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Study on Antibacterial Effect of the Neem Extracts

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Abstract: In the breeding industry, in order to control the intestinal diseases of livestock and poultry and improve the production efficiency, a large number of antibiotics are added to the feed, which has brought serious harm to the safety of livestock products and human health. Therefore, it has become one of the urgent problems to find new antibiotics substitutes in China's breeding industry. In this study, the leaves of the Neem were collected and the extract was prepared by water extraction. The paper diffusion method and tube double dilution method were used to test the antibacterial effect of the Neem extracts on Escherichia coli and Staphylococcus aureus in vitro. At the same time, mice were used as experimental animals to test the antibacterial effect of the Neem extract in vivo.

1. Introduction

The Neem (*Azadirachta Indica A .Juss.*) is a plant of the genus Azadirachta A.Juss. in the Meliaceae family. It is a tropical or subtropical tree species that loves high temperature, tolerance to drought, full of sunshine, tolerance to poor soil, and rapid growth[1]. The whole body of the Neem is a treasure, and its seeds, branches and leaves and bark have been widely used in medicine, pesticides and daily chemical products for a long time. The azadirachtin extracted from the Neem seeds has good insecticidal activity against plutella xylostella, cabbage caterpillars, locusts and other agricultural, forestry, storage and sanitation pests; in 1968, the United Nations stated that the Neem was "a great impact on local communities in this century." The greatest gift of residents", and is also praised by the US Department of Agriculture as "a tree that can solve global problems"[2-3]. The active ingredients isolated from the Neem and extracts from different parts have various pharmacological activities such as anti-inflammatory, anti-rheumatic, anti-fever, antibacterial and anti-cancer. A lot of research work has been done on synthesis, structural modification and biological activity[4-5]. The Neem has been used in folk medicine for thousands of years. Known as the "country pharmacy"[6].

As a traditional feed additive, antibiotics have been widely used in the breeding industry to prevent or treat livestock diseases. However, the long-term disordered use and abuse of antibiotics, its side effects are more and more obvious, drug residues, drug-resistant bacteria and other problems caused by antibiotic abuse seriously affect the healthy development of animal husbandry, and even

threaten food safety and human health. On July 10, 2019, the Ministry of agriculture and rural areas of the people's Republic of China issued the Announcement No.194. From July 1, 2020, feed production enterprises will stop producing commercial feed containing growth promoting drug feed additives (except Chinese herbal medicine). Since then, the era of feed antibiotics officially entered the countdown[7].

It is one of the important ways to find the active substance of bactericidal and bacteriostatic from the natural ingredients of plants. So far, many plants have been found to contain active substances which have effects on Escherichia coli, Dysentery, Staphylococcus aureus and Bacillus subtilis. The way and mechanism of bactericidal and antibacterial action are different from those of general chemical synthetic bactericides, and have no pollution to the environment safety and no residue[8]. Plant resources are widely distributed and abundant in the living environment, which can be used as an ideal material source of plant active substances. Based on this, the leaves of the Neem were collected in this experiment, and the antibacterial test of its extract against pathogenic Escherichia coli and Staphylococcus aureus was carried out, so as to make full use of the Neem resources in the prevention and control of livestock and poultry bacteria, and provide scientific basis for the further development of plant medicine products.

2. Experimental Materials

2.1 Experimental Equipment

Water proof electric thermostatic incubator, ultra clean worktable, electric thermostatic incubator, electric thermostatic blast drying oven, microscope, evaporating dish, vertical pressure steam sterilizer, electronic balance, blood cell counting board, constant temperature culture oscillator.

2.2 Tested Bacterial Strain

Both Escherichia coli and Staphylococcus aureus came from the school of life sciences, Southwest Forestry University.

2.3 Experimental Drugs

Beef extract, peptone and agar are biochemical reagents; sodium chloride, agar and absolute ethanol are analytical pure.

Azadirachta Indica A .Juss.: The samples were collected from Kunming, Yunnan Province in summer. The collected the Neem leaves were dried in a drying oven and then crushed by a traditional Chinese medicine grinder to obtain the raw powder of the Neem leaves for standby.

2.4 Experimental Mice

Experiment Healthy Five-week-old male KM mice, weighing approximately 25-35g, were purchased from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China, license number: SCXK 2019-0004). Mice were housed in the animal laboratory of Southwest Forestry University according to the guides for the care and use of laboratory animals.

3. Experimental Methods

3.1 Culture Medium Preparation

East extract 5g/L, tryptone 10g/L, NaCl 10g/L, adjust pH value to 7.2-7.4, then steam sterilize at

121℃ for 20min, keep constant temperature in water bath about 50℃. Petri dishes, pipettes, inoculation rings and forceps were sterilized at 121℃ for 30min.

