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ORIGINAL ARTICLE

Antitumor Effects of Ethanol Extracts from *Hyptis Rhomboidea* in H_{22} Tumor-bearing Mice

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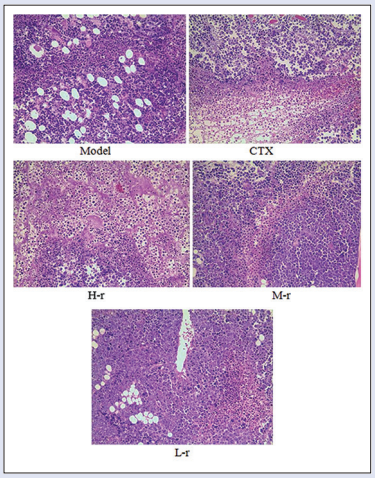
ABSTRACT

Background: Research the antitumor effects of ethanol extracts from *Hyptis rhomboidea* in H_{22} tumor-bearing mice. At the fist-stage of the experiments, the research team took MTT method to measure the antitumor activity *in vitro*, then selected the most inhibitory tumor cell strain as the test object of antitumor activity *in vivo*, established three models of a solid tumor H_{22} liver cancer, ascites tumor, and immunodeficiency in male mice. From inflammatory factor, liver toxicity, *in vivo* antioxidant index to observe antitumor activity of ethanol extracts from *H. rhomboidea*. **Materials and Methods:** Hundred and twenty ICR male mice were used to establish three models of a solid tumor H_{22} liver cancer, ascites tumor, and immunodeficiency in male mice and models group of a solid tumor H_{22} liver cancer randomly divided into six groupshe normal control group, the model control group, the positive group (cyclophosphamide), the sample treated group (high - 1.300 g/kg, medium - 0.750 g/kg, low - 0.373 g/kg). The animals were sacrificed 15d after oral administration and tumors were taken out for the tumor weights and antitumor rates. Blood in eyeball was collected for the determination of aspartate transaminase, alanine transaminase, malondialdehyde (MDA), superoxide dismutase (SOD), interleukin (IL)-2, and tumor necrosis factor (TNF)-α in serum. Sections of tumor issue were prepared, and morphological changes in tumor tissue cells were observed using hematoxylin and eosin staining technique. **Results:** The results showed that ethanol extracts from *H. rhomboidea* have a certain inhibitory effect on the digestive tumor cells. In solid tumor model, the inhibitory rate is up to 68.84% of the high dose of treated group from *H. rhomboidea*, and *H. rhomboidea* could improve the immune organ index, decrease the concentration of TNF-α and IL-2 in serum. In ascites tumor model, *H. rhomboidea* could slow down weight gain in mice and prolong the survival time; in immunodeficiency model, *H. rhomboidea* could improve the serum TNF-α and, IL-2 levels, increase SOD activity, and reduce MDA content, so as to achieve antitumor effect. **Conclusions:** Ethanol extracts from *H. rhomboidea* have obvious antitumor activity *in vivo* and can improve a tumor-burdened mice inflammation factors, improve the survival quality of H_{22} tumor mice, and enhance immunity and antitumor activity.

Key words: H_{22} tumor strains, *Hyptis rhomboidea*, MTT

SUMMARY

- Ethanol extracts from *Hyptis rhomboidea* have obvious antitumor activity without obviously liver damage
- The experiment results prompt that the antitumor activity probably caused by decreasing the inflammatory factors, improve the survival quality, enhance the abnormal cytokines and scavenging free radicals, and raising autoimmune function in H_{22} tumor bearing mice.



Abbreviations used: H. rhomboidea: Hyptis rhomboidea; AST: Aspartate transaminase; ALT: Alanine transaminase; MDA: Malondialdehyde; SOD: Superoxide dismutase; IL-2: Interleukin 2; TNF-α: Tumor Necrosis Factor-α; CTX: Cyclophosphamide.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common human cancer, and the death rate associated with cancer ranks the third in worldwide. Commonly cure method used in diseased liver is to removal of the lesions in the liver or liver transplantation, but it is just limited to a few people and is not able to relieve the suffering of the patients in general. The currently used drugs for liver cancer were sorafenib, but these could not fundamentally solve the problem of liver cancer and have the problems of recurrence of HCC, and postoperative problems at the same time, as a result, the life quality of patients were reduced and unable to undergo more surgery or chemotherapy. Therefore, it is imminent to seek for high-efficiency, natural medicine for the treatment of liver cancer.

