# IMMUNOSTIMULANT EVALUATION OF NEEM LEAVES AGAINTS NON-SPECIFIC IMMUNE OF TILAPIA INFECTED BY *A. hydrophila*

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**ABSTRACT**
**The aim of this study was to find out how neem leaf extract affected the immune systems of Tilapia (O. niloticus) that had A. hydrophila in their bodies. This included looking at its phytochemistry, antibacterial properties, bioactive compounds, and ability to make the immune system stronger. The extraction uses methanol. The phytochemical test of neem leaf extract includes flavonoids, alkaloids, tannins, saponins, and triterpenoids. We obtained the isolate A. hydrophila from the Jepara Brackish Water Aquaculture. Before use, we store these bacteria in Trypticase Soy Agar (TSA) media at 4 oC and Trypticase Soy Broth (TSB) subcultures. re use. Test antibacterial using published We confirmed flavonoids, tannins, saponins, and triterpenoids through phytochemical tests. firmed. The test against A. hydrophila bacteria found that 100% of the extract was the most effective concentration at stopping the growth of A. hydrophila bacteria. After feeding A. hydrophilla bacteria to Tilapia (O. niloticus), the extract of neem leaf (A. indica) significantly boosted the immune system. bserved.**

**Key words: Indica; bioactive; extract; neem plant; phytochemical screening; life below water**

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## مستخلص أوراق التقييم المناعي لأوراق النيم ضد المناعة غير المحددة لسمكة البلطي المصابة ببكتيريا *A. hydrophila*

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**المستخلص**

**كان الهدف من هذه الدراسة هو معرفة كيفية تأثير مستخلص أوراق النيم على الجهاز المناعي للبلطي (النيلوتيكوس) الذي يحتوي على أ.هيدروفيلا في أجسامها. وشمل ذلك دراسة في الكيمياء النباتية، والخصائص المضادة للبكتيريا، والمركبات النشطة بيولوجيا ، والقدرة على رفع فعالية الجهاز المناعي. تستخدم طريقة استخراج الميثانول. يتضمن الاختبار الكيميائي النباتي لمستخلص أوراق النيم مركبات الفلافونويد والقلويدات والعفص والصابونين والتريتربينويدات. حصلنا على عزل أ. هدروفيلا من تربية الأحياء المائية في مياه جيبارا قليلة الملوحة. قبل الاستخدام ، وتم تخزين هذه البكتيريا في تريبتيكاز أجار الصويا (تسا) وسائط زرعية في 4 اج و تريبتيكاز مرق الصويا (تسب). تم اجراء اختبار مضاد للجراثيم باستخدام عدة فحص أكدنا الفلافونويد والعفص والصابونين والتريتربينويدات من خلال الاختبارات الكيميائية النباتية. وجد الاختبار ضد بكتيريا أ. هدروفيلا أن 100 ٪ من المستخلص كان التركيز الأكثر فعالية في وقف نمو بكتيريا أ.هدروفيلا. بعد تغذية بكتيريا أ. هيدروفيلا إلى البلطي (س.نيلوتيكوس) ، استخراج أوراق النيم (أ. إنديكا) عزز بشكل كبير جهاز المناعة. مخدومة. *(O. niloticus)* بعد التحدي مع *A. hydrophila* وفقًا للمعايير المناعية غير المحددة التي تمت ملاحظتها.**

**الكلمات المفتاحية: أ .إنديكا؛ نشطة بيولوجيا .يستخرج؛ نبات النيم؛ فحص المواد الكيميائية النباتية، الحياة تحت الماء**

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## INTRODUCTION

Neem (*Azadirachta indica* A. juss), also known as the neem plant, is one of the most popular medicinal plants. It is native to India and naturalized in most tropical and subtropical countries (16, 53). Many tropical countries, such as India, Pakistan, Burma, and Indonesia, host the neem plant (A. indica Juss.), a member of the Meliaceae family (6). *Azadirachta indica*, commonly known as Indian neem or Indian lilac, is an extensively cultivated evergreen tree in the Indian subcontinent. Neem (A. indica) has been widely recognized in Ayurveda as a remedy for several illnesses, mainly because of its exceptional antimicrobial properties (16). Neem leaves serve multiple purposes, which has led to this plant being referred to as the "miracle tree." Neem leaves and seeds offer numerous advantages. Neem seeds can serve as natural pesticides, fungicides, anti-bacterials, spermicides, neem oil soap, and neem oil lubricants. Neem plants possess the ability to impede the growth of Salmonella thiposa and Staphylococcus aureus (16, 53). Neem plants exhibit antibacterial properties against both gram-positive and gram-negative bacteria (8–10). Neem trees contain many bioactive chemicals that can hinder the production of bacterial cell membranes, therefore impeding their growth (11–13). Research findings indicate that neem comprises alkaloids, flavonoids, triterpenoids, steroids, phenolic chemicals, carotenoids, and ketones (40). Neem can be used by doctors to treat inflammation, criticism, low blood sugar, fever, inflammation, acne, infections, malaria, tumors, fungi, ectacterial infections, spemicidal infections, inflammation, diuretics, and ulcers in the stomach (14–16). The existence of freshwater fish parasites is very diverse (37Aeromonas hydrophila, a pathogenic bacteria, causes Motil Aeromonas Septicemia (MAS), often known as red spot disease, in various species of freshwater fish. This disease has the potential to cause mortality in fish eggs, leading to a fatality rate of 80% to 100% during a span of one to two weeks (45, 48). Fish infected with the bacteria A. hydrophila exhibit symptoms such as anorexia, dermal sores, the appearance of erythematous areas on the body, and inflammation of the skin. The progression of these symptoms ultimately results in the development of ulcers, liver hemorrhaging, bleeding in the fins, scales becoming loose, release of the caudal fin, bleeding in the muscles, presence of bloody mucus in the rectum, and creation of bloody fluid. Bacteria A. hydrophila shown high pathogenicity towards freshwater fish (39, 14). Scientists have examined the utilization of plant materials as substances that enhance the immune response in fish for the purpose of improving their health (43, 28, 4). In order to ascertain the magnitude of its impact on A. hydrophila bacteria, it is important to do additional research and experimentation on the efficacy of neem as an antibacterial agent. This statement requires additional investigation into the impact of neem leaf extract on A. hydrophila bacteria. The objective of this investigation was to ascertain the antibacterial efficacy and identify the optimal concentration of neem leaf extract necessary to suppress the growth of A. hydrophila bacterium. The objective of this study is to examine the viability of using neem leaf as a substitute raw material for producing immunostimulants that can enhance the non-specific defense system of tilapia. Due to its rapid development, neem leaf has the potential to serve as a valuable source of bioactive chemicals.

