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Immunomodulatory effects of *Pimpinella anisum* L. (Aniseed) in Broiler Chicks against Newcastle Disease and Infectious Bursal Disease Viruses

[Efecto inmunomodulador de Pimpinella anisum L. (anís) en pollos de engorde contra la Enfermedad de Newcastle y la enfermedad viral de la Bursitis infecciosa)]

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Abstract: *Pimpinella anisum* L. (Aniseed) is mostly used as an immune stimulant, growth promoter, antifungal, antibacterial in many countries for centuries. The aim of this study was to determine the immunomodulatory effect of aniseed against Newcastle Disease (ND) and infectious bursal disease (IBD) viruses. The immunomodulatory effect of aniseed against ND and IBD viruses were determined by modifying splenic cell migration inhibition assay and differential leukocyte count for cellular immunity. Haemagglutination inhibition and indirect haemagglutination were used for measurement of humoral immune response against ND and IBD viruses, respectively. The present study suggests that the aniseed addition to basal diet at the rate of 0.5 g/kg and 1 g/kg of feed had best immunomodulatory activity both for humoral and cellular immune responses. However, at higher doses aniseed had adverse effects. Aniseed possesses significant immunomodulatory activity when it is added at lower doses i.e., 0.5 g/kg and 1 g/kg.

Keywords: *Pimpinella anisum* L., aniseed, immunomodulation, Newcaslte disease, infectious bursal disease

Resumo: *Pimpinella anisum* L. (Anís) se utiliza principalmente como un estimulante inmunológico, promotor del crecimiento, antifúngico, y antibacteriano, en muchos países durante siglos. El objetivo de este estudio fue determinar el efecto inmunomodulador de anís contra la enfermedad de Newcastle (ND) y la enfermedad de la bursitis infecciosa (IBD). El efecto inmunomodulador de anís contra los virus ND y e IBD se determinaron mediante la modificación del ensayo de inhibición de la migración de células del bazo y recuento diferencial de leucocitos de la inmunidad celular. La inhibición de la hemaglutinación y hemaglutinación indirecta se utilizaron para la medición de la respuesta inmune humoral contra el virus de ND e IBD, respectivamente. El presente estudio sugiere que la adición de anís a la dieta basal a la tasa de 0,5 g/kg y 1 g/kg de alimentación tuvo una mejor actividad inmunomoduladora tanto para las respuestas inmunes humorales como celulares. Sin embargo, a dosis más altas de anís tuvo efectos adversos. El anís posee una importante actividad inmunomoduladora cuando se añade en dosis más bajas, es decir, 0,5 g/kg y 1 g/kg.

Palabras clave: *Pimpinella anisum* L., anís, inmunomodulación, enfermedades Newcastle, bursitis infecciosa.

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INTRODUCTION

Plants and herbs (their extracts and whole products) are being used for medication against various diseases worldwide. The role of plants and their products is important in maintaining human health, improving the quality of human life and maintain animal performance (Osman *et al*., 2005). Feeding drug-dietary supplements or probiotics to birds is another novel approach to improve their intrinsic defense mechanisms (Masood *et al*., 2013). The most of the aromatic plants, herbs and their extracts consists of flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saponins, sterols, curcumins, and phthalates have been extensively used to treat different diseases both in animals and human beings (Craig, 1999).

Aniseed (*Pimpinella anisum* L.), is an aromatic plant mostly found in Iran, India, Turkey, Pakistan and many other countries having a suitable climate for this plant. Aniseed oil contains anethole as active ingredient and various important chemicals like methylchavicol, eugenol, anisaldehyde and estragole. The World Health Organization reported that more than 80% of the earth's living depends upon traditional medicine for their primary health requirements and mostly use plant extracts or their active materials as treatment (Mehmet *et al*., 2005). Aniseed, a medicinal plant can also be used to enhance the function of the digestive system and it can be administered for deworming (Cabuk *et al*., 2003), antimicrobial (Osman *et al*., 2005), antifungal (Soliman & Badea, 2002) and antipyretic effects (Afifi *et al.*, 1994).

Due to cross-resistance of pathogens against antibiotics and their residual and harmful effects in tissues, researchers have suggested for alternative herbal approaches. The aromatic plants, herbs and their essential oils have become more important due to their multiple functions including antioxidants, antiplatelet, antitumor and immune-stimulating properties (Valero & Salmeron, 2003; Raziq *et al*., 2012; Mushtaq *et al*., 2012). Aniseed is very popularly used product for human use to improve the digestion, as a dewormer an in ruminants to treat the ailments of indigestion and parasite associated problems (Mahmood *et al*., 2014).

