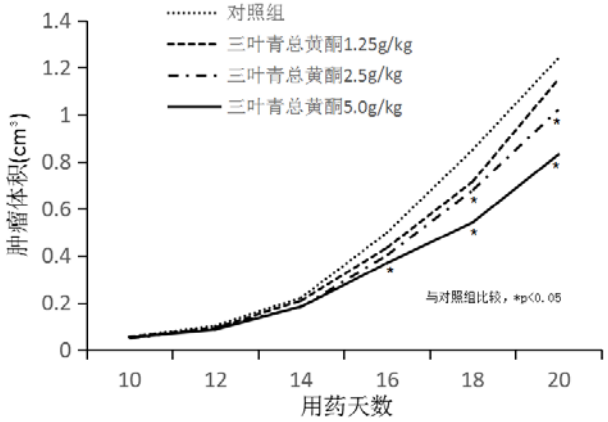
3.4 Statistical Processing

Data processing was performed using SPSS16.0 statistical software. Firstly, the normal distribution test of the measurement data and the homogeneity test of the variance are performed. If the data conforms to the normal distribution and the variance is homogeneous, it is represented by (x±s), the difference between the groups is analyzed by one-way ANOVA, and the average of multiple samples is between two and two. Comparison was performed using the q test. The difference was statistically significant at $P < 0.05$.

4. Results

4.1 Effects of total flavonoids from Trifolium repens L. on tumor growth in mice with breast cancer

Compared with the control group, total flavonoids of Trifolium repens L. at dose group of 2.5 and 5.0g/kg inhibited the growth of transplanted MCF-7 tumors in mice. Compared with the control group, *P<0.05, the inhibiting effect was dose-dependent. The results are shown in Figure 1.



Note: Compared with the control group, *P<0.05

Figure 1. Tumor growth curve of breast cancer mice in each group (x±s, n=8)

4.2 Effects of total flavonoids of Trifolium repens L. on tumor weight in breast cancer mice

Compared with the control group, the total flavonoids of Trifolium repens L. at dose group of 2.5 and 5.0g/kg had a significant inhibitory effect on the weight growth of breast cancer mice ($P < 0.05$), and there was a dose-effect relationship. The inhibition rate of the 5.0g/kg dose group was 32.18%, and the results are shown in Table 1.

Table 1. Effect of total flavonoids of Trifolium repens L. on tumor growth in breast cancer mice (x±s, n=8)

Group	Dose（g/Kg）	Tumor volume before administration (cm^3)	Tumor volume after 21 days of administration（cm^3）	Inhibition rate (%)	Tumor weight	Inhibition rate (%)
Control group	-	0.048±0.014	1.253±0.328	-	1.212±0.384	-
Total flavonoids of Trifolium repens L.	1.25	0.047±0.015	1.154±0.403	7.90	1.109±0.471	8.50
	2.50	0.048±0.016	1.027±0.355*	18.04	0.997±0.450*	17.74
	5.00	0.047±0.014	0.831±0.297*	33.68	0.822±0.403*	32.18

Note: Compared with the control group, *P<0.05

4.3 Effects of total flavonoids of Trifolium repens L. on expression of Caspase-3 in tumor tissues of breast cancer mice

According to the sum of the number of cell stains and the intensity of cell staining, the results are judged: 0 is divided into negative (-), 1 to 2 is weakly positive (+), 3 to 4 is moderately positive (+ +), and 5 to 7 is Strong positive (+ + +). The results showed that three doses of Trifolium repens L. low, medium and high dose groups had increased Caspase-3 protein expression compared with the control group ($P<0.05$, $P<0.01$), as shown in Table 2.

Table 2. Effect of Trifolium repens L. on the expression of Caspase-3 protein in tumor tissues (x±s, n=10)

Group	Dose (g/Kg)	Positive cell ratio	Fromowitz comprehensive score
Control group	-	21.24	+
Total flavonoids of Trifolium repens L.	1.25	42.95*	++
	2.50	39.08*	++
	5.00	60.06**	+++

Note: Compared with the control group, *P<0.05, **P<0.01

4.4 Effects of total flavonoids of Trifolium repens L. on liver and kidney function in mice

There was no significant difference in serum of mice ALT, AST, BUN and CRE contents between the groups of total flavonoids of Trifolium repens L. and the control group.