## MATERIAIS AND METHODS

### Neem leaf (*A. indica*) extract preparation

We acquired neem leaf (Azadirachta indica) from a farmer in Malang, located in East Java. We utilize aged foliage that has been purged of debris, cleansed with flowing water, and then dried. We employ an indoor drying method to dehydrate the leaves, allowing for proper air circulation, for a duration of roughly 7-8 days. Subsequently, we proceed to pulverize and sift the dried leaves to acquire a fine powder.

### Extraction

We collect both mature and young neem leaves, wash them thoroughly with flowing water, allow them to air dry in the absence of direct sunlight, and subsequently pulverize them using a blender (42, 17). We get bioactive constituents from neem leaves (Azadirachta indica A. juss) through the process of maceration, using ethanol as the solvent. The extraction process involved

immersing 100 grams of neem leaf flour in a solvent with a ratio of 1:3 for a total of 48 hours, divided into two periods of 24 hours each. We employed filter paper and a rotary vacuum to separate the extract. Subsequently, we utilized an evaporator at a temperature range of 40-45 oC till the condenser indicated no further evaporation (26). Subsequently, we procure a concentrated neem leaf extract in order to evaluate its antibacterial efficacy.

**Phytochemical test**

Phytochemical studies of neem leaf extracts, including flavonoids, alkaloids, tannins, saponins, and triterpenoids, are conducted using established procedures (25, 38). We employ GC-MS instruments to ascertain the chemical composition and analyze the mass spectra of leaf extract samples from A. indica. The pre-programmed database of a GC-MS equipment will analyze the acquired mass spectrum, which corresponds to a standardized mass spectrum for identified chemicals. The GC analysis was conducted using an Agilent 6890N gas chromatograph that was fitted with an Agilent 5973 selective mass detector. We employed helium (99% purity) as the carrier gas, with a flow rate of 1 mL/min. We introduced 1 liter of extract into the injector at a temperature of 250 degrees Celsius. The interface temperature was set to 270 degrees Celsius, the detector temperature was set to 230 degrees Celsius, and we maintained a split ratio of 1:20. The temperature program on the column commences at 70 oC and remains constant for 5 minutes. It then increases at a rate of 10 oC per minute until it reaches 270 oC, where it is held for an additional 5 minutes. This entire process takes a total of 30 minutes (21). The GC-MS instrument comprises of two essential elements: Gas chromatography (GC) serves as a detector for a substance's constituent, analyzing its chemical composition. Mass spectrometry (MS) is a technique used to identify the smallest structural unit of a molecule in the fractions generated by gas chromatography (GC). It then analyzes the mass spectrum of the compound to estimate its abundance and the time it takes to detect it (23).

**Bacterial preparation**

We acquired a solitary A. hydrophila strain from the Jepara Brackish Water Aquaculture Center. Before using them, we keep these bacteria stored at a temperature of 4 degrees Celsius in Trypticase Soy Agar (TSA) media and Trypicase Soy Broth (TSB) subcultures (33, 36).

