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ORIGINAL ARTICLE

Antitumor and Immunoregulation Effects and Mechanism of N-butanol Fraction from *Zanthoxylum avicennae* in H22 Mice

Hong-Xin Cui[1,2], Fang-Rong Cheng[1], Nan-Nan Zheng[1], Shan-Shan Jia[3], Ke Yuan[3]

[1]College of Pharmacy, Henan University of Chinese Medicine, [2]Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment and Chinese Medicine Development of Henan Province, Zhengzhou, 450046, China, [3]Jiyang College of Zhejiang Agriculture and Forestry University, Zhu'ji 311800, P. R. China

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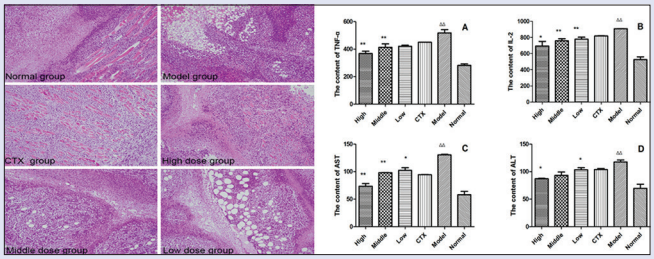
ABSTRACT

Background: The aim of this study was to study the antitumor and immunomodulatory effects and its mechanism of the n-butyl alcohol extract of the ethanol extracts in *Zanthoxylum avicennae* (Lam.) DC. for the mice with liver cancer H_{22}. **Materials and Methods:** To inoculate H_{22} tumor plant to the mice and establish three different animal models of liver cancer solid tumor ascitic tumor and immunodeficiency in mice H_{22} and then divide the mice into six groups: blank group, model group, positive group (cyclophosphamide or astragalus polysaccharide), drug dosages group of high, medium, and lower (1.28 g/kg, 0.64 g/kg, 0.32 g/kg). After 2 weeks of intragastric administration, the content of aspartate transaminase (AST), alanine transaminase (ALT), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) in the blood serum of the mice with solid cancer and the level of superoxide dismutase (SOD), malondialdehyde (MDA), TNF-α, and IL-2 in the blood serum of the immunodeficiency mice were determined. At the same time, the indexes of liver, spleen, and thymus gland organs in solid tumor mice and immunodeficiency mice were determined, the life elongation rate of the mice with liver cancer H_{22} ascitic tumor was observed, and then the changing status of the tumor tissue by the method of HE dyeing was observed. **Results:** The contents of TNF-α and IL-2 in the serum of the high-dose group of the solid tumor model were obviously higher than the normal group, with very significant difference ($P < 0.01$) and very high tumor inhibition rate of 54.61%, and the contents of transaminase AST, ALT, and the tumor inflammatory factors such as TNF-α and IL-2 in the mice serum were obviously decreased, and the tumor weights were reduced greatly; the contents of MDA, TNF-α, and IL-2 in the serum of the mice of the immunodeficiency group were obviously decreased, with obvious increase of the SOD activity and the organ indexes of the liver, spleen, and thymus gland; the life extension rate of the mice in the high-dose group of the ascitic tumor model was obviously increased. **Conclusion:** The n-butyl alcohol extract of *Z. avicennae* improved the survival quality of the H_{22} tumor-bearing mice and enhanced their immune ability to exhibit very excellent antitumor activity through improving the inflammatory factors of the tumor-bearing mice. The mechanism may be that it had the function of antitumor just by improving the immune organs' quality of the tumor-bearing mice, and at the same time increased the secretion of the cell inflammatory factors such as TNF-α and IL-2 and thus strengthened the immunocompetence and improved the lipid peroxidation in the bodies of tumor-bearing mice.

Key words: Antitumor, ascitic tumor, H22 tumor strains, immunoregulation, solid tumor, *Zanthoxylum avicennae*

SUMMARY

- n-butanol fraction from *Zanthoxylum avicennae* has obvious antitumor and immunomodulatory effects without obviously liver damage
- The antitumor and immunomodulatory activity mechanism was probably caused by decreasing the inflammatory factors of tumor necrosis factor-α and interleukin-2, which further strengthened the immunocompetence and improved the survival quality and the lipid peroxidation in the bodies of H_{22} tumor-bearing mice.



