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Antiviral Potential of *Patikan Kebo* Extract against Chicken Corona Virus through In Ovo Challenge Test

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ABSTRACT

The coronavirus, known to exist in humans and livestock for an extended period, includes variants such as the infectious bronchitis (IB) virus in chickens. As a single-stranded RNA virus, coronaviruses are prone to mutations. *Patikan Kebo* (Euphorbia hirta L) is a medicinal herb containing various compounds like Flavonoids, Mirisil, Alkaloids, Laraserol, Hentriakontanol, and Komositin, known for their antibacterial, antiviral, and anti-worm properties. This study aimed to evaluate the toxicity of *Patikan Kebo* extract on Chicken Embryo Eggs (TAB) and assess its antiviral potential against the chicken AI coronavirus. The experimental approach involved three concentrations of *Patikan Kebo* extract (0.01% - P1, 0.1% - P2, 1% - P3), a control group without *Patikan Kebo* extract (K+), and a control group without the virus (K-). Data analysis was conducted using ANOVA and Tukey HSD in the SPSS program. The results from ANOVA indicated that the dose of *Patikan Kebo* significantly impacted ($P<0.05$) its antiviral activity against the IB virus. The average HA Titer 2 (Log2) demonstrated a 47% reduction in viral activity. In conclusion, the most effective herbal antiviral dose against the IB virus was determined to be 0.1% Patikan Kebo extract.

1. Introduction

The Covid-19 pandemic in Indonesia has lasted almost two years since the first case was announced on March 2, 2020. Various attempts have been made by the government as form of anticipation to minimize the spread of Covid-19, starting from Large-Scale Social Restrictions (PSBB), disbursement of funds for hospitals and victims who died, Enforcement of Restrictions on Community Activities (PPKM) and vaccinations. One thing that has not been anticipated is if there is a mutation of the corona virus, as it is zoonotic. Zoonoses are diseases that can be transmitted from animals to humans or vice versa. The mutation of the corona virus start from the Alpha, Beta, Omicron, Delta variants and the last one is Deltacron.[1]. The World Health Organization (WHO) reported that during the pandemic, there har been mutations with various virus variants. The mutations are associated

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with higher viral loads in infected humans and experimental animals. The mutation causes an increase in the open conformation of the protein, thereby increasing the binding of the viral S protein to the receptor on the host [2].

The corona virus has actually been found for a long time in humans and animals. In animals, it infects poultry, turkeys, pigs, mice, cats, and dogs. Corona viruses that attack humans only come from the most dangerous Alpha and Beta genera. The Corona virus that attacks animals is of the genus Delta and Gama. Corona viruses that infect animals are of the Beta genus after evolving in new forms, such as SARS-Cov, MERS-Cov, Neo-cov, 2019-Ncon, FCoV and CCoV. Structurally, the three new types of corona virus have similarities in terms of structure and morphology, but are genetically and host different. This new variant is zoonotic as it is transmitted to humans. This new variant is transmitted by wild animals such as bats and snakes, but not to farmed animals such as poultry.[3]. Corona virus in animals, one of which, is the virus that causes Infectious Bronchitis (IB) in chickens. Infectious Bronchitis (IB) is an acute infectious viral disease that attacks the respiratory system of chickens. The impact of economic losses is enormous. It reduces egg production and causes abnormal egg shape, weight loss, and death[3].

Many studies on herbal plants usage to increase chicken immunity against viruses have been carried out, but limited on virus IB.[4] has conducted research on the effectiveness of herbal spices through water on the response of New Castle Diseases (ND) virus titers in broiler chickens. ND virus is the cause of ND in poultry with high mortality. Chicken immunity test against Avian influenza (AI) virus using a combination of Meniran and Temu Giring herbs was carried out by [4]. The results showed that these herbs have the potential to increase antibody titers. ND and AI viruses are viruses that attack the respiratory system of chickens.

Patikan Kebo (*Euophorbia hirta* L) is a medicinal herb containing Flavonoids, Mirisil, Alkaloids, Laraserol, Hentriakontanol and Comositin. Efficacy as a drug used by the community as a traditional medicine to treat digestive disorders and as an anti-bacterial, anti-viral, anti-worm[5]. *Patikan Kebo* has been investigated on feed rations of 5% on broiler chicken production performance [6]. The dosage form of juice, infusion and extraction can increase the number of erythrocytes, hemoglobin levels and hematocrit values in chickens infected with E. tenella [7]. Research on the use of *Patikan Kebo* herbs in ovo, using embryonic chicken eggs (TAB) to determine antibody titers (immunity) against chicken corona (IB) has never been done. In ovo research is the best method to see antibody response to virus challenge test [8].