**Bacterial test**

The user's text is a single letter "T". The initial procedure entails immersing a sterilized wooden cotton swab into a bacterial suspension that is similar to McFarland's 0.5 solution. Then, firmly apply the sterile wooden cotton to the inner wall of the tube until there is no liquid dripping. Subsequently, employing the swab method, we uniformly applied the aseptic hardwood cotton onto every surface of the Mueller Hinton Agar (MHA) medium and permitted it to remain undisturbed for a duration of 5 minutes. In order to accomplish this, we employed a micropipette to dispense varying quantities of neem leaf extract (A. indica) onto disc paper, with the greatest concentrations being 10%, 20%, 40%, 60%, 80%, and 100%. We employed a 2% solution of chlorhexidine as a positive control, while distilled water served as the negative control. Following the application of bacterial suspension onto Mueller Hinton Agar (MHA) media, position a paper disc on the surface. Apply equal quantities of neem (A. indica) leaf extract and control material onto the disc. The spacing between the paper discs must be sufficient to ensure that the clear areas do not overlap. We exert pressure on the disc paper using tweezers to establish optimal contact between the disc and the agar media. In addition, we cultured Mueller Hinton Agar (MHA) media in an environment without oxygen at a temperature of 37 °C for a duration of 48 hours. We performed the experiment on three separate occasions, each at a distinct time. Following a 48-hour period, we utilized equation (9) to quantify the extent of illumination for each neem leaf extract (A. indica) concentration. The measurement findings are analyzed and interpreted using the classification methods described in the publication by Puspita et al. (36), as shown in Table 1.

**Table 1 The intensity level of the extract is categorized according on its ability to suppress bacterial growth, as determined by Puspita (36).**

| Light Zone Diameter | Inhibited Response |
|---|---|
| >20 mm | Strong |
| <10 mm | None |
| 16-19 mm | Medium |
| 10-15 mm | Weak |

**Experimental design**

The study employed a completely randomized design (CRD) with 5 treatments and 3 replications. We intraperitoneally gave neem leaf (Azadirachta indica) extract at concentrations of 0.1, 0.2, and 0.3 mg/kg of fish weight (FW). The negative control was administered with sterile PBS, while the positive control was administered with a commercially available immunostimulant. Following a period of 24 hours for acclimation, the fish were infected by A. hydrophilla.

**Observation of immunological parameters**

We conducted an examination of non-specific immunological markers on days 0, 4, 8, and 12 following the injection. The blood of the fish was We obtained fish blood from the caudal artery and transferred it into a microtube containing a solution of 10% Na-E. We assessed general humoral immune system parameters, rather than focusing on specific ones. The measured variables were total plasma protein, superoxide dismutase (SOD), and lysozyme activity (LA). We utilized spectroscopic techniques to quantify the levels of total plasma protein and SOD. The measurements were performed at specific wavelengths of 600 nm and 505 nm, respectively. The protocols followed for these measurements were previously described (29, 50). We utilized standard protein curves using bovine serum albumin (BSA) to convert the overall absorbance measurement of plasma protein into protein mg/ml. We have also converted the absorbance value of SOD to units per milliliter (U/mml). We employed the turbidimetric assay technique to examine and quantify the activity of lysozyme. Upon completion of the feeding trial, we collected samples of the lysozyme activity assay from the fish that were slaughtered. We obtained the mucus by immersing paper in a liquid and subsequently dissected it to extract the specific tissues (kidney and liver) from the fish. Following the process of weighing and homogenizing the tissue, we introduced acetate buffer (consisting of 0.5 M acetic acid and 0.5 M sodium acetate; pH = 5) to the tissue five times. After homogenization, we subjected the tissue suspension to centrifugation at 4000 rpm for 15 minutes and obtained the supernatant. The tissue homogenates were combined with 10 µl to create a solution of 140 µl containing Aeromonas hydrophila. The Aeromonas hydrophila was in lyophilized form and had a concentration of 1 mg/ml. The solution was prepared in 0.05 M sodium phosphate buffer with a pH of 6.2. Subsequently, we measured the decrease in absorbance at a wavelength of 450 nm (delta A) at two time points: immediately and after 30 minutes of incubation at a temperature of 20 °C. We established a single unit of lysozyme activity as a decrease in absorbance of 0.001 per minute. To determine the activity, we applied the formula: A multiplied by 1000 per minute per milliliter (44, 52). *=Data Analysis* ===The data was examined using the ANOVA test. The purpose of this study is to analyze the impact of batik waste exposure on the levels of SOD (superoxide dismutase) in tilapia. The ANOVA analysis employed a confidence level of 95% and a significance level of 5%. We employed Duncan's test as a further examination.

**RESUITS AND DISCUSSION**

**Neem leaf extraction yield result**

The initial, subsequent, and final attempts to dry 1 kg of neem leaf resulted in powder yields of 188 ± 1.49 grams, 195 ± 1.87 grams, and 172 ± 1.45 grams, respectively. The initial, subsequent, and final iterations yielded 68 ± 1.29 grams, 71 ± 1.55 grams, and 65 ± 1.42 grams of extract, respectively. Table 2 presents the complete results.

**Table 1. Neem leaf extraction yield results**

| Repetition | Powder Yield (Gram) | Extract (Gram) |
|---|---|---|
| 1 | 188 ± 1.49 | 68 ± 1.29 |
| 2 | 195 ± 1.87 | 71 ± 1.55 |
| 3 | 172 ± 1.45 | 65 ± 1.42 |

Several variables impact the aforementioned value, such as the solvent type, its concentration, the particle size of the simplicia, and the time of the extraction process. The success of the separation is determined by the differences in solubility of the components in the solvent (47, 51). Polar chemicals are soluble in polar solvents, and vice versa. The yield is influenced not only by the type of solvent but also by the sample size. A reduced sample's surface area will result in greater expansion of contact and increased interaction with the solvent (12). The antioxidant activity of phenolic chemicals isolated from maize cobs is determined by the kind of solvent extraction. Antioxidant chemicals exhibit diverse chemical properties and polarity, leading to their solubility or insolubility in specific solvents (42-44).