Abbreviations used: APS: Astragalus polysaccharide; CTX: Cyclophosphamide; AST: Aspartate transaminase; ALT: Alanine transaminase; TNF-α: Tumor necrosis factor-α; IL-2: Interleukin-2; SOD: Superoxide dismutase; MDA: Malondialdehyde.

Correspondence:

Prof. Fang-Rong Cheng,
College of Pharmacy, Henan University of Chinese Medicine, Zhengzhou, P. R. China.
E-mail: chengfangrong1963@126.com

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INTRODUCTION

Zanthoxylum avicennae (Lam.) DC., also known as dog-like pepper, tang pepper, thorn-down tree, mountain pepper, etc., belongs to *Zanthoxylum* L. category, Rutaceae family, with about 250 different kinds in the world, distributed in Asia, America, Africa, Oceanica, and the tropical and subtropical areas. There are about 18 kinds of medicinal *Z. avicennae* plants in China. Its fruit, roots, stems, and leaves can be made into medicine, having the effects of easing pain, narcosis, bacteriostasis, and insect killing.[1-4] Tang *et al.*[5] found that the pepper aqueous extract can clearly influence metabolism in the mice urine samples related to the antitumor.

The chemical components of *Z. avicennae* mainly involve volatile oil, triterpenes, alkaloids, coumarin, xylogen, flavonoid glycosides, sterols, fatty acid,[6] etc. It's reported in the medical literature that the high concentration(4-16mg/mL) of *Zanthoxylum avicennae* volatile oil has the killing effects for the cell strains of human lung cancer for the A549,

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and the low concentration (1 mg/mL) of *Z. avicennae* volatile oil has the effects of inducing the cell death for the A549.[7] Furthermore, the *Z. avicennae* volatile oil can restrain the reproduction of the H_{22} liver cancer cells and can stimulate the cell apoptosis, and it does not play the part of antitumor through enhancing the body's immune function.[8] So far, the research for the *Z. avicennae* plants mainly focuses on the extracting of essential oil, volatile oil, the chemical analysis, and the mosquito-ridding function of the main volatile oil. There are rarely reports on the research for its bioactivity. This article reports the antitumor and the immunomodulatory effects of the n-butyl alcohol extract of the ethanol extracts in *Z. avicennae* for the H_{22} tumor-bearing mice so that we can reasonably develop and utilize the resources of *Z. avicennae*.

MATERIALS AND METHODS

Instruments and reagents

The instruments and reagents used were rotary evaporator (German IKA RV8), a series of multifunctional microplate reader (Swiss TECAN Infinite 200), paraffin slicing machine (German Leica company), BX20 fluorescence microscope, and KQ-250B supersonic cleaner (Supersonic Instrument Company of Kunshan City).

The raw plants of *Z. avicennae* were picked up in Sanya city, Hainan Province, China, in October 2013, and were identified by Professor Huang Shiman, who specialized in medicinal plant taxonomy, Hainan University. The samples were kept in the laboratory. They were pulverized into coarse powder after drying and kept at room temperature for later use after being sifted through the 60-eye sieve. Sixty of male ICR mice (aged 3–4 weeks and 18–22 g) and five H_{22} tumor-bearing mice were all purchased from Zhejiang Academy of Medical Sciences Laboratory Animal Center (Zhejiang, China, Certificate Number SCXK2015-0033). All procedures for animal experiments were in accordance with the guidelines of Chinese animal care, which conform with International Acceptance of the use of experimental animals. Try feeding them for 1 week adaptively (pay attention to the feeding environment, temperature, and humidity). Cyclophosphamide (CTX, Reference sample number: 100147-201502) was provided by China institute for the identification of pharmaceutical and biological products, Beijing, China). Astragalus polysaccharide (APS) was provided by Zhejiang Medical Science Academy. Aspartate transaminase (AST), Alanine transaminase (ALT), Superoxide dismutase (SOD), Malondialdehyde (MDA), GSH-Px, Tumor necrosis factor-α (TNF-α), Interleukin-2 (IL-2), and Alb ELISA kits were provided by Nanjing jiancheng institute of biological engineering. All the other reagents were analytical reagents.

