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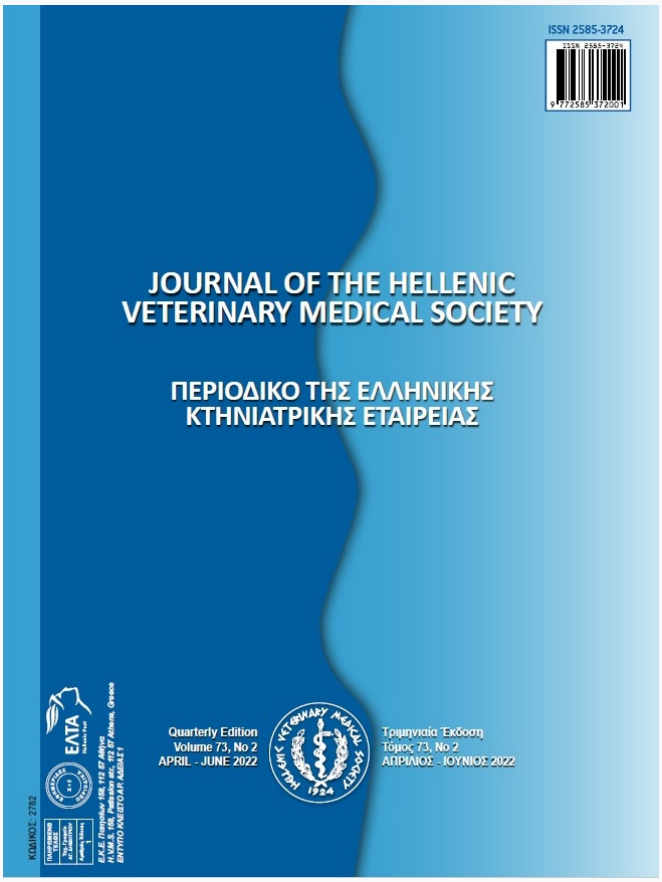
Journal of the Hellenic Veterinary Medical Society

Vol 73, No 2 (2022)



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doi: 10.12681/jhvms.26157

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To cite this article:

Talazadeh, F., Mayahi, M., & Fathi, M. (2022). Evaluation of immunostimulatory effects of a commercial herbal extract on avian influenza subtype H9N2 and Newcastle disease vaccination in chickens. *Journal of the Hellenic Veterinary Medical Society*, *73*(2), 4023–4030. https://doi.org/10.12681/jhvms.26157

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J HELLENIC VET MED SOC 2022, 73 (2): 4023-4030
ΠΕΚΕ 2022, 73 (2): 4023-4030

Research article
Ερευνητικό άρθρο

Evaluation of immunostimulatory effects of a commercial herbal extract on avian influenza subtype H9N2 and Newcastle disease vaccination in broilers

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ABSTRACT: The ability of herbal extracts to improve the immune system supports their use as immune stimulants. The present study aimed to examine the effects of barley malt extract in drinking water on humoral immunity of broiler chickens against ND and Avian Influenza (AI) disease subtype H9N2 vaccines. A total of 225 one-day-old broiler chicks (Ross 308) strain were divided into 5 groups of 3 subgroups and each subgroup had 15 chicks. Group A, B, and C chickens received 0.2%, 0.3%, and 0.5% of malt extract respectively in drinking water. Group D chickens did not get malt extract. Group E chickens did not receive malt extract and Newcastle and AI vaccines as the control group. All groups except group E were vaccinated with live Newcastle vaccine (B1 strain) intraocularly and AI-ND subtype H9N2 killed vaccine subcutaneously on the 7th day. Antibody titer against NDs and AI vaccines was considered by the Hemagglutination Inhibition test (HI test). Malt extract at 0.5% concentration, at all periods after vaccination, enhanced the systemic antibody response to ND vaccine in broiler chickens, but this extract had no significant effect on antibody response against the AI vaccine.

***Conclusion**:* Inoculation of ND vaccines with barley malt extract as an immune-boosting agent induces extensive immune responses involved in HI-NDV Ab titers**.**

Keywords: Commercial plant extract,ND vaccine (NDV), AI Virus (AIV), immune response, broiler chickens.

