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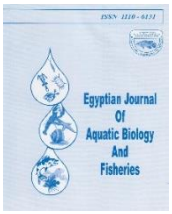

Role of Antibacterial Compounds in Neem Leaf Extract (*Azadirachta indica* A. Juss) in Inhibiting the Growth of *Vibrio parahaemolyticus* Bacteria in the *Vannamei* Shrimp

Jordi[1], Marlina Achmad[2]*, Gunarto Latama[3]

[1]Master Program of Fishery Science, Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar, 90245, South Sulawesi, Indonesia
[2]Fakulty of Vacation Studies, Hasanuddin University, Makassar, 90245, South Sulawesi, Indonesia
[3]Departement of Fisheries, Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar, 90245, South Sulawesi, Indonesia

*Corresponding Author: marlina.achmad@unhas.ac.id

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ABSTRACT

Herbal plants are increasingly being used as alternatives to synthetic antibacterials due to their cost-effectiveness and minimal adverse effects. Among various plants, the neem leaves (*Azadirachta indica* A. Juss) have been extensively utilized in traditional medicine for their antiviral, antifungal, insecticidal, and antibacterial properties. This study aimed to identify antibacterial compounds extracted from neem leaves with potential activity against *Vibrio parahaemolyticus* in the *vannamei* shrimp (*Penaeus vannamei*). The antibacterial compounds were identified using GC-MS (Gas Chromatography-Mass Spectrometry), while the antibacterial activity was evaluated through disc diffusion tests with concentrations of 50, 75, and 100 mg/mL, followed by a minimum inhibitory concentration (MIC) test. The GC-MS analysis revealed 12 peak compounds in the neem leaf extract, detected as secondary metabolites from the mycosporin, phenol, alkaloid, triterpenoid, phytosterol, and steroid groups. The antibacterial activity test showed inhibition zone diameters of approximately 12.8, 11.5, and 10.1mm at concentrations of 100, 75, and 50mg/ mL, respectively, with minimum inhibitory concentrations of 25 and 12.5mg/ mL. Based on these results, the neem leaf extract shows promise as an alternative antibacterial agent.

INTRODUCTION

Herbal plants are integral to promoting a healthy lifestyle due to significant medicinal properties, minimal adverse effects, and cost-effectiveness **(Jain *et al.*, 2018)**. According to numerous studies, bioactive compounds from herbal plants have strong antibacterial activity, underscoring the potential as alternative drugs against chemically resistant and sensitive bacteria **(Fazly *et al.*, 2018)**. Currently, about 80% of people in developing countries rely on medicinal herbs as the primary source of care **(Rupani & Chavez, 2018)**.

The neem tree *(Azadirachta indica* A. juss), commonly referred to as neem, nimtree, or indica lilac, belongs to the Meliaceae family and the *Azadirachta* genus. This tree is native to tropical and subtropical countries such as India, Bangladesh, Nepal, Thailand, and Indonesia **(Kozłowski *et al.*, 2020)**. Neem has a long history of use as a herbal medicine, with records from the Center for Traditional Medicine and Research (CTMR) library in Chennai, India, indicating that every part of the plant can be used as a cure for diseases including ulcers and smallpox **(Gupta *et al.*, 2017)**. This plant, widely distributed worldwide, is a source of various new medicinal compounds that contribute significantly to healthcare **(Tibebu *et al.*, 2018)**. Several previous studies have confirmed that neem leaves have anti-arthritis, anti-gastric, hypoglycemic, antipyretic, antifungal, antibacterial, larvicidal, and immunomodulatory activities **(Srivastava *et al.*, 2020; Fisayomi *et al.*, 2022)**.

Neem leaves serve as natural ingredients for antibacterial agents due to the presence of secondary metabolite compounds such as flavonoids, steroids, alkaloids, and saponins **(Saxena, 2015; Pratiwi, 2022)**. In addition, the extract has been widely used as a medicine for microbial and parasitic infections, especially in livestock **(Tibebu *et al.*, 2018)**. **Ibrahim and Kebede (2020)** stated that neem leaves extracts, specifically those extracted using methanol showed an antibacterial activity *in vitro* against *S. aureus, E. coli, P. aeruginosa,* and *C. albicans.*

Food-borne pathogenic bacteria such as *Vibrio* sp. are a major concern, causing food poisoning and various diseases **(Singh *et al.*, 2023)**. *Vibrio* is an inherited pathogen responsible for the occurrence of gastroenteritis and other cases of foodborne infections in humans, especially from contaminated food, such as seafood. In addition, this infection is responsible for losses in the livestock and aquaculture industry **(Heng *et al.*, 2017)**.

A common type of *Vibrio* sp. is *Vibrio parahaemolyticus*, which causes vibriosis in shrimp, potentially leading to foodborne illness **(Letchumanan *et al.*, 2015)**. Rising ocean temperatures and climate change have led to the prevalence of *V. parahaemolyticus*. The first case was detected in 1951 by Tsunesa-buri Fujino at the Research Institute of Microbial Disease (RIMD), Osaka University, from an outbreak of acute gastroenteritis **(Letchumanan *et al.*, 2019)**. These bacteria have a single flagellum, are curved rod-shaped **(Yeoh *et al.*, 2021; Fu *et al.*, 2023)**, and attack *vannamei* shrimp **(Chunguang *et al.*, 2023)**.

V. parahaemolyticus is a negative halophilic bacteria distributed worldwide and causes damage to the hepatopancreas of infected shrimp **(Thammatinna *et al.*, 2020)**. It is the causative agent of early mortality syndrome, now called acute hepatopancreatic necrosis (AHPND), which is hard to be treated in the shrimp farming industry **(Kalatzis *et al.*, 2018)**. The bacteria infect shrimp through oral transmission, colonizing the gut and hepatopancreas **(Lee *et al.*, 2015)**. *V. parahaemolyticus* is also highly resistant to antibiotics widely used in aquaculture activities **(You *et al.*, 2021)**. This is due to the high adaptability to hyperosmotic and high-alkaline environments by using various carbon

sources, amino acids, and substrates **(Zhang *et al.*, 2021)**. Therefore, this research aimed to determine the antibacterial compounds of neem leaf extract that have the potential as antibacterial against *V. parahaemolyticus* in *vannamei* shrimp *(Panaeus vannamei).*

MATERIALS AND METHODS

Preparation of samples

Neem leaves were collected from trees growing wild in Makassar City, and then treated with water until becoming clean, followed by drying at room temperature. Subsequently, the completely dry leaves were mashed using a blender to powder, which was weighed **(Vicencio, 2020)**.

