NANO PROPOLIS COMPOSITE ADJUVANT FOR POULTRY AND ITS APPLICATION

Field of the Invention

The present invention belongs to the field of veterinary medicine and specifically relates to a nano propolis composite adjuvant for poultry and its application.

Background to the Invention

Vaccination is one of the effective measures to prevent animal diseases. Its purpose is to induce immune responses against specific pathogenic microorganisms in the body through immunization with vaccines, including attenuated live vaccines, inactivated vaccines, and subunit vaccines, in order to prevent specific diseases. Attenuated live vaccines can stimulate the body to produce comprehensive systemic immunity and local immunity, with long-lasting immunity. However, this type of vaccine carries the risk of scattered virus and resurgence of virulence. Inactivated vaccines are prepared by inactivating pathogenic microorganisms through physical and chemical methods. These vaccines induce weak immune responses in the body, require large doses of immunity, and have high production costs. Therefore, immune adjuvants are needed to enhance their immune efficacy. Especially in recent years, with the continuous development and emergence of weakly immunogenic vaccines such as genetic engineering subunit vaccines, recombinant vaccines, and synthetic peptide vaccines, the high purity of their antigens has made the use of immune adjuvants even more necessary. However, the existing immune adjuvants are no longer able to meet the rapid development needs of new vaccines, and the development of new immune adjuvants with high immune activity and low side effects is particularly urgent.

Propolis adjuvant refers to a propolis solution obtained by ethanol extraction of propolis. The propolis solution contains various immune active substances such as flavonoids, phenolic acids, and their esters. It is a natural and efficient immune enhancer that can increase phagocytic cell activity, promote the increase of white blood cell count and the production of specific antibodies, and enhance the immune function of the body. Most of the currently developed propolis adjuvants are ethanol extracts of propolis, which have low antibody titers when used with vaccines prepared from enveloped viruses. This may be due to a component in the propolis adjuvant that destroys the outer membrane antigen of enveloped viruses; In addition, the vaccine prepared by mixing propolis ethanol extract as an adjuvant with inactivated bacterial antigen produces precipitation during storage, which accumulates at the bottom of the vaccine bottle and is not easily dispersed. When used, it needs to be shaken vigorously, which brings inconvenience to use.

Statement of Invention

In order to solve the above problems, the present invention provides a nano propolis composite adjuvant for poultry and its application. The nano propolis composite adjuvant for poultry provided by the present invention has the characteristics of non-toxic side effects, extremely low residual levels, and good immune effects, and can be used as an adjuvant for inactivated poultry vaccines and a diluent for live vaccines; The nano propolis composite adjuvant for poultry can be used to prepare vaccines against enveloped viruses such as Newcastle disease virus and avian influenza virus. The prepared vaccines have good stability and excellent characteristics of inducing both cellular and humoral immunity.

The first aspect of the present invention provides a nano propolis composite adjuvant for poultry, the nano propolis composite adjuvant for poultry is composed of the following volume percentages of components: 10% -30% propolis ethanol extract, 30% -45% immune enhancer, and 30% -50% surfactant; the dry matter content of propolis in the ethanol extract of propolis is 50mg/mL~70mg/mL.

Furthermore, the immune enhancer is composed of a mixture of immune enhancer A and immune enhancer B; The mixed volume ratio of immune enhancer A and immune enhancer B is 5-8:1.

Furthermore, the immune enhancer A is white oil or vitamin D, the immune enhancer B is a mixture of grass mycobacterial extract and dimethyl bis octadecylammonium bromide, or the immune enhancer B is a saponin.

Furthermore, the volume ratio of the mixture of the grass mycobacterial extract and dimethyl bis octadecylammonium bromide is 100:1~30.

Furthermore, the extract of grass mycobacteria is obtained by lysing inactivated grass mycobacteria cells under 800-1000 bar conditions and centrifuging at 11000rpm-13000 rpm for 25-35 minutes.

Furthermore, the cracking process needs to be repeated twice.

Furthermore, the saponin is soapberry saponin A or soapberry saponin QS-21.

Furthermore, the surfactants are polyglycerol fatty acid esters, Span-80, and polyoxyethylene hydrogenated castor oil.

Furthermore, the volume ratio of polyglycerol fatty acid ester, Span-80, and polyoxyethylene hydrogenated castor oil is 7~9:3~5:2.

The second aspect of the present invention provides an application of the above-mentioned nano propolis composite adjuvant for poultry in the preparation of inactivated poultry vaccines, subunit vaccines, or live vaccine dilutions.

The third aspect of the present invention provides a nano propolis composite adjuvant vaccine for poultry, comprising the above-mentioned nano propolis composite adjuvant for poultry.

Furthermore, the vaccine further comprises inactivated antigens or subunit antigens of pathogenic microorganisms infecting poultry.

Furthermore, the antigen is Newcastle disease virus, avian influenza virus, or Haemophilus parahaemolyticus.

Furthermore, the volume ratio of the antigen to the avian nano propolis composite adjuvant is 2-4:1.

Preferably, the volume ratio of the antigen to the avian nano propolis composite adjuvant is 3:1.

Overall, compared with existing technologies, the present invention has the following beneficial effects:

The present invention provides a nano propolis composite adjuvant for poultry, consisting of propolis ethanol extract, immune enhancer, and surfactant. The propolis ethanol extract is rich in bioactive ingredients such as flavonoids and terpenes, which can effectively regulate the poultry immune system, stimulate immune cell proliferation, and enhance specific and non-specific immune functions; The addition of immune enhancers further strengthens the immune response, synergizes with propolis extract, significantly enhances the resistance of poultry to pathogens, and improves the immune effect of vaccines; The surfactant added in the nano propolis compound adjuvant for poultry can reduce the surface tension of the nano propolis compound adjuvant for poultry, form a stable nano lotion structure, improve the stability of vaccine preservation, and the nano preparation significantly increases the specific surface area, making the combination of adjuvant and immune cells more efficient, and improving the bioavailability.

The specific non-ionic surfactant polyoxyethylene hydrogenated castor oil, Span-80 and polyglycerol fatty acid ester compound system is used to form a stable nanometer lotion structure, effectively preventing the precipitation and aggregation after the propolis extract is mixed with the inactivated bacterial antigen, and ensuring that the vaccine has good stability in the storage process without precipitation.