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Date of initial submission: 15-02-2021
Date of acceptance: 18-10-2021

INTRODUCTION

Influenza viruses are segmented negative-sense RNA viruses and are a member of the family Orthomyxoviridae, and they have three types, A, B, and C (Wright et al., 2007). In humans, all three types of influenza are found, but in birds, only the type A viruses can cause AI disease. H9N2 virus can infect humans. Besides, H9N2 AIV infection in chickens may be latent and easily neglect, and so has more chance to infect humans. In chickens and turkeys, clinical symptoms include disorders in the respiratory, reproductive, digestive, and urinary systems. In layers and breeders, may decrease the production of eggs and increase broodiness. Clinical symptoms in domestic poultry include dropped activity, decreased consumption of feed and water, diarrhoea, ruffled feathers, huddling, listlessness, and lethargy (Swayne and Halvorson, 2008). H9N2 is a low pathogenic avian influenza (LPAI) and two H5 and H7 subtypes are highly pathogenic viruses or highly pathogenic avian influenza (HPAI) in poultry (OIE, 2015). Despite its low pathogenicity, if H9N2 influenza outbreaks are caused by concurrent bacterial and viral infections, they will be very lethal and lead to economic losses due to reduced egg production and reduced feed intake. Moreover, it is the most important sub-type of influenza in poultry in endemic countries (Thuy et al., 2016).The H9N2 influenza virus is the most common subtype isolated from non-aquatic birds in Asia and Europe. This disease is endemic to the poultry industry in the Middle East and Asia, including Iran, and causes significant damages (Nili and Asasi, 2003). Vaccination with a killed virus (killed AI vaccine (subtype H9N2) or AI-ND killed vaccine (subtype H9N2)) is the original choice to prevent AI, but using the vaccine alone may produce a poor antibody titer and result in some negative consequences in the vaccinated host (Talazadeh *et al.*, 2016).Newcastle disease is a fatal viral diseaseof poultry. Due to its high mortality and morbidity, which is adjusted from 90-100% regulated by the ND virus type, it is also considered as one of the most important economic threats to the poultry population (Brandly, 2010). It is an acute and contagious infection of free-living birds, pets, and domestic birds. Paramyxovirus type 1 (APMV-1) serotype causes ND (Meulemans, 1988). Lesions affecting the neurological, gastrointestinal, respiratory, and reproductive systems are most often observed. The control of ND must include strict biosecurity that prevents virulent NDV from contacting poultry, and also proper administration of efficacious vaccines (Meulemans, 1988).Safe and appropriate use of the vaccine is crucial in controlling NDV. The most commonly used ND vaccines are live vaccine viruses formulated with strains isolated in the 1940s and 1960s (Meulemans, 1988). Viruses circulating in poultry were the source of the LaSota, B1, and VG/GA vaccines. All of those viruses belong to genotype II and are genetically and antigenically highly related among themselves (>98% nucleotide identity) (Meulemans, 1988). The main differences among those vaccines are the tropism and the capacity to replicate in naïve chickens, which is highest in LaSota and results in higher levels of neutralizing antibodies compared to other strains (Meulemans, 1988). Thus, the LaSota strain is nearly always used in countries where virulent NDV is endemic (Diel et al., 2012). The VG/GA strain is normally sold as an enterotropic vaccine, and the B1 strain as the most attenuated vaccine to be used in cases of low challenges or very young birds (Meulemans, 1988).While live vaccines provide both mucosal and humoral immunity and can be administered using mass application techniques, they may cause clinical respiratory disease, drop in egg production, and are easily inactivated when not kept at the required temperature (commonly 4º C) (Winterfield and Dhillon, 1981). The second group of traditional vaccines that are widely used is vaccine strains from class II genotype I (i.e. I2, V4, and PHY-LMV42), which are avirulent and safely used in chickens of all ages (Cardenas Garcia et al., 2013). Strains of NDV that have increased stability to heat are especially advantageous in rural areas of the world with limited refrigeration. The I-2 strain has improved thermostability in comparison to the V4 ND vaccine strain it was derived from and is mainly used in areas with higher ambient temperatures (Alders, 2014). Inactivated ND vaccines have the disadvantage of requiring a withdrawal period before vaccinated birds can be processed for human consumption, and each vaccine requires individual administration by subcutaneous or intramuscular injection. Even though birds vaccinated with inactivated vaccines tend to have higher humoral antibody levels, they do not develop a strong cell-mediated response (Schijns et al., 2013), and shed larger amounts of virulent challenge virus compared to birds vaccinated with live ND vaccines (Miller et al., 2013). Although live and inactivated vaccines protect against clinical disease in SPF chickens, there are continuous reports of vaccine failures under field conditions (Rehmani et al., 2015). One of the possible reasons for these failures may be poor vaccination response