Procedures

Neem leaves extract

Extraction was carried out based on the method of **Nyalo *et al.* (2023),** namely modified maceration. The fine powder was soaked with methanol solvent in a ratio of 1:3 and allowed to stand for 24h in a shaker incubator. The solution was then filtered using Whatman No. 1 filter paper to separate the filtrate and debris. The same procedure was carried out three times until a non-concentrated filtrate with a light green color indicator was obtained. Furthermore, the filtrate was concentrated separately using a rotary evaporator at 90rpm under vacuum to ensure perfect evaporation of the solvent. To obtain the amount of extract yield, the formula proposed by **Dhanani *et al.* (2017)** was used. The neem leaves extract obtained was stored in a cooler at 8°C for further use.

$$Yield(\%) = \frac{Weight\ of\ Extracth}{Weight\ of\ Starting\ Material} \times 100$$

To determine the antibacterial compounds, GC-MS analysis was carried out using GCMS-QP2010 ultra (Shimadzu). Following the procedures referenced by **Khayyat and Roselin (2018)**, particle-free neem leaves extract was taken up to 1µL and injected into the injector with a separation ratio of 10:1. All data were obtained by collecting a full-scan mass spectrum with a range of 40–55µm. The constituent composition of the crude extract was expressed as a percentage by peak area. Furthermore, the identification and characterization of the compounds in various extracts were based on GC runtimes. These mass spectra have been computer-matched (>70%) to the standards available in the NIST 08 mass spectrum library.

Bacteria preparation

A pure bacteria stock of *V. parahaemolyticus* on thiosulfate citrate bile salt sucrose (TCBSA) media was cultured on tryptic soy agar (TSA) media tilted using a single dose aseptically, then incubated for 24h at 30°C. The sample was further cultured on tryptic soy broth (TSB) media with a volume of 5ml in a test tube and incubated for 24h **(Hikmawati *et al.*, 2019)**, followed by the inhibition and minimum inhibitory concentration tests.

a. Inhibition level test

The antibacterial activity test was carried out using the Kirby-Bauer method, namely disc diffusion **(Singha *et al.*, 2023)**, with slight modifications. The solid extract was dissolved using methanol, homogenized with a vortex, and then diluted with concentrations of 100, 75, and 50mg/ mL^{-1}. Subsequently, about 20µL were dripped on a 6mm-diameter paper disc and incubated to evaporate the solvent at 45°C. The same procedure was carried out for the positive control, oxytetracycline (OTC) 0,5mg.mL^{-1}, and the negative control, methanol.

Stock culture of *V. parahaemolyticus* bacteria was taken up to 10^7 cells/mL, then mixed homogeneously into TSA media, and slowly poured on a Petri dish medium **(Basir *et al.*, 2020)**. Subsequently, the incubated disc paper was placed on a Petri dish containing TSA media and *V. parahaemolyticus* bacteria followed by incubation for 24h at 37°C. After the incubation period, the inhibition zone around the disc paper was measured using a caliper, with each measurement written in mm from the edge of the disc, and antibacterial activity was determined when the inhibition zone was ≥1mm. The determination of the inhibition zone strength followed the method proposed by **Davis and Stout (1971)**.

Table 1. Inhibitory response according to **Davis and Stout (1971)**

Diameter of clear zone (mm)	Inhibitory response
<5	Weak
5-10	Moderate
10-20	Strong
>20	Very Strong

b. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined following the procedure described by **Nikolic *et al.* (2014)** and **Nyalo *et al.* (2023)**, adjusted to an agar diffusion with disc paper. About 20µL of each neem leaves extract including 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, and 0.390mg/ mL^{-1}, was dripped onto the disc paper, then dried in an

incubator at 30°C before being placed on TSA media previously inoculated with *V. parahameolyticus* bacteria and incubated for 24h. The lowest concentration of active extract that produced inhibition zone was considered the MIC value. The assessment was only carried out on extracts that have the potential to inhibit the test pathogenic bacteria.

The MIC is defined as the lowest concentration of an antibiotic at which no bacterial growth is observed, disregarding the growth of one or two colonies **(Singha *et al.,* 2023)**. The lowest concentration of each extract that showed no visible growth was recorded as the MIC.

Data analysis

The data obtained included GC-MS analysis, inhibition strength, and minimum inhibitory concentration (MIC), which were then analyzed descriptively using tables and figures.

.

RESULTS

Extraction

The neem leaves extraction results (*A. indica* A. juss) are presented in Table (2).

Table 2. Result of maceration extract

Sample type	Simplified weight (g)	Extract weight (g)	Yield (%)
neem leaves	1000	110.19	11.01

Based on Table (2), from 1000g of extracted simplicia, it can produce 110.19g of thick extract with a percentage yield of 11.01%.

GC-MS analysis

Gas chromatograph has a wide range of applications, including the separation and analysis of mixtures containing several components. The results obtained during gas chromatograph of neem leaves extract are presented in Fig. (1).

