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Comparative Study between Mushroom-extracted and Commercial Lectins: Impact of Immune Response to H5N1, NDV, and IBD Vaccines in Broiler Chicken

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Abstract

This study's objective was to look into the impact of lectin on the immune response to live and inactivated vaccines of Avian influenza, Newcastle disease virus, and Gumboro in broiler chicken. Moreover, a comparison between mushroom-extracted lectin and commercial lectin is investigated. Accordingly, 100 broiler chicken were divided into 4 groups each one consisting of 25 chicken: the first group is -ve control, and the second one was treated with extracted lectin only. Followed by a third group, which is treated with commercial lectin only. Finally, the fourth group was treated with both extracted and commercial lectins. The vaccination program was VAXXITEK® HVT+IBD in the hatchery, then ValleyVac® Penta-Pro injection on the sixth day, and Nobilis® Gumboro 228E on the 16th day in drinking water. Lectin dose is 0.25 ml/L of drinking water for 2 days before live vaccines and 2 days after inactivated vaccines. After that, samples were collected before and after 48 hrs lectin administration in case of Infectious Bursal Disease (IBD). Otherwise, samples were taken 48 hrs before and 10 days after lectin administration in case of Highly Pathogenic Avian Influenza (H5N1) and Newcastle Disease Virus (NDV). Then, 3 samples from each group in each measurement are extracted for testing. The results obvious that there is no change in immune response in four groups to the Newcastle vaccine before and after lectin, indicating no effect of lectin on NDV immunity. Additionally, the titre of avian influenza after lectin administration is the same as before lectin, indicating no effect of lectin on H5N1. On the other hand, there is a superior impact of mushroom-extracted lectin on the immune response of IBD that the antibodies titre of the extracted lectin increased four times rather than the commercial lectin.

Keywords: Volvariella Volvacea lectin, Newcastle Disease Virus, Infectious Bursal Disease, Avian Influenza.

1. Introduction

Because of rising human consumption, Egypt's poultry sector and its modern technology are looking for quick and efficient ways to meet the country's rising food demands. There are several strategies to get around these challenges given the variety of illnesses and infectious agents that the chicken business encounters, so advanced poultry vaccine technologies are mandatory [1, 2]. Immunostimulants represent one of the best ways to enhance the chicken business and lessen issues that this sizable sector faces. Besides, it can protect poultry infection from avian influenza viruses (AIV) and other diseases, resulting in a higher poultry production rate [3, 4]. One of the key immunostimulants used in the poultry industry is called lectin, which will be briefly discussed in this paper. The lectin name is derived from the Latin term "Lego", which means "choose out" or "to choose" [5]. Further, sugar-binding proteins, neither enzymes nor antibodies, are known as lectins [6]. *Rudiger and Gabius* state that in order for a glycoprotein to be classified as a lectin, it has to fulfill three different conditions. First off, a lectin is a kind of protein or glycoprotein that binds carbohydrates. Secondly, immunoglobulins (antibodies) are not lectins. Lastly, the carbohydrates that lectins attach to are not biochemically changed by them [7]. Bioactive natural proteins and glycoproteins with the ability to selectively bind sugars are called lectins [8]. These non-immune sugar-binding proteins have the ability to precipitate glycoconjugates or agglutinate cells [9]. From ancient times, it has been considered that some plant and animal products are hazardous to humans. In the late 1800s, the study of bacteriology greatly influenced scientific methods and assumed that the toxicity of seeds originated from bacterial toxins.

Considering that lectins are made by a broad variety of living things, including microbes and animals, they may be divided into multiple groups according to the species from which they originated including fungal, animal, plant, bacterial, as well as algal lectins.

Algal lectins commonly referred to as phycolectins, exhibit a preference for glycoproteins over monosaccharides. Fungal lectins, there are now several new lectins with distinct carbohydrate specificities to be found in fungus. Additionally, Fungal lectins are truly widely distributed; 82 percent are found in mushrooms, 15 percent are found in microfungi or molds, and 3 percent are found in yeasts [10]. Bacterial lectins are also known as adhesins because, during an infection, they help bacteria adhere to host cells. These bind via the carbohydrate-recognition domain to the glycan receptor [11]. Moreover, Animal lectins are proteins that bind carbohydrates and have wildly

different amino acid sequences. Every animal lectin has a distinct domain of carbohydrate recognition with a 115–130 amino acid residue sequence pattern [12]. Followed by, Plant lectins that have at least one non-catalytic domain that can attach to a specific oligo- or monosaccharide in a reversible way [13]. There are now 500 distinct plant lectins that have been identified and described [14].