Labiatae plants can be found everywhere in the world, and there are about 220 genera and 6000 species, nearly one-half species are in the Mediterranean and the near east central Asia. China has 99 genera and

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800 species and abundant of plant resources. *Rabdosia rubescens* belongs to the *Rabdosia* (Bl.) Hassk genus. According to the clinical curative observation, *R. rubescens* crude extract has cure effects on esophageal and cardia cancer, liver cancer, breast cancer, and colorectal cancer. Its main anticancer active ingredients are oridonin[1] and ponicidin.[2]

At the initial test found that *Hyptis rhomboidea* has good inhibitory effect on HCC using MTT method to measure the antitumor activity. *H. rhomboidea* belongs to *Hyptis* which has about 300 species, but only four species in China, and this plant originates from South America. *H. rhomboidea* for treatment or adjuvant treatment of liver cancer in Guangdong folk of China is also used in the treatment of gastrointestinal infection, cramp, pain, skin infection,[3] and so on. Xu *et al.*[4] find that volatile oils in *H. rhomboidea* have good antioxidant, antibacterial, and cytotoxicity activities using gas chromatography–mass spectrometer analysis and activity evaluation. Li *et al.*[5] found that ethyl acetate extract in *H. rhomboidea* has an antioxidant activity significantly in the research of the antioxidant activity of *H. rhomboidea* crude extracts. The article researches the antitumor effect in H_{22} tumor-bearing mice by *H. rhomboidea* ethanol extracts using animal experiment *in vivo* according to the basis of the screening of many tumor cells experiments *in vitro*.

MATERIALS AND METHODS

Instruments and reagents

Rotary evaporator (Shanghai Yarong Biotechnology Limited Company); paraffin section machine (Germany Leica Company); BX20 type fluorescence microscope camera; GNP-9080 type exclusion of water constant temperature incubator (Shanghai Jinghong Medical Equipment Limited Company in China); TECAN Infinite 200 series multifunction enzyme mark instrument (Switzerland).

H_{22} cell strain was provided by Zhejiang Academy of Medical Sciences Laboratory Animal Center. They were cultured in 37°, 5%CO_2 and saturated humidity incubator with RPMI 1640 culture medium containing 10% fetal bovine serum (100 U·mL^{-1} Penicillin, 100 μg·mL^{-1} Streptomycin). Used 0.25% trypsin digestion cell to serial passage, and then refreshed culture medium after one or two days. Took logarithmic growth phase cell in the experiment. ICR male mice (18–22 g) were purchased from Zhejiang Academy of Medical Sciences Laboratory Animal Center. Animal Certificate of Quality No: SCXK (ZJ) 2008-0033. They were fed on 25°C ± 1°C, humidity of 60% ± 10% and allowed free access to standard laboratory pellet diet and water during the experiments. Cyclophosphamide (CTX) by injection (H22022234, Tonghua Maoxiang Pharmaceutical Limited Company) and Astragalus polysaccharide injection (AP, 190782712, Guangdong Zijinzhengtian Pharmaceutical Limited Company) were used. RPMI1640 (America GIBCO Company); fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Limited Company); trypsin (America GIBCO Company); PBS (prepared by cell culture); DMSO (Beijing Beihua Fine Chemicals Company); penicillin and streptomycin (Huabei Pharmaceutical Group Company); MTT kit (America Sigma Company); superoxide dismutase (SOD), malondialdehyde (MDA), alanine transaminase (ALT), aspartate transaminase (AST), tumor necrosis factor (TNF)-α, interleukin (IL)-2, and hematoxylin and eosin (H and E) Dyeing kit (Nanjing Jiancheng Bioengineering Institute) were used.

Collection and preparation of sample

The whole grass of *H. rhomboidea* that was collected from Sanya in Hainan in August 2012 was identified as *H. rhomboidea* by the plant taxonomy professor Shiman Huang in Hainan University. The specimens were preserved in the laboratory. They then were smashed into coarse powder (40 mesh sieve) after drying, then get them stored in airtight package by cold storage. Took the whole grass after drying of *H. rhomboidea* 2 kg, and cold-soaked extracted for 24 h with three times 70% ethanol. Extracted three times and get 360 g ethanol extracts after vacuum concentration. The extraction rate of ethanol extracts was 18%.

