Phytochemical Constituents Propolis Flavonoid, immunological enhancement,

Anti-porcine parvovirus Activities of isolated from the Propolis

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Abstract:

Propolis was widely used in health preservation and disease healing, it contains many ingredients. The previous study had been revealed that the propolis has a wide range of efficacy, such as antiviral, immune enhancement, anti-inflammatory and so on, but its antiviral components and underlying mechanism of action remain unknown. In this study, we investigated the chemical composition, and anti-PPV and immunological enhancement of Propolis Flavonoid(PF). Chemical composition of PF was

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distinguished by UPLC-Q/TOF-MS/MS analysis.The presence and characterized of 26 major components was distinguished in negative ionization modes.To evaluate the effects of PF used as adjuvant on the immune response porcine parvovirus (PPV). Thirty Landrace-Yorkshire hybrid sows were randomly assigned to 3 groups, and the sows in adjuvant groups were intramuscular injected PPV vaccine with 2.0 mL PF adjuvant (PA), oilemulsion adjuvant (OA), respectively. After that, serum hemagglutination inhibition antibody titers, IgM and IgG subclasses, eripheral lymphocyte proliferation activity, and concentrations of cytokines were measured. Results indicated an enhancing effect of PA on IgM, IL-2, IL-4, IFN-γ and the IgG subclass responses. These findings suggested that PA could significantly enhance the immune responses. Furthermore, we screened the chemical components the effective of anti-PPV, Ferulic acid have an excellently anti-PPV effective.

Keywords: Propolis Flavonoid, UPLC-Q/TOF-MS/MS , immunological enhancement, Ferulic acid, Anti-PPV

1. Introduction：

Nature product and natural product derivative have been drawn more attentions due to its wide spectrum of biological and pharmaceutical properties. Propolis is a gelatinous solid substance with aromatic smell, which is collected from plant trunks and flower bracts by bees and mixed with the secretions of their maxillary glands and beeswax.[1-2] Propolis have been widely marketed by the pharmaceutical industries as an alternative medicine and as the health-food in various parts of the world.[3]Bioactive constituents isolated from the Propolis, Flavonoids, terpenes, organic acids, fatty acids, amino acids, enzymes, vitamins, trace elements and minerals, have application in treating diseases due its anticancer, anti-inflammatory, antioxidant, antibacterial, antimycotic, antifungal, and immunomodulatory properties.[4-7] So fare, more than 500 chemical compounds have been isolated from propolis.[8] Also, we previous studies revealed that propolis and its constituents exert immunological enhancement in vivo

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,as well as anti-PPV effect in vitro.

Porcine parvovirus (PPV) is one of main pathogens that cause swine reproductive disorder and increasing economic losses in the world.[9-12] Several studies have pointed out that vaccination is an effective method for controlling this disease.[13-14] The successful vaccination depends on their association with potent adjuvant which can increase the immunogenicity of vaccine. A better adjuvant can activate specific effectors of the immune system, such as cytotoxic or auxiliary T cells (Th1/Th2) and strengthen the humoral and/or cellular immune responses against that antigen.[15-16] On the other hand, suitable adjuvant should have lower toxicity and side effects.[17] But there are some shortages in commonly used adjuvants, for example, oilemulsion adjuvant (OA) can cause inflammation, induration or necrosis in the local, and disseminated granulomas in lungs, liver, kidneys, heart, lymph nodes and skeletal muscles in rabbits or rats.[18] Aluminum salt adjuvant (AA) is a widely used adjuvant in human vaccines licensed by the US FDA,[19] but it is a poor inducer of Th1 cellular immune response and easy to induce immunoglobulin E antibody response associated with some allergic reactions.[20-21]Thus,nature product Propolis as a immunologic adjuvant has attracted more and more attention due to its minor side effects and more pharmacological efficacy.

Most of the studies in the literature have investigated the anti-PPV and immunological enhancement effective of the propolis flavonoid isolated from the Propolis.[22] Propolis flavonoid (PF), a kind of ingredient extracted from propolis, as a harmless natural adjuvant has been used in chickens vaccinated with activated vaccine, and the results showed that PF could improve the immune-enhancing activity in the humoral and cellular immune response.[23-24] Since the major effective of propolis are derived from active ingredient, the anti-PPV and immunological enhancement effective is highly dependent on its extraction method and content of active ingredients of propolis flavonoid. Ethanol extracted propolis is still the main method for propolis flavonoids.However，still little is known regarding the anti-ppv effective of propolis flavonoids compounds,such as Ferulic acid,Quercetin,Chrysin and so on.

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Thus,this study a ultra-performance liquid chromatography-quadrupole/time-of-flight mass (UPLC-Q/TOF-MS) spectrometry was used to distinguish and exhibit the Standard chemical map of propolis flavonoid.In addition,this study was to evaluate the adjuvant effect and characteristic of PF on the humoral and cellular immune response of immunized pigs and screen out good anti-PPV propolis flavonoids compounds.

2. Materials and Methods

2.1 Propolis Samples Collection, Extraction and Processing

Propolis samples were collected in September of 2018 in liaocheng apiculture research institute,Shandong province,China.The ethanolic extract was prepared as reported by Park et al.[25] 10g of the powder was mixed with 100 mL of 75% ethanol in a sealed container protected from light (to avoid loss of volatile and photosensitive compounds), under agitation in a water bath at 70 ℃ for 30 min.After extraction, the mixture was filtered.The filtrated liquid was combined with the original liquid. The ethanol was recovered by rotary evaporation and left for overnight to obtain the crude propolis extract in the upper layer and the crude propolis extract in the lower layer.