Many lectins from different sources exhibited immunomodulatory properties, including activation of T helper type 17 (Th17), type 2 (Th2), or type 1 (Th1) responses, as well as mitogenic activity. It has been shown that fungus lectins are powerful immune response modulators. In addition, Tricholoma Mongolicum Lectin 1 (TML-1) and Tricholoma Mongolicum Lectin 2 (TML-2) are lectins derived from the Tricholoma mongolicum fungus. These lectins activated macrophages treated with lectins, inducing the Th1 response through the activation of macrophages. Additionally, they increased the synthesis of nitrite ions and the cytokine Tumor Necrosis Factor (TNF) by macrophages. Lectins induced mouse macrophage activation in vivo, which in turn inhibited the development of tumour cells [15]. Purified lectin from the edible Volvariella Volvacea fungus, also known as Volvariella Volvacea Lectin (VVL), increased the transcriptional expression of interleukin 2 (IL-2) and caused the proliferation of splenic lymphocytes and interferon-alpha (INF alpha), results in activating the Th1 response in vitro [16].

It is well established that the quantity of Mannose-Binding Lectin (MBL) present affects the health of chickens [17]. Where MBL possesses a variety of antibacterial properties, such as promoting bacterial phagocytosis and causing bacterial aggregation. MBL is a crucial protein for pattern recognition in innate immunity. It is a member of the collagenous C-type lectin family, sometimes referred to as "collectins". Further, MBL is mostly found in blood serum and is produced in the liver. It binds to carbohydrates on the surface of a variety of pathogens, including bacteria, viruses, fungi, and protozoa, and is crucial to the host's first line of defense [18].

The earlier mentioned studies and other literature stated that rarely research discusses the effects of Highly Pathogenic Avian Influenza (H5N1), Newcastle Disease Virus (NDV), and Infectious Bursal Disease (IBD) on immune response in broiler chicken for mushroom-extracted lectin and commercial lectin. Therefore, this paper presents a comprehensive comparison between commercial and mushroom-extracted lectins to extract the impact of lectin on the immune response to live and inactivated vaccines of Avian influenza, NDV, and Gumboro in broiler chicken. Lastly, the merits of mushroom-extracted lectin are presented in comparison to commercial lectin.

2. Material and methods

2.1. Experimental chicks

Commercial broiler chicks, one day old, were bought from a local Egyptian Poultry Company and reared at the same room for 35 days and they were given regular broiler ration free of antibiotics and constant lighting, then finally cervical dislocated. Notably, all animals were treated and approved by the following ethical approval from the Faculty of Veterinary Medicine's Ethical Committee, which approved all experiments under No. (BUFVTM 03-01-24).

2.2. Vaccine and medication

1) VAXXITEK® HVT+IBD vaccine (Bursal Disease-Marek's Disease vaccine serotype 3, live MD vector): It was acquired from MERIAL SELECT, INC. Gainesville, GA, 30503 USA, U.S.VET.
2) ValleyVac® Penta-Pro vaccine (High concentration of Inactivated high pathogenic avian influenza clade 2.2.1.2 and 2.3.4.4b, low pathogenic H9N2 G1-Lineage, Newcastle disease virus GVII (Whole virus) and Newcastle disease virus GII (Whole Virus)) it was obtained from VACCINE VALLEY, 6 October City, Egypt.
3) Nobilis® Gumboro 228E vaccine GUMBORO VACCINE NOBILIS 228E Vaccine – lyophilized live vaccine obtained from an intermediate strain of IBDV (228E) from INTERVET.
4) Lector50® (Microbiotech INT. INC, USA), 15,000 mg of lectin, 5000 mg of xylitol, 15,000 mg of fructo-oligosaccharide, and 30,000 mg/liter of NaCl were presented in a commercial product that was used.

2.3. Material and chemicals

The edible straw mushroom, i.e., V. volvacea, was bought at a local market. Notably, all chemicals were of reagent grade.