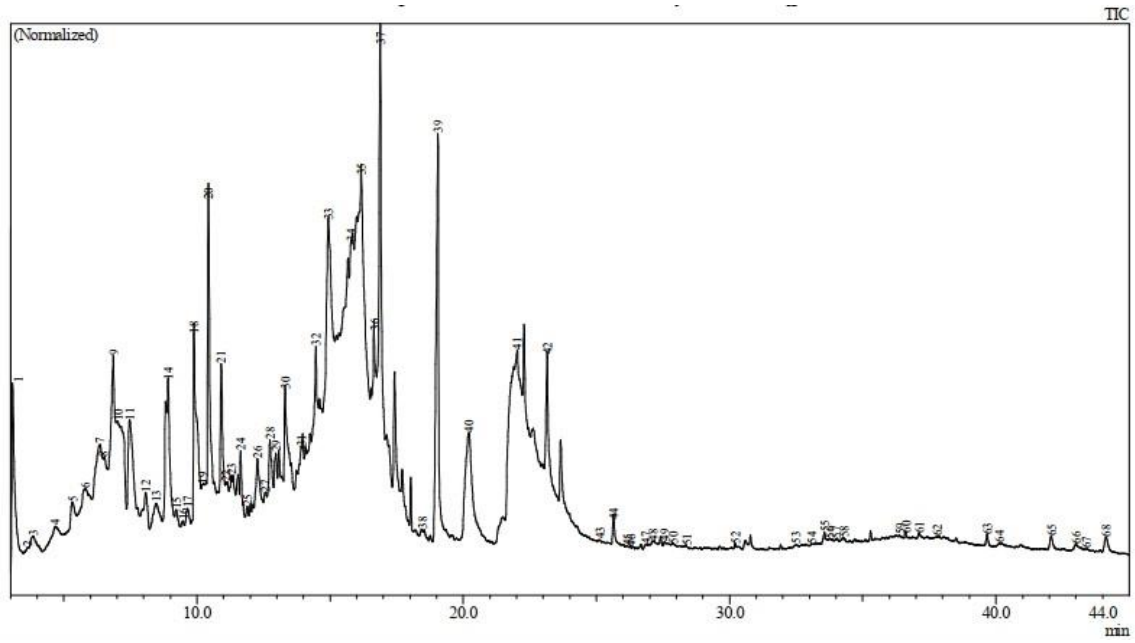

Fig. 1. GC-MS analysis results neem leaf extract

Based on the GC-MS analysis of neem leaves (*A. indica* A. juss), about 68 peaks were detected, and after descriptive analysis, 12 peaks were identified as secondary metabolite compounds (Table 3).

Table 3. Secondary metabolite compounds of neem leaf extracts

Peak	Name of compound	Other names	Molecular formula	Retention time	%Area	Compound class
3	*3-Cyclopropyl-4-hydroxy-4,5,5-trimethyl-oxazolidin-2-one*	*Mycosporine*	$C_{14}H_{24}N_2O_6$	3.844	0.62	*Mycosporines*
8	*Phenol, 2-methoxy-*	*Guaciacol*	$C_7H_8O_2$	6.492	0.75	*Fenol*
20	*Phenol, 2-methoxy-4-(1-propenyl)-(CAS)*	*Acetyleugenol*	$C_{12}H_{14}O_3$	10.438	2.72	*Fenol*
24	*l-Proline, N-propoxycarbony*	*Strigosine*	$C_{14}H_{25}O_4$	11.644	1.22	*Alkaloid*

	l-, isobutyl ester					
28	*2-Hydroxy-1-(1'-pyrrolidiyl)-1-buten-3-one*	*Arekolin*	$C_8H_{13}NO_2$	12.744	1.03	*Alkaloid*
30	*Phenol, 4-ethenyl-2,6-dimethoxy-*	*Dihyclroconifery*	$C_{10}H_{14}O_3$	13.315	2.27	*Fenol*
38	*1,2(equat)-dimethyl-trans-decahydroquinol-4-one*	*Lupinine*	$C_{10}H_{19}NO$	18.455	0.06	*Alkaloid*
62	*Cholest-5-en-3-ol, 24-propylidene-, (3.beta.)-*	*Lupeol*	$C_{30}HS_0O$	37.792	0.03	*Tripterpenoid*
64	*.beta.-Sitosterol acetate*	*Beta-sitosterol acetate*	$C_{31}H_{52}O_2$	40.129	0.05	*Fitosterol*
65	*BUFA-20,22-DIENOLIDE, 14,15-EPOXY-3,5,16-TRIHYDROXY-, (3.BETA.,5.BETA.,15*	*Cinobufagin*	$C_{26}H_{34}O_6$	42.087	0.08	*Steroid*
67	*Campesterol*	*Campesterol*	$C_{28}H_{48}O$	43.392	0.04	*Fitosterol*
68	*STIGMASTA-5,22-DIEN-3-OL*	*Stigmasterol acetate*	$C_{31}H_{50}O_2$	44.146	0.15	*Fitosterol*

Table (3) shows that the most prominent antibacterial compound of neem leaves methanol extract was *Phenol, 2-methoxy-4-(1-propenyl)- (CAS)* located at peak 20 with a retention value of 2.72%, followed by *Phenol, 4-ethenyl-2,6-dimethoxy-*, at peak 30 with a retention value of 2.27% and *l-Proline, N-propoxycarbonyl-, isobutyl ester* at peak 24 with a retention value of 1.22%. The three compounds are included in the class of phenols and alkaloids.

Inhibition level test

The antibacterial test results of neem leaves extract (*A. indica* A. juss) on *V. parahaemolyticus* bacteria are presented in Table (4).

Table 4. Inhibition level test results

Concentration (mg/mL^{-1})	**Inhibition diameter (mm)**			**Average (mm)**	**Category**
	1	**2**	**3**		
100	12.8	13.2	12.4	12.8	strong
75	10.9	11.7	12.0	11.5	strong
50	10.0	10.2	10.2	10.1	strong
control +	23.6	24.1	23.4	23.7	very strong
control -	0	0	0	0	none

As shown in Table (4), the antibacterial activity test results indicated that the largest diameter was produced at a concentration of $100mg.mL^{-1}$, with an average inhibition zone diameter of 12.8mm. At concentrations of 75 and $50mg/ mL^{-1}$, the average inhibition zone diameter was 11.5 and 10.1mm, respectively. The positive control, oxytetracycline (OTC) 0.5mg.mL-1 produced an average inhibition zone diameter of 23.7mm, and the negative control, methanol solvent, had no inhibition zone, as presented in Fig. (2).