Preparation of samples

Weigh dried *Z. avicennae* samples 3 kg, pulverized them and sifted through 20-eye sieve and extract them three times by impregnation method with 8 times of 70% ethyl alcohol (each time 10 h). Combine the filtrate solution and to concentrate them by the device of vacuum thin-film concentration to without taste of ethanol. Disperse the concentrated solution into water by ultrasonic method and extract them with diethyl ether, ethyl acetate, and n-butyl alcohol. The extracted solutions are then concentrated, respectively, by thin vacuum thin-film device. Then, put all the concentrated solutions to the rotary evaporators and concentrate and dried them to powder under the temperature of 45°C. Different parts can be obtained, among which the n-butyl extracting part gets 132 g. Prepare the n-butyl alcohol part into three doses of concentration 1.28 g/kg, 0.64 g/kg, and 0.32 g/kg using normal saline, and thus three groups of high dosage, medium dosage, and low dosage were constituted.

Determination of the antitumor activity

This experiment adopts the solid tumor, ascites tumor, and the immunodeficiency model mice to determine the antitumor activity *in vivo*.

Establishment of the tumor-bearing H_{22} mice solid tumor model: Put to death of tumor-.bearing H_{22} mice with liver cancer by dislocation of their cervical vertebra, which have survived 10 days by the passage and grow well and have no ulceration. Then, put the mice bodies on the super-clean worktable. Take out the milk-white tumor liquid from their abdomen under the aseptic condition. Use the sterile-physiological saline solution to dilute them to tumor-cell suspension liquid using the normal saline with the concentration ratio of l: 7 and pick out 60 male mice in ICR series, and choose 10 of them as the blank group randomly, and the other 50 mice are waiting to be molded. Inoculate 0.2 mL of the liver cancer cell suspension liquid into the inside armpits of the right front limbs of each mouse. The next day, divide the mice randomly into the model group, positive group of (CTX 20 mg/kg), and the drug groups of different dosages of high, medium, and low 1.28 g/kg, 0.64 g/kg, 0.32 g/kg, respectively, ten mice in every group. The administration method is one time each day for consecutive 15 days. For the blank group and model group, give them an equivalent amount of normal saline. Weighing after 24 h of administration daily and observing the mice's hair color, food taking, activity, spiritual and defecating status. The mice were weighed 24 hours after the last injection. Take some blood from the eyeball, kill them by dislocation of their cervical vertebra, and strip the tumor tissue and organs of the liver, thymus gland, and spleen, weigh them, respectively, and calculate the tumor weight, tumor inhibition rate, and the indexes of each organ. Calculate each indexes according to the following equation: organ indexes (%) = organ weight/the mice's body mass/th, tumor inhibition rate (%) = (the average tumor weight of the model group-average tumor weight of the drug-administered group)/the average tumor weight of the model group × 100%, separate the serum from the blood sample and according to the instructions in the reagent kit, adopt the microplate reader to determine the contents of AST, ALT, TNF-α, IL-2 in the blood serum, and make pathological section for the tumor tissues to have an observation with HE dyeing method.

Building of the immunodeficiency mice models: Take 60 male mice of ICR series and choose 10 of them randomly as a blank group, and injection CTX (80 mg/kg) into the abdominal cavity of the other 50 mice for three days consecutive. Afterward, inject them with CTX (40 mg/kg) every 2 days so that they are made into the immunodeficiency models. The positive group was APS (0.1 g/kg). Later, administer the drug to mice for 2 weeks by consecutive intragastric administration. Give the mice normal saline oral isopyknic in normal group and model group. Meanwhile, observe the various status of the administered mice daily. After 24 h of administration, take blood by removing eyeball and kill them after ether anesthesia. Take out the tissue of tumor, liver, thymus gland, and spleen and weigh them, respectively. Calculate each index according to the above equation. Separate the serum from the blood sample and according to the instructions in the reagent kit, adopt the microplate reader to determine the contents of SOD, MDA, TNF-α, and IL-2 in the blood serum.