2.2 Mass Spectrum Analysis and Verification ofMethodology

Propolis flavone samples analysis, managed by consisted of SIL-30SD auto sampler,Shimadzu UPLC (Kyoto, Japan), CTO-30A column oven, an LC-30AD Binary liquid pump , DGU-20A5R On-Line Solvent Degasser, AB SCIEX Triple TOF 5600+ system, and ESI source. Chromatographic conditions were as follows: performed on a C18 reversed-phase LC column (Agilent ZorBax SB-C18 50 mm × 2.1 mm, 1.8 mm), and the column temperature was maintained at 25℃.The mobile phase consisted of 0.1% (v/v) formic acid water (solvent A), and an acetonitrile (solvent B) using a gradient program as follows. Gradient elution program: 5 – 25% B,0 – 1 min; 25 – 30% B,1 – 4 min; 30 – 55% B,4 – 12 min; 55 – 70% B,12 – 18min; 70 – 100% B,18 – 25 min;100 – 5% B, 25 – 28 min; flow rate: 0.3 mL/min;

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column temperature: 35℃; injection volume: 1 μL. Mass spectrometer condition: ESI source, data collection in positive and negative ion mode, respectively. The source parameters were set as follows: Ion spray voltage floating: +4500/−4500; declustering potential: +60/−60 V; source temperature: 550 ◦C; the atomizing gas is N2, curtain gas: 35 psi; gas1(nebulizer gas): 55 psi; gas2(heater gas): 55 psi; collision energy: +35/−35e V; using MS/MS secondary mass spectrometry mode: The MS spectrometer ion scanning range was m/z 100 – 2000. The MS/MS spectrometer ion scanning range was m/z 50 – 1000; turn on dynamic background subtraction.

2.3 Identification of chemical constituents

The UHPLC-Q/TOF-MS data of all of the studied samples were analyzed using MarkerView and PeakView software (AB Sciex, Massachusetts, USA) for the purpose of identifying PF chemical constituents.

2.4 Preparation of adjuvant and vaccine

PF was prepared in our laboratory in a final purity of 925 mg •g^{-1}. The PF adjuvant (PA) was prepared as previoulsy described. The PF was dissolved in phosphate buffer solution (PBS, pH 6.2), in a final concentration of 40 mg • mL^{-1}. PPV was supplied by China Institute of Veterinary Drug Control. After propagated in vitro in PK-15 cell cultures, the virus contained 2048 haemagglutination units (HAU) • mL-1 was inactivated with formaldehyde and stirring 24 h at 37 ℃. The inactivated virus contained 512 HAU • mL^{-1} was used as vaccine antigen. Three adjuvant vaccines containing PA and OA, were prepared by Lvdu Veterinary Biologicals Co. Ltd., Binzhou, China. Their virus contents were the same.

2.5 Animals, Housing and Treatment

Thirty Landrace-Yorkshire hybrid sows (aged from 198 to 204, and average weight is 64.5 kg) were randomly assigned to one of 3 groups, receiving intramuscular injection of PPV vaccine with 2.0 mL PA, OA or physiological saline (blank control group,BC), respectively. The animals were kept in ten pigpens divide

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equally under standard conditions in the Experimental Animal Center of Binzhou Animal Science and Veterinary Medicine Academy, Shandong province (NO. SYXK (lu) 20110066). They were maintained in an air-conditioned room with light from 06:00 h to 18:00 h. The room temperature (24 ± 3℃) and humidity were controlled automatically. They were fed water and food ad libitum. All procedures related to the animals and their care conformed to the internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the government of China. The antibody against PPV were negative before the experiment.

Before vaccination and on days 7, 14, 21, 28, 35 and 49 after vaccination, the blood of 6 pigs randomly from each group were sampled for determination of serum hemagglutination inhibition (HI) antibody titer of ppv by micro-method dynamically and continuously. On days 7, 14, 21 and 35 after vaccination, the blood of 4 pigs randomly from each group were sampled for examination of lymphocyte proliferation by MTT assay[18] and analyzing specific IgM and IgG1, IgG2, IgG3, IgG4, IL-2, IL-4 and IFN-γ in serum by enzyme-linked immunosorbent assay (ELISA) kit.

2.6 HI antibody titre of ppv determination

Blood samples (1.0 mL per pig) from ear vein were drawn into Eppendorf tubes and allowed to clot at 37 oC for 1 h. Serum was separated by centrifugation for determination of HI antibody. Briefly, twofold serial dilution of 50 μL serum, after inactivated at 56 oC for 30 min, were made in a 96-well V-shaped bottom microtiter plate containing 50 μL of CMF-PBS in all wells, then 50 μL of PPV antigen (4 HA units) was added into all wells except for the last row served as the controls. serum dilutions ranged from 1:2 to 1:2048. The antigen-serum mixture was incubated at 37 oC for 10 min. Fifty microlitres of 1% rooster erythrocytes suspension was added into each well and re-incubated for 30 min. A positive serum, a negative serum, erythrocytes and antigens were also included. The highest dilution of serum causing complete inhibition was considered the endpoint. The geometric mean titer was expressed as reciprocal log2 values of the highest dilution that displayed HI antibody titer.