The research objectives were: 1. To test the toxicity of *Patikan Kebo* extract against TAB, 2. To measure the antiviral activity of *Patikan Kebo* against the corona virus that causes AI in chickens, 3. To evaluate zoonotic predictions based on the antiviral potential of *Patikan Kebo* as a measurement from antibody titers of chicken embryos.

2. Review of Related Literature

2.1. Corona Virus Infectious Bronchitis

IB corona virus, better known as Infectious Bronchitis is a disease that attacks the respiratory system of chickens caused by the Infectious Bronchitis virus. Infectious Bronchitis is a member of the Coronavidae family (Order Nidovirales) and genus C viruses that cause respiratory, intestinal, and various other neurological disorders.[9]. IB disease in Indonesia is still a serious problem in chickens due to the many variants that arise due to mutations of the IB virus[3]. The corona virus is easy to mutate because its genetic material is RNA. RNA is easily mutated in the host cell to fool the host cell's immune system. One of the factors that can cause mutations is because the virus enters the body of an intermediate animal. Changes in the viral nucleic acid sequence caused by insertion, deletion, replacement, or rearrangement of bases (nucleotides) in the host can change the phenotype of the

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organism as it produces new proteins. Corona viruses undergo various mutations in the spike protein, including in the receptor-binding domain, which causes an increase in affinity for the ACE-2 receptor, resulting in an increase in the virulence of the virus and its transmission or possibly zoonoses.[10].

Corona poultry cause IB has a structure similar to the corona virus in humans. IB is an infectious disease of the respiratory tract. AI is very detrimental to farmers because it can cause urogenital tract disorders and reduce egg production accompanied by a decrease in egg quality in the form of irregular egg shapes, soft egg shells and liquid egg albumin. IB disease attacks can harm the livestock industry, both laying hens and broilers. IBs are spread all over the world, including Indonesia. The IB virus spreads through the respiratory route (droplets) which are released during coughing and coughing and are also excreted in the feces. The spread of disease through flocks of birds in one flock quickly. The incubation period is relatively short between 18-36 hours[11].

2.2. HA test

The HA test is a serological test to determine whether a virus has the ability to agglutinate erythrocytes and also to determine the virus titer. Observation of agglutination by looking at the bottom of the most recent wells which was indicated by positive hemagglutination. The interpretation of the HA test results is that hemagglutination is clearly visible in the form of an evenly distributed layer of erythrocytes (diffuse) at the bottom of the well and purification of the upper fluid without deposition of erythrocytes in the form of dots in the middle of the well.[12].

Virus challenge test with Hemagglutinin (HA) test. The purpose of the HA test is to detect the development of viruses containing Hemagglutinin through erythrocyte samples, either by fast testing or slow testing. The material being challenged by the virus is chicken Red Blood Cell (RBC). Hemagglutination indicates the presence of viral antigen in sufficient concentration to agglutinate red blood cells. The antigen titer was determined at the highest dilution of the antigen that was still able to completely agglutinate chicken red blood cells[13]. HA test performed on chickens such as AI, AI virus hemagglutinin, plays a role in the induction of immunity, determining host specificity, and determining the pathogenicity of VAI. Avian influenza is caused by type A influenza virus, has RNA genetic material, enveloped in a lipid bilayer. The result was there were 8 HA test positive isolates. The HA titer number shows an interval between 28 -211. The HA titer of 28 is already high (Natih et al., 2010). This HA titer principally agglutinates red blood cells so that red dots appear in the well. The HA titer was the highest dilution that still showed complete agglutination. The HA test is a test to determine the presence of viral antigens that can agglutinate HR (Red Blood Cells). Based on the results of the study showed that the viral HA titer was quite high at more than 28 (Natih et al., 2010). To be able to perfectly agglutinate it takes about 107 virus units. The HA titer in this study was 28 -210 which can be said to be quite high[12].

Tetelo virus hemagglutinin has the ability to bind specifically to sialic acid receptors found on the plasma membrane of chicken red blood cells (HR), in addition to poultry RBCs, and to agglutinate guinea pig and human erythrocytes. Certain strains have the ability to agglutinate mammalian HR, namely cattle, horses, sheep and pigs, white rats, rabbits, and cats. These properties can be used as a marker of tetelo virus strain although it does not affect the difference in serological tests. The hemagglutination process occurs because the HR is mixed with the tetelo virus in a balanced proportion. This coteteloisi can occur because of the match between the tetelo virus and the receptors on the surface of the HR. Several factors that influence the HA test, among others: HR concentrations ranged from 1%, solvents containing electrolytes (0, 85% NaCl) and viral particles up to 105 to 106 per mL of suspension. Tetelo virus that shows a positive HA test can be inhibited by specific antibodies produced by tetelo virus hemagglutinin. This hemagglutination inhibitory activity can be used as a basis for enabling the identification of tetelovirus[14].