Table 3. Effect of total flavonoids of Trifolium repens L. on liver and kidney function in mice with breast cancer (x±s, n=8)

Group	Dose (g/Kg)	ALT (IU/L)	AST (IU/L)	BUN (mmol/L)	CREA (μmol/L)
Control group	-	41.51	117.71	5.44	35.36
Total flavonoids of Trifolium repens L.	1.25	43.23	140.34	6.87	36.61
	2.50	44.86	105.08	7.15	36.02
	5.00	30.72	128.44	6.10	38.77

Note: Compared with the control group, there was no significant difference in the flavonoids of Trifolium repens L. group, P>0.05.

5. Discussion

As a kind of commonly used Chinese herbal medicine, Trifolium repens L. has high medicinal value. Traditional Chinese medicine believes that it is cold nature, return to the spleen and stomach meridians, has the effect of clearing away heat and detoxification, internal use is mostly used to treat febrile convulsions and fever in children; external use is mostly used for bruises and sputum. Researchers found that TCM flavonoids of Trifolium repens L. extract have good anti-tumor effect [8], and the existing researches mostly focus on the inhibition of in vitro tumor cells by total flavonoids of Trifolium repens L., and the inhibition of animal xenografts. It mainly focuses on lung cancer [9], colon cancer [10], gastric cancer [11], liver cancer [5] and other aspects, and its inhibitory effect on breast cancer xenografts has rarely been reported.

In this study, the total flavonoids of Trifolium repens L. were extracted by condensing reflux method to study its inhibitory effect on transplanted tumors of MCF-7 breast cancer mice. Drawing the growth curve of tumors and comparing the weight of tumors of breast cancer mice in different concentrations of total flavonoids of Trifolium repens L. and control groups, the results showed that the medium dose and high dose of clover flavonoids of Trifolium repens L. can significantly inhibit the growth of transplanted tumors. Immunohistochemically assay showed that Caspase-3 in tumor tissue was activated after total flavonoids of Trifolium repens L. were administered. To explore the

protein level of total flavonoids of Trifolium repens L. may inhibit the proliferation of breast cancer cell MCF-7 by promoting cell apoptosis. Caspase-3 is one of the most important executioners of caspase family. It can cleaves itself and is responsible for the enzymatic cleavage (activation or inactivation) of all or part of the key proteins during the execution phase of apoptosis, which is a key factor in the apoptotic signaling process in mammals. Caspase-3 activation is generally considered to be a typical manifestation of apoptosis. Therefore, Caspase-3 was selected as an indicator, and the anti-tumor effect and mechanism of clover flavonoids of Trifolium repens L. on MCF-7 tumor-bearing mice were credible.

Through the biochemical indicators detection, there was no significant effect on serum ALT, AST, BUN and CRE of mice in different dose groups of total flavonoids of Trifolium repens L. It suggests that it does not damage the liver and kidney function of mice, but due to the limited means and indicators, the toxicity of the extract of Trifolium repens L. to animal organs has not been clearly confirmed.

Subcutaneous transplanted tumor are a common experimental model for anti-tumor drug screening at home and abroad [12], which has the advantages of easy observation and judgment of curative effect. In this study, McF-7 tumor-bearing mice subcutaneous transplantation model was established to observe the effect of total flavonoids of Trifolium repens L. on the growth rate of tumor cells and the expression of Caspase-3 protein in tumor-bearing mice.

The results showed that compared with the experimental control group, the total and high doses of total flavonoids from C. chinensis could significantly increase the expression of Caspase-3 in mouse tumor tissues in a dose-dependent manner, suggesting that C. sinensis can enhance Caspase. The expression of -3 promotes tumor cell apoptosis and exerts an anti-tumor effect. In addition, this experiment did not find that the total flavonoids of Trifolium repens L. produced liver and kidney toxicity in mice. The results showed that, compared with the control group, moderate and high doses of total flavonoids of Trifolium repens L. could significantly increase the expression of caspase-3 in tumor tissues of mice, and there was a certain dose dependence, suggesting that total flavonoids of Trifolium repens L. could play an anti-tumor role by promoting the expression of caspase-3 and promoting the apoptosis of tumor cells. In addition, the present study did not find that the total flavonoids of Trifolium repens L. had hepatic and renal toxicity on mice.

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