The nano propolis composite adjuvant for poultry can synergistically activate the multi-channel response of the poultry immune system with propolis ethanol extract by adding vitamin D, white oil, dimethyl bis (octadecylammonium bromide), saponins or grass mycobacteria extract, significantly improving the antibody potency against enveloped viruses (such as avian influenza virus and Newcastle disease virus), avoiding the

destruction of the virus outer membrane by propolis components, and enhancing the immune effect of vaccines.

The nano propolis composite adjuvant for poultry provided by the present invention has the characteristics of non-toxic side effects, extremely low residual amount, good immune effect, and good safety. It can be used as an adjuvant for inactivated poultry vaccines and a diluent for live vaccines.

Brief Description of the Drawings

FIG. 1 shows the appearance of propolis ethanol extract. The two bottles of samples from left to right are distilled water and propolis ethanol extract, respectively.

FIG. 2 shows the absorption spectrum of propolis ethanol extract.

FIG. 3 shows the measurement results of particle size and particle size distribution of the nano propolis composite adjuvant prepared in Embodiment 1.

FIG. 4 shows the measurement results of particle size and particle size distribution of the nano propolis composite adjuvant prepared in Embodiment 2.

FIG. 5 shows the measurement results of particle size and particle size distribution of the nano propolis composite adjuvant prepared in Embodiment 3.

FIG. 6 shows the stability test results of the avian nano propolis composite adjuvant vaccine. Figures A, B, C, D, E, and F show the stability test results of the avian nano propolis composite adjuvant vaccines prepared in Embodiments 4, 5, 6, Comparative Embodiments 1, 2, and 3, respectively.

Detailed Description

In order to enable technical personnel in this field to better understand the technical solution of the present invention and implement it, the present invention will be further explained in conjunction with specific embodiments and accompanying drawings.

In some embodiments of the present invention, propolis is purchased from Shandong Ludu Biotechnology Co., Ltd., Span 80, polyglycerol fatty acid esters, polyoxyethylene hydrogenated castor oil, and Tween 80 are all purchased from the British company, polyoxyethylene hydrogenated castor oil is purchased from BASF in Germany, vitamin D is purchased from Shanxi Jinyang Pharmaceutical Accessories Co., Ltd., white oil is purchased from Mobil Corporation, soapberry saponin A, soapberry saponin QS-21, and dimethyl bis octadecylammonium bromide (DDA) are purchased from Sigma aldrich, and other chemical reagents are analytical grade and purchased from China National

Pharmaceutical Group.

In the description of the present invention, unless otherwise specified, all reagents used are commercially available and all methods used are conventional techniques in this field.

Most of the currently developed propolis adjuvants are directly ethanol extracts of propolis, which have low antibody titers when used with vaccines prepared from enveloped viruses. This may be due to a component in the propolis adjuvant that destroys the outer membrane antigen of enveloped viruses; In addition, the vaccine prepared by mixing propolis ethanol extract as an adjuvant with inactivated bacterial antigen produces precipitation during storage, which accumulates at the bottom of the vaccine bottle and is not easily dispersed. When used, it needs to be shaken vigorously, which brings inconvenience to use.

The present invention provides a nano propolis composite adjuvant for poultry and its application. The present invention provides a nano propolis composite adjuvant for poultry, which is obtained by compounding ethanol extract of propolis, immune enhancer, and surfactant. The characteristics of the nano propolis composite adjuvant for poultry are characterized, and the safety of the nano propolis composite adjuvant for poultry is tested; The present invention further utilizes the nano propolis composite adjuvant for poultry to prepare a nano propolis composite adjuvant vaccine for poultry, and tests the characteristics, safety, and antibody potency after immunization of the vaccine; It was found that the nano propolis composite adjuvant for poultry of the present invention can be used to prepare vaccines against enveloped viruses such as Newcastle disease virus and avian influenza virus. The prepared vaccines not only have good stability, but also significantly improve the antibody potency against enveloped viruses.

Embodiment 1: A preparation method of nano propolis composite adjuvant for poultry , comprising the following preparation steps:

S1、 Preparation of propolis ethanol extract

Prepare ethanol extract of propolis according to GB/T 24283-2018 "Propolis". The specific preparation steps are as follows: freeze crude propolis at -15 ℃ for 36 hours, then grind and screen it with a freeze grinder. Add 95% v/v ethanol to the screened crude propolis, with a weight ratio of 1:4. Extract at 37 ℃ for 60 hours, cool, filter, centrifuge to remove sediment, and then ultrafiltration and concentration to obtain pure ethanol extract of propolis.

The present invention tests the properties, pH value, relative density, oxidation time, dry matter content, and total flavonoid content of propolis ethanol extract.

Testing of propolis ethanol extract:

(1)Characteristic testing

As shown in Figure 1, the prepared propolis ethanol extract was placed in a colorless and transparent container. Under natural light conditions, it was observed that the propolis ethanol extract was a brown or dark brown glossy transparent liquid with the unique aroma of propolis.

As shown in Figure 2, the propolis ethanol extract solution was scanned in the wavelength range of 300-600nm, and the results showed that the propolis ethanol extract solution had a maximum absorption peak at 490nm.

Take an appropriate amount of this product and use the light scattering method to detect the propolis ethanol extract sample according to the detection method in General Rule 0982 of the Pharmacopoeia of the People's Republic of China (2020 Edition) (Part IV). The particle size should be less than 100nm.

After testing, the particle size of three batches of samples (propolis ethanol extract Lot.2021001, propolis ethanol extract Lot.2021002, propolis ethanol extract Lot.20210033) is below 100 nanometers, which meets the standard.

(2)PH value determination

According to Appendix 3101 of the Pharmacopoeia of the People's Republic of China (2020 Edition) (Part III), the pH value of propolis ethanol extract samples should be measured within the range of 6.0-7.0.

As shown in Table 1, the pH values of the three batches of samples are within the range of 6.2~6.6, which meets the standard.