2.3. Patikan Kebo

Patikan Kebo (Euphorbia hirta L) is a vine that lives on the ground, especially in tropical areas. In Indonesia, this traditional medicinal plant can be found among the grass on the side of the road, garden or yard. This plant is still in the family with Chinese Patikan, namely in the Euphorbiaceae

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family.[15]. *Patikan Kebo* contains many compounds with various benefits. The ability of the *Patikan Kebo* plant in treating various kinds of diseases is because it involves chemical compounds in it. These compounds are antiseptic, anti-inflammatory, antifungal, antiviral and antibacterial. The bioactive ingredients in *Patikan Kebo* are tannins, lavanoids (especially quercitrin and myricitrin) and saponins.[16]

Bioactive substances in plants are secondary metabolites, such as enol or polyphenols, saponins, flavonoids, curcumin, artemisinin, tannins. Phenol and saponin bioactive substances inhibit or slow the growth of bacteria, fungi, parasites by targeting the membrane or cytoplasm[17]. Phenolic bioactive substances are one of the chemical components of plants that have benefits for the plant itself and also for humans. In general, phenols are divided into monovalent and polyvalent groups (polyphenols).[18]. Some examples of bioactive substances that include phenols are curcumin, flavonoids, lignin, melanin, tannins, monocyclic phenols, phenyl propanoids, phenolic quinones, gallic acid, caffeic acid, phenyl propanoids, phenolic quinones, and catechins including carbolic acids which are weak acids and can protein coagulation[19]. Protein coagulation properties can be used as a prevention of viral replication.

The use of *Patikan Kebo* in livestock has often been used either singly or in combination. Hemorrhoids, Rita, Nurhayati and Nelwida reported that the use of yogurt fermented pineapple peel in rations containing medicinal weeds with a concentration of 15% *Patikan Kebo* gave the best results on nutrient consumption of broiler chickens. *Patikan Kebo* can also function as an immunostimulator. Immunostimulators are compounds that can enhance the immune system and can reactivate the immune system in various ways, such as increasing the number and activity of T cells, NK cells and macrophages and releasing interferons and interleukins. The results of Aristika's research (2015) showed that Euphorbia hirta L. flavonoids could act as immunostimulators in chickens as indicated by an increase in the expression of CD4+ cell expression, TLR-2 expression and IL-2 secretion in chicken PBMCs and the optimum dose showed an increase of 10 g[20].

3. Method

The research method is experimental. The measured variable is toxicity dose of *Patikan Kebo* and the Ha Titer of Corona Virus IB. This research was carried out in June – July 2022. The research location is at the Integrated Microbiology and Biochemistry Laboratory, Islamic University of Malang. The tools used in this research are: Bunsen, candler, Patri Cup, syringe 1, tweezers, scissors, spatel, mortar, well, stamper, incubator. While the materials used are: Phosphate buffered saline (PSB), cotton, embryonic chicken eggs (TAB), Alcohol, sterile Aquadest, *Patikan Kebo* Extract, Seed Virus IB. In this study, the treatment in the form of a dose of *Patikan Kebo* extract injected into Embryo Chicken Eggs (TAB) aged 10 days consisted of five treatments and five replications with each concentration of 0.01% (P1), 0.1% (P2), 1 % (P3), control without *Patikan Kebo* extract (K+) and control without virus (K-). To determine the antiviral activity of *Patikan Kebo* against Corona Virus IB, a test was carried out Hemagglutination (HA).