2.4. Lectin extraction and identification

2.4.1. Extraction of Lectin

Lectin was extracted according to the method described by [16] as follows:
homogenization of V. volvacea acetic acid containing mercaptoethanol, centrifugation at 10,000 g for 20 min, saturation, stirring, centrifugation at 20,000 g for 30 min, dissolving, and dialysis, respectively.

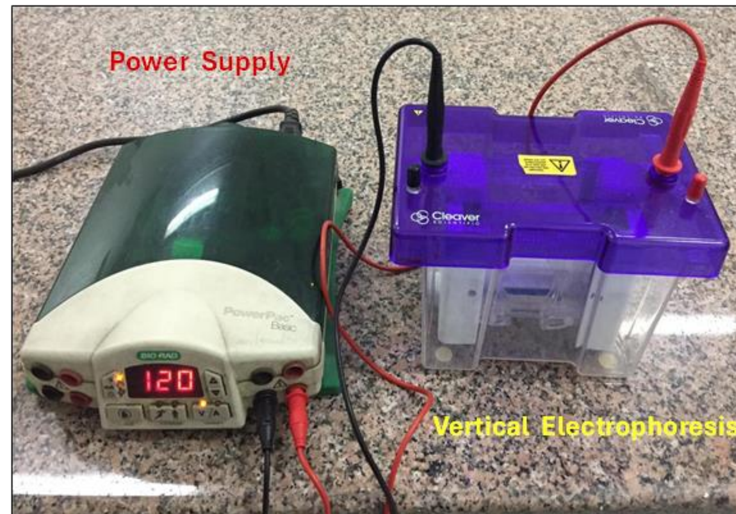

Figure 1. The schematic diagram of experimental setup.

2.4.2. Hemagglutination test

The test was performed according to *Osman et al*. with slight modifications [19]. By collecting chicken blood on trisodium citrate, the 1 percent Red Blood Cells (RBC) solution was made. Subsequently, the citrated blood was diluted to 1 percent using Phosphate Buffer Saline (PBS) and rinsed three times with PBS. To conduct the Haemagglutination (HA) test, a V-bottomed microtiter plate containing 50 µl of the lectin extract was serially diluted twice in PBS. Next, each well received 50 µl of the 1 percent RBC solution. To create the negative RBC control, mix 50 µl of the 1 percent RBC solution with 50 µl of PBS. Afterward, the plate was incubated for 30 minutes at 25 ºC, and the outcomes were noted and recorded. Besides, an RBC dot was regarded as negative, and a hemagglutination sheet as positive. Ultimately, the greatest positive dilution's reciprocal was used to compute the HA titer.

2.4.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Regarding the Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it was carried out according to Ref. [20].

After dilution with an equivalent volume of 2X sample buffer (i.e., 1% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 1% bromophenol blue in tris buffer pH = 6.8), the extracted lectin was ready for SDS-PAGE. The mixture was then incubated for 5 minutes at 95 ºC. Further, a 10 percent discontinuous SDS-PAGE gel was loaded with 10 µl of the prepared sample and a pure gene protein ladder (Genetix Biotech Asia Pvt. Ltd., India). In addition, a vertical electrophoresis chamber was used to perform the electrophoresis (Cleaver Scientific, UK). Finally, Coomassie Brilliant Blue R-250 was used to stain the gel, and a solution of methanol, water, and acetic acid was used to destain the background (30:60:10). The experimental setup is shown in Figure 1.

2.5. Experimental design

Regarding the experimental work, the samples of 100 chicks were divided into 4 groups as follows: **H1** (-ve control), **H2** (Group treated with extracted lectin), **H3** (Group treated with commercial lectin), and **H4** (Group treated with both).

The vaccination program was VAXXITEK® HVT+IBD in the hatchery, ValleyVac® Penta-Pro intramuscular on the sixth day, and Nobilis® Gumboro 228E in drinking water on day number 16th. Notably, the lectin dose is 0.25 ml/L drinking water – 2 days before live vaccines and 2 days after inactivated vaccines.

2.6. Blood sampling

Samples were collected for 48 hrs before and after lectin administration in case of live vaccines and 48 hrs before, and 10 days after lectin administration in case of inactivated vaccines. Where 3 samples from each group in each measurement are extracted for testing.