Table 1 pH values of propolis ethanol extract

Sample	Production Date	pH value
Propolis ethanol extract Lot.2021001	November 2021	6.2
Propolis ethanol extract Lot.2021002	December 2021	6.5
Propolis ethanol extract Lot.20210033	November 2021	6.6

(3) Relative density measurement

According to Appendix 0601 of the Pharmacopoeia of the People's Republic of China (2020 Edition), the relative density determination method should be used to determine the ethanol extract sample of propolis, which should be within the range of 0.75~0.95.

As shown in Table 2, the relative density values of the three batches of samples are within the range of 0.80-0.86, which meets the standard.

Table 2 Relative Density of Ethanol Extract of Propolis

Sample	Production Date	Relative density
Propolis ethanol extract Lot.2021001	November 2021	0.80
Propolis ethanol extract Lot.2021002	December 2021	0.85
Propolis ethanol extract Lot.2021003	December 2021	0.86

(4) Oxidation time

The content of reducing substances in propolis is represented by the decay time of potassium permanganate purple red solution. The propolis ethanol extract sample is tested according to the Chinese national standard (GB/T 24283-2018) for propolis oxidation time, and the oxidation time should be ≤ 22 seconds.

As shown in Table 3, the oxidation time of the three batches of samples is within the range of 18-20, which meets the standard.

Table 3 Oxidation time of propolis ethanol extract

Sample	Production Date	Oxidation time/s
Propolis ethanol extract Lot.2021001	November 2021	20
Propolis ethanol extract Lot.2021002	December 2021	18
Propolis ethanol extract Lot.2021003	December 2021	18

(5) Dry matter content of propolis ethanol extract

Take the ethanol extract solution of propolis, weigh the dry matter after ethanol evaporation, obtain the weight of ethanol extract, and calculate its percentage in the sample weight.

As shown in Table 4, the dry matter content of propolis in the three batches of samples is within the range of 55-62mg/mL, which meets the standard.

Table 4 Dry matter content of propolis ethanol extract

Sample	Production Date	Dry matter content （mg/mL）
Propolis ethanol extract Lot.2021001	November 2021	55
Propolis ethanol extract Lot.2021002	December 2021	60

Propolis ethanol extract Lot.2021003	December 2021	62

(6) Determination of total flavonoid content

According to the national standard of the People's Republic of China (GB/T 24283-2018) for propolis, the total flavonoid content in propolis was determined using spectrophotometric colorimetry. The total flavonoid content in every 100 milliliters of propolis should be 20-40mg.

As shown in Table 5, the total flavonoid content in the propolis of the three batches of samples is within the range of 29~35mg/100mL, which meets the standard.

Table 5 Total flavonoid content of propolis ethanol extract

Sample	Production Date	Total flavonoid content （mg/100mL）
Propolis ethanol extract Lot.2021001	November 2021	29
Propolis ethanol extract Lot.2021002	December 2021	35
Propolis ethanol extract Lot.2021003	December 2021	33

S2、 Extraction of Bacillus subtilis extract: Inoculate *Mycolicibacterium phlei* (purchased from Shenzhen Aiyi Biotechnology Co., Ltd.) into a 2% glycerol supplemented nutrient broth medium (purchased from Qingdao Haibo Biotechnology Co., Ltd.), ventilate at 37 °C for 72 hours, and heat at 100 °C for 30 minutes to inactivate the bacterial solution. Centrifuge the inactivated bacterial solution at 8000rpm for 30 minutes to obtain the bacterial cells. Resuspend the bacterial cells in physiological saline solution and wash them twice to remove impurities. Calculate the weight of the bacterial cells and resuspend them in physiological saline solution to make the solution concentration 10%. Use a high-pressure homogenizer (model SCIENTZ-150A, Ningbo Xinzhi Biotechnology Co., Ltd.) to crush the bacterial cells under 900 bar conditions, repeat twice. Centrifuge the crushed bacterial cells at 12000rpm for 30 minutes, and collect the supernatant as the extract of grass mycobacteria.

S3、 Preparation of nano propolis composite adjuvant for poultry use

Mix 30mL of white oil, 20mL of Span 80, 10mL of polyglycerol fatty acid ester, and 5mL of polyoxyethylene hydrogenated castor oil evenly. Then add 20mL of propolis ethanol extract prepared in step S1 and mix well. Add 0.1g of soapberry saponin A, and finally make up to 100mL with injection water. Stir at 800rpm for 20 minutes, mix thoroughly and emulsify, filter and sterilize with a 0.22 μ m filter to obtain a nano propolis composite adjuvant for poultry.

Embodiment 2: A preparation method of a nano propolis composite adjuvant for poultry, comprising the following preparation steps:

Mix 30mL of vitamin D, 20mL of Span 80, 10mL of polyglycerol fatty acid ester, and 5mL of polyoxyethylene hydrogenated castor oil evenly. Then add 20mL of propolis ethanol extract prepared in step S1 of Example 1 and mix well. Add 0.1g of soapberry saponin QS-21, and finally make up to 100mL with injection water. Stir at 800rpm for 30 minutes, mix thoroughly and emulsify, filter and sterilize with a 0.22 μm filter to obtain a nano propolis composite adjuvant for poultry.

Embodiment 3: A preparation method of a nano propolis composite adjuvant for poultry, comprising the following preparation steps:

Mix 30mL of vitamin D, 20mL of Span 80, 10mL of polyglycerol fatty acid ester, and 5mL of polyoxyethylene hydrogenated castor oil evenly. Then add 20mL of propolis ethanol extract prepared in S1 of Embodiment 1 and mix well. Then add 0.1g of DDA and 5mL of grass branching bacteria extract prepared in S2 of Embodiment 1. Finally, make up to 100mL with injection water, stir at 800rpm for 30 minutes, mix thoroughly and emulsify, filter and sterilize with a 0.22 μ m filter to obtain a nano propolis composite adjuvant for poultry.

The present invention tested the particle size, safety and other indicators of the nano propolis composite adjuvant for poultry prepared in Embodiments 1 to 3. The test results are as follows.

(1) Determination of particle size and particle size distribution of adjuvants

According to the detection method provided in General Rule 0982 of the Pharmacopoeia of the People's Republic of China (2020 Edition) (Part IV), the particle size and particle size distribution of the nano propolis composite adjuvant for poultry prepared in Embodiments 1 to 3 of the present invention.