Building of models of the tumor-bearing H_{22} mice's ascitic tumor: The liver cancer H_{22} tumor-bearing mice that had survived for 10 days, grew vigorously, no ulceration were killed by cervical dislocation. Then, put the mice bodies on the super-clean worktable. Take out the milk-white tumor liquid from their abdomen under the aseptic condition. Use the sterile normal saline solution to dilute them to tumor-cell suspension liquid by using the normal saline with the concentration ratio of l: 7

and pick out 60 male mice in ICR series and choose 10 of them as the blank group randomly, and the other 50 mice are waiting to be molded. Inoculate 0.2 mL of the liver cancer cell suspension liquid into the enterocoelia. The next day, divide the mice randomly into model group, CTX positive group (20 mg/kg), and the groups of different dosages of high, medium, and low 1.28 g/kg, 0.64 g/kg, 0.32 g/kg, respectively, ten mice in every group, and the administration method is one time of each day for consecutive 15 days. Observe the average survival time of mice in each group, and after they die naturally, dissect the mice and observe their ascetic fluid and internal organs and use Kaplan–Meier method to draw their lifetime curve and calculate their increase of life span.

Data processing

Adopt pairing test to analyze between different groups and take $P < 0.05$ to be the significance testing standard, the data are shown by average value ± standard deviation, and "*": $P < 0.05$ is to show the obvious difference, whereas "**": $P < 0.01$ is to show extreme difference. All the data analyses are done by GraphPad Prism 5.0 data processing software.

RESULTS AND DISCUSSION

Effects of the samples for the organ index of H_{22} solid tumor mice and its serum biochemical indices

The experimental results are shown in Table 1 and Figure 1. It can be seen that compared with the mice in model group, the tumor body mass in the positive groups of CTX and each drug-administered group are greatly reduced with extremely higher differences ($P < 0.01$), meanwhile their indexes of the spleen, liver, and thymus gland are increased remarkably ($P < 0.05$). The tumor inhibition rate in the positive group of CTX and the drug-administered groups of dosages of high, medium, and low are, 59.29%, 54.61%, 41.57%, and 39.53%, respectively. The tumor inhibition rate in the high-dosage group reaches 54.61%, much near to that of the positive group, the tumor inhibition activity of which can be considered equivalent to that of the positive group. The physical and spiritual activities of the mice in the drug-administered group are very excellent, whereas those mice in the positive group are low spirited, anorexic, and weight losing. These indicate that the positive groups of CTX have toxic and side effects on the mice's bodies.

Table 1: Effect of sample on viscera index and tumor inhibitory rate in H_{22} solid tumor mice

Group	Dose/g/kg	Liver index	Thymus index	Spleen index	Tumor index	Inhibitory rate/%
Normal	-	9.48±0.31	0.36±0.01	1.15±0.05	-	-
Model	-	3.73±0.08$^{\Delta\Delta}$	0.12±0.01$^{\Delta\Delta}$	0.39±0.06$^{\Delta\Delta}$	4.59±0.31	-
CTX	0.02	2.13±0.11	0.07±0.01	0.31±0.01	1.87±0.10**	59.29
High	1.28	5.04±0.04**	0.18±0.01**	0.50±0.01*	2.09±0.32**	54.61
Middle	0.64	5.68±0.03**	0.21±0.01**	0.71±0.03**	2.68±0.21**	41.57
Low	0.32	3.66±0.03*	0.13±0.01	0.37±0.01	2.78±0.46	39.53

$^{\Delta\Delta}P<0.01$ versus normal; **$P<0.01$ versus model; *$P<0.05$ versus model. CTX: Cyclophosphamide