Model preparation and group treatments

Adjust H_{22} cell strain to 1×10^6 by normal saline (ice-cold) to reserve. Injected 0.2 mL the H_{22} cell strains into the male mice by serial passage for 3-4 days. Then adjust H_{22} cell l strain number to 1×10^6 by normal saline (ice-cold) for usage. Study its antitumor activity by solid tumor model, ascites tumor model, and immunosuppression model.

Preparation of solid tumor model group

Randomly selected ten mice to the blank group in the sixty ICR male mice, the other ten mice were treated by subcutaneous injection in medial right fore with 0.1 mL tumor cells liquid completed in 30 min. After 24 h, mice divided them into five groups including model group, positive group (cyclophosphamide, 0.20 mg/10 g), high, middle, and low concentration group (H-r: 13.00 mg/10 g, M-r: 7.50 mg/10 g, and L-r: 3.73 mg/10 g). The blank group and model group were treated by equivalent normal saline. All groups were treated once a day and administrated 2 weeks successively. Weigh them daily and observed their appearance, hair color, stool, activities, and appetite at the same time. After 24 h of the last administration (fasted but given water), weighted and anaesthetized by ethyl ether. Blood samples were drawn from orbit, and then they were killed by cervical dislocation. Striped tumors, thymus, and splenic tissue completely under aseptic condition and weighted accurately after blotting residual blood with filter paper. Each index was calculated according to the following formulas.

Tumor inhibitory rate (%) = (the average weight of tumors in model group – the average weight of tumors in administration group)/the average weight of tumors in model group × 100%, and made an observation of tumor tissues that made H and E staining pathological sections. Viscera index = organ weight/body weight.[6] The four indexes (TNF-α, IL-2, AST, and AST) were determined to mice serum of the solid tumor group by ELISA kit.

Preparation of ascites tumor model group

Randomly selected ten mice to the blank group in the sixty ICR male mice. The others were treated by intraperitoneal injection with 0.2 mL tumor cells liquid completed in 30 min. After 48 h, mice divided them into five groups including model group, positive group (cyclophosphamide, 0.20 mg/10 g), high, middle, and low concentration group (H-r: 13.00 mg/10 g, M-r: 7.50 mg/10 g, and L-r: 3.73 mg/10 g). The blank group and model group were treated by equivalent normal saline. All groups were treated once a day and administrated 2 weeks successively. Weigh daily and timely and observed their diet, mental status, and somatotype change at the same time. Draw curve of the body weight changes in mice. Observed ascites and viscera after natural death and the anatomy of mice. Draw curve of the survival time in mice by Kaplan–Meier method. Calculated the median survival time (MST) value.[7]

Preparation of immunosuppression model group

Randomly selected ten mice to the blank group in the sixty ICR male mice. The others were treated by intraperitoneal injection with CTX (200 mg/kg) completed in 30 min. After 48 h, mice divided them into five groups, including model group, positive group (AP, 0.1 g/kg), high, middle, and low concentration group (H-r: 13.00 mg/10 g, M-r: 7.50 mg/10 g, and L-r: 3.73 mg/10 g). The blank group and model group were treated by equivalent normal saline. All groups were treated once a day and administrated 2 weeks successively. Weigh daily and timely and

observed their appearance, hair color, stool, activities, and appetite at the same time. After 24 h of the last administration (fasted but given water), weighted and took ether anesthesia.

Blood sample was drawn from orbit, and then they were killed by cervical dislocation. Exposed tumor thymus and splenic tissue completely under aseptic condition and weight accurately after blotting residual blood with filter paper. Each index was calculated according to the formulas (viscera index = organ weight/body weight). The four indexes (TNF-α, IL-2, SOD, and MDA) were determined to mice serum of the solid tumor group by ELISA kit.

Made single factor data analysis of variance among the different groups by Graphad prism 5.0. showed a significant difference, and $^{**}P < 0.01$ showed extremely significant difference.