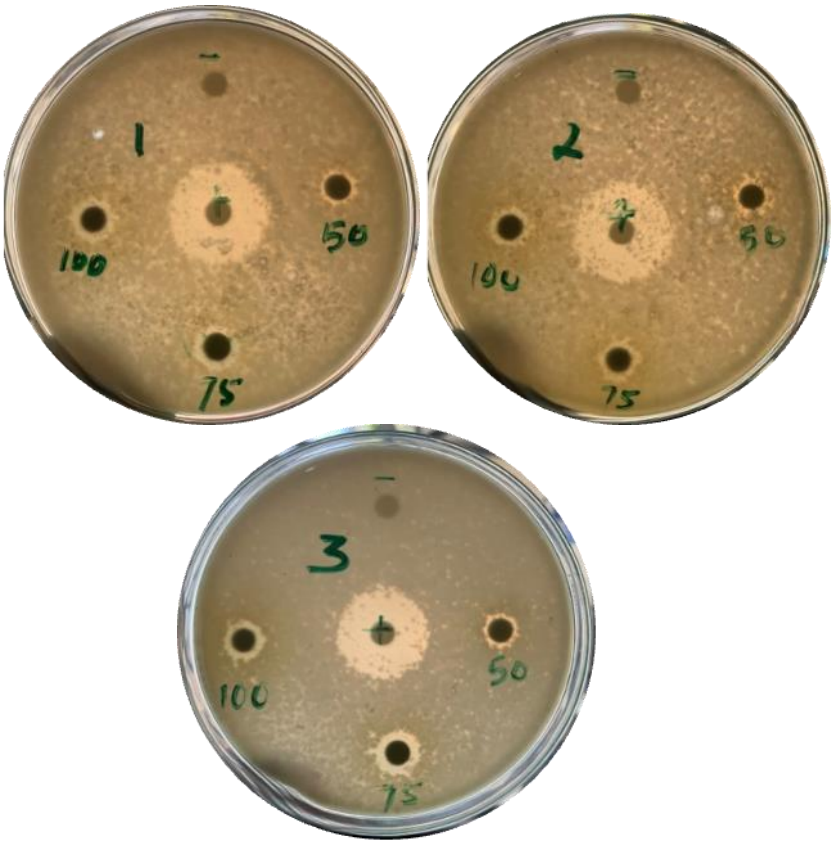

Fig. 2. Inhibition level test result

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration test data obtained are depicted in Table (5).

Table 5. MIC test results

Extract concentration ($mg.mL^{-1}$)	Results		
	Repeat 1	**Repeat 2**	**Repeat 3**
50	+	+	+
25	+	+	+
12.5	+	+	+
6.25	-	-	-
3.12	-	-	-
1.56	-	-	-
0.78	-	-	-
0.39	-	-	-

Note : (+) : Zone of inhibition formed, (-) : No zone of inhibition.

As shown in Table (5), inhibition zones were formed at three concentrations, namely 50, 25, and 12.5mg.mL^{-1}. Based on these results, the minimum concentration that could inhibit the growth of *V. parahaemolyticus* bacteria was determined to be 12.5 and 25mg.mL^{-1}. At this concentration, an inhibition zone was formed around the disc paper, but it was less clear. The inhibition zone resembled a circle inside a clear zone that did not grow bacteria. Different results were found at concentrations of 6.25, 3.12, 1.56, 0.78, and 0.39mg.mL^{-1}, where no inhibition zone was formed.

DISCUSSION

Neem leaves contain various antibacterial compounds with numerous biological applications for effective curative and healing roles **(Aisha, *et al.*, 2022)**. According to several studies, neem is rich in various compounds that have pharmacological potential as insecticidal and antimicrobial agents **(Islas *et al.*, 2020)**. The maceration method is a cold extraction method and is the simplest method. Additionally, the use of the maceration method was chosen because neem leaves have a soft texture, and the extraction process does not use heating, which will affect the content of compounds in neem leaf extract **(Wahyulianingsih *et al.*, 2016)**. Meanwhile, methanol was chosen as a solvent because it has universal properties so that it can attract most of the chemical compounds contained in simplisia (polar and non-polar) **(Salamah & Widyasari, 2015)**.

The research of **Hasnaeni *et al.* (2019)** revealed that the use of the maceration method with 70% methanol on beta-beta wood stems obtained higher yields of 2.352% compared to reflux and sokletasi methods, whose results were only 1.611 and 0.960%. **Ibrahim and Kebede (2020)** suggests that neem leaf extract extracted by maceration method using methanol solvent has the highest antibacterial activity on bacteria *staohylococcus aureus, salmonella typhi,* and *streptococcus agalactiae* compared to using solvents in the form of aquadest. Moreover, the heat treatment given can reduce the effectiveness of the plant material.

After GC-MS testing, 12 types of secondary metabolite compounds were obtained, and there were similarities in several previous studies in the form of mycosporine, phenols, alkaloids, tripterpenoids, Phytosterols and steroids. Secondary metabolite compounds were found in neem leaf extract in the form of *3-Cyclopropyl-4-hydroxy-4,5,5-trimethyl-oxazolidin-2-one, Phenol, 2-methoxy-, Phenol, 2-methoxy-4-(1-propenyl)-(CAS), l-Proline, N-propoxycarbonyl-, isobutyl ester, 2-Hydroxy-1-(1'-pyrrolidiyl)-1-buten-3-one, Phenol, 4-ethenyl-2,6-dimethoxy-, 1,2(equat)-dimethyl-trans-decahydroquinol-4-one, Cholest-5-en-3-ol, 24-propylidene-, (3.beta.)-, .beta.-Sitosterol acetate, BUFA-20,22-DIENOLIDE, 14,15-EPOXY-3,5,16-TRIHYDROXY-, (3.BETA.,5.BETA.,15, Campesterol,* dan *STIGMASTA-5,22-DIEN-3-OL*. Some research studies on neem leaves also show the results of the same compound content, such as phytosterols **(Virshette *et al.*, 2020)**, mycosporine **(Lepidoptera *et al.*, 2021)**, dan

steroids **(Binta *et al.,* 2022)** phenols **(Fisayomi *et al.,* 2022)**, triterpenoids **(Manerlin *et al.,* 2023)**, and alkaloids **(Bolaji *et al.,* 2024)**.