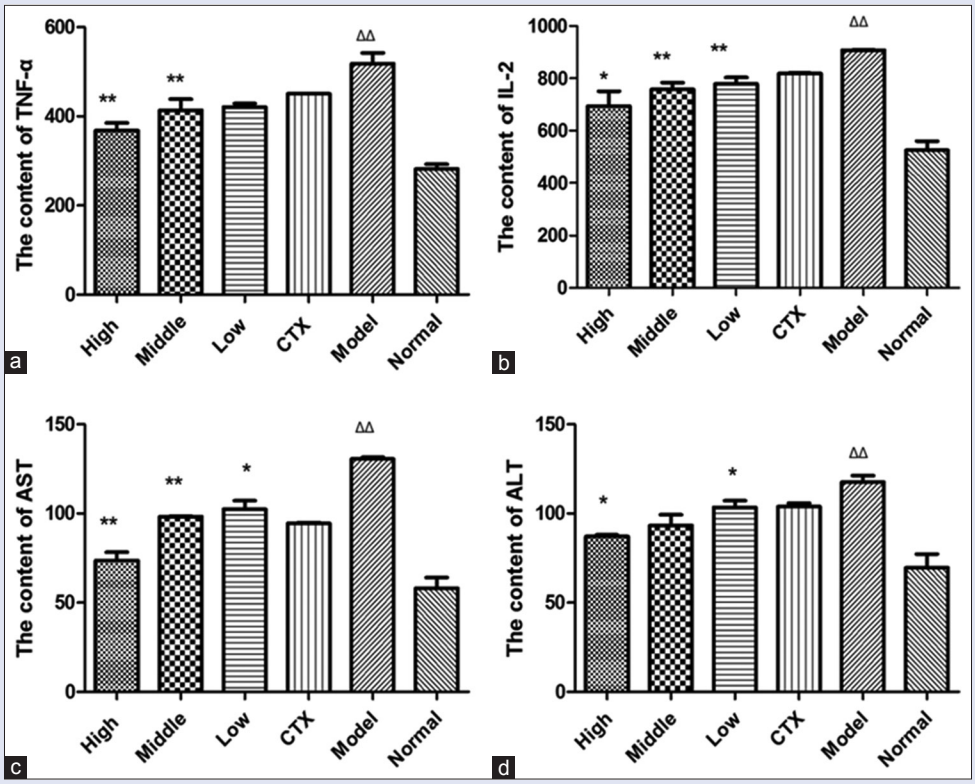

Figure 1: Effect of sample on serum tumor necrosis factor-α, interleukin-2, alanine transaminase and aspartate transaminase in H_{22} solid tumor mice. Data denoted were mean ± standard deviation ($n = 10$)

Abnormal liver function is an important indicator for clinical diagnosis of liver tumors, and AST and ALT are indicators to reflect the degree of liver damage. In solid tumor mice, severe pathological changes occurred in the liver, and the increase of AST and ALT in the positive group was extremely significant. After taking the drug, the recovery of damaged liver in mice could be understood by observing AST and ALT indexes. Compared with the mice in the control group, the contents of the inflammatory factors TNF-α and IL-2 in the blood serum of the mice in the model group are increased obviously ($P < 0.01$), whereas the contents of the inflammatory factors in the positive group of CTX and the drug-administered groups are greatly decreased, and the differences are obvious. Compared with the control group, the blood serum ALT and AST in the model group increased greatly, which shows that the positive group of CTX has strong effects of antitumor but has destructive effects on the liver. Compared with model group, the mice of the drug-administered group serum AST and ALT are decreased greatly, with the most obvious in the high-dose and medium-dose groups being the most remarkable ($P < 0.01$). The above experimental results indicate that the n-butyl alcohol extract of *Z. avicennae* can effectively decrease the inflammatory factors in the tumor-bearing mice and thus improve the serum biochemical index and the organ index of H_{22} mice with solid tumor.