The detection results are shown in Figures 3, 4, and 5. The particle sizes of the avian nano propolis composite adjuvant prepared in Embodiments 1 to 3 are 145-221nm, 145-255nm, and 126-294nm, respectively, all within the range of 100-300nm, indicating that the avian nano propolis composite adjuvant prepared in the present invention is a nano grade product.

(2) Aseptic testing

According to Appendix 3306 of the Pharmacopoeia of the People's Republic of China (2020 Edition) (Part III), the sterile test method for semi-finished products was used to test the sterility of the nano propolis composite adjuvant for poultry prepared in Embodiments

1 to 3.

As shown in Table 6, there was no bacterial growth observed.

Table 6 Aseptic test of nano propolis composite adjuvant

Sample	Production Date	37℃		25℃		
		TG	GA	TG	GA	TSB
Embodiment 1	November 2021	-	-	-	-	-
Embodiment 2	December 2021	-	-	-	-	-
Embodiment 3	December 2021	-	-	-	-	-

Note: TG represents thioglycolate fluid medium, GA represents casein agar medium, TSB represents pancreatic casein soy peptone liquid medium, and - represents sterile growth.

(3) Safety inspection

Conduct safety tests on the nano propolis composite adjuvant for poultry prepared in Embodiments 1 to 3.

Mouse experiment: 10 female mice weighing 18-22 g were randomly divided into a blank control group and an experimental group. Each mouse in the blank control group was injected with 0.5 mL of sterile physiological saline through muscle injection. In the experimental group, each mouse was injected with 0.5 mL of the avian nano propolis composite adjuvant solution prepared in Examples 1 to 3 through muscle injection, and observed for 7 days. Weigh the animals separately before and after the experiment.

Guinea pig experiment: Four guinea pigs weighing 250-350 g were randomly divided into a blank control group and an experimental group. The blank control group injected 1.0 mL of sterile physiological saline into each guinea pig muscle. The experimental group injected 1.0 mL of the avian nano propolis composite adjuvant solution prepared in Embodiments 1 to 3 into each guinea pig muscle, and observed for 7 days.

The experimental results are shown in Tables 7 to 10. During the observation period after injection of the nano propolis composite adjuvant solution for poultry, all mice and guinea pigs survived without any abnormal reactions, and there was no swelling, necrosis, or abscess in the injection site. At the end of the observation period, the weight of each mouse and guinea pig increased significantly, and there was no significant difference compared to the blank control group, indicating that the adjuvant is safe in mice and guinea pigs.

Table 7 Safety Test of Mice

Group	Mouse body weight (g)								
	-1d	0d	1d	2d	3d	4d	5d	6d	7d
Embodiment 1	20.2	21.3	21.3	22.3	23,0	23.3	24.1	24.2	24.8
Blank comparative group	20.5	21.5	22.1	22.3	23.0	23.3	24.3	24.4	24.4

Table 8 Safety Test of Guinea Pigs

Group	Guinea pig weight (g)								
	-1d	0d	1d	2d	3d	4d	5d	6d	7d
Embodiment 1	342.1	347.9	343.8	344.8	353.7	356.1	361.9	371.5	373.4
Blank comparative group	344.4	345.4	350.9	351.4	358.2	363.6	369.8	369.8	376.5

Table 9 Safety Test of Mice

Group	Mouse body weight (g)								
	-1d	0d	1d	2d	3d	4d	5d	6d	7d
Embodiment 2	21.0	21.5	21.5	22.0	22.7	23.4	23.5	24.2	24.6
Embodiment 3	21.5	22.3	22.2	22.7	23.6	24.1	24.1	24.5	25.1
Blank comparative group	20.4	21.2	21.6	22.5	22.8	23.4	24.1	23.9	24.1

Table 10 Safety Test of Guinea Pigs

Group	Guinea pig weight (g)								
	-1d	0d	1d	2d	3d	4d	5d	6d	7d
Embodiment 2	334.3	331.2	326.6	332.4	340.7	349.4	357.1	360.3	366.8
Embodiment 3	340.5	347.0	340.1	346.8	355.9	360.3	369.4	380.1	381.5
Blank comparative group	337.6	340.0	342.1	346.4	347.8	356.4	366.5	367.9	375.9

Embodiment 4: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

S1. Preparation of Newcastle Disease Virus Solution

1. Preparation of Newcastle Disease Virus Antigen Liquid

The production strain is the Newcastle disease virus LaSota strain, which is identified, stored, and supplied by the China Veterinary Medical Products Administration.

(1)Vaccination: Take the production strain, dilute it 103 times with sterile physiological saline, and inoculate 10 day old susceptible chicken embryos into the bladder cavity. Inoculate 0.1mL per embryo, seal the needle hole after inoculation, and continue incubation at 37 ℃.

(2) Incubation and observation: After inoculation of chicken embryos, the embryos should be photographed every 24 hours, and any dead embryos within 48 hours should be discarded. Afterwards, the embryos were photographed every 24 hours, and any dead embryos were removed at any time until 96 hours. Regardless of whether they died or not, all embryos were removed with the chamber facing upwards and cooled at 4 ℃ for 12 hours.

(3) Harvest: Remove the cooled chicken embryos and harvest the chicken embryo liquid (first collect live embryos, then collect dead embryos). The harvested chicken embryo liquid is placed in a sterilized container and sampled to determine the HA potency. HA potency below 1:256 should be discarded. According to the current Chinese Veterinary Pharmacopoeia, aseptic testing should be conducted to ensure sterile growth. The harvested chicken embryo liquid should be stored below -15 ℃ before inactivation.

(4) Concentration: The harvested chicken embryo virus liquid is centrifuged at 2-8 ℃ to remove residues and other particulate impurities from the chicken embryo liquid. Then, it is concentrated using an ultrafiltration concentrator until the HA potency is not less than 1:2048, and the concentration is stopped. At the same time, sterility testing is conducted according to the current Chinese Veterinary Pharmacopoeia, and it should grow aseptically. And a sample was retained for testing the virus value, and the concentrated chicken embryo virus solution was immediately inactivated.