RESULTS AND DISCUSSION

Effect analysis on H_{22} solid tumor-bearing mice

The weights of tumors in CTX group and the three sample concentration groups were decreased significantly compared with the model group by Figure 1a and b.

The tumor inhibitory rate of CTX group was 75.27%. The tumor inhibitory rate of H-r group was 68.83%. The tumor inhibitory rate of M-r group was 59.24%. The tumor inhibitory rate of L-r group was 46.40%. The weights of tumors in the H-r and M-r group were decreased compared with the CTX group, but there were not significant differences ($P > 0.05$). Hence, the antitumor efficacy of them is similar. The antitumor efficacy was positively related with the concentration of the three sample concentration groups [Figure 1c and d].

The inflammatory factors (TNF-α and IL-2) of the model group were increased very significantly compared with the blank group by Figure 1e and f. The inflammatory factors (TNF-αand IL-2) of the CTX group and the three sample concentration groups were decreased compared with the model group, but there were not significant differences compared with the blank group. The results of the experiment showed that *H. rhomboidea* ethanol extracts could decrease the inflammatory factors in H_{22} tumor-bearing mice, alleviating inflammation *in vivo* to exert antitumor efficacy.

The ALT and AST of the model group were increased very significantly compared with the blank group by Figure 1g and h. It showed that the liver lesion was very serious. The ALT in CTX, H-r, and M-r group was decreased significantly compared with the model group. The AST of the CTX group was increased very significantly compared with the model group, showing that CTX was destructive to the liver or could not protect the liver even if it had antitumor efficacy. However, the AST in the H-r and M-r groups was decreased significantly compared with the model group. It showed that *H. rhomboidea* ethanol extracts were not destructive to the liver, decreasing the inflammatory factors in H_{22} tumor-bearing mice.

The high and medium dose groups could obviously elevate the thymus and spleen indexes and the levels of IL-2 and TNF-a and decrease the IL-10. The indicated that *H. rhomboidea* ethanol extracts may indirectly play the role of antitumor activity by improving immunologic function. It could ameliorate the levels of AST and ALT, increase the concentrations of SOD, and decrease the MDA level in serum obvious, which indicating that it could improve the antioxidant activity *in vivo*

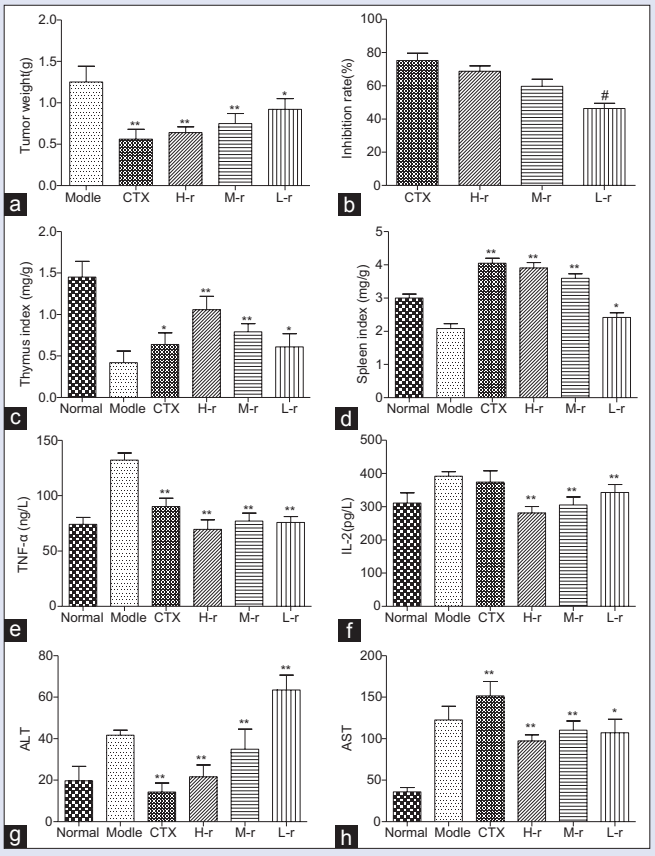

Figure 1: The various indexes of solid tumor group in a~h (a) Tumor weight, (b) Inhibitory rate, (c) Thymus index, (d) Spleen index, (e) TNF-α, (f) IL-2, (g) ALT, (h) AST