Poultry sector is one of the major and swiftly growing segments in Pakistan (Awais & Akhter, 2012; Eila *et al*., 2012; Javaid *et al*., 2012). It generates employment (direct/indirect) for both male and female of rural as well as urban areas (Islam *et al*., 2012).

The poultry in Pakistan specifically, but worldwide in general encounters many immunosuppressive factors. They need to administer immunostimulants along with vaccinations because vaccines are not only expensive, but their availability in downtowns is not paced sumptuously. Keeping the safety and efficacy of herbal products and medicinal value of aniseed in view the present study aimed to determine the immunomodulatory effects of aniseed on humoral and cellular immune responses.

MATERIALS AND METHODS

Plant material

The seeds of *Pimpinella anisum* (L.) were purchased from the local herbal market and identified for authentication by a botanist in the department of Botany, University of Agriculture, Faisalabad-Pakistan and powdered using an electric grinder.

Test animals

A total of 300 day-old broiler chicks were purchased from a local hatchery. The birds were kept in a clean, well ventilated and disinfected shed. The duration of the experiment was 42 days. Feed and water were given to all groups *ad-libitum*. All biosecurity measures were adopted according to standard protocol. After one week of acclimatization, the chicks were divided into five equal groups A-E randomly having twenty birds in each pen with three pens (replicates) per treatment. Treatments were randomized within blocks. The chickens of all the groups were immunized against ND and IBD viruses.

Treatment

The chickens of allocated groups were given aniseed at different ratios as follows:

The aniseed was given to birds with basal diet at 0.5, 1.0, 1.5 and 2.0 g/kg diet to all the replicates groups A- D, respectively. The chickens of group E were kept negative control. The treatment was continued from 7^{th} to 42^{nd} days of age.

Parameter to study

Humoral immune response

The blood samples were collected to determine the antibody titer using haemagglutination inhibition and indirect haemagglutination test to determine the humoral immune response against Newcastle disease virus and infectious bursal disease virus, respectively

(Soltan *et al.*, 2008). For this purpose, five birds from each experimental group were slaughtered at 14, 24, 34 and 42 days of age. The blood was collected and kept in refrigerator for 3-4 hours. Serum was collected in plastic vials and frozen at -20° C for further analysis. The sera were used for the antibody titration and Geometric Mean Titer (GMT) was calculated (Benjamin, 1978).

Cellular immune response

Differential leukocyte count

Blood smears were made by using a drop of blood on clean microscopic glass slides and the smears were fixed with methanol. Slides were stained using Giemsa's stain (Benjamin, 1978). A total of 100 leukocytes was counted and their percentage was calculated under oil immersion lens and categorized as monocytes, lymphocytes, eosinophils, basophils and hetrophils.

Modified splenic cell migration inhibition assay

Spleen collected at 7th day post vaccination were used in the modified splenic cell migration inhibition test to determine the cellular immune response following the method of Morita *et al*. (1973) with some modification as described by Akhtar *et al.* (1999). Briefly, spleens were immersed immediately in the PBS after removal from the birds and were labeled separately and minced into small pieces (0.3-0.5 mm) with the help of a pair of sterilized scissors in a sterilized Petri-dish containing HBSS.

Statistical analysis

Data were analyzed by randomized complete block design two factorial analysis of variance and further compared with LSD.

Table 1
Lymphocytes (A), Monocytes (B), Eosinophils (C), Heterophils (D) and Basophils (E) percentage of broiler chicks treated with aniseed

Table 1A

Treated Groups	Lympocytes (%age)			
	1st	2nd	3rd	4th
A	64.50 ± 0.65^{bc}	74.00 ± 2.74^{a}	75.50 ± 1.85^{a}	71.00 ±1.96^{a}
B	64.50 ± 1.19^{bc}	71.00 ± 1.58^{ab}	70.50 ± 1.66^{b}	71.25 ±2.39^{a}
C	65.25 ± 1.32^{bc}	65.75 ± 3.01^{c}	65.50 ± 2.90^{c}	65.75 ±2.21^{b}
D	61.75 ± 2.78^{d}	64.75 ± 2.06^{d}	58.75 ± 1.55^{d}	60.25 ±1.11^{c}
E	62.25 ± 1.32^{cd}	69.50 ± 2.50^{ab}	59.50 ± 0.65^{d}	59.50 ±1.66^{c}