that is also dependent on field-associated factors unrelated to the vaccines, such as immunosuppression (Meulemans, 1988) from infections before ND vaccination.Vaccines that are co-expressing antigens of different pathogens and are simultaneously inducing immunity against several avian diseases would be of great value (Lancaster, 1966). In chickens with maternal immunity, the best response to live ND vaccine is achieved through conjunctival and intranasal routes of administration, perhaps due to the development of local immunity induced by these vaccines (Lancaster, 1966). However, immunity induced by inactivated vaccines was less affected by the presence of maternal antibodies (Lancaster, 1966).The immune-stimulating activity of many plant extracts has been analyzed in chicken, human, and mouse cell lines (Shan et al., 1999). The use of immune stimulants is one of the ways to strengthen the immunity of animals and reduce their sensitivity to infectious diseases (Liu, 1999). Someherbs that are full of flavonoids such as thyme (*Thymus vulgaris*) increase the activity of vitamin C, and act as antioxidants, and seem to improve immune function (Talazadeh *et al.*, 2016).Principally (80-90%) barley production is for animal feeds and malt (Giraldo *et al.*, 2019). There are increasing affections in barley yields because of their high levels of phenolic acids (cinnamic and benzoic acid), tannins, chalcones, flavanones, proanthocyanidins, flavonols, flavones, and amino phenolic compounds (Carvalho *et al.*, 2015). Malt contains different complexes from the malting process (Maillard reaction products) or barley (phenolic compounds) (Gąsior *et al.*, 2020). Due to the high levels of antioxidant contents in barley and malt, they are used as ingredients for functional food production.

While the antioxidant activity of malt or barley has been studied, there is a lack of information about the effect of barley malt extract in drinking water on the immune response against AI and ND vaccines in broiler chickens. Thus, this experiment aimed to evaluate the effects of the different doses of malt extract in drinking water on antibody response against AI and ND vaccines in broilers.

MATERIAL AND METHODS

Ethics statement

All ethical standards have been respected in the preparation of this experiment. Ethical permission was granted by the Shahid Chamran University of Ahvaz Ethical Commission for Animal Experiments.

Chickens and housing

A total of 245 one-day-old (average weight about 45 g), Ross 308 broilers were obtained from the South Sahraye Jonoob's broiler breederfarm (Pirmoradi Co., Khuzestan, Iran).

Chickens in each group were kept in floor pens with suitable temperature, light, and humidity at the department of avian medicine (the faculty of veterinary medicine of Shahid Chamran University of Ahvaz, Iran). Chickens were provided with free access to water and broiler diets and received feed (Table 1) and water ad libitum during the experiment. A standard basal diet in pellet form was formulated which was mainly composed of corn and soybean meal. The birds were reared under similar conditions from one day old to 47 days of age.