(5) Inactivation: The concentrated chicken embryo virus solution of Newcastle disease is introduced into the inactivation tank, and 10% formaldehyde solution is metered in. The mixer is turned on to stir thoroughly, and the final concentration of formaldehyde is 0.1%. After adding formaldehyde solution, introduce it into another inactivation tank to avoid the virus adhering near the tank mouth from not contacting the inactivation agent. Inactivate at 37 ℃ for 16 hours (start timing when the temperature inside the tank reaches 37 ℃, and start the mixer for continuous stirring), then remove and store at 4 ℃.

2. Newcastle disease virus antigen liquid test

(1) HA potency: Take the Newcastle disease virus antigen solution and determine it according to the current Appendix of the Chinese Veterinary Pharmacopoeia. The HA potency should not be less than 1:1024.

(2) Virus content: Dilute the concentrated chicken embryo virus solution taken out before inactivation in a 10 fold series, taking 10^{-7}, 10^{-8}, and 10^{-9}3 dilutions. Inoculate 5pieces of 10 days old SPF chicken embryos into each bladder cavity, with 0.1mL per embryo, and continue incubation at 37 ℃. Discard the chicken embryos that died 48 hours ago, and take out the chicken embryos that died within 48-120 hours at any time. Harvest the chicken embryo solution and observe for 120 hours. The HA potency should be measured for both dead and live embryos. If the HA potency is not less than 1:128, it is considered infected. Calculate EID50 according to the Reed Muench method. When the virus content is ≥ 108.3 EID50 per 0.1mL, it can be used for seedling production.

(3) Aseptic test: Take inactivated concentrated chicken embryo virus solution and conduct aseptic test according to the current Appendix of the Chinese Veterinary Pharmacopoeia. It should grow aseptically.

(4) Inactivation test: Take 5 pieces SPF chicken embryos at 10 days old, inoculate inactivated virus solution into the bladder cavity, inoculate 0.2mL per embryo, and continue incubation at 37 ℃. Take care of the embryos twice a day and observe for 120 hours. There should be no more than 1 non-specific death of chicken embryos. Measure the HA potency of all chicken embryo liquid separately, which should be negative, and blindly pass it for 1 generation. Observe for 120 hours and measure the HA potency. If there is still no coagulation phenomenon, it is considered complete inactivation.

S2、 Avian influenza virus (H9 subtype) antigen solution

1. Preparation of avian influenza virus (H9 subtype) liquid

The avian influenza virus (H9 subtype) JY strain was isolated, identified, stored, and supplied by Shandong Ludu Biotechnology Co., Ltd.

(1)Vaccination: Take the production strain, dilute it 10^3 times with sterile physiological saline, and inoculate 10 day old susceptible chicken embryos into the bladder cavity. Inoculate 0.1mL per embryo, seal the needle hole after inoculation, and continue incubation at 37 ℃.

(2) Incubation and observation: After inoculation of chicken embryos, the embryos should be photographed every 24 hours, and any dead embryos within 48 hours should be discarded. Afterwards, the embryos were photographed every 24 hours, and any dead embryos were removed at any time until 96 hours. Regardless of whether they died or not, all embryos were removed with the chamber facing upwards and cooled at 4 ℃ for 12

hours.

(3) Harvest: Remove the cooled chicken embryos and harvest the chicken embryo liquid (first collect live embryos, then collect dead embryos). The harvested chicken embryo liquid is placed in a sterilized container and sampled to determine the HA potency. HA potency below 1:256 should be discarded. According to the current Chinese Veterinary Pharmacopoeia, aseptic testing should be conducted to ensure sterile growth. The harvested chicken embryo liquid should be stored below -15 ℃ before inactivation.

(4) Concentration: The harvested chicken embryo virus liquid is centrifuged at 2-8 ℃ to remove residues and other particulate impurities from the chicken embryo liquid. Then, it is concentrated using an ultrafiltration concentrator until the HA potency is not less than 1:2048, and the concentration is stopped. At the same time, sterility testing is conducted according to the current Chinese Veterinary Pharmacopoeia, and it should grow aseptically. And a sample was retained for testing the virus value, and the concentrated chicken embryo virus solution was immediately inactivated.

(5) Inactivation: The concentrated chicken embryo virus solution of avian influenza virus (H9 subtype) is introduced into the inactivation tank, and 10% formaldehyde solution is metered in. The mixer is turned on to stir thoroughly, and the final concentration of formaldehyde is 0.1%. After adding formaldehyde solution, introduce it into another inactivation tank to avoid the virus adhering near the tank mouth from not contacting the inactivation agent. Inactivate at 37 ℃ for 16 hours (start timing when the temperature inside the tank reaches 37 ℃, and start the mixer for continuous stirring), then remove and store at 4 ℃.

2. Avian influenza virus (H9 subtype) antigen liquid test

(1) HA potency: Take concentrated chicken embryo virus solution and determine it according to the current Appendix of the Chinese Veterinary Pharmacopoeia. The HA potency should not be less than 1:1024 before it can be used for vaccine preparation.

(2) Virus content: Dilute the concentrated chicken embryo virus solution before inactivation in a 10 fold series, taking three dilutions of 10^{-6}, 10^{-7}, and 10^{-8}. Inoculate 5 SPF chicken embryos at 10 days of age into each bladder cavity, each embryo inoculated with 0.1mL, and continue incubation at 37 ℃. Discard the chicken embryos that died 24 hours ago, take care of the embryos twice a day, and take out the chicken embryos that died within 24-20 hours at any time. Harvest the chicken embryo solution and observe for 120 hours. The HA titer should be measured for both dead and live embryos. If the HA titer is not less than 1:16, it is considered infected. Calculate the EID_{50} according to the Reed Muench method. When the virus content is ≥ 107.7 EID_{50} per 0.1mL, it can be used for vaccine production.

(3) Aseptic test: Take inactivated concentrated chicken embryo virus solution and conduct aseptic test according to the current Appendix of the Chinese Veterinary Pharmacopoeia, it should grow aseptically.

(4) Inactivation test: Take 5 pieces SPF chicken embryos at 10 days old, inoculate inactivated virus solution into the bladder cavity, inoculate 0.2mL per embryo, continue incubation at 37 ℃, and observe the embryos twice a day for 120 hours. Non specific death of chicken embryos should not exceed 1. Measure the HA potency of all chicken embryo liquid separately, which should be negative, and blindly pass it for 1 generation. Observe for 120 hours and measure the HA potency. If there is no coagulation phenomenon, it is considered complete inactivation.