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2.7. Peripheral lymphocyte proliferation assay

The blood sample from precaval vein was diluted in Hanks' solution. After centrifugation (800 g at 4 oC for 10 min), the cells were washed three times with PBS and resuspended in RPMI-1640. The cells were counted with a haemocytometer by trypan blue dye exclusion technique and they viability exceeded 95%. Briefly, lymphocyte at 5.0×10^{6} cell • mL^{-1} were seeded into a 96-well flat-bottom microtiter plate, 100 μL each well, then Con A (final concentration 5 μg • mL^{-1}) or medium were added, respectively. The plates were incubated in a humid atmosphere with 5% CO2 at 37℃ for 48 h. All the tests were carried out in quadruplication. The cell proliferation was evaluated by MTT methods. Briefly, 30 μL of MTT solution (5 mg • mL^{-1}) were added into each well at 4 h before the end of incubation. The plates were centrifuged (1000 g, 5 min) at room temperature and MTT was removed carefully. After 150 μL DMSO was added into each well, the plates were shaken for 5 min to dissolve the formazan crystals completely, and the absorbance at 570 nm (A570 value) was determined by ELISA reader (BIO-TEK. Instruments INC) as the index of lymphocytes proliferation.

2.8 Cytokine and immunoglobulin determination

Serum concentrations of cytokines (including IL-2, IFN-γ and IL-4) and specific IgM and IgG subclasses were assayed by ELISA according to manufacturer’s instructions. Briefly, a 96-well flat bottom microtiter plate (Nunc, USA) was coated with capture antibody specific for each cytokine. The plate was washed and blocked before 10 μL of the serum and serially diluted specific standards were added to the respective wells. Following a series of washing, the captured cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm using an ELISA reader (BIO-TEK. Instruments INC). Cytokine concentrations and IgM and IgG subclasses were accomplished by a standard curve plotting A450 value against each dilution of standards concentration.

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2.9 Screening of flavonoids in propolis against PPV in vitro

PK-15 cells (presented by Bin zhou animal husbandry and veterinary research institute of Shandong province) were cultured in DMEM medium containing 10% fetal calf serum (FBS,South American origin) in 5% CO_2 cell incubator at 37 ℃. 0.25% trypsin (0.02% EDTA) digestion passage.

PK-15 cells with trypsin digestion, 1 x 10^5 / ml to vaccination in 96 well plates, 100 μL per hole, under the condition of 37 ℃ and 5% CO_2 training 24 h; abandon the supernatant, PBS washing, except the blank group added DMEM, containing the rest every hole to join MOI = 1.0 PPV virus cultures of 100 μL, training after 4 h, abandon the supernatant, except the blank group and model group with DMEM, in the remaining Wells, 100 μL medium was added with 50 μM concentration of galangin, kaolin, quercetin, ferulic acid, aspen and apigenin, and 4 replicates were set for each drug. After 48h, the supernatant was discarded, and 10 μL CCK-8 medium was added to 100 μL DMEM medium per well for 30min culture, and the absorbance value of CCK-8 was detected.

2.10 Statistical analysis

Data analysis was performed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences in mean values among the polysaccharide and control groups were analyzed by a one-way analysis of variance (ANOVA). The data were expressed as mean ± standard error. Values of $P < 0.05$ were considered to be statistically significant.

3. Result

3.1 Identification of active components of alcohol extract by UHPLC-Q/TOF-MS.

The crude total propolis flavonoids was about 80% in the alcohol extraction. According to the analytical results for the chemical constituents(as shown in Figure 1A),the different components in the Supernatant and Substratum of propolis extract are mainly small polar components.We analyzed the chemical fingerprint

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(UHPLC-Q/TOF-MS) of PF (Figure 1B).The analysis in negative ionization modes revealed the presence and characterized of 26 major components(Table 1).