Table 1. Ingredient (%) and chemical composition of broiler diets

Ingredient	Starter diet (1-21 days)	Finisher diet (22-47 days)
Corn	55.2	60.81
Soybean meal	37.47	31.60
Soybean Oil	3	3.60
Oyster powder	1.42	1.13
Dicalcium Phosphate	1.6	1.50
Sodium chloride	0.3	0.23
Coccidiostate (Salinomycin, Robenidine)	0.05	0.05
Limestone	0.23	0.23
Mineral supplements	0.25	0.25
DL-methionine	0.23	0.24
lysine	0	0.11
Nutrient composition %		
Metabolizable energy (kcal/kg)	2969.20	3086.91
Crude protein, %	21.3	19.35
Calcium, %	1	0.85
Available phosphorus %	0.45	0.43

Barley Malt extract: Barley malt extract was acquired commercially as the solution from Gorgan Malt Zarrin Co. (Golestan province, Iran). (Table 2) https://companylist.org/Details/11547828/Iran/Gorgan_Malt_Zarin/

Table 2. Chemical analysis of barley malt extract

pH	3.8-4.2
Water soluble solid substances % (brix)	60
Reducing sugars (maltose)%	At least 45
Acidity (acid lactic)	0.6
Crud Protein%	1.5
Moisture%	38
Total solid substances%	62
Specific Weight at 20 degree	1.3
refractive index at 20 degree	1.4

Experimental design: Two hundred forty-five one-day-old broiler Ross 308 strain chickens were purchased and 20 out of them randomly bled to assess the titer of the maternal antibody for determination of the vaccination time, and the residual chickens were divided into 5 equal groups of 3 subgroups and each subgroup had 15 chicks. During the breeding period, the chicks of groups A, B, and C received 0.2%, 0.3%, and 0.5% of malt extract in drinking water, respectively. The chicks of group D did not receive malt extract but they were vaccinated against ND and AI viruses. The chicks of group E were kept as the control group and did not take malt extract, ND, and AI vaccines. The chicks of groups A, B, C, and D were vaccinated with live ND (B1 strain) intraocularly and AI-ND killed vaccine (subtype H9N2) subcutaneously into the dorsal of neck region at the age of 7 days.

Vaccination program: At 7 days of age, the chicks of groups A, B, C, and D were vaccinated with live Newcastle B_1 strain (commercial vaccines Avishield® ND B1 was provided by Genera Inc. (Croatia)) via eye-drop and, they were also injected subcutaneously with killed Newcastle + Influenza (H_9N_2) commercial vaccine (Gallimune 208 ND+ Flu H9 ME was provided by Merial Inc. (France))into the dorsal of the neck region.

Blood collecting

Ten chicks of each group bled randomly and blood samples were collected before vaccination (0 days) and also on days 14, 21, and 28 after vaccination from the brachial vein. Sera were isolated and frozen at -20˚ C until the serological trials were done. Serum samples were examined by the hemagglutination inhibition test (HI) to identify antibody titers against ND and AI vaccines (TABARI *et al.*, 2020).

Serological evaluation

Haemagglutination (HA) assay

Phosphate buffered saline (PBS) (50 µl) was added to every well in U-bottomed 96-well microtiter plates. Then, 50 µL of AI antigen was added to the first well in each row. This resulted in a 1:2 dilution of the test material. Then the test material was diluted and the contents of the first well were mixed by pipetting up and down. 50 µl from the first well was placed in the second well to make two-fold dilutions of the virus suspension across the entire row and the excess 50 µl after the last row was discarded. All wells had a final volume of 50 µl. Then 50 µl of 1% erythrocyte suspension was added to every well and was allowed 20-30 min for the erythrocytes to settle.The HA assay plate was read when the erythrocytes in the cell control wells settled to form a solid button at the bottom of the well. The endpoint of the virus titration is the highest dilution causing complete hemagglutination and considered 1 hemagglutination unit (HAU), and the number of HAUs/50 µl is the reciprocal of the highest dilution. A similar test was performed for the ND virus. 4 hemagglutinating units (4 HA units) of NDV and 4 HA units of AI viruses were used in the HI assay (Spackman, 2008).

Haemagglutination Inhibition (HI) assay

The beta procedure of the micro-plate HI test was carried out in U-bottomed 96-well microtiter plates to find out the titer of antibody in sera of different groups. One percent chicken erythrocytes were used in this test. The test was organized using constant 4 hemagglutinating units (4 HA units) of NDV and 4 HA units of AI viruses (Mohammad Mostafijur *et al.*, 2017).