S3、 Preparation of nano propolis composite adjuvant vaccine for poultry use

Mix the Newcastle disease virus antigen solution prepared in S1 and the avian influenza (H9 subtype) antigen solution prepared in S2 in a 1:1 volume ratio as the antigen phase.

Mix the antigen phase with the avian nano propolis composite adjuvant prepared in Embodiment 1 in a volume ratio of 3:1 and stir evenly to obtain the Newcastle disease avian influenza (H9 subtype) dual inactivated vaccine, namely the avian nano propolis composite adjuvant vaccine, which is packaged 250mL per bottle.

Embodiment 5: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

The antigen prepared in S3 of Embodiment 4 was mixed with the avian nano propolis composite adjuvant prepared in Example 2 in a volume ratio of 3:1, and stirred evenly to obtain the Newcastle disease avian influenza (H9 subtype) dual inactivated vaccine, namely the avian nano propolis composite adjuvant vaccine, which is packaged 250mL per bottle.

Embodiment 6: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

The antigen prepared in S3 of Embodiment 4 was mixed with the avian nano propolis composite adjuvant prepared in Embodiment 3 in a volume ratio of 3:1, and stirred evenly to obtain the Newcastle disease avian influenza (H9 subtype) dual inactivated vaccine, namely the avian nano propolis composite adjuvant vaccine, which is packaged 250mL per bottle.

Comparative Example 1: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

Mix the antigen phase and physiological saline prepared in S3 of Embodiment 4 with the

ethanol extract of propolis prepared in Step S1 of Example 1 in a volume ratio of 75:20:5 to obtain the Newcastle disease avian influenza (H9 subtype) dual inactivated vaccine, namely the avian nano propolis composite adjuvant vaccine, which is packaged 250mL per bottle.

Comparative Example 2: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

S1. Preparation of nano propolis composite adjuvant for poultry: Mix 30mL of white oil, 20mL of Span 80, and 15mL of Tween-80 evenly, then add 20mL of propolis ethanol extract prepared in step S1 of Embodiment 1 and mix evenly. Add 0.1g of soapberry saponin A, and finally make up to 100mL with injection water. Stir at 800rpm for 30 minutes, thoroughly mix and emulsify, filter and sterilize with a 0.22 μ m filter to obtain the nano propolis composite adjuvant for poultry.

S2. Preparation of avian nano propolis composite adjuvant vaccine: The antigen prepared in step S3 of Embodiment 4 and the avian nano propolis composite adjuvant prepared in step S1 of this comparative example were stirred and mixed uniformly in a volume ratio of 3:1 to obtain the Newcastle disease avian influenza (H9 subtype) dual inactivated vaccine, namely the avian nano propolis composite adjuvant vaccine, which is packaged 250mL per bottle.

Comparative Example 3: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

S1. Preparation of nano propolis composite adjuvant for poultry:

Mix 30mL of vitamin D, 20mL of Span 80, and 15mL of Tween-80 evenly, then add 20mL of propolis ethanol extract prepared in step S1 of Example 1 and mix evenly. Add 0.1g of soapberry saponin QS-21, and finally make up to 100mL with injection water. Stir at 800rpm for 30 minutes, mix thoroughly and emulsify, filter and sterilize with a 0.22 μ m filter, and obtain a nano propolis composite adjuvant for poultry.

S2. Preparation of avian nano propolis composite adjuvant vaccine: The antigen prepared in step S3 of Embodiment 4 and the avian nano propolis composite adjuvant prepared in step S1 of this comparative example were stirred and mixed uniformly in a volume ratio of 3:1 to obtain the Newcastle disease avian influenza (H9 subtype) dual inactivated vaccine, namely the avian nano propolis composite adjuvant vaccine, which is packaged 250mL per bottle.

The present invention tested the characteristics and other indicators of the avian nano propolis composite adjuvant vaccine prepared in Embodiments 4 to 6 and Comparative Examples 1 to 3. The results are as follows:

The results of the trait and loading inspection are shown in Figure 6. The appearance of the nano propolis composite adjuvant vaccine for poultry in Embodiment 4 to 6 presents a light yellow, uniform, and semi transparent suspension; The appearance of the nano propolis composite adjuvant vaccine for poultry in proportion 1 is light yellow, and precipitation appears after being stored at 4 ℃ for 2 weeks; The avian nano propolis composite adjuvant vaccines of Comparative Example 2 and Comparative Example 3 showed stratification after being stored at 4 ℃, without the characteristic testing standards for composite vaccines. Therefore, no further tests were conducted on viscosity, formaldehyde residue, loading, safety, and efficacy of Comparative Example 2 and Comparative Example 3 vaccines.

(1) The viscosity of the avian nano propolis composite adjuvant vaccine prepared in Embodiments 4 to 6 and Comparative Example 1 is less than 20cP, the residual formaldehyde content is below 0.05%, and the loading volume is between 251.3mL and 252.0mL.

(2) Aseptic testing: After testing, the avian nano propolis composite adjuvant vaccines prepared in Embodiments 4 to 6 and Comparative Example 1 all showed aseptic growth.

(3) Safety test: 14 day old SPF chickens were subcutaneously injected with 1mL of the avian nano propolis composite adjuvant vaccine prepared in Embodiments 4 to 6 and Comparative Example 1 at neck points, respectively, for safety testing of the vaccine, and a blank control group without treatment was set up. After vaccination, all experimental chickens were observed for 14 days, during which their mental state, food intake, and water intake were normal. After 14 days of culling, no abnormalities were found in any organs, and there were no unabsorbed vaccines or swelling or ulceration at the injection site. The results showed that the avian nano propolis composite adjuvant vaccines prepared in Embodiments 4 to 6 and Comparative Example 1 exhibited good safety.

(4) Efficacy test: Use 21 day old SPF chickens to test the efficacy of the avian nano propolis composite adjuvant vaccine.