The research stages include: 1. TAB Setup**,** Embryoed Chicken Eggs (TAB) used as media were 10 days old eggs. Before use, the TAB is checked first to make sure the embryo is alive or dead by examining it using an egg candler or egg binoculars.2. Making extracts of *Patikan Kebo* by maceration method using methanol. Then the results of the *Patikan Kebo* extract sample from the maceration process were evaporated using a rotary evaporator to obtain a paste-shaped extract. The extract was then dissolved with Aqua pro to obtain a concentration of 1%, 0.1% and 0.01%. 3. High Toxicity TestThis study was conducted to determine whether the *Patikan Kebo* extract was toxic to TAB or not by injecting the *Patikan Kebo* extract and then incubating for 3×24 hours. 4. Preparation of IB virus suspension was carried out by grinding the liver organs taken from IB positive chickens as much as 1 gram with a ratio of 1 gram of PBS and 5 grams of water. Then added PPG (Procaine Penicillin-G) antibiotics as much as 0.01 g and Streptomy (Streptomycin Sulfate Meiji) 0.01 mg. 5. Corona IB virus antiviral test using *Patikan Kebo* extract with concentrations of 0.01% (P1), 0.1% (P2) and 1% (P3). IB virus that was put in the freezer was thawed at room temperature 370 C, then TAB was treated with *Patikan Kebo* extract and Virus IB suspension with each concentration inoculated into 0.1 ml allantoic

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chamber. As a positive control, TAB was inoculated with IB virus without extract treatment. While the negative control, TAB was injected with the highest concentration of virus-free *Patikan Kebo* extract and was non-toxic with five replications for each treatment. Then incubated in an incubator for three days. After incubation, the Allantois fluid was harvested in each treatment group and then the HA test was performed. Then after knowing the HA titer, calculate the inhibition of antiviral activity.

$$\text{Percentage of inhibition of antiviral activity} = \frac{\text{HA titer without treatment - HA titer with treatment}}{\text{HA titer without treatment}} \times 100\ \%$$

Then the antiviral effect data were statistically analyzed with Anova and Tukey HSD with SPSS program.

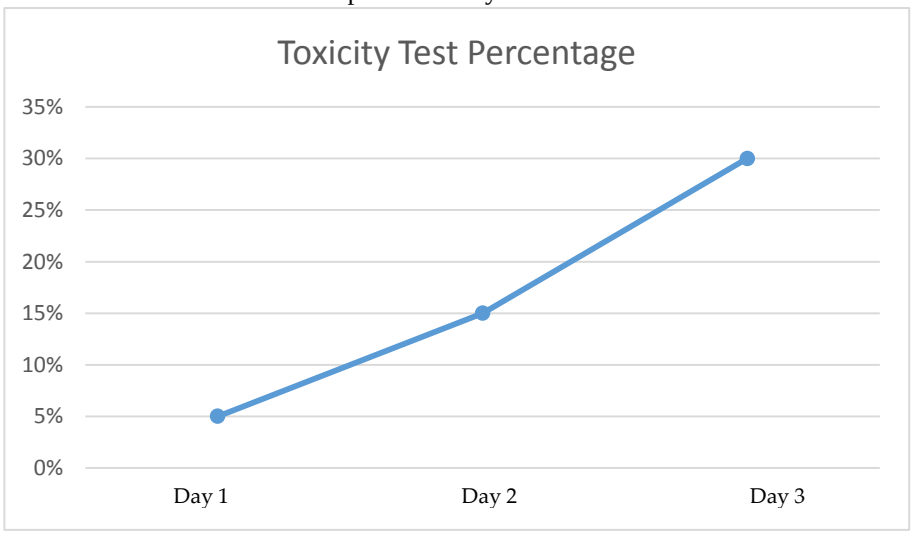
4. Results and Discussion

4.1 Toxicity Test

Determination of the toxic effect of a compound can be determined by a toxicity test. Toxicity test is a test carried out to detect the toxic effect of a compound on a biological system and to obtain specific dose or response data from the test preparation. Toxicity test results can be used to prove the safety of a substance or preparation when applied to humans or animals. Toxicity tests can determine the relative toxicity and help identify toxic effects[21].

Toxicity test of *Patikan Kebo* extract was made for comparison concentration by using concentration variations of 1%, 0.1%, 0.01% and control. *Patikan Kebo*'s toxicity is known through injection of *Patikan Kebo* extract in chicken eggs embryo (TAB) aged 9-10 days as many as 5 grains/concentration, then incubated for 3 days. *Patikan Kebo* toxicity by observing dead embryos is characterized by dark blood vessels. The results of the *Patikan Kebo* extract toxicity test with stratified treatment can be seen in the following graph.