The highest percentage area of secondary metabolite compounds in neem leaf extracts after GC-MS testing is in the type of phenol compounds with a range of 0.75–2.75%. Phenol is a secondary metabolite compound that exhibits many diverse biological activities and is recognized for its ability to change the characteristics and structure of proteins, including antibacterial activity **(Shahidi & Dissanayaka, 2023)**. Phenol acts as an antibacterial by damaging bacterial cell membranes, inhibiting virulence factors such as enzymes and toxins, and suppressing bacterial biofilm formation **(Majdanik *et al.,* 2018)**, resulting in the disruption of plasma membrane stability **(Górniak *et al.,* 2019)**. Phenol compounds are also referred to as reactive oxygen species (ROS), which affect various cellular mechanisms, such as proliferation and metabolism, or damage to biomolecules that cause cell and tissue injury **(Naz *et al.,* 2022)**.

Aside from neem leaves, other herbal plants also contain similar secondary metabolite compounds, such as papaya **(Candra *et al.,* 2024)**, *Verbascum sinaiticum* (Qetetina) **(Legesse *et al.,* 2024)**, Congou black tea **(Liu *et al.,* 2024)**, and butterfly flower **(Tunggal *et al.,* 2024)**. These plants contain flavonoids, tannins, phenols, alkaloids, saponins, and steroids, differing only in terms of concentration, presumably due to the use of different polar solvents.

The inhibitory levels of the compounds, as shown in Table (4), indicate that neem leaves methanol extract has antibacterial activity against *V. parahaemolyticus* bacteria with an average diameter of 12.8, 11.5, and 10.1mm. According to **Davis and Stout (1971)**, an inhibition zone diameter greater than 10mm is considered strong in inhibiting the rate of bacteria growth **(Yunita *et al.,* 2023)**. Another study also found that the neem inhibited various types of bacteria, including *Vibrio alginoticus* and *Escherichia coli* with a diameter of 8.2 and 7mm, respectively, as well as *Helicobacter pylori* with a diameter of 11mm **(Saxena *et al.,* 2021; Singh *et al.,* 2023)**. In other studies, green tea leaves extract produced an inhibition zone of 14.4 to 16.4mm against *V. parahaemolyticus* bacteria **(Kongchum *et al.,* 2016)**, while basil leaves produced an inhibition of about 8.67mm **(Snoussi *et al.,* 2016)**. Disparities in the diameter may be caused by both the method and the concentration of bacteria used **(Yunita *et al.,* 2023)**.

Differences in the diameter of the inhibition zone can also be caused by variations in the solvents used and the incubation temperature. For example, **Ibrahim and Kebede (2020)** found that extraction using ethanol showed the highest activity compared to aquadest solvent. Additionally, neem leaves extract showed an increased activity after exposure to 45°C for 30 minutes using pathogenic test bacteria such as *Staphylococcus aureus, Streptococcus agalctiae, Salmonella Typhi,* and *Shigella boydii*. In a study conducted by **Prateek *et al.* (2023)**, ethanol extract showed an antibacterial activity of 19mm on *Staphylococcus aureus* and 16mm on *Escherichia coli*. Factors such as crop

age, harvest time, drying, and processing of ingredients also influence the efficacy of plant extract **(Ibrahim & Kebede, 2020)**.

Based on the results, the MIC was found to be 12.5 and 25mg.mL-^{1}, determined by observing the lowest extract concentration that can fully inhibit the growth of the test bacteria used, as detected by the naked eye **(Ibrahim & Kebede, 2020)**. Similarly, **Ali *et al.* (2021)** found that concentrations of 12.5 and 25mg.mL^{-1} inhibited the growth of *Pasteurella multocida*. **Ibrahim *et al.* (2023)** also demonstrated similar results for *Aeromonas sobria* bacteria. Other results were obtained when testing neem leaves extract using the agar well diffusion method with *Mycrosporum gypsum* and *Trichophyton mentagrophytes*, where MIC ranged from 125–250µg/ mL **(Thakur *et al.*, 2019)**. Furthermore, the use of well diffusion method can inhibit bacteria growth at a concentration of 10.42µg, indicating that the method also affects the results of MIC test. Differences were observed when the neem leaves extract was combined with essential oil, showing MIC in the range of 0.25–5.0g/ mL **(Tiple *et al.*, 2023)**. According to **Ibrahim and Kebede (2020)**, a higher MIC value indicates that the plant extract has a weaker activity.

CONCLUSION

In conclusion, the neem leaves extract has antibacterial compounds, supporting the potential as a natural ingredient in the treatment of microbial infections. Based on the results, the extract can be developed as an antibacterial agent against *V. parahamolyticus* attack on *vannamei* shrimp farming.

REFERENCES

Ali, E.; Islam, M.; Hossen, M.; Khatun, M. and Islam, M. A. (2021). Extract of neem *(Azadirachta indica)* leaf exhibits bactericidal effect against multidrug resistant pathogenic bacteria of poultry. *Veterinary Medicine and Science*, *7*(5): 1921–1927. https://doi.org/10.1002/vms3.511

Basir, B.; Isnansetyo, A.; Istiqomah, I. and Jabbar, F. B. A. (2020). Toksisitas Daun Miana *(Coleus scutellarioides (L) Benth)* Sebagai Antibakteri Pada Udang Vannamei *(Litopenaeus vannamei)*. SIGANUS: Journal of Fisheries and Marine Science, 1(2): 56–61. https://doi.org/10.31605/siganus.v1i2.653

Binta, B.; Dematus, O. K. and Dadi, M. H. (2022). Nematicidal Activity of Methanolic Extracts of Leaf , Stem Bark , and Root of Nematicidal Activity of Methanolic Extracts of Leaf , Stem Bark , and Root of *Azadirachta Indica* (Neem) Against Haemonchus Contortus. June, 130–136. https://doi.org/10.47191/ijpbms/v2-i6-04