2.7. Assay method

2.7.1. Haemagglutination inhibition for NDV and H5N1

The Haemagglutination Inhibition (HI) test is performed as described in [21] as follows:

Preparation of the antigen solution

1) Determine the volume of antigen solution required based on the quantity of 96-well plates utilized (i.e., 25 µL antigen for each well × 96 = 2,400 µL antigen for each 96-well plate).
2) Using PBS, prepare the appropriate dilution of 4 HA units for the determined volume.

Preparation of the RBC suspension

1) Determine how much RBC suspension is required based on how many 96-well micro-titer plates are being used (50 µl RBC as suspension for each well × 96 = 4,800 µl suspension of RBCs per 96-well plate).
2) To get the right concentrations, dilute the RBC stock suspension with PBS for mammalian and avian red blood cells of 0.75% and 1%, respectively.

Preparation of the 96-well micro-titer plate

The procedures are described below:

1) For all wells other than the first well of the back titration row, there is 25 µl of PBS.
2) The first well of rows 1-11 contains a 25 µl RDE (Receptor destroying enzyme) treated serum.
3) The first well in the back titration row contains 50 µl of diluted antigen.
4) Serial twofold dilution of every row, every dilution step, transfer 25 µl and discard 25 µl of the final wells.
5) Add a diluted antigen of 25 µl to each well in rows 1-11.
6) PBS (with 25 µl) was added to the back titration row.
7) Plate tapping and a 30-minute incubation period at 25 °C.
8) 50 microliters of RBC suspension (0.75% or 1%) in each well.
9) Plate tapping and incubation.
10) Readout.

Reading the plate

1) Give the plate a 25-second, 90° tilt.
2) On a printed schematic of the 96-well plate, note the results right away while the plate is still inclined, as explained in Figure 2.

2.7.2. Enzyme-linked immunosorbent assay for IBD

All procedures of Enzyme Linked Immunosorbent Assay (ELISA) for IBD are applied according to BIOCHEK KIT CK113 as described in the following ten steps. Besides, the illustration of the indirect ELISA method is described in Figure 3.

1) Take the IBD-coated plate out of the bag and mark the samples' placement on the template.
2) Fill wells A1 and B1 with 100 µl of the negative control.
3) Fill wells C1 and D1 with 100 µl of positive control.
4) Fill the relevant wells with 100 µl of diluted samples. For 30 minutes, incubate the plate at room temperature (22–27 ºC) with the cover on.
5) Aspirate the contents of the wells, then use the wash buffer four times (350 µl per well). Turn over the plate and give the absorbent paper a firm smack.
6) Fill the corresponding wells with 100 µl of the conjugate reagent. For 30 minutes, incubate the plate at room temperature (22–27 ºC) with the cover on.
7) Proceed with the wash process once more, just like in step 5.
8) Fill the relevant wells with 100 µl of the substrate reagent each. For 15 minutes, incubate the plate at room temperature (22–27 ºC) with the cover on.
9) Pour 100 µl of the Stop solution into the proper wells to halt the process.
10) Read the absorbance of the samples and controls at 405 nm after blanking the micro titre plate reader with air.

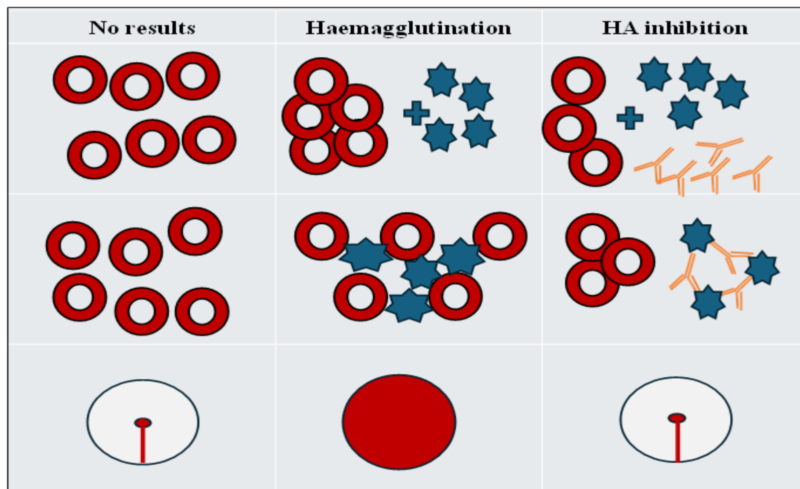

Fig. (2) Principle of the HI assay.