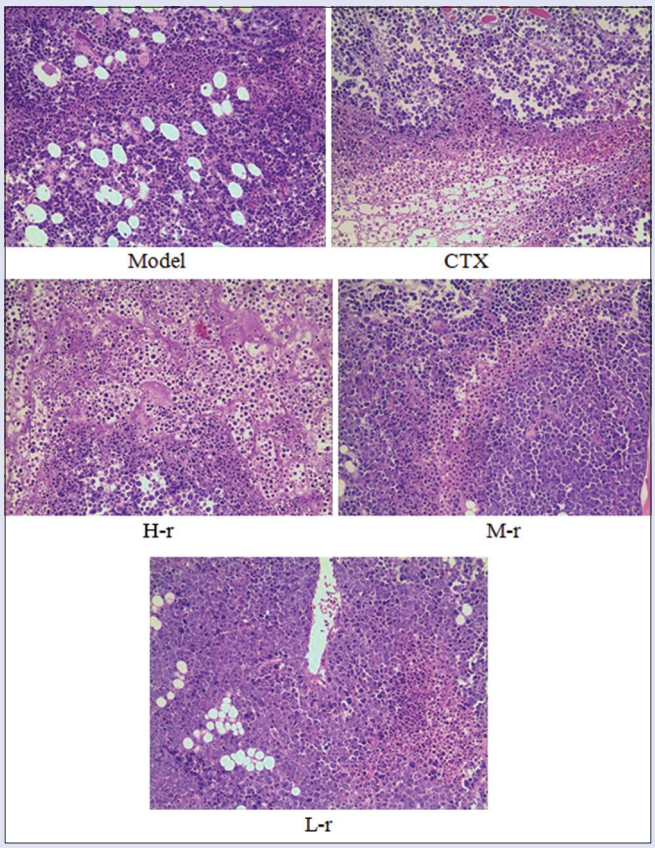

Figure 2: Results of histological observation of solid tumor group (H and E, ×200)

of H_{22} tumor-bearing mice, which proved that *H. rhomboidea* ethanol extracts possess indirectly antitumor activity.

Figure 2 showed that there were many round tumor cells in the model group which had many vacuoles, but there were a few tumor cells in the M-r and L-r groups and few in the CTX group and H-r group. The results of the experiment obviously showed that *H. rhomboidea* ethanol extracts had antitumor efficacy obviously *in vivo*.

Cell pathological change in the tumor tissue can be shown directly in Figure 2; in the model group, there are many tumor cells with large vacuoles. Compared with the model group, the number of tumor cells of high and medium dose groups decreased obviously, almost no normal tumor cells were found, but in low dose groups, there are part tumor cells with small vacuoles.

Effect analysis on H_{22} ascites tumor-bearing mice

The MST value of the blank group, model group, CTX group, H-r group, M-r group, and L-r group was 29, 8, 23, 28, 20.5, and 14 days in sequence. The results of CTX group and the three sample concentration groups had statistical significance compared with the model group. The survival stage of the H-r group increased significantly compared with CTX group. Figure 3 was the curve of the survival time.

After intraperitoneal injection with H_{22} tumor cells liquid, the mice weight increased rapidly from the 5th day and the abdomen swell gradually in the model group, CTX group, and the three concentration groups, compared with the blank group, as was shown in Figure 4. The body weight of CTX group and the three sample concentration groups increased slowly, compared with the model group.

Effect analysis on immunosuppressive mice

The thymus index and spleen index of the CTX group decreased significantly ($P < 0.05$), compared with the model group. The thymus index and spleen index of the H-r group increased significantly ($P < 0.05$), and there was no significant difference in the decreased liver index ($P > 0.05$), compared with the CTX group.

The thymus index and spleen index of the AP group increased significantly ($P < 0.05$), compared with the model group. The thymus index and spleen index of the H-r group increased significantly ($P < 0.05$), compared with the AP group. These results were consistent with the solid tumor group, which showed that *H. rhomboidea* ethanol extracts could improve immunity.