Bolaji, O.; Abolade, Y. A.; Aduwa, S.; Isiaka, A. B.; Durodola, O.; Adeoye, A.; Adeoye, T. O., Bamidele, A. A.; Akagbue, B. O.; Aminu, M. B. and Siame, T. (2024). Potential health and environmental benefits of the identified phytochemicals

screening of *(Azadirachta indica)* neem leaves in Bauchi Metropolis , Bauchi Potential health and environmental benefits of the identified phytochemicals screening of (Azadirac. March. https://doi.org/10.30574/gscbps.2024.26.3.0037

Candra, A.; Fahrimal, Y.; Yusni, Y.; Azwar, A. and Santi, T. D. (2024). Phytochemistry and antifatigue activities of Carica papaya leaf from geothermal, coastal and urban areas, Indonesia. Narra J, 4(1), e321. https://doi.org/10.52225/narra.v4i1.321

Chunguang, W.; Jingyun, F.; Ying, L.; and Zengguo, H. (2023). Isolation, identification, and characterization of Vibrio parahaemolyticus phage Vpas_PP24 WU. March. https://doi.org/10.13344/j.microbiol.china.220316

Davis, W. W. and Stout, T. R. (1971). Disc plate method of microbiological antibiotic assay. I. Factors influencing variability and error. Applied Microbiology, *22*(4): 659–665. https://doi.org/10.1128/aem.22.4.659-665.1971

Dhanani, T.; Shah, S.; Gajbhiye, N. A. and Kumar, S. (2017). Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of Withania somnifera. Arabian Journal of Chemistry, 10(June 2014), S1193–S1199. https://doi.org/10.1016/j.arabjc.2013.02.015

Fazly Bazzaz, B. S.; Khameneh, B.; Reza, M.; Ostad, Z. and Hosseinzadeh, H. (2018). In vitro evaluation of antibacterial activity of verbascoside, lemon verbena extract and caffeine in combination with gentamicin against drug-resistant *Staphylococcus aureus* and *Escherichia coli* clinical isolates Eradication of bacterial resistance by com. Original Research Article AJP, 8(3), 246–253.

Fisayomi, O. O.; Adekanmi Adeyinka, A.; Adegboye, M. A.; Oyindamola, O. F. and S., A. A. (2022). Comparison of Neem Leaf (*Azadirachta Indica* A . Juss) and Scent Leaf (*Ocimum Gratissimum*) Phytochemical and Nutritional Properties. October 2021.

Fu, J.; Li, Y.; Zhao, L.; Wu, C. and He, Z. (2023). Characterization of vB _ ValM _ bacteriophage infecting *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. May, 1–20. https://doi.org/10.3389/fmicb.2023.1105924

Górniak, I.; Bartoszewski, R. and Króliczewski, J. (2019). Comprehensive review of antimicrobial activities of plant flavonoids. In Phytochemistry Reviews (Vol. 18, Issue 1). https://doi.org/10.1007/s11101-018-9591-z

Gupta, S. C.; Prasad, S.; Tyagi, A. K.; Kunnumakkara, A. B. and Aggarwal, B. B. (2017). Neem *(Azadirachta indica)*: An indian traditional panacea with modern molecular basis. Phytomedicine, 34(May), 14–20. https://doi.org/10.1016/j.phymed.2017.07.001

Heng, S. P.; Letchumanan, V.; Deng, C. Y.; Ab Mutalib, N. S.; Khan, T. M.; Chuah, L. H.; Chan, K. G.; Goh, B. H.; Pusparajah, P. and Lee, L. H. (2017). Vibrio vulnificus: An environmental and clinical burden. *Frontiers in Microbiology*, *8*(MAY). https://doi.org/10.3389/fmicb.2017.00997

Hasnaeni.; Wisdawati.; and Usman, S. (2019). Effect of Extraction Method on Yield and Phenolic Content of Beta-Beta Wood Plant Extract *(Lunasia amara Blanco)*. *5*(2): 175–182. https://doi.org/10.22487/j24428744.2019.v5.i2.13149

Hikmawati, F.; Susilowati, A. and Ratna, S. (2019). Number Detection and Pathogenicity Test of Vibrio spp. in Green Shells *(Perna Viridis)* in Yogyakarta Beach Tourism Area. Jurnal Biodiv Indonesia, 5(2): 334–339. https://doi.org/10.13057/psnmbi/m050234

Ibrahim, N. and Kebede, A. (2020). In vitro antibacterial activities of methanol and aqueous leave extracts of selected medicinal plants against human pathogenic bacteria. Saudi Journal of Biological Sciences, 27(9): 2261–2268. https://doi.org/10.1016/j.sjbs.2020.06.047

Ibrahim, R. E.; Elshopakey, G. E.A.; bdelwarith, A. A.; Younis, E. M.; Ismail, S. H.; Ahmed, A. I.;, El-Saber, M. M.; Abdelhamid, A. E.; Davies, S. J.; El-Murr, A. and Abdel Rahman, A. N. (2023). Chitosan neem nanocapsule enhances immunity and disease resistance in nile tilapia (Oreochromis niloticus). Heliyon, 9(9), e19354. https://doi.org/10.1016/j.heliyon.2023.e19354

Islas, J. F.; Acosta, E.; G-Buentello, Z.; Delgado-Gallegos, J. L.; Moreno-Treviño, M. G.; Escalante, B. and Moreno-Cuevas, J. E. (2020). An overview of Neem *(Azadirachta indica)* and its potential impact on health. Journal of Functional Foods, 74(8): 104171. https://doi.org/10.1016/j.jff.2020.104171

Jain, A.; Sengupta, S. and De, S. (2018). Effect of process parameters on aqueous extraction of thymol and other phytonutrients from herbal seed Ajwain *(Trachyspermum ammi L.)*. Journal of Applied Research on Medicinal and Aromatic Plants, 11(9): 27–36. https://doi.org/10.1016/j.jarmap.2018.09.001

Kalatzis, P. G.; Castillo, D.; Katharios, P. and Middelboe, M. (2018). Bacteriophage interactions with marine pathogenic vibrios: Implications for phage therapy. Antibiotics, *7*(1): 1–23. https://doi.org/10.3390/antibiotics7010015