**Phytochemical test of neem leaves**

The qualitative phytochemical analysis revealed the presence of many compounds in the positive neem leaf extract, including flavonoids, tannins, saponins, and triterpenoids. Table 3 has further information.

**Table 2. The results of a qualitative phytochemical analysis**

| Parameter | Result | Information |
|---|---|---|
| **Flavonoid** | +++ | Forms a dark red color |
| **Alkaloid** | | |
| **- Meyer** | - | A white precipitate is formed |
| **- Dragendroff** | - | An orange precipitate is formed |
| **- Bouchardat** | - | A brown precipitate forms |
| **Tanin** | +++ | A blackish green color forms |
| **Saponin** | + | Permanent foam is formed |
| **Terpenoid** | +++ | A brownish orange color form |

Flavonoids constitute a significant category of organic compounds known as phenols, which occur naturally in abundance. Flavonoids are frequently found in plants, where they are either attached to sugar molecules as glycosides or exist as aglycones. Flavoplants generally consist of a blend of flavonoids, never in a solitary state. Compounds found in plants, including floral pigments, serve the purpose of attracting birds and flower pollinating insects. Furthermore, flavonoids serve as growth regulators, photosynthetic regulators, and possess antimicrobial and antiviral properties. Flavonoids in the body act as inhibitors of the enzyme lipoxygenase, which is involved in the production of prostaglandins. The reason for this is that flavonoids are effective reducing agents that impede oxidation processes (10). Tannin is typically described as a polyphenol molecule with a molecular weight more than 1000, capable of forming complexes with proteins. Tannin is a complex component of organic compounds. Tannin is composed of phenolic chemicals, which pose challenges in terms of separation and crystallization. These chemicals also extract proteins from their solutions and form complexes with them (41). Tannins are classified into two categories: hydrolyzed tannins and condensed tannins. Tannins play diverse biological activities, including protein precipitation and metal chelation. Tannins can also serve as innate antioxidants (25). Saponins are amphiphilic chemicals that exhibit high surface activity and produce abundant foam when agitated in water. Certain saponins function as antimicrobial agents. Upon hydrolysis, saponin undergoes decomposition into carbohydrates (glycans) and non-sugars (aglycones), specifically a sugar and an organic hydroxyl molecule. Saponins are composed of two

distinct categories: triterpenoid saponins and steroid saponins. Terpenoids (15, 26) are a class of active chemicals with the potential to act as anti-bacterial agents. Terpenoids are chemical substances that have undergone modifications to their isoprene structure. Further investigation is required to fully explore the utilization of terpenoid chemicals in red galangal rhizomes, as their potential remains mostly untapped.

**Identification of compounds on neem leaves extracts:** The neem leaf extract contains several chemicals that display a wide array of bioactivities, such as antifungal, antibacterial, antioxidant, and anti-inflammatory effects. This discussion will thoroughly examine the existing literature in order to provide a comprehensive understanding and evidence for the observed bioactivities.

**Tabel 4. Identification of compounds from neem leaves (*A. Indica*) Extracts by GC-MS**

| Retention Time | Name of the compound | Chemical formula | Bioactivity |
|---|---|---|---|
| 12.048 | Pentadecane | $C_{15}H_{32}$ | Antifungi Antibacterial |
| 16.672 | Neophytadiene | $C_{20}H_{38}$ | Antioxidant |
| | Phytol | $C_{20}H_{40}O$ | Antioxidant |
| 18.021 | Palmitic Acid | $C_{16}H_{32}O_2$ | Antioxidant |
| | Methyl Palmitate | $C_{17}H_{34}O_2$ | Antibacterial, antioxidant |
| 19.071 | Palmitic Acid | $C_{16}H_{32}O_2$ | Antioxidant |
| 20.968 | Methyl elaidate | $C_{19}H_{36}O_2$ | Antiinflammatory |
| | Oleic acid | $C_{18}H_{34}O_2$ | Antioxidant |
| 21.217 | Phytol | $C_{20}H_{40}O$ | Antioxidant |
| 21.789 | Oleic acid | $C_{18}H_{34}O_2$ | Antioxidant |
| | Cis-vaccenic acid | $C_{18}H_{34}O_2$ | Antiinflammatory |
| 21.938 | Oleic acid | $C_{18}H_{34}O_2$ | Antioxidant, |
| | Cis-vaccenic acid | $C_{18}H_{34}O_2$ | antinflammatory |