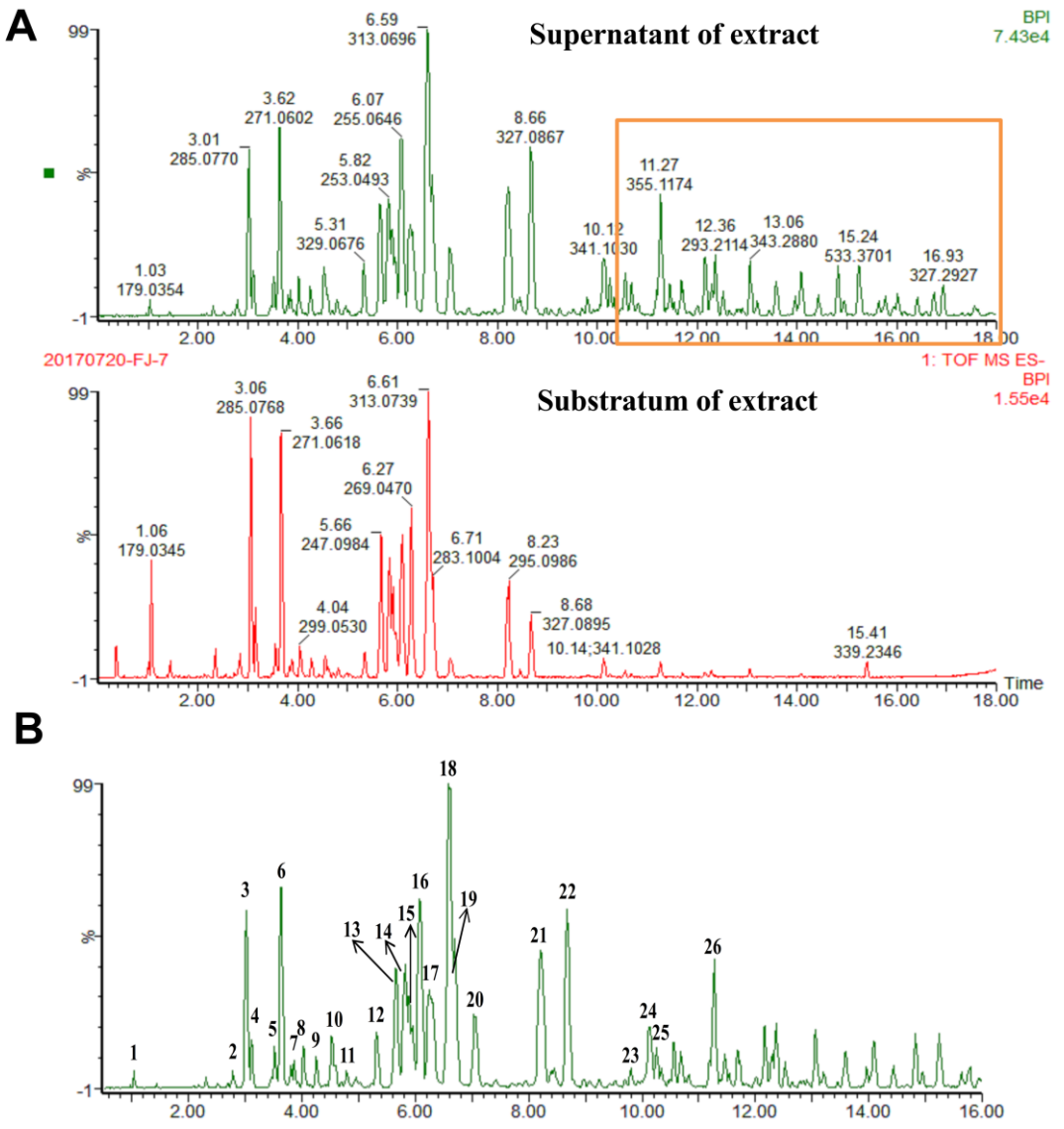

Figure1.UHPLC-Q/TOF-MS chemical fingerprints (TIC chromatography) of propolis extract

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Table1 Identification of compounds in ethanolic crude extract from the propolis of M. quadrifasciata by UPLC-MS analysi

Peak No.	Rt(min)	Tentative identification	Chemical formula	[M-H]- (m/z)		
				Mean measured mass(Da)	Theoretical exact mass(Da)	Mass accuracy (ppm)
1	1.03	Caffeic acid	C9H8O4	179.0345	179.0344	0.6
2	2.78	Quercetin	C15H10O7	301.0348	301.0350	0.7
3	3.01	5-Methoxypinobanksin	C16H14O5	285.0768	285.0763	1.8
4	3.10	3-Methoxyquercetin	C16H12O7	315.0507	315.0505	0.6
5	3.51	Apigenin	C15H10O5	269.0455	269.0450	1.9
6	3.62	Pinobanksin	C15H12O5	271.0602	271.0606	-1.5
7	3.86	Isorhamnetin	C16H12O7	315.0499	315.0505	-1.9
8	4.02	5-Methoxyluteolin	C16H12O6	299.0541	299.0556	-5.0
9	4.25	5,7-Dimethoxyquercetin	C17H14O7	329.0666	329.0661	1.5
10	4.52	5-Methoxygalangin	C16H12O5	283.0610	283.0606	1.4
11	4.78	7-Methoxyquercetin	C16H12O7	315.0497	315.0505	-2.5
12	5.31	7-Methoxyquercetin-X-methyl ester	C17H14O7	329.0653	329.0661	-2.4
13	5.63	Isoprene caffeic acid ester	C14H16O4	247.0974	247.0970	1.6
14	5.82	Chrysin	C15H10O4	253.0493	253.0501	-3.2
15	5.89	Caffeic acid benzyl ester	C16H14O4	269.0825	269.0814	4.1
16	6.07	Pinocembrin	C15H12O4	255.0646	255.0657	-4.3
17	6.24	Galangin	C15H10O5	269.0457	269.0450	2.6
18	6.59	3-O-Acetylpinobanksin	C17H14O6	313.0696	313.0712	-5.1
19	6.69	Caffeic acid phenethyl ester	C17H16O4	283.0966	283.0970	-1.4
20	7.04	Methoxychrysin	C16H12O5	283.0602	283.0606	-1.4
21	8.20	Cinnamate caffeic acid	C18H16O4	295.0970	295.0970	0.0
22	8.66	Pinobanksin-3-O-propanoate	C18H16O6	327.0867	327.0869	-0.6
23	9.80	P-Cinnamyl coumarate	C18H16O3	279.1031	279.1021	3.6
24	10.12	Pinobanksin-3-O-butyrate	C19H18O6	341.1030	341.1025	1.5
25	10.25	Pinobanksin-3-O-Pentenoic acid ester	C20H18O6	353.1035	353.1025	2.8
26	11.27	Pinobanksin-3-O-Valerate	C20H20O6	355.1174	355.1182	-2.3

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3.2 Identification and characterized of propolis flavonoids

Fig.2A results showed that the molecular composition of the compound was C10H10O4. In the anion mode, m/z 194 [M-H]-, typical fragment ion peaks such as 178 and 164 were found in the secondary mass spectrogram, and m/z 194 lost OCH3 to obtain m/z 178, which was speculated to be Ferulic Acid by comparing the mass spectrogram information with the reference substance.