The content of TNF-α of the model group and AP group in serum decreased significantly ($P < 0.05$), compared with the blank group. The content of TNF-α of the H-r and M-r groups in serum increased significantly ($P < 0.05$) compared with the positive group, and there was no significant difference ($P > 0.05$) compared with the blank group. The content of IL-2 of the model group increased very significantly ($P < 0.01$), compared with the blank group. The content of IL-2 of the AP group and the three sample concentration groups in serum decreased very significantly ($P < 0.01$) compared with the model group, and there was no significant difference ($P > 0.05$) compared with the blank group. The content of SOD of the three sample concentration groups increased differently, in which the content of SOD of the H-r group increased significantly ($P < 0.05$), compared with the AP group. The content of MDA of the model group in serum increased significantly, compared with the blank group. The content of MDA of the AP group and the three sample concentration groups decreased differently ($P < 0.05$), compared with the model group. There was no significant difference in the H-r and M-r groups compared with the AP group.

According to the reports, the tumor inhibitory rate which is lower than 40% was considered ineffective, but the tumor inhibitory rate which is more than 40% was considered effective by a statistical process. By the above mentioned, the tumor inhibitory rate of the H-r group was 68.83%, and there was a significant difference ($P < 0.05$) compared with the model group. Hence, the H-r group could obviously inhibit the growth of H_{22} tumor in mice and improve the thymus and spleen indexes.

TNF-α is inflammatory factor of participating in systemic inflammatory response, and it is also an endogenous pyrogen that causes fever and induces apoptosis, causing inflammation by producing IL-1, IL-2, IL-6, IL-8, and so on. TNF-α can stimulate monocytes and megakaryocytes to form and release IL-1 and IL-8 to further expand the inflammation.[8,9] Inflammatory mediators and cellular effect are the important parts in the local environment of tumors. In some malignant tumors, the inflammation is existent in previous canceration. In the other cancers, the different causes of factor change of inflammatory microenvironment could further promote the development of tumor.[10] TNF-α is the indispensable strong link[11] between inflammation and cancer. TNF-α is involved transformation and proliferation of cells, which is also a chronic inflammatory factor in tumor metastasis. This study found that the content of TNF-α and IL-2 of the model group and CTX group increased significantly. However, the contents of TNF-α and IL-2 of the three sample concentration groups decreased, and there was a significant difference compared with the CTX group. Hence, *H. rhomboidea* ethanol extracts can reduce inflammation in H_{22} tumor-bearing mice, improving the abnormal cytokines and autoimmune to exert the antitumor effect.