Graph 1. Toxicity Test Data



The results of the toxicity test of *Patikan Kebo* extract against TAB which were incubated for 3×24 hours showed that the embryos died of 30% of all concentrations. Toxicity limit is maximum TAB mortality of 50% or LD50 (Lethal dose 50) [21]. Characteristic features a live embryo can be seen from the movement of the embryo and the blood vessels that still appear red, while a dead embryo is marked by dark and red blood vessels. The embryo's heart is no longer beating. If the death of the embryo occurs less than 24 hours after injection, it is considered as death due to non-specific factors and is not

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ready for injection [17]. Toxicity testis one of the important preclinical trials. This test is used to determine the toxic effect of a compound that will occur in a short time after its administration in a certain dose. Quantitative data obtained from the toxicity test is LD50 (Lethal dose 50). From the LD50 data, the compound can be classified as very toxic (extremely toxic) to non-toxic (practically non-toxic) [22].

4.2 Hemagglutination Test

Corona Virus Hemagglutinin (HA) Test Results *Infectious Bronchitis* (IB) describing the average HA titer and antiviral activity of *Patikan Kebo* extract can be seen in the following table.

Table 1. HA Virus IB test results and % antiviral activity *Patikan Kebo*

No	Treatment	Average Titer HA (Log2)	Antiviral Activity (%)
1	K+	1.8	53±4.91
2	K-	3.8	0
3	P1	2.2	42±6.83
4	P2	2	47±6.83
5	P3	2.4	37±4.24

ANOVA test analysis showed that *Patikan Kebo* extract had an effect ($P<0.05$) on the antiviral activity of corona IB. The test results show the P2 treatment, namely *Patikan Kebo* with a dose of 0.1% has the best antiviral properties, namely 47±6.83% with an average HA titer of 2 $Log^2$the measurement of the average HA titer has a limit value of Log4 which means the average HA titer exceeds Log4 then the antiviral activity is considered to be still low[23]. This shows that the P2 treatment at a dose of 0.1% has a lower parameter value compared to the other groups, which means that the *Patikan Kebo* extract (Euphorbia hirta L) contains bioactive phenols and saponins which have antiviral activity, with the content of bioactive *Patikan Kebo* leaf extract. Phenol and Saponins can activate the antiviral activity of the Infectious Bronchitis (IB) virus that attacks the respiratory tract. The content of bioactive substances including phenols contained in the *Patikan Kebo* extract are flavonoids, tannins, lavanoids (especially quercitrin and myricitrin) and saponins.[23]. known to damage viral envelope lipids. Hemagglutinin is a protein on the surface envelope of the virus that is able to hemagglutinate erythrocytes, resulting in damage to the surface structure of the virus, resulting in no replication. In the treatment of Infectious Bronchitis (IB) virus with *Patikan Kebo* extract with a concentration of 0.01%, the HA titer was 2.2 with 42% antiviral activity and IB virus with 1% treatment had an average HA titer value of 2.4 with 37% antiviral activity. can also have antiviral activity because it is lower than the control without extract treatment which has a titer value of 3.8. Limit measurement of viral antiviral activity poultry corona i.e. 26%, which means that if the antiviral activity value is 26%, the extract can inhibit the attachment of the avian corona virus [18].

Contents of *Patikan Kebo (Euphorbia hirta L)* have many with various benefits. The ability of the *Patikan Kebo* plant in treating various kinds of diseases is because it involves chemical compounds in it. These compounds are antiseptic, anti-inflammatory, antifungal, antiviral and antibacterial. The bioactive ingredients in *Patikan Kebo* are tannins, lavanoids (especially quercitrin and myricitrin) and saponins.[16].

Bioactive substances in plants are secondary metabolites, such as enol or polyphenols, saponins, flavonoids, curcumin, artemisinin, tannins. Phenol and saponin bioactive substances inhibit or slow the growth of bacteria, fungi, parasites by targeting the membrane or cytoplasm[17]. Phenolic bioactive substances are one of the chemical components of plants that have benefits for the plant itself and also for humans. In general, phenols are divided into monovalent and polyvalent (polyphenols) groups.[18]. Some examples of bioactive substances that include phenols are curcumin,

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flavonoids, lignin, melanin, tannins, monocyclic phenols, phenyl propanoids, phenolic quinones, gallic acid, caffeic acid, phenyl propanoids, phenolic quinones, and catechins including carbolic acids which are weak acids and can protein coagulation[19]. Protein coagulation properties can be used as a prevention of viral replication. With this, *Patikan Kebo* can inhibit IB virus replication in TAB.

5. Conclusions

Based on the findings from in-ovo research, it has been determined that a 0.1% concentration of *Patikan Kebo* extract is the most effective herbal antiviral against Infectious Bronchitis (IB) in chickens, surpassing both the 0.01% and 1% treatments in terms of HA titer values. Further investigations into the impact of *Patikan Kebo* extract on chickens in vivo are warranted.

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