Fig.2B results showed that the molecular composition of the compound was C15H10O7. In the anion mode, m/z 301 [M-H]-, the secondary mass spectrogram showed typical fragment ion peaks such as 273, 179, 151 and 107, m/z 301 lost CO to obtain m/z 273, aglycone RDA reaction produced m/z179, 151 and 107, and compound B was speculated to be Quercetin by comparing the mass spectrogram information with the reference substance.

Fig.2C results showed that the molecular composition of the compound was C15H10O5. In the anion mode, typical fragment ion peaks such as 151 and 117 were found in the secondary mass spectrogram of m/z 269[M-H]-, and compared with the mass spectrogram of the reference substance, compound C was speculated to be Apigenin.

Fig.2D results showed that the molecular composition of the compound was C15H10O6. In the anion mode, typical fragment ion peaks such as m/z 284 [M-H]- and 255 and 227 were found in the secondary mass spectrogram. m /z 284 lost COH to get m/z 255, while m/z 255 lost CO to get m/z 227. By comparing the mass spectrogram information with the reference substance, the compound D was speculated to be Kaempferol.

Fig.2E results showed that the molecular composition of the compound was C15H10O4. In the anion mode, m/z 253 [M-H]-, secondary mass spectrometry, 143, 63 and other typical fragment ion peaks were found. By comparing the mass spectrometry information with the reference substance, it was speculated that compound E was Chrysin.

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Fig.2F results showed that the molecular composition of the compound was C15H10O5. In the anion mode, the ion peaks of typical fragments such as 213, 171 and 169 were found in the secondary mass spectrogram of m/z 269 [M-H]-, and the mass spectrogram information was compared with the reference substance, suggesting that compound E was Galangal.

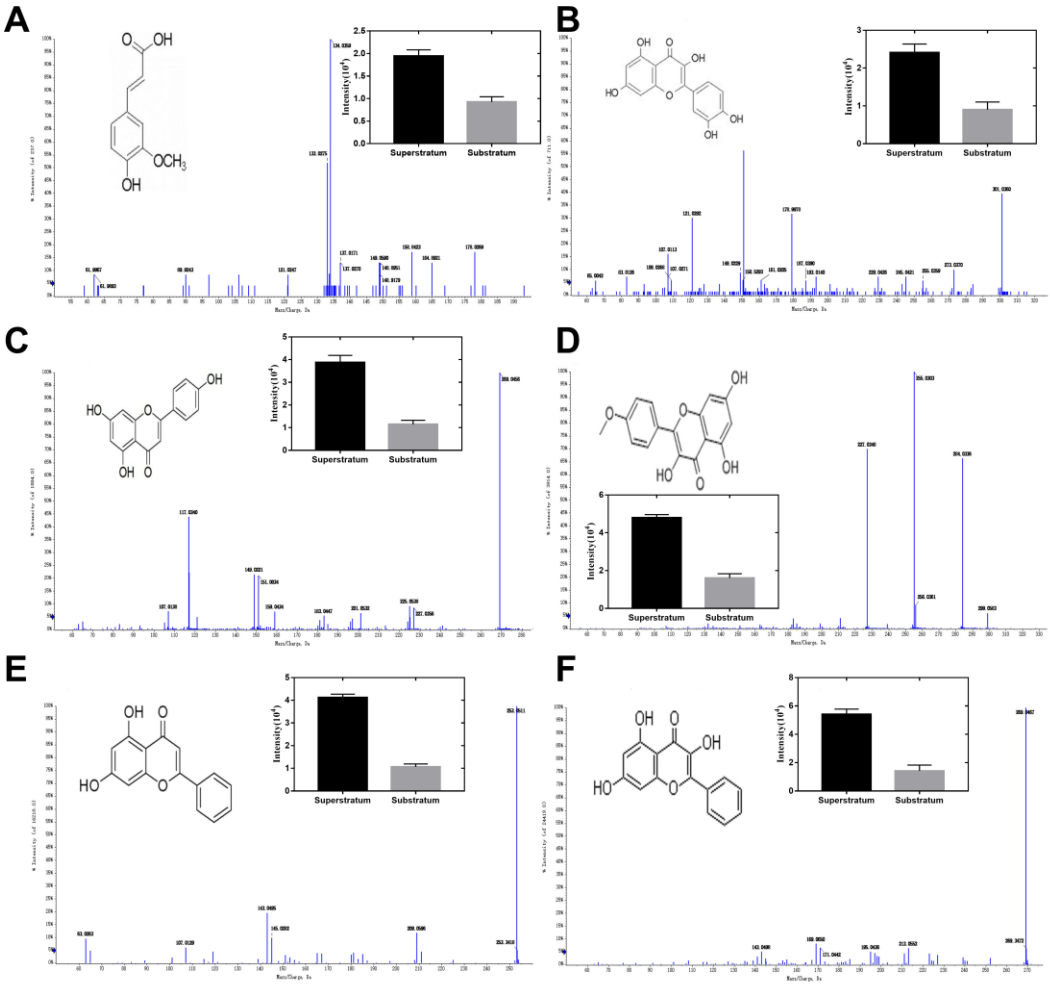

Figure2. Ion chromatogram of flavonoids in propolis

3.3 Serum IgM and IgG subclasses detected by Elisa assay.

As illustrated in Fig. 3A, on days 7 and 35 after vaccination, IgM level of PA group was higher than those of other groups; while on days 14 and 21 after