Mean ± SE showing the same superscript within a column differs non-significantly from each other. 1st, 2nd, 3rd and 4th in the columns mean sampling on day 14, 24, 34 and 42 of age, respectively

Table 1B

Treated Groups	Monocytes (%age)			
	1st	2nd	3rd	4th
A	8.25 ± 1.11^{a}	6.00 ± 1.08^{bc}	8.50 ± 0.65^{a}	8.25 ± 1.11^{a}
B	6.50 ± 0.65^{ab}	6.00 ± 0.91^{bc}	7.25 ± 0.85^{ab}	7.50 ± 1.19^{ab}
C	6.25 ± 1.11^{ab}	6.00 ± 1.47^{bc}	5.75 ± 0.85^{c}	6.50 ± 1.04^{c}
D	5.00 ± 0.91^{bc}	5.50 ± 0.65^{bc}	3.50 ± 0.65^{c}	3.50 ± 0.29^{c}
E	5.75 ± 1.25^{bc}	5.75 ± 0.85^{bc}	3.50 ± 0.65^{c}	4.50 ± 0.65^{bc}

Mean ± SE showing the same superscript within a column differs non-significantly from each other. 1st, 2nd, 3rd and 4th in the columns mean sampling on day 14, 24, 34 and 42 of age, respectively

Table 1C

Treated Groups	Eosinophils (%age)			
	1st	2nd	3rd	4th
A	3.25 ± 0.48^{cd}	4.00 ± 0.41^{cd}	5.50 ± 0.65^{ab}	6.00 ± 1.08^{a}
B	3.00 ± 0.41^{cd}	4.25 ± 0.48a^{cd}	6.00 ± 0.91^{a}	5.50 ± 0.65^{ab}
C	2.75 ± 0.48^{cd}	4.50 ± 0.65^{cd}	5.50 ± 0.65^{ab}	5.00 ± 0.71^{bc}
D	3.00 ± 0.71^{cd}	3.50 ± 0.65^{cd}	3.75 ± 0.48^{cd}	3.25 ± 0.75^{cd}
E	2.50 ± 0.65^{d}	3.25 ± 0.63^{cd}	3.25 ± 0.48^{cd}	2.75 ± 0.75^{d}

Mean ± SE showing the same superscript within a column differs non-significantly from each other. 1st, 2nd, 3rd and 4th in the columns mean sampling on day 14, 24, 34 and 42 of age, respectively

Table 1D

Treated Groups	Heterophils (%age)			
	1st	2nd	3rd	4th
A	29.50 ± 1.56^{c}	27.50 ± 1.85^{cd}	25.25 ± 1.11^{e}	24.50 ± 1.04^{f}
B	29.25 ± 0.85^{cd}	29.25 ± 0.85^{cd}	24.75 ± 0.85^{f}	23.50 ± 1.04^{g}
C	28.50 ± 2.40^{de}	30.50 ± 0.65^{b}	29.50 ± 0.65^{c}	26.00 ± 1.08^{d}
D	31.00 ±1.47^{ab}	28.50 ± 0.65^{bc}	26.50 ± 1.19^{cd}	25.50 ± 1.32^{e}
E	32.00 ± 1.08^{a}	28.50 ± 0.65^{bc}	26.00 ± 1.08^{d}	26.00 ± 1.08^{d}

Mean ± SE showing the same superscript within a column differs non-significantly from each other. 1st, 2nd, 3rd and 4th in the columns mean sampling on day 14, 24, 34 and 42 of age, respectively

Table 1E

Treated Groups	Basophils (%age)			
	1st	2nd	3rd	4th
A	2.00 ± 0.41^{a}	1.75 ± 0.48^{b}	2.00 ± 0.41^{a}	2.25 ± 0.63^{a}
B	2.00 ± 0.41^{a}	2.00 ± 0.41^{a}	2.25 ± 0.25^{a}	2.50 ± 0.65^{a}
C	1.75 ± 0.48^{b}	1.75 ± 0.25^{b}	1.75 ± 0.48^{b}	2.25 ± 0.25^{a}
D	1.50 ± 0.29^{b}	1.50 ± 0.29^{b}	1.25 ± 0.25^{c}	1.75 ± 0.48^{b}
E	1.50 ± 0.29^{b}	1.25 ± 0.25^{c}	1.25 ± 0.25^{c}	1.25 ±0.25^{c}