Reference antigens must be standardized to a concentration of 4 HAU/50 µl.The initial concentration of undiluted reference antigen is determined by the hemagglutination assay (HA assay). The number of HAU present is equal to the endpoint of the hemagglutination titration, which is the highest dilution of the antigen/virus causing complete hemagglutination.

Serum samples (50 µl) were double diluted serially in 50 µl PBS in U-bottomed 96-well microtiter plates. Then, 4HA unit AIV (50 µl) was added to each well, and plates were incubated for 45 min at room temperature. Eventually, 1% chicken red blood cells were added and plates incubated for 30 min. HI, antibody titer of the sera was the reciprocal of the last serum dilution with haemagglutination inhibition (Talazadeh *et al.*, 2016). A similar test was performed for the ND virus with 4HA units of NDV.

Statistical analysis

SPSS Statistics 18.0 software was used for statistical analysis, and a one-way ANOVA LSD test was performed to demonstrate the significant differences in HI titers of each group of chickens after vaccination. The data were represented as mean ± SD. Differences were assessed as statistically significant if $P < 0.05$.

RESULTS

The results of table 3 indicated that 14, 21, and 28 days after vaccination, there were significant differences between all groups and group E ($P < 0.05$). . This suggests that the ND vaccines (group A, B, C, D) were able to provide protective immunity compared to the non-vaccinated control group (group E).Also at these periods, there was a significant difference between group C and group D ($P < 0.05$).Chickens that received the highest dose of malt extract, had the highest antibody titer in 14, 21, and 28 days after vaccination (C › B › A) but there was no significant difference between any doses of malt extract at all periods.

Chickens had maternal immunity at the first days of life, as seen in the results because their breeders received the killed ND-AI (H9N2) vaccine,four weeks before egg production.

The results of Table 4, indicated that 14, 21, and 28 days after vaccination, there were significant differences between all groups and group E ($P < 0.05$). This suggests that the AI vaccine (group A, B, C, D) was able to provide protective immunity compared to the non-vaccinated control group (group E).But there was no significant difference between receiving malt extract groups (A, B, C) and the non-receiving malt extract-vaccinated control group (group D). This suggests that the malt extract, at all periods after vaccination, could not enhance the systemic antibody response to AI vaccine in broiler compared to the control group (group D).

Chickens that received the highest dose of malt extract, had the highest antibody titer in 14, 21, and 28 days after vaccination (C › B › A) but there was not any significant difference between any doses of malt extract at all periods.

DISCUSSION

In the poultry industry, it is important to boost the immune system for reasons such as vaccination failure, immunosuppressive diseases, and antibiotics misuse. Today, research on substances that are likely to have immune-boosting effects is increasing. In the case of birds, which can be achieved through a combination of measures such as vaccination and the use of conventional chemical drugs has almost reached its maximum level (TABARI *et al.*, 2020). Phytotherapy, or herbalism, is defined as the usage of plants or herbs as medication to treat or prevent diseases in humans and animals (Yasmin *et al.*, 2020). The usage is gaining more attention among medical practitioners as well as large-scale livestock producers (Yasmin *et al.*, 2020). Some reports have shown the positive effects of herbal extracts as an antiviral agent used in

Table3. Effect of barley malt extract on HI antibody titer against ND vaccine

Days post-vaccination groups	**0 (** maternal antibody)	**14**	**21**	**28**
A (0.2% of malt extract)	6.75±0.63	5.11±0.35 ^{ab*}	5.71±0.41 ^{ab}	6.15±0. 39 ^{ab}
B (0.3% of malt extract)		5.21±0.44 ^{ab}	5.8±0.32 ^{ab}	6.23±0.22 ^{ab}
C (0.5% of malt extract)		5.32 ±0.26 ^{a}	5.9 ±0.54 ^{a}	6.32 ±0.24 ^{a}
D (vaccine -no malt)		5.09 ±0.35 ^{b}	5.58 ±0.27 ^{b}	6.05 ±0.53 ^{b}
E (no vaccine- no malt)		1.9 ±0.3^{c}	- ^{c}	- ^{c}