The literature has reported the potential antifungal and antibacterial activities of pentadecane (C15H32), a long-chain hydrocarbon. Long-chain hydrocarbons, such as pentadecane, are known to disrupt microbial membranes, leading to their antimicrobial effects (22). The neem leaf extract identifies neophytadiene and phytol (C20H38 and C20H40O) as having antioxidant properties. Particularly, phytol has demonstrated strong antioxidant activity, aiding in neutralizing reactive oxygen species and shielding cells from oxidative damage (50–52). The compounds palmitic acid and methyl palmitate (C16H32O2 and C17H34O2) exhibit both antioxidant and antibacterial activity. Palmitic acid and its methyl ester derivative, methyl palmitate, are known for their antioxidant properties. Palmitic acid possesses antioxidant properties, while methyl palmitate possesses both antibacterial and antioxidant properties. These two chemicals work together to make the neem leaf extract bioactive (18, 30). Anti-inflammatory and Antioxidant Activity: Oleic acid and its methyl ester derivative, methyl elaidate, have anti-inflammatory and antioxidant properties (C18H34O2). Researchers have reported the anti-inflammatory effects of oleic acid, a monounsaturated fatty acid, and both compounds contribute to the antioxidant profile of the neem leaf extract (5, 13). Antioxidant and Anti-inflammatory Activity: The neem leaf extract contains cis-vaccenic acid, which has antioxidant and anti-inflammatory properties. These properties make it a potential contributor to the overall anti-inflammatory effects of the neem leaf extract (32, 7). The identified compounds, as discussed in the context of relevant literature, underscore the multifaceted bioactivity of neem leaf extract. The fact that neem leaf extract is antifungal, antibacterial, antioxidant, and anti-inflammatory suggests that it could be a useful natural source for treating microbial infections and conditions related to oxidative stress.

**Neem leaf extract on the growth of *A. hydrophila:*** Employing varying concentrations of neem (Azadirachta indica) leaf extract as positive or negative controls effectively inhibited the growth of A. hydrophila in the experiment. The disk exhibited distinct clear zones surrounding it, thereby indicating this. The categorization of published studies (59–62) directs the understanding of the average inhibition zone measurement outcomes. The concentrations of

the extracts that were tested included 10%, 20%, 40%, 60%, 80%, and 100%. A positive control, which was 2% chlorhexidine, resulted in the creation of a clear zone around the disc. On the other hand, a negative control, which was distilled water, did not exhibit a clear zone around the disc. The anti-bacterial test findings of the neem leaf extract are presented in Table 4.

**Table 5. Test Results of Neem Leaf Extract on the Growth of *A. hydrophila***

| Extract Concentration | Inhibitions Zone | | | Average | Note |
|---|---|---|---|---|---|
| | Repeatition 1 | Repeatition 2 | Repeatition 3 | | |
| 10 | 8.4 | 8.6 | 8.8 | 8.60 ± 0.20 | Weak |
| 20 | 12.1 | 12.6 | 11.7 | 12.13 ± 0.45 | Weak |
| 40 | 13.0 | 14.1 | 13.7 | 13.60 ± 0.56 | Weak |
| 60 | 17.9 | 17.6 | 17.7 | 17.73 ± 0.15 | Medium |
| 80 | 18.0 | 18.4 | 18.6 | 18.33 ± 0.31 | Medium |
| 100 | 20.1 | 20 | 20.4 | 20.17 ± 0.21 | Strong |
| Chx 2% | 27.6 | 27.2 | 28.4 | 27.73 ± 0.61 | Strong |
| Aquades | 6 | 6 | 6 | 6.00 ± 0.00 | None |

According to the analysis of the table above, the neem leaf extract has the smallest inhibitory zone at a concentration of 10%, and the largest inhibitory zone at a concentration of 100%. Chlorhexidine at a concentration of 2% acts as a positive control by inhibiting the growth of A. hydrophila. It achieves this by disrupting the bacterial cell walls and creating openings in the cell membrane. Hydrophilic and lipophilic-charged chlorhexidine molecules interact with phospholipids and lipopolysaccharides in cell membranes. The breakdown of the membrane enables the entry of chlorhexidine into the cell, leading to intracellular toxicity. The negative control, consisting of pure water, does not exhibit any zone of bacterial suppression. This implies that it lacks any antibacterial characteristics that could inhibit the proliferation of A. hydrophila. The investigation determines that the extract from the neem leaf (A. indica) possesses antibacterial properties and is capable of inhibiting the growth of A. hydrophila. The phytochemical test revealed that the neem (A. indica) leaf extract contains steroids, saponins, phenolic chemicals, and triterpenoids, which possess antibacterial properties. The phytochemical test results do not align with the findings of published studies, which indicate that neem leaf extract contains alkaloids, flavonoids, triterpenoids, phenolic compounds, carotenoids, steroids, and ketones (27). The lack of alkaloid and flavonoid components in neem leaf extract diminishes its antibacterial efficacy. This is because the presence of alkaloids and flavonoids in the neem leaf extract results in the formation of highly intricate active compounds that possess potent antibacterial properties. Neem leaf extract contains chemical components that have an antibacterial effect through a distinct method. Plant-derived antibacterial chemicals have the ability to inhibit the growth of bacteria (11, 31). Triterpenoids interact with porin proteins located on the outer membrane of bacterial cell walls, forming robust polymer linkages that degrade porin. Damage to porins decreases the permeability of the bacterial cell wall, leading to a decrease in the flow of nutrients into the bacterium. This, in turn, hinders or eliminates bacterial growth. Saponins have bactericidal

effects by binding to cell membranes via hydrogen bonding, resulting in the formation of intricate compounds. These chemicals have the ability to inhibit bacterial entry into the cell wall, ultimately leading to its destruction.