Khayyat, S. A. and Roselin, L. S. (2018). Recent progress in photochemical reaction on main components of some essential oils. Journal of Saudi Chemical Society, 22(7): 855–875. https://doi.org/10.1016/j.jscs.2018.01.008

Kongchum, P.; Chimtong, S.; Chareansak, N. and Subprasert, P. (2016). Effect of Green Tea Extract on Vibrio Parahaemolyticus Inhibition in Pacific White Shrimp (Litopenaeus Vannamei) Postlarvae. Agriculture and Agricultural Science Procedia, 11, 117–124. https://doi.org/10.1016/j.aaspro.2016.12.020

Kozłowski, R. M.; Talarczyk, M. and Bedoya, J. (2020). New emerging natural fibres and relevant sources of information. In *Handbook of Natural Fibres: Second Edition* (Vol. 1). https://doi.org/10.1016/B978-0-12-818398-4.00022-0

Lee, C.; Te, Chen, I. T.; Yang, Y. T.; Ko, T. P.; Huang, Y. T.; Huang, J. Y.; Huang, M. F.; Lin, S. J.; Chen, C. Y.; Lin, S. S.; Lightner, D. V.; Wang, H. C.; Wang, A. H. J.; Wang, H. C.; Hor, L. I., and Lo, C. F. (2015). The opportunistic marine

pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences of the United States of America, *112*(39): E5445. https://doi.org/10.1073/pnas.1517100112

Legesse, A. B.; Emire, S. A.; Dadi, D. W. and Tadesse, M. G. (2024). Impact of Drying Method and Solvent Extraction on Ethiopian *Verbascum sinaiticum* (Qetetina) Leaves : Metabolite Profiling and Evaluation of Antioxidant Capacity Impact of Drying Method and Solvent Extraction on Ethiopian Verbascum sinaiticum (Qetetina . April. https://doi.org/10.3390/pr12050914

Lepidoptera, J. E. S.; Kofi, S.; Adjei, F.; Abraham, J. and Addo, E. (2021). Potential of neem extracts as natural insecticide against fall armyworm (Spodoptera frugiperda (J. E. Smith) *(Lepidoptera: Noctuidae)*. 4.

Letchumanan, V.; Loo, K. Y.; Law, J. W.-F., Wong, S. H., Goh, B.-H., & Lee, N.-S. A. M. L.-H. (2019). *Vibrio parahaemolyticus:* The Protagonist Causing Foodborne Diseases.

Letchumanan, V.; Yin, W. F.; Lee, L. H. and Chan, K. G. (2015). Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. Frontiers in Microbiology, 6(JAN), 1–11. https://doi.org/10.3389/fmicb.2015.00033

Liu, Z.; Ran, Q.; Luo, J.; Shen, Q.; Zhang, T.; Fang, S.; Pan, K. and Long, L. (2024). Correlation analysis of secondary metabolites and disease resistance activity of different varieties of Congou black tea based on LC-MS/MS and TCMSP. Food Chemistry: X, 101331. https://doi.org/10.1016/j.fochx.2024.101331

Manerlin, G. S. K.; Limanan, D.; Ferdinal, F. and Yulianti, E. (2023). Phytochemical identification and total antioxidant capacity of neem leaves *(Azadirachta indica A. Juss)* and toxicity test against Artemia salina Leach larvae. 5(1), 59–66.

Majdanik, M.; Kępa, M.; Wojtyczka, R. D.; Idzik, D. and Wąsik, T. J. (2018). Phenolic compounds diminish antibiotic resistance of staphylococcus aureus clinical strains. International Journal of Environmental Research and Public Health, 15(10). https://doi.org/10.3390/ijerph15102321

Naz, H.; Akram, N.; Ashraf, M. Ingo Hefft, D. and Latief Jan, B. (2022). Leaf extract of neem *(Azadirachta indica)* alleviates adverse effects of drought in quinoa *(Chenopodium quinoa willd.)* plants through alterations in biochemical attributes and antioxidants. Saudi Journal of Biological Sciences, 29(3): 1367–1374. https://doi.org/10.1016/j.sjbs.2022.01.038

Naz, H.; Aisha, N.; Ashraf, M.; Ingo, D. and Latief, B. (2022). Leaf extract of neem *(Azadirachta indica)* alleviates adverse effects of drought in quinoa *(Chenopodium quinoa Willd.)* plants through alterations in biochemical attributes and antioxidants Hira. Saudi Journal of Biological Sciences, 29(3), 1367–1374. https://doi.org/10.1016/j.sjbs.2022.01.038

Nikolic, M.; Vasic, S., Djurdjevic, J.; Stefanovic, O. and Comic, L. (2014). Antibacterial and anti-biofilm activity of ginger (*Zingiber officinale* (Roscoe)) ethanolic extract. Kragujevac Journal of Science, 36(36): 129–136. https://doi.org/10.5937/kgjsci1436129n

Nyalo, P. O.; Omwenga, G. I. and Ngugi, M. P. (2023). Antibacterial properties and GC-MS analysis of ethyl acetate extracts of Xerophyta spekei (Baker) and Grewia tembensis (Fresen). Heliyon, *9*(3), e14461. https://doi.org/10.1016/j.heliyon.2023.e14461

Prateek, V. S.; Neralla, M., V, B. and Satheesh, T. (2023). Comparative Extraction and Bioactive Potential of the Leaf Extracts of Azadirachta indica for Combatting Postoperative Head and Neck Infections: An In Vitro Study. Cureus, *15*(12), 1–8. https://doi.org/10.7759/cureus.51303

Rupani, R. and Chavez, A. (2018). Medicinal plants with traditional use: Ethnobotany in the Indian subcontinent. Clinics in Dermatology, 36(3), 306–309. https://doi.org/10.1016/j.clindermatol.2018.03.005

Saxena, A.; Arivaradarajan, P.; Mukhopadhyay, A. K. and Nandi, S. P. (2021). Bactericidal effect of Neem (Azadirachta indica) leaf extract on Helicobacter pylori. Journal of Environmental Biology, 42(6), 1591–1597. https://doi.org/10.22438/jeb/42/6/MRN-2070

Salamah, N. and Widyasari, E. (2015). Antioxidant Activity of Methanolic Extract of Longan Leaf *(Euphoria longan* (L) Steud.) by Capture Radikan Method 2,2'-Difenil-1-Pikrilhidrazil. *Pharmaciana, 5*(1): 25–34.