The columns which have no common superscripts are significantly different ($P < 0.05$).
* Mean of antibody titer according to log 2 ± standard deviation

Table 4. Effect of barley malt extract on HI antibody titer against AI vaccine

Days post-vaccination groups	**0 (** maternal antibody)	**14**	**21**	**28**
A (0.2% of malt extract)	5.9±0.8	2.91±0.3 ^{a}	4.8±0.52 ^{a}	5.05±0. 42 ^{a}
B (0.3% of malt extract)		3±0.47 ^{a}	4.87±0.55 ^{a}	5.11±0.33 ^{a}
C (0.5% of malt extract)		3.2 ±0.5 ^{a}	4.96 ±0.52 ^{a}	5.2 ±0.28 ^{a}
D (vaccine -no malt)		2.8 ±0.63 ^{a}	4.7 ±0.27 ^{a}	4.9 ±0.46^{a}
E (no vaccine- no malt)		1.4 ±0.32^{b}	- ^{b}	- ^{b}

The columns which have no common superscripts are significantly different ($P < 0.05$).
* Mean of antibody titer according to log 2 ± standard deviation.

animal feed or as prophylaxis and remedy (Yasmin *et al.*, 2020). Besides being a cheaper and safer alternative, the use of herbs may reduce the incidence of drug resistance and may modulate the immune system in preventing viral-related diseases (Yasmin *et al.*, 2020). The use of plants as traditional medicine against viral diseases in the production of animals has been described and practiced worldwide. The use of herbs and their extracts as antiviral agents began following World War II in Europe, and the research was later developed worldwide (Yasmin *et al.*, 2020).

Barley malt extract showed high antioxidant activities both in vitro and in vivo, and the phenolic compounds in the extract were responsible for their effective antioxidant properties (Thomas *et al.*, 2005). This antioxidant property could be explained by a free-radical scavenging ability (such as scavenging hydroxyl-radical, superoxide-radical, and carbon-centered free radicals) (Thomas *et al.*, 2005). It has been suggested that hydroxyl was the most important factor in determining the antioxidant activities of phenolic compounds (Thomas *et al.*, 2005). It seems that the antioxidant effects of phenolic compounds in malt extract can strengthen immunity against diseases (Thomas *et al.*, 2005). The most destructive avian viral diseases are Newcastle disease virus (NDV), avian influenza virus (AIV), infectious bursal disease virus (IBDV), infectious bronchitis virus (IBV), egg drop syndrome avian adenovirus, and fowlpox virus. Vaccination programs against these viruses have been applied in many countries worldwide (Marangon and Busani, 2006). However, the problems arise from backyard-reared chicken infections, which are normally not vaccinated, but still prevalent, leading to the spread of the virus that eventually causes an outbreak in the community (Bagust, 2008). Modern treatments of the infected avian species are laborious and expensive. Treatments with medicinal plants have been practiced traditionally to overcome the virus infection.

The effect of the herbal extracts on NDV

While the information on the immune response of birds to NDV is limited, both antibodies and cell-mediated immunity play an important role in protecting and clearing NDV after infection (Miller *et al.*, 2013). Antibodies can be detected against the ND virus about 6-10 days after infection, whereas stimulation of antigen-specific cytotoxic T-cells generally requires about 7-10 days (Miller *et al.*, 2013).Since the average time to death after infection with ND virus is 2-6 days, the presence of antibodies before infection seems to be crucial for protection against clinical disease (Miller *et al.*, 2013). In this study, for the first time, we used barley malt extract with ND and AI vaccines as an immune booster and investigated whether it could improve the humoral immunity against these vaccines. The results of this survey exhibited that taking malt extract at 0.5% concentration 14, 21, and 28 days after vaccination, can increase the specific antibody titer against the ND vaccine. Therefore, malt extract at 0.5% concentration, at all periods after vaccination, improved the systemic antibody response to ND vaccine in broiler chickens.Prescribing products containing antioxidants may be a vital way to delay or inhibit the oxidation of vulnerable cell substrates and prevent some diseases. Due to the high content of flavanones, flavones, amino phenolic compounds, phenolic acids (cinnamic and benzoic acid), tannins, chalcones, and proanthocyanidins in barley yields (Goupy *et al.*, 1999). It seems that high levels of these antioxidant compounds in barley malt extract could promote immunity against the ND vaccine.While the antioxidant activity of barley or malt has been studied, there is no publication documenting the effects of barley malt extract on immune response against NDs vaccine in broiler chickens. Studies have been done on the effect of compounds in malt extract on various parameters such as feed conversion ratio and gastrointestinal health. Talazadeh *et al.* stated in a study that adding malt extract to drinking water increases the average feed intake and increases the weight of chickens during the breeding period, but has no effects on the feed conversion ratio, carcass percentage, and weight of crop, liver, and heart (Talazade*h et al.*, 2019). Bamforth *et al.* stated that vitamin E (alpha-tocopherol) is a monophenolic compound found in barley that is produced during the germination process during malt production. Therefore, part of the antioxidant process of malt extract is due to vitamin E, and this can protect the intestinal villi and improve the feed conversion ratio (Bamforth *et al.*, 1993).