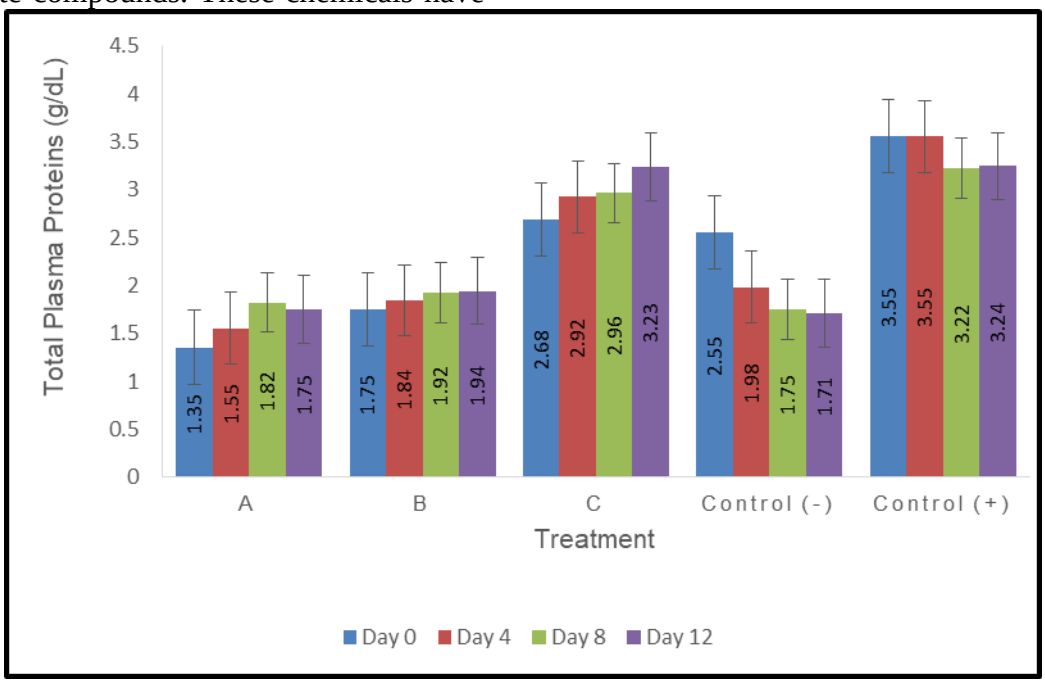


**Figure 1. Total Plasma Protein (TPP) of Tilapia (O. Niloticus) during the research**

Multiple studies have demonstrated that phenolic compounds and tannins have the ability to interact with cell membranes, inhibit the activity of reverse transcriptase and DNA topoisomerase enzymes, and disrupt or halt the functioning of bacteria's genetic material (49, 8). Steroids possess antibacterial activities due to their ability to induce permeability in bacterial cell walls (33). An inhibitory zone was observed surrounding the disc paper in the tests conducted to evaluate the antibacterial properties of neem (A. indica) leaf extract at various concentrations. Evidence demonstrates that neem leaf extract possesses antibacterial properties capable of impeding bacterial proliferation.

**The immunostimulant evaluation in tilapia (*O. niloticus*)**

**Total plasma protein:** Figure 1 illustrates the effect of the A. hydrophilla challenge on the concentration of total plasma protein (TPP) in Tilapia (O. niloticus). Total plasma protein refers to the aggregate amount of protein found in the liquid portion of blood, called plasma. These proteins encompass various types, including albumin, fibrinogen, and globulin. Plasma, often known as blood plasma, has a pale yellow or straw-like tint. Plasma is the liquid part of blood, and it shares many functions with blood. Plasma performs various functions, one of which is vital in the body's immune response to bacteria, viruses, fungi, and parasites. Organisms, including fish, can use the protein concentration in their plasma as a benchmark to assess their immunological condition. Figure 1 depicts the average concentration of total protein in the blood plasma of Tilapia. Based on the study, treatment C, which involved administering an extract of 0.3 mg/kg body weight, had the highest concentration of total plasma protein. After a maintenance period lasting 12 days, the average concentration of plasma proteins was determined to be 3.23 g/dL ($p < 0.05$). In contrast, treatment A (control) showed the lowest overall plasma protein level when the extract dosage was not present. The presence of A. hydrophilla bacteria resulted in a significant alteration in the protein contents within the blood of the tilapia. The analysis of variance clearly demonstrated this effect. The study demonstrated that the compounds included in neem leaf extract (A. indica) has immunostimulant properties, enabling them to enhance and repair the immune systems of fish. Immunostimulants have a double impact: they enhance immune system activity and strengthen resistance to diseases, hence enhancing an organism's capacity to survive in the face of dangerous pathogens. Published