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vaccination, IgM level of PA group only was numerically higher than the other adjuvant groups, and higher than that of BC group.In addition,IgA serotype is monomer, and the immune function is weak.The IgA level of all groups have not significant difference, due to the sample was the serum(Figure 3B). IgG1 levels in three adjuvant groups were higher than that of BC group when the samples were collected after immunization. On days 7, 14, and 35 after vaccination, IgG1 levels of PA and OA groups were higher than that of BC group; on day 21 after vaccination, IgG1 level of PA group was higher than that of BC group(Figure 3C).PA triggered stronger IgG2 level than the OA and BC groups on days 14, 21 and 35, respectively(Figure 3D).The IgG3 level was higher in the pigs of PA group than those of the pigs immunized with other adjuvant groups. However, no significant difference was observed for the responses of IgG3 between OA and BC groups (Figure 3E). As illustrated in Fig 3F, on days 7 and 35 after vaccination, IgG4 level of PA group was higher than those of other adjuvant and BC groups; while on days 14 and 21 after vaccination, IgG4 level of PA group was only numerically higher than the other adjuvant groups, and higher than that of BC group.

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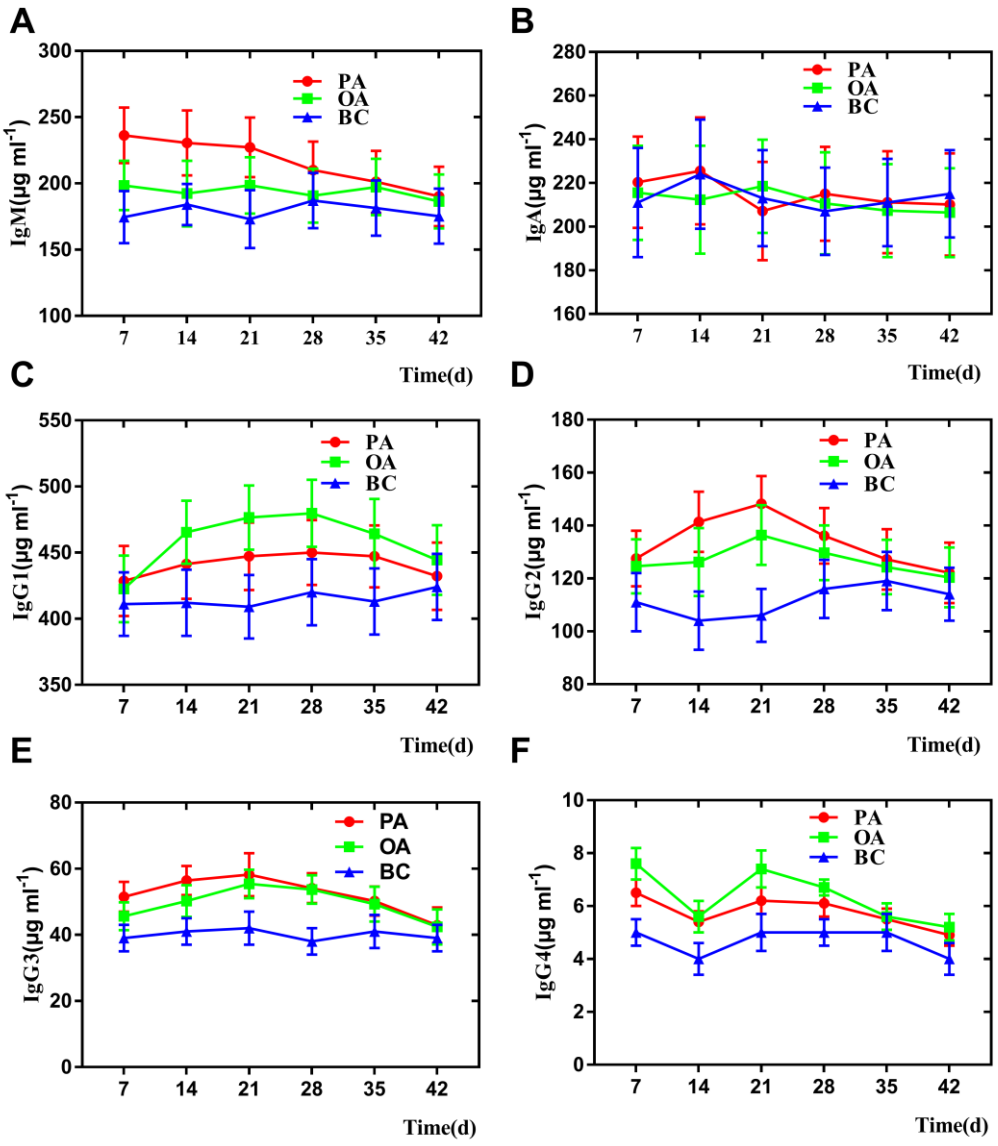

Figure3. The change of serum IgM and IgG in each group

3.4 PF promoted the activity of peripheral lymphocyte proliferation

The lymphocyte proliferation activities are shown in Table 2. In all adjuvant groups, the peak values of lymphocyte proliferation activity were on day 7 after vaccination. The A570 values in PA group were significant highest and significantly higher than those of the other two adjuvant groups from days 7 to 21 . On day 35, the A570 values in three adjuvant groups were significant higher than that of BC group.