Mean ± SE showing the same superscript within a column differs non-significantly from each other. 1st, 2nd, 3rd and 4th in the columns mean sampling on day 14, 24, 34 and 42 of age, respectively

RESULTS

Differential leukocytes count

On the 14th day Mean ± SE value of lymphocytes and monocytes in groups A and B was non-significantly different from each other but increased from other groups. However, on the 24th day of the experimental period the lymphocyte count of groups A and B was significantly greater (Table 1 A-E). The Mean ± SE value of eosinophils in group A was significantly greater as compared to all other groups. The Mean ± SE value of heterophils in groups D and E was significantly greater as compared to all other groups. While the number of hetrophils in groups A, B and C were non-significantly different from each others. Basophils in groups A and B were significantly raised as compared to all other groups.

Humoral immune response

On day 14th Mean values of antibody titers against ND and IBD (1st sampling) were non-significantly different from each other in groups A, B, C and significantly increased from group E. At 24th day (2nd sampling) the antibody titer against ND was significantly greater in groups A and B as compared

to all other groups. In groups C and D titer was non-significantly different from each other, but non-significantly greater than group E. On day 34th (3rd sampling) antibody titer was similar in groups A, B, C and greater than groups D and E, whereas differences in antibody titers between groups D and E was non-significant. At 42nd day (4th sampling) antibody titer was significantly higher in groups A and B as compared to all other groups, while an antibody titer in groups C and D was non-significantly different from each other and non-significantly greater than group E (Table 2).

Table 2A
Antibody titer against ND and IBD viruses in broiler chickens treated with aniseed

Sampling	HI				
	A	B	C	D	E
1st (14th days)	7.00 ± 0.41[b]	7.00 ± 0.41[b]	6.75 ± 0.25[b]	6.75 ± 0.25[b]	6.50 ± 0.29[c]
GMT	125.89	125.89	105.92	105.92	89.12
2nd (24th days)	8.50 ± 0.29[a]	8.25 ± 0.25[a]	7.50 ± 0.29[b]	7.25 ± 0.25[b]	6.25 ± 0.48[c]
GMT	177.83	149.62	89.12	89.12	74.98
3rd (34th days)	7.50 ± 0.29[b]	7.25 ± 0.25[b]	7.25 ± 0.25[b]	6.50 ± 0.29[c]	5.75 ± 0.25[c]
GMT	177.83	149.62	149.62	89.12	53.09
4th (42nd days)	7.25 ± 0.25[b]	7.00 ± 0.41[b]	6.50 ± 0.29[c]	6.25 ± 0.25[c]	5.50 ± 0.29[d]
GMT	149.62	125.89	89.12	74.99	44.67

Table 2B
Antibody titer against ND and IBD viruses in broiler chickens treated with aniseed

Sampling	IHA				
	A	B	C	D	E
1st (14th days)	7.75 ± 0.48[b]	7.50 ± 0.29[b]	8.00 ± 0.41[a]	6.00 ± 0.41[bc]	5.00 ± 0.41[c]
GMT	105.92	89.12	125.89	53.09	31.62
2nd (24th days)	9.00 ± 0.41[a]	9.25 ± 0.25[a]	8.25 ± 0.25[a]	6.75 ± 0.25[b]	6.00 ± 0.41[bc]
GMT	125.89	149.62	149.62	74.99	63.09
3rd (34th days)	8.75 ± 0.25[a]	8.50 ± 0.29[a]	7.50 ± 0.29[b]	6.75 ± 0.25[b]	6.50 ± 0.29[bc]
GMT	211.35	177.83	89.12	74.99	74.98
4th (42nd days)	7.75 ± 0.25[b]	7.25 ± 0.25[b]	6.75 ± 0.20[bc]	6.25 ± 0.25[bc]	5.75 ± 0.25[c]
GMT	149.62	125.89	89.12	74.99	53.09

Migration index

The mean migration distance from the edge of the splenic fragment with and without antigen in group A was significantly higher followed by group B, C, D and E, respectively. There was a nonsignificant difference of migration index of splenic cells in group B and C as compared to each other (Table 3).