The analysis on the pathological section of the mice tissues with solid tumor

The pathological section of the tumor tissue can have a direct observation of the cytopathic effects in the tumor tissue as in Figure 2. It can be seen from the observation of the tumor tissue pathological change that there are more cancer cells and physalides in the model group. Compared with that of the model group, the tumor cell counts in the high dose and low dose in the drug-administered group are relatively small but still with some bubbles. However, there are almost no normal tumor cells in the positive group of CTX and high-dose drug-administered groups. The pathological section of the tumor tissue has more directly proved that the n-butyl alcohol extract of *Z. avicennae* has very obvious antitumor activity *in vivo*.

Effects of the samples on the immune organ index of the immunodeficient mice and its serum biochemical indices

For the selection of drug dosage, this article referred to the literature[9] and carried out preliminary test screening. The high-, medium-, and low-dose groups were selected according to the effective drug dosage. The experiment chose APS as the positive group because APS are well-known as effective traditional Chinese medicine to regulate immunity and significantly enhance the body's immunity. The data of SOD and MDA can indirectly reflect whether the body's immunity is improved or not. Studies have shown that almost all tumor cells have imbalance of oxidation–reduction system, which is the main cause of excess free radical in the body. SOD content reflects the ability of the body to resist oxidation. MDA content can indirectly reflect the degree of oxidative damage of the body. By observing SOD and MDA indexes, it can be understood whether the antioxidation ability of cells is increased, and the body's immunity is improved after taking the drug, thereby delaying cell aging and slowing down the occurrence and development process of tumor cells.[10]

The experiment results can be seen in Table 2 and Figure 3, which indicate that compared with the mice in the normal control groups, the indexes of the liver, spleen, and thymus gland in the model groups are all greatly decreased ($P < 0.01$), and this shows that the immunity of the drug-administered mice is lowered, and the modeling is successful. Compared with those of the model group, the indexes of the liver, spleen, and thymus gland in the positive group of APS and the different doses of drug-administered groups are increased greatly ($P < 0.01$). Among them, visceral organs indexes in the high-dose and medium-dose groups are nearly equivalent to those in the positive group of APS, and this is because through increasing the visceral indexes of the H_{22}, tumor-bearing mice can increase their immune ability in a certain degree and thus inhibit the growth of the H_{22} tumor cells. The serum biochemical indices are shown in Figure 3, compared with the control group; the contents of TNF-α, IL-2, and MDA of the mice in the model group are somewhat increased, whereas the contents of SOD in the blood serum of the model group and the positive group of APS are lower than that of the normal control group. Compared with the model group, the contents of TNF-α, IL-2, and MDA in the blood serum of the drug-administered group and the positive group of APS are lowered relatively ($P < 0.01$), and the SOD content increased greatly ($P < 0.01$). This indicates that the n-butyl alcohol extract of *Z. avicennae* can increase the index of the mice's immune organs, decrease the contents of TNF-α, IL-2, and MDA in the blood serum, and increase the SOD content.

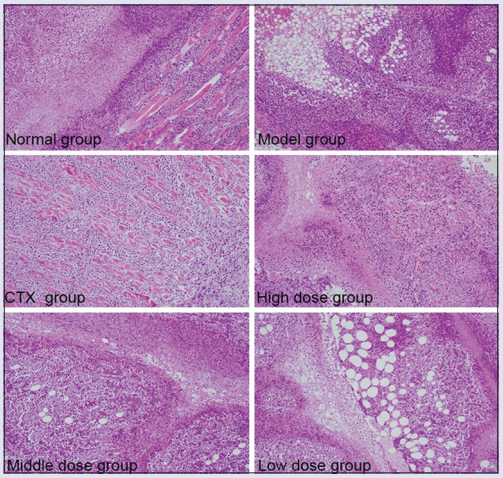

Figure 2: Results of histological observation in all groups (H_{22} solid tumor mice, H and E, ×200)

Table 2: Effect of sample on immune organs in H_{22} immunodeficiency mice

Group	Dose/g/kg	Liver index	Thymus index	Spleen index
Normal	-	9.47±0.31	0.36±0.01	1.15±0.05
Model	-	3.65±0.09$^{\Delta\Delta}$	0.16±0.01$^{\Delta\Delta}$	0.31±0.01$^{\Delta\Delta}$
APS	0.02	5.41±0.10	0.32±0.01	0.53±0.02
High	1.28	5.52±0.22**	0.29±0.01**	0.41±0.01**
Middle	0.64	5.14±0.22**	0.21±0.01**	0.43±0.02**
Low	0.32	4.27±0.06**	0.19±0.01**	0.38±0.01**