After immunizing 10 SPF chickens with the avian nano propolis composite adjuvant vaccines prepared in Examples 4 to 6, the HI antibody titers of Newcastle disease virus in chickens ranged from 1:16 to 1:64, with average values of 1:29.9, 1:27.9, and 1:26, respectively, meeting the protection standards of Newcastle disease vaccines; After immunizing SPF chickens with the vaccine prepared in Comparative Example 1, the HI antibody titer of Newcastle disease virus in chickens ranged from 1:2 to 1:8, with an average value of 1:4, which did not meet the protection standard of Newcastle disease vaccine. Through statistical t-test analysis, there was no significant difference between Embodiments 4 to 6. The p-value between Examples 4 and 5 was 0.773, between

Embodiments 4 and 6 was 0.535, and between Embodiments 5 and 6 was 0.764. In addition, the p-values of Embodiments 4 to 6 and Comparative Example 1 were all less than 0.001, indicating a significant difference in the immune effect of the avian nano propolis composite adjuvant vaccine prepared in Examples 4 to 6 compared to the avian nano propolis composite adjuvant vaccine prepared in Comparative Example 1 using propolis ethanol extract. In addition, the HI antibody titers of the blank control group were all ≤ 1:2 (Table 11).

Table 11 HI antibody titers of Newcastle disease virus in chickens after vaccination

Group	HI antibody titer of Newcastle disease virus in chickens	
	Individual value	Average
Embodiment 5	1: 16, 1: 32, 1: 32, 1: 16, 1: 64, 1: 32, 1: 32, 1: 64, 1: 16, 1: 32	1: 29.9^{a***}
Embodiment 6	1: 16, 1: 64, 1: 16, 1: 32, 1: 32, 1: 64, 1: 16, 1: 16, 1: 32, 1: 32	1: 27.9^{a}
Embodiment 7	1: 16, 1: 32, 1: 32, 1: 16, 1: 64, 1: 32, 1: 32, 1: 16, 1: 16, 1: 32	1: 26^{a}
Comparative example	1: 2, 1: 4, 1: 4, 1: 2, 1: 8, 1: 4, 1: 4, 1: 8, 1: 4, 1: 4	1: 4^{b}
Blank comparative group	<1: 2, <1: 2, <1: 2, <1: 2, <1: 2	<1: 2

Note: The same letters in the table represent insignificant differences between groups, while different letters represent significant differences between groups. * * * indicates $p<0.001$.

After immunizing SPF chickens with the avian nano propolis composite adjuvant vaccines prepared in Examples 4-6, the HI antibody titers of avian influenza (H9) virus ranged from 1:64 to 1:256, with average values of 1:104, 1:97, and 1:147, respectively, meeting the protection standards of avian influenza (H9) vaccines; After immunizing SPF chickens with the vaccine prepared in Comparative Example 1, the HI antibody titer of avian influenza (H9) virus ranged from 1:1 to 1:4, with an average value of 1:3, which did not meet the protection standard of avian influenza (H9) vaccine; Through statistical analysis t-test, there was no significant difference between Embodiments 4-6. The p-value between Embodiments 4 and 5 was 0.79, between Embodiments 4 and 6 was 0.105, and

between Embodiments 5 and 6 was 0.06. In addition, the p-values of Embodiments 4-6 and Comparative Example 1 were all less than 0.001, indicating a significant difference in the immune effect of the avian nano propolis composite adjuvant vaccine prepared in Embodiments 4-6 compared to the avian nano propolis composite adjuvant vaccine prepared in Comparative Example 1 using propolis ethanol extract. In addition, the HI antibody titers of the blank control group were all ≤ 1:2 (Table 12).

Table 12 HI antibody titers of avian influenza (H9) after vaccine immunization

Group	HI antibody titer of avian influenza (H9)	
	Individual value	Average
Embodiment 5	1: 64, 1: 64, 1: 128, 1: 128, 1: 128, 1: 128, 1: 64, 1: 256, 1: 128, 1: 64	1: 104^{a***}
Embodiment 6	1: 64, 1: 128, 1: 128, 1: 128, 1: 128, 1: 64, 1: 64, 1: 64, 1: 128, 1: 256	1: 97^{a}
Embodiment 7	1: 64, 1: 128, 1: 128, 1: 128, 1: 128, 1: 128, 1: 256, 1: 256, 1: 128, 1: 256	1: 147^{a}
Comparative example	1: 1, 1: 4, 1: 2, 1: 4, 1: 4, 1: 4, 1: 4, 1: 4, 1: 4, 1: 2	1: 3^{b}
Blank comparative group	<1: 2, <1: 2, 1: 2, <1: 2, <1: 2	⩽1: 2

Note: The same letters in the table represent insignificant differences between groups, while different letters represent significant differences between groups. * * * indicates $p<0.001$.

Embodiment 7: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

S1. Preparation of vaccine culture for chicken infectious rhinitis (type A)

The strain used for production and inspection is Haemophilus parainfluenzae type A C-

Hpg-8 strain, which is identified, stored, and supplied by the China Veterinary Drug Administration.

Preparation of seeds for production:

Inoculate the bacterial strain onto chicken soup agar plates by streaking, and incubate at 37 ℃ for 16-18 hours in an environment containing 5% CO_2. Select typical fluorescent colonies and inoculate them into the yolk sac of 5-day-old chicken embryos. Continue incubation at 37 ℃, collect the yolk solution of chicken embryos that have died within 30 hours as primary seeds, and store at -20 ℃.

Take the yolk solution of infected chicken embryos, streak and inoculate it onto chicken soup agar plates. Incubate at 37 ℃ for 16 hours under conditions containing 5% CO_2. Select typical fluorescent colonies and inoculate them into the chicken soup medium. Incubate at 37 ℃ for 16 hours as secondary seeds.

Add the secondary seed solution to a semi synthetic medium (1000mL semi synthetic medium containing 5g of peptone, 30g of casein peptone, 15g of monosodium glutamate, 5g of yeast extract, 3g of glucose, 15g of sodium chloride, and the remaining amount of distilled water), adjust the pH to 7.2-7.4, and add 5% inactivated healthy chicken serum and 0.8% 0.5% coenzyme I before use. Incubate at 37 °C for 18 hours, shake the culture bottle twice during this period, and confirm no bacterial contamination after pure testing. Add 0.05% formaldehyde solution and store at 2 ° C.