DISCUSSION

The number of lymphocytes in the present study was significantly raised in groups A and B compared to all other groups. The results of our study regarding increases in lymphocyte cell number are in accordance with previous reports (Merz *et al*., 1981; Adel & Sahar, 2003; Ziaran *et al*., 2005; Kong *et al*., 2006). The similar findings were also observed in rats by the (Ivanovska *et al*., 1995). The monocytes population at day 14 in group A was significantly higher as compared to all other groups and also at day 34 the value of monocytes significantly raised in groups A and B. On the 42nd day of experimental period the number of monocytes was significantly higher in groups A and B as compared to C, D and E groups, however, our findings are in contrast to the reports of Ziaran *et al*. (2005). At day 14 in groups D and E the hetrophils number was significantly grater as compared to all other groups. Our observations regarding hetrophils are similar to the findings of

previous researchers (El-Deek *et al*., 2001; Ziaran *et al.,* 2005) who reported that the hetrophils number increases in untreated birds along with higher doses. The value of basophils at 14th day in groups A and B was significantly higher as compared to other groups. Mean values of antibody titers against ND on 24th day was significantly greater in groups A and B. The findings of this study are similar to the results of previous studies (Nie & Zhang, 1999; Chen *et al*., 2002; Guo *et al*., 2004, Jiang & Yu, 2005) who found that plant extracts in feed improve the humoral immune response against ND. At 42nd day antibody titers were significantly higher in groups A and B. These results are in contrast to the findings of previous researchers (Al-Ankari *et al*., 2004; Durani *et al*., 2007; Soltan *et al*., 2008) who found non-significant increase in antibody titer against after a feed of aniseed with basal diet. Ziaran *et al*. (2005) reported that lower doses of plant extract had a positive antibody titer against ND, while the negative effects at higher levels. At 24th day the antibody titer against IBD was significantly greater in groups A, B and C. On day 34 antibody titer against IBD was similar in groups A and B but greater than group C and D. Zho *et al*. (1993), Ilsley *et al*. (2005), Kong *et al*. (2006), Dong *et al*. (2007) found that addition of plant extracts in broiler feed enhanced the immunity, which supports our findings. These results are in contrast to the findings of Durani *et al*. (2007) who disclosed that lower doses of aniseed given to broiler birds had non-significant effect on antibody titers against IBD. However, our findings are in line to the investigation of Durani *et al*. (2007) at higher levels. At 42 day antibody titers against IBD significantly raised in groups A and B as compared to all other groups, while an antibody titer against IBD in groups C and D was non-significantly different from each other. At day 7 the mean migration distance from edge of splenic fragment was significantly greater in group A followed by the groups B, C and D. The results of splenic cell migration inhibition essay in the present study are similar to the findings of previous studies (Taki *et al*., 2003; Lee *et al*., 2005; Lee *et al*., 2007; Lee *et al*., 2008).

Table 3
Splenic cell migration inhibition assay on 7th and 14th days post-vaccination

Groups	7th day post vaccination			14th day post vaccination		
	Migration distance with antigen (µm)	Migration distance without antigen (µm)	Migration Index (%)	Migration distance with antigen (µm)	Migration distance without antigen (µm)	Migration Index (%)
A	16.50 ± 1.35^{b}	25.56 ± 1.58^{a}	64.55	18.15 ± 0.95^{b}	28.05 ± 0.95^{a}	64.71
B	14.85 ± 0.95^{c}	23.93 ± 0.83^{ab}	62.06	14.85 ± 0.95^{c}	23.93 ± 0.83^{a}	62.06
C	14.85 ± 0.95^{c}	24.75 ± 0.95^{ab}	60.00	16.50 ± 1.35^{b}	28.05 ± 0.95^{a}	58.82
D	13.20 ± 1.35^{c}	23.93 ± 0.83^{ab}	55.16	13.20 ± 1.35^{c}	23.93 ± 0.83^{ab}	55.16
E	13.20 ± 1.35^{c}	27.23 ± 0.83^{a}	48.48	13.20 ± 1.35^{c}	26.40 ± 1.35^{a}	50.00

Mean ± SE showing the same superscript with in a column row differs non-significantly from each other
A: Group treated with aniseed @ 0.5 g/kg; B: Group treated with aniseed @ 1.0 g/kg
C: Group treated with aniseed @ 1.5 g/kg; D: Group treated with aniseed @ 2.0 g/kg
E: Group kept as control

In conclusion, based on the finding of the present study, we suggest that addition of aniseed at the rate of 0.5 g/kg to 1 g/kg affects the broiler chickens as immunomodulant particularly against ND and IBD viruses. The same product may be tested for its efficacy against other viruses too.

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