$^{\Delta\Delta}P<0.01$ versus normal; **$P<0.01$ versus model. APS: Astragalus polysaccharide

Effects of the samples on the survival time and the organ indexes of the H_{22} ascitic tumor mice

The experimental results are shown in Table 3 that compared with the model group; the survival time of the mice in the drug-administered group is greatly increased ($P < 0.05$); the survival time of the mice in the positive group of CTX is much lower than those in the model group ($P < 0.05$), which indicates that the positive chemotherapeutic drug has some side effects; and the high-dose group of the n-butyl

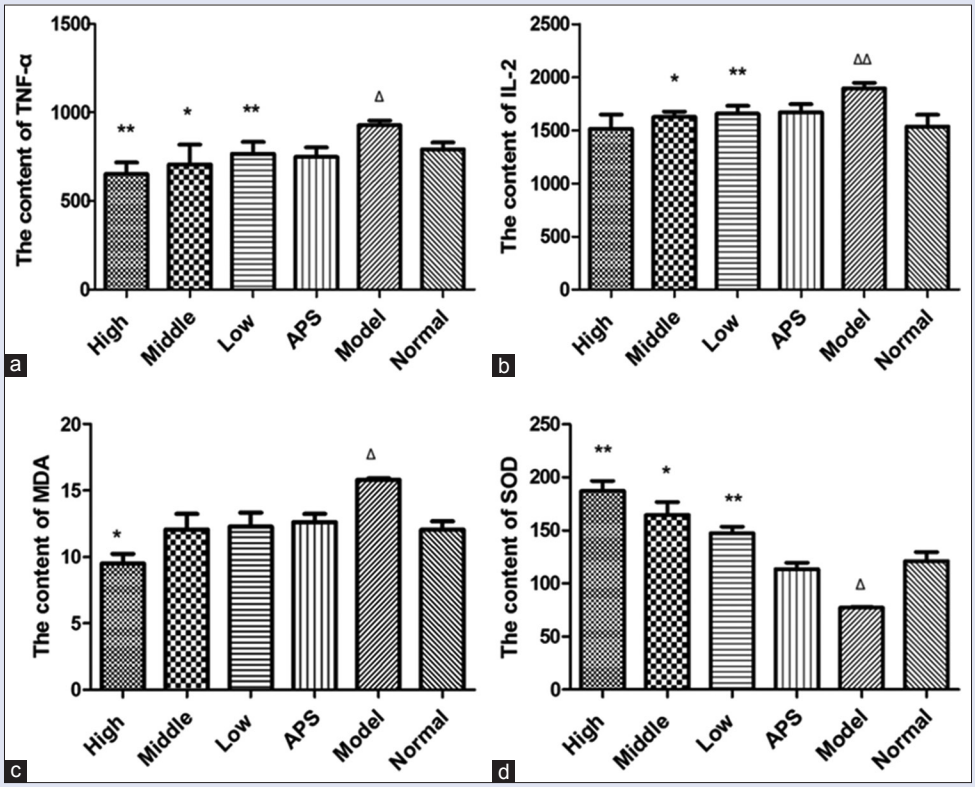

Figure 3: Effect of sample on serum tumor necrosis factor-α, interleukin-2, malondialdehyde, and superoxide dismutase in H_{22} immunodeficiency mice. Data denoted were mean ± standard deviation (n = 10)

Table 3: Effect of sample on survival time and life extension rate in H_{22} ascites tumor mice

Group	Dose/g/kg	MST/day	Life extension rate/%
Normal	-	29.16±1.35	-
Model	-	9.534±0.23*	-
CTX	0.02	23.57±0.34*	
High	1.28	28.46±0.22**	20.75
Middle	0.64	25.19±0.14**	6.87
Low	0.32	21.52±0.29*	−8.69

***P*<0.01, versus model, **P*<0.05, versus model; MST: Median survival time; CTX: Cyclophosphamide

alcohol extract of *Z. avicennae* prolongs the survival time of the mice with the liver cancer H_{22} ascitic tumor.