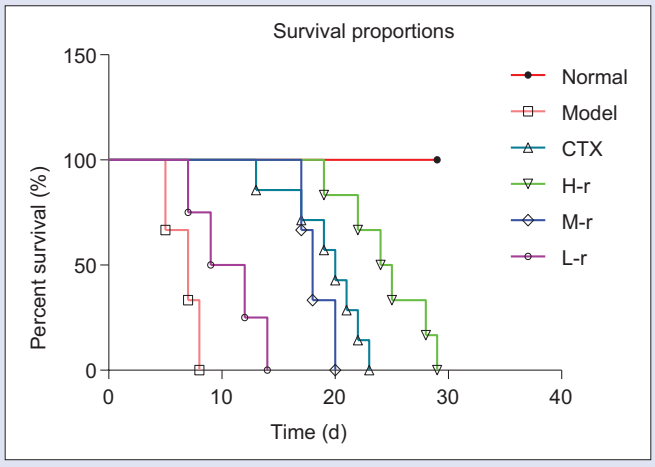

Figure 3: The curve of the survival time in ascites tumor group

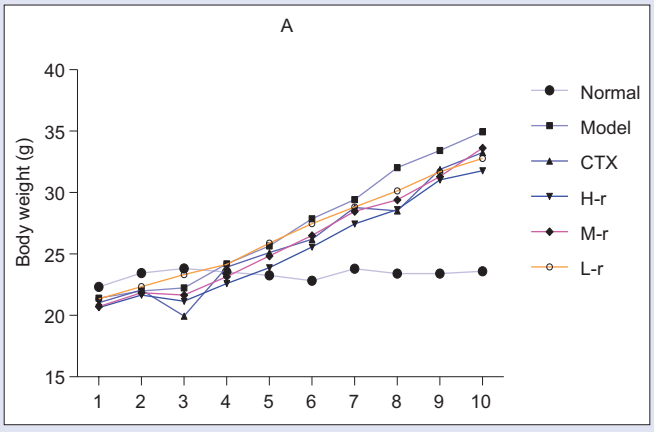

Figure 4: The body weight of the ascites tumor group

ALT and AST are the important indexes of liver injury. The contents of the two indexes are proportional to the extent of the damage of liver cells. The results of the experiment showed that the content of the ALT of the three sample concentration groups increased, but there was no significant difference compared with the CTX group. The contents of the AST of the model group and CTX group increased significantly compared with the blank group. Moreover, there was no significant difference between the three sample concentration groups and the positive group. It showed that the function of the samples was achieved by reducing the level of inflammatory factors and improve immunity rather than by protecting liver of the H_{22} tumor-bearing mice. *H. rhomboidea* ethanol extracts could inhibit the growth of H_{22} tumors and do not have liver toxicity compared with CTX.

The thymus is the body's central lymphoid organs, which generates the initial T-cells to the peripheral lymphoid organs. The spleen is an important peripheral lymphoid organs, and it is a place for immune cell proliferation differentiation. They increase of the body weight shows that immune cell proliferation differentiation, TNF-α, IL-2 with dual biological effects, low concentration sample are mainly involved in resistance to bacterial virus and parasite infection. They promote tissue repair and regulate inflammation and induce tumor cell apoptosis, etc. High concentration sample can destroy the body's immune balance caused inflammation.

The study of oncology professor Chi Dang in Johns Hopkins School found that, to undergo treatment, we must increase the free radicals[12,13] of tumor-burdened bone cancer cells in mice with hypoxia inducing factor (HIF-1). The growth of tumor cells will consume much oxygen. Then, HIF-1 under anoxic conditions will transform sugar into energy. Moreover, it begins to build new blood vessels to replenish the fresh oxygen, which provides favorable conditions for tumor cell proliferation. However, HIF-1 can play a role only under the condition of the presence of a large number of free radicals, once the antioxidant inhibits the generation of free radicals, as a result it will prevent the function of HIF-1,[14] which inhibits tumor growth. SOD indirectly reflects the ability of scavenging free radicals, if SOD decreased, the ability of scavenging free radicals will be weakened, and thus it will lead to more cell damages,[15] MDA indirectly reflects the severity of the attacks of the free radicals on the body cells, and if MDA value increases, it showed that cells, especially the cell membrane lipid peroxidation, will get severe damage.

CONCLUSIONS

After the treatment of the experiment-built immunodeficiency model mice, their immune organ weights are significantly increased compared with the model group, which proves that *H. rhomboidea* ethanol extracts play a role of the immune adjustment and can increase the mice's quality of life. *H. rhomboidea* ethanol extract has the role of improve abnormal low immunity of mice. It also has the function of regulating the body immunity. It increased the level of oxygen-free radical and can change of antioxidant enzyme activity in cell, so it leads to tumor produced, and tumor patients usually show the body's imbalance of oxidation and reduction. Hence, SOD is increased significantly compared with the model group, and the MDA decreases significantly, but there was no significant difference from that of the blank group. This shows that *H. rhomboidea* ethanol extract has certain antioxidant capacity *in vivo*.

The high-dose and medium-dose groups could obviously elevate the thymus and spleen indexes and the levels of IL-2 and TNF-a and decrease the IL-10. They indicated that *H. rhomboidea* ethanol extracts may indirectly play the role of antitumor activity through improving immunologic function. At the same time, it could ameliorate the levels of AST, ALT, and increase the concentrations of SOD, and decrease the MDA level in serum obvious, which indicating that it could improve the antioxidant activity *in vivo* of H_{22} tumor-bearing mice and has no toxicity to body weight and the liver, which further proved that *H. rhomboidea* ethanol extracts possess indirectly antitumor activity *in vivo*, particularly is high-dose and medium-dose groups.

Ethanol extracts from *H. rhomboidea* possess antitumor activity *in vivo* may by reducing the level of inflammatory factors, improve the quality of life of H_{22} tumor-bearing mice, and it can improve immunity and scavenging free radicals in body inside to exerting antitumor activity, but we need further studies on the mechanism of antitumor effect of ethanol extracts from *H. rhomboidea*.

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Conflicts of interest

There are no conflicts of interest.

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