3.2 Preparation of Bacterial Suspension

Under aseptic condition, the second generation of Escherichia coli and Staphylococcus aureus were inoculated in the test tube of inclined medium, cultured at 37℃ for 18-20h. Take out and add 5ml sterile normal saline, shake fully, wash the bacterial spores into the test tube to get the bacterial suspension, and measure the number of live bacteria in the bacterial suspension by plate colony counting method. The freshly prepared bacterial suspension was diluted to 106cfu/mL and 107cfu/mL by 10 fold dilution method for standby.

3.3 Preparation of Drug Sensitive Tablets

Take the qualitative filter paper, use a punch to make the qualitative filter paper into a small round piece of paper with a diameter of 6 mm, sterilize it for 30 minutes under high pressure, and then put it in a 37℃ oven to dry it completely.

1g of the Neem extract was dissolved in 1mL absolute ethanol, filtered by 0.22μm sterile filter membrane, stored in 4mL centrifuge tube, and stored in 4℃ refrigerator for standby. Put Azadirachta azedarach solution in the sterilized and dried culture dish, put the dried filter paper discs into the solution one by one, and soak them in the refrigerator at 4℃ for 1-2 hours. At the same time, pay attention to turning the discs to fully absorb the solution. After soaking, put the drug sensitive tablets into another dry culture dish and dry them in the oven at 37℃ (each drug sensitive tablet contains 10μg Azadirachta azedarach extracts)[9].

3.4 Extraction of the Neem Extract

Weigh 100g powder of the Neem leaves, add 80℃ distilled water according to the ratio of material to liquid of 1:10, reflux for 1h, three times in total, filter and take the filtrate, reduce pressure and concentrate at 80℃ on rotary evaporator, and obtain the Neem extract after concentration and drying.

3.5 Antibacterial Effect of the Neem Extract

3.5.1 Determination of Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) of the Neem extract against Escherichia coli and Staphylococcus aureus in vitro was determined by tube double dilution method. Take 6 sterilized test tubes, add 2mL of culture medium respectively, add 2ml of the Neem extract to the first tube, mix well, then draw 2mL and add it to the second tube, and so on, until the fourth tube, discard 2mL of extract from the fourth tube, make it into a concentration gradient of 1:2, 1:4, 1:8, 1:16, then add 107cfu/ml of bacterial solution 50μL to the first tube to the fourth tube, compare the culture medium with the fifth tube and the sixth tube, and place 6 test tubes The cells were cultured at 37℃ for 24 hours. Then the growth of bacteria was observed, and the minimum dilution concentration of aseptic growth was the minimum inhibitory concentration (MIC)[10].

3.5.2 Determination of Minimum Bactericidal Concentration (MBC)

Take 0ml and 1 ml of culture from each tube and inoculate them on the common nutrient agar plate. Then put them in the constant temperature incubator at 37℃ for 24 hours and count the colonies.

The minimum bactericidal concentration (MBC) of the drug was the lowest concentration without bacterial growth[11].

3.5.3 Bacterial Inoculation

100μL of 106cfu/mL test bacteria suspension was sucked to nutrient agar plate by pipette gun, and evenly smeared on the plate surface for 3 times, then smeared around the edge of the plate, covered the culture dish, dried at room temperature for 5 minutes, and then the drug sensitive tablets were gently pasted on the surface of the culture dish with sterile tweezers, and each plate was pasted with 3 pieces. At the same time, the control plate with sterile saline filter paper was set.

3.5.4 Bacterial Culture and Determination of Inhibition Zone

Put the culture dish in 37℃ incubator for 18h, observe and measure the diameter of inhibition ring with vernier caliper, repeat the test for 3 times, and take the average value as the result[12].

3.6 Determination of Minimum Lethal Dose of Escherichia Coli and Staphylococcus Aureus in Mice

114 mice were divided into 19 groups with 6 mice in each group. Mice in each group were intraperitoneally injected with 107cfu/ml fresh culture solution of Escherichia coli and Staphylococcus aureus, 0.1ml, 0.2ml, 0.3ml, 0.4ml and 0.5ml, respectively. The mice were isolated and reared. The mice were continuously observed for 72h, and the death rate was recorded. The results showed that the concentration that made the death rate of mice reach 90% was the minimum lethal dose of Escherichia coli and Staphylococcus aureus to mice[13].