Sanders *et al.* stated that malt extract is high in maltose due to starch breakage during malting and strongly promotes the growth of probiotics (Sanders *et al.*, 1999).

Several other medicinal plants have been used by farmers/owners in treating NDV in diseased birds such as Aloe species (Abd-Alla *et al.*, 2012)**,** *Azadirachta indica* (neem) (Gupta *et al.*, 2017), and *Commiphora swynnertonii* (Burtt) (Bakari *et al.*, 2013).

The effect of the herbal extracts on AIV

Avian influenza virus (AIV) is classified into highly pathogenic avian influenza virus (HPAIV) and low pathogenic AIV (LPAIV) based on its pathogenicity in poultry. Vaccination against AIV has not been very successful as multiple subtypes are co-circulating (i.e., H5, H7, and H9); hence, vaccination against multiple HA subtypes is required. Alternatively, medicinal plants could be used to overcome the infection (Talazadeh *et al.*, 2017).

The results of the present study showed that none of the three doses of malt extract had a significant effect on antibody response against the AI vaccine. Since no study has been performed on the effects of malt extract on immunity against the influenza virus, we cite studies on the effect of other plant extracts containing phenolic compounds on immunity against the influenza virus.

In disagreement with this result, Talazadeh *et al.* reported that pediatric cough syrup including thyme extract at 0.2%, increased the specific antibody response against the Influenza vaccine virus compared to all groups (Talazadeh *et al.*, 2017).In disagreement with this result, Talazadeh *et al.* reported that receiving Antibiofin® (mostly including *Thymus vulgaris*) at 0.1% and 0.2% concentrations, 14 and 28 days after vaccination, could increase the specific antibody titer against AI subtype H9N2 vaccine compared to the control group (Talazadeh *et al.*, 2016).The no influence of malt extract on systemic antibody response to AI vaccine in our study may be associated with the type of virus (killed vaccine) in which antibody response against it was determined. Since few reports are available on the impact of malt extract on poultry immune response, more studies will be needed to study the malt extract's immunomodulatory properties on broiler health.

Several other medicinal plants have been used by farmers/owners in treating AIV in diseased birds such as *Camellia sinensis* (green tea) (Song *et al.*, 2005;Kim *et al.*, 2013;Lee *et al.*, 2012), *Eugenia jambolana Lam*. (Sood *et al.*, 2012),NAS preparation (Shang *et al.*, 2010), *Echinacea purpurea* (purple cone flower) (Hudson, 2012 andKarimi *et al.*, 2014), and *Sambucus nigra L.* (elder berries) (Krawitz *et al.*, 2011;Roschek *et al.*, 2009).

In conclusion,this study showed that the barley malt extract could increase the immune response against the ND vaccine virus compared to the control group (vaccinated but did not receive malt extract). The findings suggest the use of barley malt extract during chicken breeding especially along with ND vaccination programs.

Acknowledgment

Shahid Chamran University of Ahvaz, Ahvaz, Iran supported this study by grant number: SCU. VC99.372.

CONFLICT OF INTEREST

None declared by the authors.

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