studies have demonstrated that providing immunostimulants through injections can improve the resistance of fish to bacterial infections (67–69). The study concluded that the optimal dosage of the extract to increase the overall plasma protein level was 0.3 mg/kg of body weight. We contend that this specific dosage is the most suitable to endow Tilapia with optimal protection against A. hydrophilla bacteria. However, if a dosage that is too large is administered, it can also have a harmful effect on fish (33) and cause immunosuppression, which reduces their non-specific immune systems. Figure 1 illustrates the effect of the A. hydrophilla challenge on the concentration of total plasma protein (TPP) in Tilapia (O. niloticus). Total plasma protein refers to the total amount of protein found in the liquid part of blood, called plasma. These proteins encompass various types, including albumin, fibrinogen, and globulin. Plasma, often known as blood plasma, has a pale yellow or straw-colored look. Plasma is the liquid part of blood, and it shares many functions with blood. Plasma performs various functions, one of which is vital in the body's immune response to bacteria, viruses, fungi, and parasites. Living creatures, including fish, can use the protein concentration in plasma as a benchmark to assess their immunological condition. Figure 1 depicts the average concentration of total protein in the blood plasma of Tilapia. Based on the study, treatment C, which supplied an extract of 0.3 mg/kg body weight, had the highest concentration of total plasma protein. After a maintenance period lasting 12 days, the average concentration of total plasma protein was determined to be 3.23 g/dL ($p < 0.05$). In contrast, treatment A (control) showed the lowest overall plasma protein level when the extract dosage was not present. A. hydrophilla bacteria significantly altered the protein contents in the blood of the tilapia. The analysis of variance clearly demonstrated this effect. The study shown that the compounds present in neem leaf extract (A. indica) possess immunostimulant properties, enabling them to enhance and restore the immune systems of fish. Immunostimulants have a double impact: they enhance immune system activity and strengthen resistance to diseases, hence enhancing an organism's capacity to survive in the face of dangerous pathogens. Published research have demonstrated that providing immunostimulants by injections can improve the fish's resistance to bacterial infections (67–69). The study established that the optimal dosage of the extract to augment the overall plasma protein level was 0.3 mg/kg of body weight. We contend that this specific dosage is the most suitable to endow Tilapia with optimal protection against A. hydrophilla bacteria. However, if a dosage that is too large is administered, it can also have a harmful effect on fish (33) and cause immunosuppression, which reduces their non-specific immune systems. Figure 1 illustrates the effect of the A. hydrophilla challenge on the concentration of total plasma protein (TPP) in Tilapia (O. niloticus). Total plasma protein refers to the aggregate amount of protein found in the liquid portion of blood, called plasma. These proteins encompass various types, including albumin, fibrinogen, and globulin. Plasma, often known as blood plasma, has a pale yellow or straw-like tint. Plasma is the liquid part of blood, and it shares many functions with blood. Plasma performs various functions, one of which is vital in the body's immune response to bacteria, viruses, fungi, and parasites. Organisms, including fish, can use the protein concentration in their plasma as a benchmark to assess their immunological condition. Figure 1 depicts the average concentration of total protein in the blood plasma of Tilapia. Based on the study, treatment C, which involved administering an extract of 0.3 mg/kg body weight, had the highest concentration of total plasma protein. After a maintenance period lasting 12 days, the average concentration of plasma proteins was determined to be 3.23 g/dL ($p < 0.05$). In contrast, treatment A (control) showed the lowest overall plasma protein level when the extract dosage was not present. The presence of A. hydrophilla bacteria resulted in a significant alteration in the protein contents within the blood of the tilapia. The analysis of variance clearly demonstrated this effect. The study demonstrated that the compounds included in neem leaf extract (A. indica) has immunostimulant properties, enabling them to enhance and repair the immune systems of

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**Superoxide dismutase (SOD)**

The results of the sublethal toxicity test in Figure 2 demonstrate that the superoxide dismutase (SOD) activity of each treatment increases on day 4 and subsequently decreases on days 8 and 12. An elevation in superoxide dismutase (SOD) levels serves as an indication that the fish's organism is initiating its immune response to combat bacterial infections. The purpose of this elevation in the SOD enzyme is to mitigate cellular superoxide bursts during the immune response to viral infection and safeguard shrimp cells from harm. It can be inferred that the fish's immune system has concluded its battle against the bacterial illness and has reverted to its usual state on days 8 and 12. The study found that administering an extract dose of 0.3 mg per body weight resulted in a significant and quick decline in SOD levels. After 12 days of maintenance, the average SOD value was measured to be 0.13 units mL-1, which was statistically significant ($p < 0.05$). Based on our assumption, administering this dosage is considered the optimal amount to combat bacterial illness and enhance the fish's immune system. This is due to its ability to expedite the reduction of SOD levels and promote the fish's overall health and return to a normal state (19, 31).