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Table 2 The changes in lymphocyte proliferation of blood (A_{570} value)

Group	D_7	D_{14}	D_{21}	D_{35}
PA	0.158±0.015[a]	0.140±0.013[a]	0.142±0.009[a]	0.133±0.010[a]
OA	0.120±0.010[b]	0.127±0.015[b]	0.137±0.012[b]	0.130±0.027[a]
BC	0.100±0.021[c]	0.108±0.012[c]	0.120±0.013[c]	0.112±0.012[b]

PA= propolis adjuvant, OA= oilemulsion adjuvant, BC=Blank control, the same as follows.

[a – c] Data within a column without the same superscripts differ significantly (p<0.05).

3.5 Serum antibody titer

The serum HI antibody titers of each group are shown in Table 3. As compared with the BC group, the antibody titers in all vaccination groups increased (p<0.05) from days 7 to 35. On days 7 and 14 after vaccination, the antibody titers in PA and OA groups were significant higher than that of AA groups. On days 21, 28, 35 and 42, the antibody titers in PA group were significant lower than those of OA group, while significant higher than those of AA group.

Table 3 The dynamic variation of HI antibody titer after vaccination (log_2)

Group	D_0	D_7	D_{14}	D_{21}	D_{28}	D_{35}	D_{49}
PA	0±0[a]	4.5±0.4[a]	7.4±0.5[a]	7.5±0.4[b]	7.8±0.7[b]	7.3±0.5[b]	6.5±0.5[b]
OA	0±0[a]	4.3±0.5[a]	7.3±0.5[a]	8.5±0.6[a]	8.8±0.5[a]	8.3±0.7[a]	7.5±0.7[a]
BC	0±0[a]	0±0[c]	0±0[c]	0±0[c]	0±0[c]	0±0[c]	0±0[c]

[a - c] Data within a column without the same superscripts differ significantly (p<0.05).

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3.6 Serum cytokine level detected by Elisa assays

Serum cytokine levels were listed as Figure 4.The serum IL-2,IL-4 and IFN- γ levels in three adjuvant groups were significant higher when compared with the BC group(Figure 4A,4B and 4F).Interestingly, the serum IL-6,IL-10,IL-12 levels in three groups have no significant higher when compared with the BC group(Figure 4C,4D and 4E).IL-6 and IL-12 could promote the express level of TNF-α.In addition.In spire of this found,we think PF could immune enhancement in vivo.

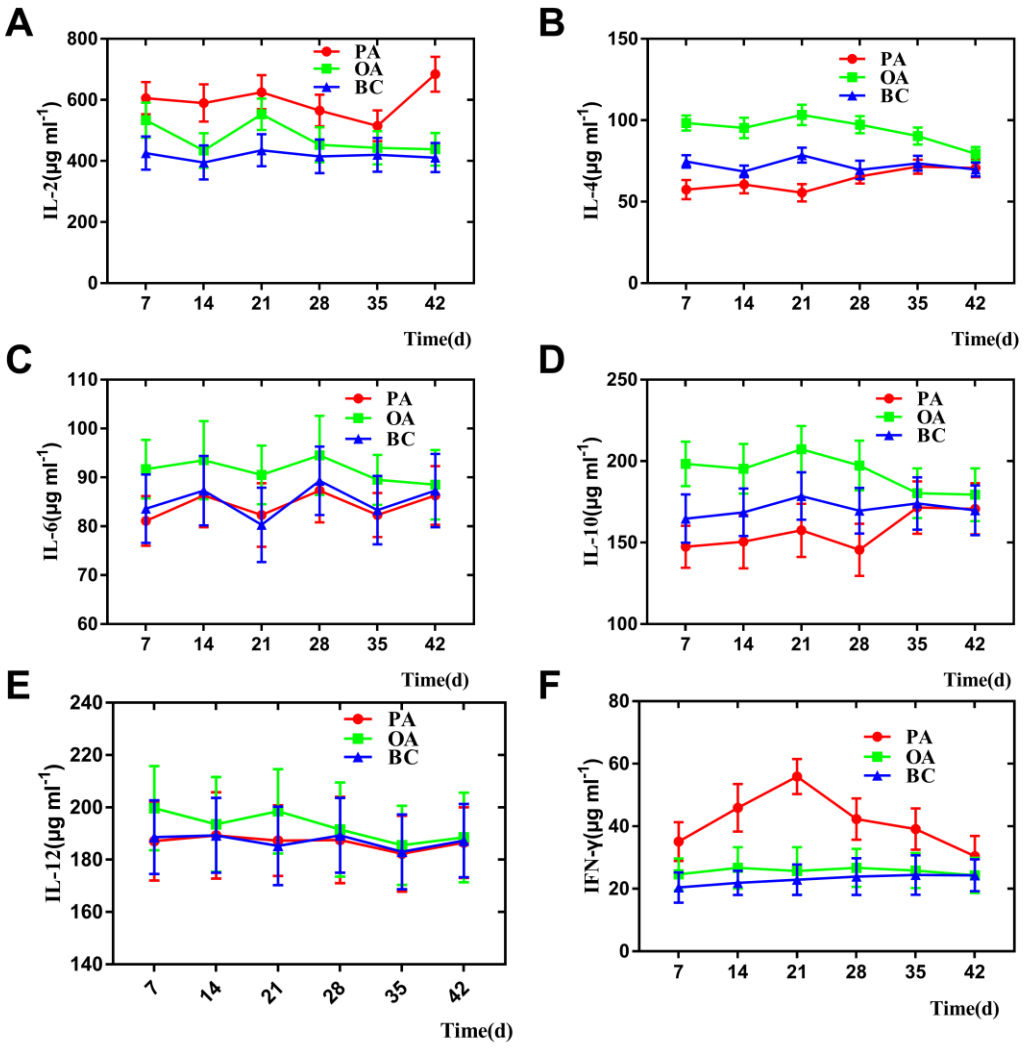

Figure4.The change of serum cytokine in each group

3.7 Screening of flavonoids in propolis against PPV in vitro

As shown in Figure 5A and 5B, Ferulic Acid, Chrysin, Kaempferol and Galangal

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had a good inhibitory effective on PPV-induced PK-15 cells,but Ferulic Acid and Chrysin had the best effective.