Shahidi, F. and Dissanayaka, C. S. (2023). Phenolic-protein interactions: insight from in-silico analyses – a review. Food Production, Processing and Nutrition, 5(1). https://doi.org/10.1186/s43014-022-00121-0

Singh, A. A.; Naaz, Z. T.; Rakaseta, E., Perera, M.; Singh, V.; Cheung, W.; Mani, F. and Nath, S. (2023). Antimicrobial activity of selected plant extracts against common food borne pathogenic bacteria. Food and Humanity, 1(4), 64–70. https://doi.org/10.1016/j.foohum.2023.04.002

Singha, A. A.; Zafiar.; Edward, R.; Perera, M.; Vrinda, S.; Wilson, C.; Francis, M.; Swastika, N. and A. (2023). Antimicrobial activity of selected plant extracts against common food borne pathogenic bacteria. The Virtual Museum of Bacteria, 1(April), 1. https://doi.org/10.1016/j.foohum.2023.04.002

Snoussi, M.; Dehmani, A.; Noumi, E.; Flamini, G. and Papetti, A. (2016). Chemical composition and antibiofilm activity of Petroselinum crispum and Ocimum basilicum essential oils against Vibrio spp. strains. Microbial Pathogenesis, 90, 13–21. https://doi.org/10.1016/j.micpath.2015.11.004

Srivastava, S. K.,; Agrawal, B.; Kumar, A. and Pandey, A. (2020). Phytochemicals of *AzadirachtaIndica* Source of Active Medicinal Constituent Used for Cure of Various Diseases: A Review. Journal of Scientific Research, 64(01), 285–290.

https://doi.org/10.37398/jsr.2020.640153

Thakur, B. K.; Kumar, A. and Kumar, D. (2019). Green synthesis of titanium dioxide nanoparticles using *Azadirachta indica* leaf extract and evaluation of their antibacterial activity. South African Journal of Botany, 124, 223–227. https://doi.org/10.1016/j.sajb.2019.05.024

Thammatinna, K.; Egan, M. K. E.; Htoo, H. H.; Khanna, K.; Sugie, J.; Nideffer, J. F.; Villa, E., Tassanakajon, A.; Pogliano, J.; Nonejuie, P. and Chaikeeratisak, V. (2020). A novel vibriophage exhibits inhibitory activity against host protein synthesis machinery. Scientific Reports, 10(1): 1–14. https://doi.org/10.1038/s41598-020-59396-3

Tibebu, A.; Haile, G. and Kebede, A. (2018). Review On Medicinal Value And Other Application Of Neem Tree: Senior Seminar On Animal Health. Biomedicine and Nursing, 4(1): 62–69. https://doi.org/10.7537/marsbnj040118.10

Tiple, R..; Jamane, S.; Khobragade, D. and Sharma, S. (2023). Synergistic Antifungal Bioassay of Neem Leaves Extract with Essential Oils against Dermatophytes. International Journal of Pharmaceutical Quality Assurance, *14*(4): 1144–1150. https://doi.org/10.25258/ijpqa.14.4.49

Tunggal, T.; Prihatanti, R. and Sofia, N. (2024). Phytochemical Scrining And Determination Of Secondary Metabolitical Capacity Of Butterfly Leaves *(Bauhinia Purpurea L)* As A Guide For Wound Healing In Women After *(Bauhinia Purpurea L)* Como Guia Para A Cicatrização De. 1–30.

Vicencio, M. C. G. (2020). Antibacterial efficacy of leaf extracts of Paederia foetida Linnaeus. Journal of Chemical Research Advances, 02(01): 01–05.

Virshette, S. J.; Patil, M. K.; Udgir, A. S.; Shaikh, J. R. and Dhas, M. S. (2020). Phytochemical constituents of different extracts of Azadirachta indica leaves in urine solvent of non- pregnant cow. August.

Yang, L.; Wen, K. S.; Ruan, X.; Zhao, Y. X.; Wei, F. and Wang, Q. (2018). Response of plant secondary metabolites to environmental factors. Molecules, 23(4), 1–26. https://doi.org/10.3390/molecules23040762

Yeoh, H. I.; Izzatty, R.; Furusawa, G.; Amirul, A. A. A.; Shu-Chien, A. C. and Sung, Y. Y. (2021). The Vibrio-predatory filamentous bacteria effectively removed acute hepatopancreatic necrosis disease (AHPND) causative Vibrio parahaemolyticus in vitro. Aquaculture Reports, 21(8), 100910. https://doi.org/10.1016/j.aqrep.2021.100910

You, H. J.; Lee, J. H.; Oh, M.; Hong, S. Y.; Kim, D.; Noh, J., Kim, M. and Kim, B. S. (2021). Tackling *Vibrio parahaemolyticus* in ready-to-eat raw fish flesh slices using lytic phage VPT02 isolated from market oyster. Food Research International, 150(PA), 110779. https://doi.org/10.1016/j.foodres.2021.110779

Yunita, M.; Ohiwal, M.; Elfitrasya, M. Z. and Rahawarin, H. (2023). Antibacterial activity of Paederia foetida leaves using two different extraction procedures against

pathogenic bacteria. Biodiversitas, 24(11): 5920–5927. https://doi.org/10.13057/biodiv/d241110

Wahyulianingsih.; Handayani, S. and Malik, A. (2016). Determination of Total Flavonoid Content of Clove Leaf Extract *(Syzygium aromaticum (L.) 3*(2).

Zhang, X.; Sun, J.; Chen, F.; Qi, H.; Chen, L.; Sung, Y. Y.; Huang, Y.; Lv, A. and Hu, X. (2021). Phenotypic and genomic characterization of a *Vibrio parahaemolyticus* strain causing disease in penaeus vannamei provides insights into its niche adaptation and pathogenic mechanism. Microbial Genomics, 7(5). https://doi.org/10.1099/MGEN.0.000549