2.8. Statistical analysis

For statistical analysis, a one-way analysis of variance (ANOVA) and the Duncan multiple tests were applied to the gathered data. For every study, the social science statistics program was utilized (i.e., SPSS software, version 13.0) [22]. P-values less than 0.05 were considered statistically insignificant; the mean of (± SE) is used to represent all data.

3. Results and explanation

Hemagglutinating Activity; since neither VAG (lectin from fruit bodies of Volvariella Volvacea) nor the Flammulina lectin can be inhibited by a simple specific sugar, the hemagglutinating activity of VAG is similar to that of the lectin obtained from Flammulina veltipes but very different from that of the lectins from Agricus campestris and Agricus bisporus [23]. The hemagglutinating properties of VAG were examined using chicken red blood cells. Hemagglutination assay is performed following lectin extraction to determine its concentration. Figure 4 shows the HA titre concentration 1:2 (2^1) of the extracted, and this is the same result of [23] that is described. That means the concentration of the extracted lectin used in this study equal 2^1 (i.e., 1 HA unit). When it comes to VAG hemagglutinating activity, red blood cells from dogs or cats have the highest titer whereas those from ducks or chicks have the lowest titer or exhibit no hemagglutinating activity at all.

Isolated mushroom lectins range widely in molecular mass and oligomeric form, from 10 to 190 kDa [24]. Although this is from fruiting bodies, it revealed a modest band at 65 kDa along with three large bands at 33, 66, and 100 kDa [25]. Molecular weight—VAG gave many bands on polyacrylamide gel electrophoresis, i.e., 32000, 43000, 45000, and 50000, as shown in Figure 5.

Fig. (4) Concentration of HA titre.

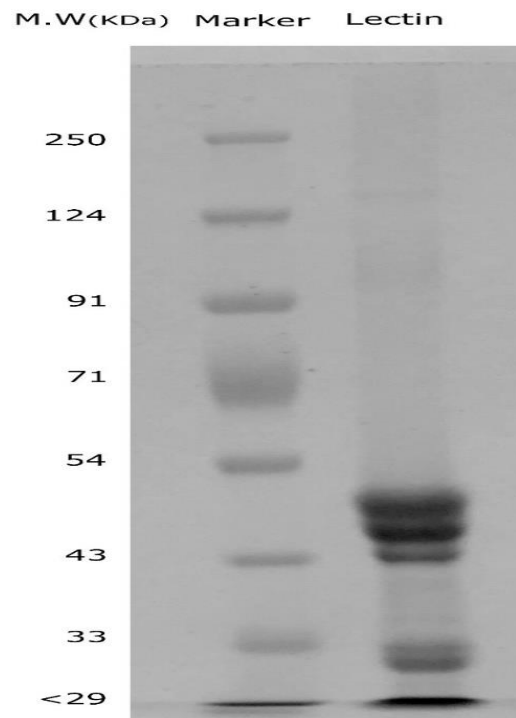

Fig. (5) SDS-PAGE for various output gel bands.

Table (1) HI titre for Newcastle Disease Virus (mean ±SE)

	Titre of HI	
	Before lectin	**After lectin**
H1	7.33 ± 0.67abA	6.33 ± 0.33abB
H2	6.67 ± 0.33bA	5.67 ± 0.33bB
H3	7.00 ± 0.00abA	6.67 ± 0.33aA
H4	7.67 ± 0.67aA	6.33 ± 0.33abB

The data presented in Table 1 show that the groups do not differ significantly from one to another before using lectins. However, after using lectins, there were also no significant differences between H1, H3, and H4, while there was a significant decrease in group H2 compared to group H3. It is concluded also that the value of group H2 was the lowest, while the value of group H3 was the highest.

Table (2) HI titre for H5N1 (mean ±SE)

	Titre of HI	
	Before lectin	**After lectin**
H1	8.67 ± 0.67aA	5.67 ± 0.33bB
H2	8.00 ± 0.00aA	5.33 ± 0.33bB
H3	8.00 ± 1.00aA	7.33 ± 0.33aA
H4	8.33 ± 0.33aA	6.00 ± 0.58bB

Prior to the use of lectins, there were no discernible differences between the groups, according to the data presented in Table 2. But there were also no appreciable changes between H1, H3, and H4 after employing lectins. Group H2 experienced a notable decline in comparison to group H3. Moreover, group H2 had the lowest value, and group H3 had the greatest value.