Compared with those in the normal control group, the body weights of the mice in the model group, CTX group, drug-administered groups of high-dose, medium-dose, and low-dose increased gradually with the passing of time. The abdomen of the mice gradually swell, but compared with those in the model group, the body mass of the mice in CTX group and all the different doses groups increased slowly. From the 8th day, the mice gradually became low spirited, slow response, loss of appetite, and later their abdomen hunched very high. Dissect the bodies after their death, and it can be seen that the mice's abdominal ascitic fluid became bright red, and the organs in the body degenerated greatly.

It is pointed out in the literature that inflammation exists in all the growing stages of tumor. During the process of the growth of tumor, the persistent inflammation can stimulate the strong antitumor function of the inflammatory cells and thus inhibit the growth of the tumor cells.[11-14] TNF-α and IL-2 are the important cell factors and the main inflammatory media, and they are the powerful link between inflammation and tumor. In the inflammatory reaction, TNF-α can not only directly take part in the inflammatory reaction but also can induce the generation and release of the other cell factors and hence lead to the spreading of inflammation and do harm to the organs.[15] IL-2 has multifunctions of immune modulation which can strengthen the body's immune-modulating ability through enhancing the cytotoxicity and proliferation ability of NK cells, T–lymphocytes, and B-lymphocytes.[16-19] In this experiment, the contents of the TNF-α and IL-2 of mice serum in the H_{22} liver cancer model group are obviously higher than those of the blank control group, whereas the contents of TNF-α and IL-2 in the serum of the drug-administered group are obviously lower. The results show that the n-butyl alcohol extract of *Z. avicennae* has clear removing function for the TNF-α in the mice bodies and inhibits the harmful effects resulting from its inflammatory reaction on the visceral organs. This can be regarded as the antitumor function by stimulating its inflammatory cells through strengthening the body's inflammatory reaction.

The generation of free radicals caused by oxidative stress and the reduction of the target cells and the antioxidation level are seen as the important accelerating factors for the occurrence of tumors.[20-23] The contents of MDA and SOD in the blood serum can reflect the peroxidation speed and degree in physical bodies.[9,24] The n-butyl alcohol extract of *Z. avicennae* can increase the SOD content of the mice with immunodeficiency and reduce the content of MDA, which indicate that it can fight against the oxidation reaction in the bodies of the immune-deficient mice induced by CTX, inhibit oxidative stress reaction in the bodies, increase the antioxidation ability in the bodies cell, and reduce the generation and growth of tumors through fighting against the cell proliferation.

CONCLUSION

The experiment studied the effects of n-butyl alcohol extract of *Z. avicennae* on the organ indexes and the blood serum indexes of H_{22} model mice of solid tumor, the survival time of ascitic tumor mice, immune organ indexes, and the blood serum indexes of the immunodeficiency mice. This indicates that the n-butyl alcohol extract of *Z. avicennae* has the effects of tumor inhibiting, prolonging the survival time of the mice with ascitic tumor and improving the index of the immune organs of the mice with immunodeficiency. The n-butyl alcohol extract of *Z. avicennae* also has definite effects of antitumor and immunomodulation, and its mechanism may be that it can slow down the generation and growing process of the tumor cells, strengthen the body immune ability, increase the antioxidation ability of the cells through regulating the cell factors TNF-α and IL-2, and delay the aging of the cells to acquire the antitumor effect. The n-butyl alcohol extract of *Z. avicennae*, as a very excellent reagent of antitumor and immunomodulation, has the characteristics of relative safety and very little side effects. It can overcome the untoward reaction of the common chemotherapeutic medicines, such as the obvious weight loss, the inhibition of the hematopoietic system and immune system, and the damage of liver and renal function. This is really a drug resource worthy of further research and development.

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Conflicts of interest

There are no conflicts of interest.

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