3.7 The Antibacterial Effect of Mice in Vivo

The extract of 1g/mL Azadirachta indica was diluted to 30mL of 0.5g/mL, 0.25g/mL, 0.125g/mL and 0.0625g/mL with distilled water, respectively, and stored at 4℃. Forty two mice were randomly divided into 7 groups with 6 mice in each group: blank control group, infection control group, experimental prevention group and experimental treatment group. The experimental prevention group was given different doses (0.3mL/10g) by gavage according to the germicidal efficacy of various extracts, three times a day for 3 consecutive days. In the morning of the fourth day, each mouse was intraperitoneally injected with the minimum lethal dose of bacterial solution to cause infection, and then continued to give the drug by gavage, three times a day, for three consecutive days; one hour after the injection of the minimum lethal dose of bacterial solution at the same time in the experimental treatment group and the prevention group, different doses were selected by gavage according to the germicidal efficacy of various extracts, for treatment, three times a day, for three consecutive days; the mice in the infection control group were injected with the minimum lethal dose of bacterial solution The control group was injected with the same dose of normal saline. The number of dead mice in each group was counted on the 7th day after infection[14].

4. Results

4.1 Determination of Minimal Inhibitory Concentration (MIC)

The smaller the minimum inhibitory concentration (MIC), the stronger the antibacterial activity. Different concentrations of the Neem extract were used to determine the minimum inhibitory concentration (MIC) of Escherichia coli (2000μg/mL) and Staphylococcus aureus

(1000-2000μg/mL). The MIC of the Neem extract to Staphylococcus aureus was lower than that of Escherichia coli.This indicates that Azadirachtin has stronger inhibitory effect on Gram-positive bacteria than on Gram-negative bacteria. The results of MIC determination of azadirachtin extract are shown in Table 1.

Table 1: Minimum inhibitory concentration of the Neem extract (μg/mL).

Number	Concentration (μg/mL)	Escherichia coli	Staphylococcus aureus
1	1000	500	550
2	2000	650	625
3	3000	1000	1010
4	4000	1500	1480
5	5000	1800	1850

4.2 Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration is generally higher than or equal to the minimum inhibitory concentration. The minimum bactericidal concentration of the Neem extract is shown in Table 2.

Table 2: minimum bactericidal concentration of the Neem extract (μg/mL).

Number	Concentration (μg/mL)	Escherichia coli	Staphylococcus aureus
1	1000	500	520
2	2000	1200	1200
3	3000	2000	2010
4	4000	2500	2500
5	5000	3000	3000

4.3 Antibacterial Effect of the Neem Extract

It can be seen from the table that the higher the concentration of the Neem extract is, the larger the diameter of inhibition zone is. There is little difference in the inhibition effect of the Neem extract on Escherichia coli and Staphylococcus aureus. The determination results of inhibition zone size of the Neem extract are as follows (Table 3, Figure 1, Figure 2).

Table 3: Antibacterial activity of the Neem extract.

Number	Concentration (μg/mL)	Escherichia coli (mm)	Staphylococcus aureus (mm)
Penicillin	1000	9.52±0.22	9.47±0.24
The Neem extract	1000	6.26±0.17	6.34±0.23
	2000	6.51±0.15	6.67±0.17
	3000	6.85±0.25	6.79±0.18

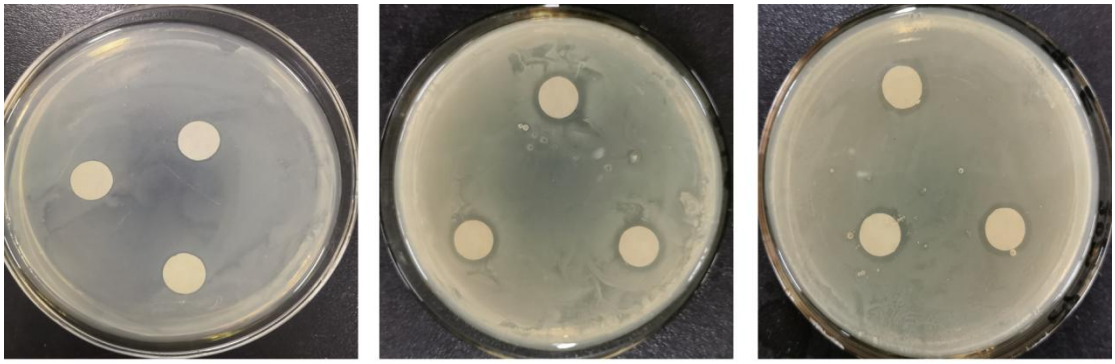

Figure1: Antibacterial activity of the Neem extract against Escherichia coli.

Figure2: Antibacterial activity of the Neem extract against Staphylococcus aureus.