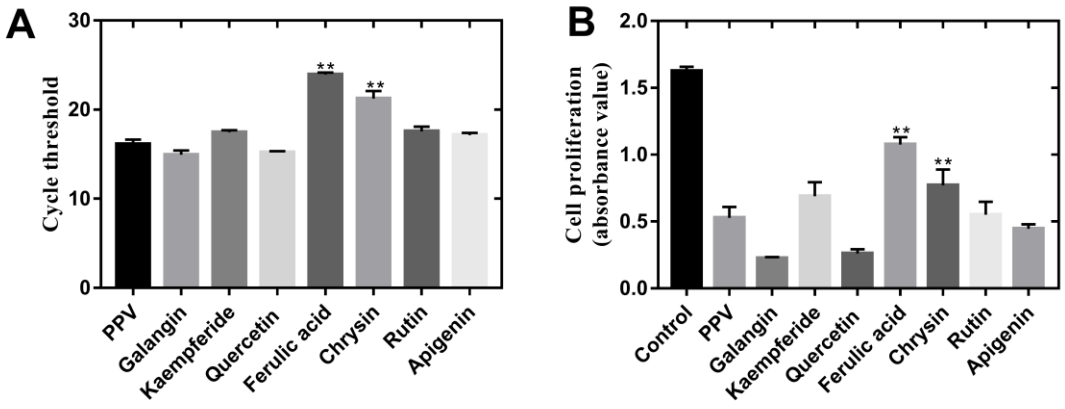

Figure5.PF isolated from propolis inhibited PPV-induced cell death of PK-15 cells

4.Discussion

Propolis is an important medicinal material with a variety of pharmacological activities. The chemical analysis of Propolis alcohol extract revealed the presence of flavonoids compounds.The traditional method of extracting and purifying traditional Chinese medicine extract first, and then identifying the structure by NMR after the monomer is obtained is complicated and requires a large amount of work. UPLC-MS techniques combined with HPLC rapid high separation ability and high sensitivity and high accuracy of high resolution mass spectrometry, to avoid the disadvantages of traditional component separation analysis method, not only can fast qualitative analysis of complex components, but also has the characteristics of simple operation, large amount of information, saving solvent, thus increasing in the study of Chinese herbal medicine composition.

Current study revealed that PF from propolis had antivirus activity and immune enhancement and antioxidant activity. PF enhances immune responses in various ways such as formation of the immunostimulating complex, activation of helper and cytotoxic T cells.In addition to stimulating the humoral immune response, PF also

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increased cellular immune responses.[26-27] In this study, PF was various in regards to their adjuvant effects on the immune responses stimulated the immune system cells to produce cytokines. As shown in Fig 4, PF significantly increased the production of both Th2 cytokines IL-4 and Th1 cytokines IL-2 and IFN-γ, which suggested that PF simultaneously elicited a Th1 and Th2 immune response. These findings are consistent with Blonska's study which demonstrated that PF could enhance the activity of T lymphocyte, promote the secretion of some cytokines, such as IL-2 and TNF-α, thus improve immune function of organism.[28]

Th1 activation contributes to cell-mediated immunity whereas Th2 activation favours the humoral immune response.[29] Th1/Th2 balance is a prerequisite for the functionality of immune system against infections. PF immunomodulatory action has been widely investigated lately, both in vitro and in vivo.[30-31] PF has been suggested to be a promising adjuvant substance in duck inactivated vaccines.[32] Important functional properties of immune cells are their capability to synthesize and secrete soluble polypeptide factors referred to as cytokines. Most cytokines are secreted and then bind to specific receptors on the surface of target cells. Upon binding they act to regulate growth and/or differentiation and to optimize the immune response. Using a Polish sample, Ansorge[33] found that propolis has immunoregulatory effects that may be mediated by Erk2 MAP kinase signals that promote cellular growth. Oršolić suggested that propolis stimulated macrophages thus influencing specific and non-specific immune defence mechanism [34]. Activation of macrophages is important for the modulating property of tested compounds, since it leads to the production of factors regulating activities of B and T cells. During a T cell dependent immune response, there is a progressive change in the predominant immunoglobulin class of the specific antibody produced. This change, isotype switch, is influenced by T cells and their cytokines. IL-4 preferentially switches activated B cells to the IgG1 isotype (Th2 type immune response), IFN-γ enhances IgG2 and IgG3 responses (Th1 type)[35-36].

In summary, PF significantly increased the serum levels of IgG subclasses, as well

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as T lymphocyte proliferation when administered in pigs with an inactivated vaccine against PPV. The enhanced IgG subclasses levels paralleled the increased serum levels of IFN-γ and IL-2.This adjuvant activity was evident in the increase in both cellular and humoral immune responses. In addition, PF could inhibit PPV-induce apoptosis by immune enhancement.Interestingly, FA as a PF components significance inhibited the PPV replication.And FA is a potential antiviral adjuvant, which could widely use in veterinary clinical.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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