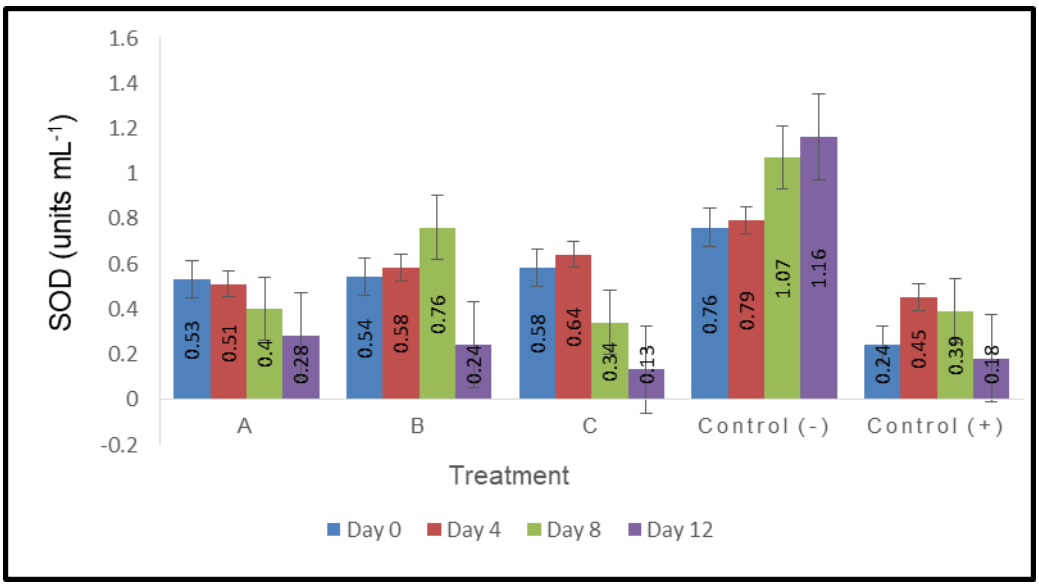


**Figure 2. Superoxide dismutase (SOD) of tilapia (O. Niloticus) during the research**

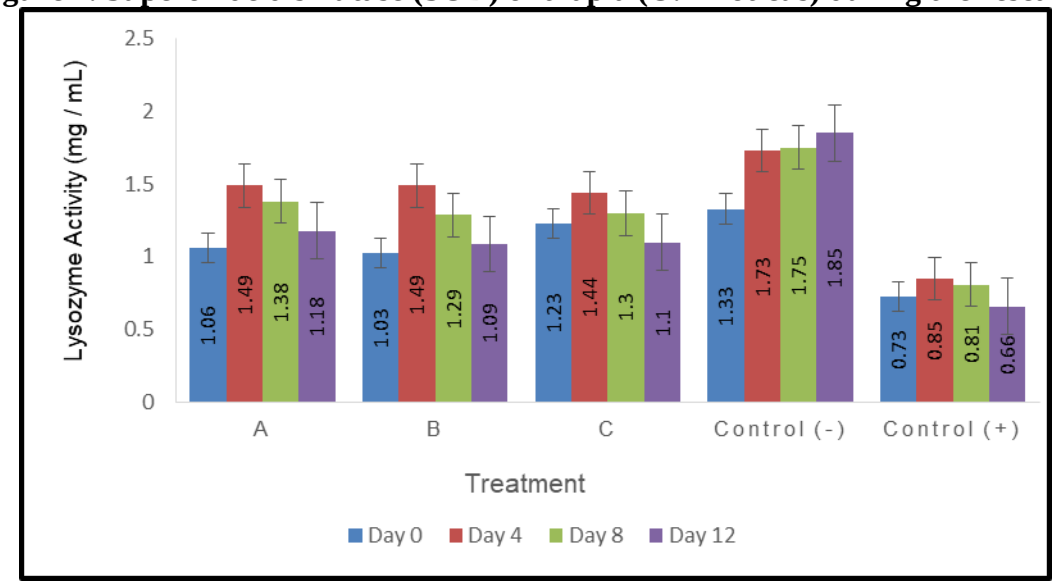


**Figure 3. Lysozyme activity of tilapia (O. Niloticus) during the research**

**Lysozyme activity**

Lysozyme is a vital bactericidal enzyme in the innate immune system. This innate immune response, which occurs during infection or stressful situations, acts as an acute-phase protein, assisting in the defense against fish disease infections (70–72). Figure 3 depicts the lysozyme activity observed during the course of the research. The data shown in Figure 3 demonstrates that treatment extracts derived from neem leaf (Azadirachta indica) exhibit a reduction in lysozyme activity at every dosage. It is postulated that this enzyme enhances the immunological response of Tilapia against A. hydrophilla bacterial infection. Lysozymes are hydrolytic enzymes found in the mucus, serum, and phagocytic cells of different fish species. Multiple investigations have indicated that this enzyme can offer significant protection against microbial infections. Fish neutrophils and monocytes possess lysozyme within their cytoplasm, while leukocytes obtain serum lysozyme (20). Lysozyme is a hydrolytic enzyme that has been used as a prototype for studying protein biochemistry. It functions as an antibacterial agent by disrupting the links inside the peptidoglycan layer on the surface of the bacterial cell wall. Phagocytosis can either directly eliminate germs or opsonize them. At that juncture, the presence of anti-bacterial lysozyme in fish blood indicates the activation of the immune system. This suggests that the neem leaf extract contains anti-microbial substances that can improve the functioning of macrophages, resulting in an increase in the process of phagocytosis. Consequently, this improves the body's

immune system by increasing the concentrations of lysozyme enzymes. In addition to its direct impact on antibacterial action, lysozyme also enhances phagocytosis. In order to further investigate this topic, we recommend doing future investigations utilizing a native or non-native aquatic animal test as described in a published publication. Examples of suitable animals include mangrove snails (24), snakeheads (35), and genggehek fish (46). Utilizing an intelligent vision system model, we can accurately determine the genus of the fish at hand (34).

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