4.4 Minimum Lethal Dose of Bacterial Solution

The results showed that the concentration of Escherichia coli and Staphylococcus aureus was 0.29×10^{7}cfu/mL and 0.35×10^{7}cfu/mL, respectively, which made the mortality rate of mice more than 90%.

4.5 Antibacterial Activity in Mice

It can be seen from table 4 that the antibacterial effect of the Neem extract in the prevention group is better than that in the treatment group. The survival rate of mice could reach more than 80% after 3 days of continuous administration before and after E.coli infection. The survival rate of mice in the treatment group was more than 80% after continuous administration for 3 days.

Table 4: preventive and therapeutic test results of the Neem extract against Escherichia coli.

Group	Drug concentration / (g/mL)	Dose (g/kg)	Number of mice	Number of deaths	Survival number
Control	—	—	6	0	6
E.coli infection	Normal saline	0.3mL/10g	6	6	0
Staphylococcus aureus infection	Normal saline	0.3mL/10g	6	6	0
Escherichia coli prevention	0.065	1.88	6	0	6
	0.125	3.75	6	0	6
	0.25	7.5	6	1	5
	0.5	15	6	0	6
E.coli treatment	0.065	1.88	6	0	6
	0.125	3.75	6	0	6
	0.25	7.5	6	0	6
	0.5	15	6	2	4
Staphylococcus aureus prevention	0.0625	1.88	6	0	6
	0.125	3.75	6	1	5
	0.25	7.5	6	0	6
	0.5	15	6	0	6
Staphylococcus aureus treatment	0.0625	1.88	6	0	6
	0.125	3.75	6	0	6
	0.25	7.5	6	2	4
	0.5	15	6	0	6

5. Discuss

The use of antibiotics has played a great role in promoting large-scale breeding, mainly by inhibiting or killing pathogenic microorganisms, reducing the occurrence of animal digestive tract diseases, improving the digestibility of animal nutrition, so as to promote the growth of animals. However, the long-term disordered use and abuse of antibiotics, its side effects are more and more obvious, drug residues, drug-resistant bacteria and other problems caused by antibiotic abuse seriously affect the healthy development of animal husbandry, and even threaten food safety and human health[15]. With the transformation of animal husbandry, the breeding industry is facing strict environmental requirements. The restriction or prohibition of antibiotics in feed has become an inevitable trend. In particular, EU member countries and the United States and other countries have already explicitly banned the use of antibiotics in animal breeding, so the implementation of non-antibiotic breeding is also an effective means to achieve healthy breeding[16]. Therefore, looking for new antibiotic substitutes, improving animal immunity, increasing animal disease resistance and reducing the harm of residual antibiotics to human health has become one of the urgent problems to be solved in China's breeding industry[17].

It can be seen from the experimental results that the Neem extract has inhibitory effect on Escherichia coli and Staphylococcus aureus, and the difference is not significant; the higher the concentration of the Neem extract, the stronger the antibacterial effect. Due to the influence of

digestive fluid, enzymes, hormones and other chemical components in the body, there are some differences in the antibacterial effect. At the same time, natural plants contain bioactive substances, which have multiple functions of prevention, treatment and nutrition. Therefore, the antibacterial effect of plant drugs is very different in vivo and in vitro. Plant medicine can achieve the purpose of disease prevention and treatment by mobilizing animal immunity. In vivo antibacterial test of drugs, due to the participation of organic factors, is the result of the interaction of drugs, pathogenic bacteria and the body, which can better reflect the anti-infection effect of drugs[18]. Therefore, this experiment should be carried out in vivo. The results showed that the experimental mice had a good effect of antibacterial and disease prevention by continuous administration of drugs for 3 days before E.coli infection and then for 3 days after infection. The survival rate of Azadirachta indica extract was over 80% when the dosage of Azadirachta indica extract was 1.88-7.5g/kg. This study provides a theoretical basis for veterinary clinical use of the Neem in the prevention and treatment of some infectious diseases. At the same time, the Neem can be used as a substitute of antibiotics in livestock and poultry production to prevent the occurrence of infectious diseases, but the specific practical application needs further research and discussion.

6. Conclusion

The extract of the Neem leaves showed obvious inhibitory effect on Escherichia coli and Staphylococcus aureus with the increase of extract concentration in vitro; the antibacterial test of Neem extract in mice showed obvious antibacterial and disease prevention effect, suggesting that Neem can achieve the effect of antibacterial infection by regulating the immune function of the body. These results suggest that Azadirachta indica can be used as a substitute for antibiotics to prevent diseases in livestock and poultry production.

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