Table (3) ELISA titre for Infectious Bursal Disease (mean ± SE)

	Titre of ELISA	
	Before lectin	**After lectin**
H1	776.67 ± 180.86aA	1465.67 ± 408.44cA
H2	259.00 ± 157.06aB	5823.00 ± 228.63aA
H3	292.33 ± 94.32aB	2651.67 ± 679.76bcA
H4	527.33 ± 419.08aB	3634.67 ± 1784.08bA

The data presented in Table 3 show that there are significant differences between the groups before using lectins. Furthermore, after using lectins, there were also significant differences between H1, H2, H3, and H4. It is obvious that also the value of group H2 was the highest, while the value of group H1 was the lowest.

4. Discussions

The main purpose of the present study was to compare mushroom-extracted lectin and commercial lectin and extract the impact of lectin on the immune response to inactivated vaccines of H5N1, NDV, and IBD in broiler chicken. Prior research has demonstrated that human MBL binds to a variety of viruses, including HIV [26] and SARS [27, 28]. On the other hand, it was discovered that giving chickens the vaccine together with an MBL ligand specifically, fructooligosaccharide (FOS) seemed to increase the development of Immunoglobulin G (IgG) antibodies specific to Infectious Bronchitis Virus (IBV). Moreover, the overall number of clusters of differentiation 4+ (CD4+) cells rose when FOS was added to the vaccine. When an MBL ligand was added to the vaccination, chickens also displayed an increase in cluster of differentiation 4 (CD4) − cluster of differentiation 8 alpha (CD8α) − gamma delta T-cells (γδ T-cells). Further, most pronouncedly after the first vaccination indicating that CD4 − CD8α − γδ T-cells might be involved in the immunological reaction against IBV as well [29]. By either inducing a more cellular immune response (Th1 versus Th2) or neutralizing the virus through complement system-mediated means, MBL influences the pathophysiology of IBV infection and the production of specific antibodies [30]. In vitro, lectin isolated from the edible mushroom Volvariella Volvacea, or VVL, promoted the transcriptional production of interleukin 2 (IL-2) and interferon-alpha (INF alpha), inducing the Th1 response and inducing the proliferation of murine spleen cells [16].

From the researcher's point of view, there is no change in immune response in four groups to the Newcastle vaccine before and after lectin which indicates no effect of lectin on NDV immunity. In addition, the titre of avian influenza after lectin administration is the same as before lectin and that indicates no effect of lectin on H5N1. After these results, this study found that there is no immunological importance between the lectin and the immunity response of the Newcastle virus and avian influenza. On the other hand, there is an excellent effect of extracted lectin on the immune response of IBD that the antibodies titre of H1 (representing the group without immunostimulant) increased by 2.5%. While H2 (that treated with extracted lectin) showed a 23% increase, otherwise H3 (that treated with commercial lectin) increased by 5.7% and the last group in which we used a mixture of extracted and commercial lectin showed an enhancement of 5.2%.

5. Conclusions

This study discovered that there is no relationship between the lectin and the avian influenza and Newcastle virus immune responses. In addition, the remarkable impact of extracted lectin on IBD immune response is evidenced by a 2.5 percent increase in H1 antibody titres, which represent the group without immunostimulant. Further, H2 treated with extracted lectin showed a 23 percent rise. Besides, H3 treated with commercial lectin showed a 5.7 percent increase, and the last group H4 treated with a combination of extracted and commercial lectin showed a 5.2 percent increase and this is due to lectin extracted

from the edible mushroom Volvariella Volvacea, or VVL, stimulated the transcriptional synthesis of interleukin 2 (IL-2) and interferon-alpha (INF alpha), triggering the Th1 response and encouraging the growth of spleen cells. For future work, additional studies are required to clarify the significance of lectin on the immune response of other live and inactivated vaccines.

Ethical approval and consent to participate

All animals were treated and approved by the following ethical approval from the Faculty of Veterinary Medicine's Ethical Committee, which approved all experiments under No. (BUFVTM 03-01-24).

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