Collect bacterial cells by centrifugation or concentrate them using a hollow fiber ultrafiltration device, and then use pH 7.2 phosphate buffer to make a suspension containing at least 5 billion bacterial cells per 1mL (compared with the national standard for biological products "China Bacterial Turbidity Standard", with a standard tube containing 2.8 billion/mL of Haemophilus parahaemolyticus). After passing the purity test, inactivate the suspension.

Add 0.15% formaldehyde solution and 0.01% thiomersal to the concentrated solution of the seedling culture according to the volume ratio, sterilize at 2 ℃ for 7 days, and after inspection, grow aseptically as the seedling antigen.

Inspection of semi-finished products:

Aseptic test: Take inactivated concentrated seedling bacterial solution and conduct aseptic test according to the current Appendix of the Chinese Veterinary Pharmacopoeia, and find aseptic growth.

The inspection results of the bacterial solution for seedling production: After 18 hours of cultivation, two batches (batch 001 and batch 002) of bacterial solution were randomly

selected for inspection. The harvested amounts of bacterial solution for batch 001 and batch 002 were 2700mL and 11000mL, respectively, with viable bacterial counts of 12 × 10^8CFU/mL and 18 × 10^8CFU/mL, and total bacterial counts of 56 × 10^8CFU/mL and 58 × 10^8CFU/mL, respectively. Centrifuge the bacterial solution and collect it, then resuspend the bacterial cells in PBS. The yield of concentrated bacterial solution for batch 001 and batch 002 was 180mL and 730mL, respectively. The total bacterial count (turbidity bacteria count) was 840 × 108CFU/mL and 870 × 108CFU/mL, respectively, which met the requirements for seedling production.

The inactivation test results of concentrated bacterial solution for seedling production: After inactivation with formaldehyde solution, the concentrated bacterial solution for seedling production was inoculated with semi synthetic liquid culture medium and solid culture medium, and both grew aseptically after cultivation.

Aseptic test results of semi-finished bacterial liquid for seedling production: According to the current Appendix of the Chinese Veterinary Pharmacopoeia, the sterile test of semi-finished products is carried out by inoculating the concentrated bacterial liquid for seedling production, which has been inactivated by formaldehyde solution, with Fluid Thioglycollate Medium (TG), Peptone from casein Agar Medium，GA, and Tryptic Soy Broth (TSB) for sterile growth.

S2、 Add the nano propolis composite adjuvant prepared in Embodiment 3 and the chicken infectious rhinitis inactivated antigen prepared in S1 of this Embodiment into a mixing tank in a ratio of 1:3, and turn on the mixing motor of the mixing tank to stir for 40 minutes until the mixture is evenly mixed.

Comparative Example 4: A preparation method of a nano propolis composite adjuvant vaccine for poultry, comprising the following preparation steps:

Add the ethanol extract of propolis prepared in Step S1 of Embodiment 1, the inactivated antigen of chicken infectious rhinitis prepared in S1 of Embodiment 7, and physiological saline solution in a volume ratio of 5:75:20 into the mixing tank, and turn on the mixing motor of the mixing tank to stir for 40 minutes until the mixture is evenly mixed.

The present invention further tested the correlation between the avian nano propolis composite adjuvant vaccine prepared in Embodiment 7 and Comparative example 4, and the results are as follows:

(1) Vaccine trait testing and filling quantity inspection

The avian nano propolis composite adjuvant vaccines prepared in Embodiment 8 and Comparative Example 4 are uniformly, pale yellow, and semi transparent water-soluble suspensions; The viscosity is 30.2cP and 36.3cP respectively; the formaldehyde content

is below 0.04%, and the loading amount meets the standard requirements.

(2) Aseptic test results

According to the method in the appendix of the current Chinese Veterinary Pharmacopoeia, the sterile test was conducted on the nano propolis composite adjuvant vaccine for poultry, and the results showed that it grew aseptically.

(3) Security testing results

Eight SPF chickens aged 70 days were injected subcutaneously with 1.0 mL of avian nano propolis composite adjuvant vaccine for 14 consecutive days. No local or systemic adverse reactions were observed due to the avian nano propolis composite adjuvant vaccine, and the results were deemed qualified.

(4) Effectiveness test results

Twenty SPF chickens aged 70 days were randomly divided into three groups, with 8 chickens in each group of Experiment 1 and Experiment 2, and 4 chickens in the blank control group. Experiment 1 injected 0.5mL of the avian nano propolis composite adjuvant vaccine from Example 8 subcutaneously into each chicken, while Experimental group 2 injected 0.5mL of the avian nano bee glue composite adjuvant vaccine from Comparative Example 4 subcutaneously into each chicken, with no treatment as the blank comparative group; After one month, experimental group 1, experimental group 2, and blank control group chickens with the same conditions were injected with 0.2mL of C-Hpg-8 bacterial solution (viable cell count of 5.0 × 105CFU) into the antrum below the frame of each chicken, and observed for 14 days. All four chickens in the blank comparative group developed symptoms, characterized by swelling of one or both infrarenal sinuses and their surroundings, as well as runny nose or tears; Among the 8 chickens in Experimental group 2, 1 chicken developed the disease, while the remaining 7 chickens showed any clinical symptoms due to immunity; However, none of the 8 chickens in Experimental group 1 showed any clinical symptoms and were all protected; The immune effect of the avian nano propolis composite adjuvant vaccine prepared in Example 8 is stronger than that of the avian nano propolis composite adjuvant vaccine prepared in Comparative Example 4.

It should be noted that when the claims of the present invention involve numerical ranges, it should be understood that the two endpoints of each numerical range and any value between the two endpoints can be selected. To avoid repetition, the present invention describes preferred embodiments. Although preferred embodiments of the present invention have been described, those skilled in the art may make additional changes and modifications to these embodiments once they have knowledge of the basic inventive

concept. Therefore, the attached claims are intended to be interpreted as including preferred embodiments and all changes and modifications falling within the scope of the present invention.

Obviously, technicians in this field can make various modifications and variations to the present invention without departing from the spirit and scope of the invention. Thus, if these modifications and variations of the present invention fall within the scope of the claims and their equivalent technologies, the present invention is also intended to include these modifications and variations.