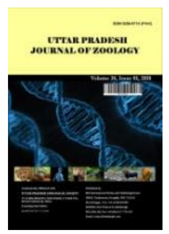

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GC-MS Analysis of Bioactive Compounds in *Azadirachta indica* Extracts and their Antibacterial Effect against Fish Pathogen *Aeromonas hydrophila*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

The bioactive chemicals found in herbal plants are responsible for their antibacterial properties against many infections. In this present study, *Azadirachta indica* extracts of isopropyl alcohol, ethanol, ethyl acetate, and hexane were tested for antibacterial activity against *Aeromonas hydrophila* at concentrations of 0.25 µl, 0.5 µl, 0.75 µl, and 1.0 µl, respectively. In antibacterial activity tests, isopropyl alcohol and ethanol extracts of *A. indica* leaves showed maximum zones of

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inhibition of 14.2 ± 0.2 mm, 14.7 ± 0.5 mm, 15.4 ± 0.3 mm, and 15.9 ± 0.2 mm; 14.0 ± 0.2 mm, 14.6 ± 0.5 mm, 15.2 ± 0.3 mm, and 15.6 ± 0.2 mm at 0.25 µl, 0.5 µl, 0.75 µl, and 1.0 µl concentrations, respectively, against the fresh water fish pathogen *A. hydrophila*. The phytochemical study of isopropyl alcohol and ethanol extracts of *A. indica* leaves revealed the existence of bioactive substances. The bioactive components of *A. indica* leaf isopropyl alcohol and ethanol extracts were analysed using a Perkin Elmer Gas Chromatography-Mass Spectrometer. The chemicals found in these extracts were matched to those of the National Institute of Standards and Technology (NIST-2008). The findings of the GC-MS analysis revealed that the isopropyl alcohol extract of *Azadirachta indica* had one major (Diethyl phthalate) and two minor (Methoxy acetic acid, pentyl ester and 2-hexanol, 3-methyl-) compounds, while the ethanol extract contained one major (Diethyl phthalate) and one minor (2-hexanol, 3-methyl-) compound. The findings of this study indicate that bioactive chemicals found in isopropyl alcohol and ethanol extracts of *A. indica* leaves may have anti-inflammatory and anti-bacterial properties.

Keywords: Bioactive; Azadirachta indica; GC-MS; antibacterial; anti-inflammatory.

1. INTRODUCTION

The development of different bacterial illnesses, ornamental fish farming leads in serious economic loss due to excessive mortality in fishes [1]. The freshwater Gram-negative bacteria *Aeromonas hydrophila* caused various diseases, including, red fin disease, haemorrhagic septicaemia, and motile *Aeromonas* septicaemia in fishes [2]. The inorganic antibiotics are widely used to control fish diseases, however, the prolonged application induced drug-resistant and affect the environment [3]. The medicinal plants serve as a best alternative source for antibiotics in the treatment of various diseases in aquaculture [4]. Phytochemical compounds present in herbal plants are effective against various microbial diseases in fishes [5]. These herbal plants stimulated immune response in fishes and improve disease-resistance in fish [6]. The bioactive compounds present in medicinal plants increases the specific and nonspecific immune response in fish [7]. The GC-MS technique is used to identify bioactive chemicals found in plant extracts. The most effective method for separating and identifying volatile and semi-volatile chemicals for both qualitative and quantitative examination is gas chromatography-mass spectrometry (GC-MS) [8,9,10]. In this present study the different extracts of *A. indica* leaves were tested for antibacterial effect against fresh water fish pathogen *A. hydrophila* and its phytochemical compounds area analysed using GC-MS.

2. MATERIALS AND METHODS

2.1 Isolation of Bacteria

The disease-infected Molly fish, *Poeciliasphenops*, was taken alive and brought to the laboratory in an oxygenated plastic bag from a nearby ornamental fish store in the Kanyakumari region of Tamilnadu. There were clinical symptoms and the fish was lethargic. Using a sterile mortar and pestle, the contaminated epidermal layer and gill tissues were removed from the molly fish and homogenised by adding Phosphate Buffer Saline (PBS) at a 1:10 ratio, or 1 g of tissue mixed with 10 ml of PBS. A sterilised L glass rod was used to spread the inoculum equally throughout the nutrient agar plates after the dilutedsamples were inoculated. After that, the infected plates were incubated at 35°C for 24 hours. Colonies were detected on agar plates following incubation. To create pure cultures, the colonieswere streaked onto new nutrient agar plates and slants using a sterile loop. They were then kept at 4°C to allow the desired bacterial strain to be identified.

2.2 Collection and Preparation of Herbal Plant Extract

The herbal plants *A. indica* was collected from the south villages in Nagercoil, Kanyakumari district, Tamil Nadu. The leaves were washed in distilled water and were allowed to dry under shade. The leaves were finely powdered once they had completely dried. Plant extracts were prepared using solvents such as hexane, ethanol, isopropyl alcohol, and ethyl acetate. In a conical flask, 10 g of plant powder and 100 ml of the intended solvent were added. i.e., 100 ml of isopropyl alcohol + 10 g of powdered *A. indica* leaves. To stop the solvent from evaporating, silver foil was placed over the mouth of the conical flask.

2.3 Determination of Mass/Volume Ratio (Concentration)

Concentration

$= \frac{Mass\ of\ solute}{Volume\ of\ solvent} = \frac{10\ gm}{100\ ml}$ = 0.1 gram

The plant leaf powder solution had a concentration of 100 mg/ml when converted to milligrams. For 48 hours, this combination was stored in a mechanical shaker. As a result, the solvent and plant leaf powder blend well. The combination was passed through Whatman No. 1 filter paper after 48 hours, and the extract was then gathered in sterile containers. The plant extracts in these containers were left open at room temperature for a full day to allow the solvent to evaporate, leaving behind the crude extract. The crude herbal extract was stored at 4°C for further studies.

2.4 Identification of *A. hydrophila*

The bacterial pathogen was cultured on nutrient agar plates and incubated for 24 hours at 327ºC. The physical and biochemical characteristics of bacteria were analysed using the standard approach described by Bergey [11].

2.5 Determination of Antibacterial Activity against *A. hydrophila*

The Mueller Hinton Agar agar plate was prepared, and bacterial culture was obtained from overnight broth culture using a cotton swab and streaked in Mueller Hinton agar plate. Then, by using a well cutter four wells were made with a diameter of 6 mm. The crude extract was loaded at four different concentrations. The plates were then incubated at 37°C for 24 hours for bacterial growth.

2.6 Phytochemical Analysis

The phytochemical analysis of freshly synthesised herbal extracts was carried out using Harbone [12] standards. A qualitative phytochemical study of selectedplant extracts revealed the existence of severalbioactive chemicals.

2.7 GC-MS Analysis

The Clarus 680 GC was utilised in the study, employing a fused silica column packed with Elite-5MS (5% biphenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID × 250μm df). The components were separated using Helium as a carrier gas at a constant flow of 1 mL/min. The injector temperature was set to 260°C for the chromatographic run. After injecting 1μL of extract sample into the device, the oven temperature was as follows: The temperature was set to 60°C for 2 minutes, then increased to 300°C at a rate of 10°C per minute, and finally kept at 300°C for 6 minutes. The mass detector was set to the following conditions: transfer line temperature of 240°C, ion source temperature of 240°C, ionisation mode electron impact at 70 eV, scan period of 0.2 seconds, and scan interval of 0.1 second. The fragments range from 40 to 600 Da. The component spectrums were compared to a database of known component spectra included in the GC-MS NIST 2008 library.

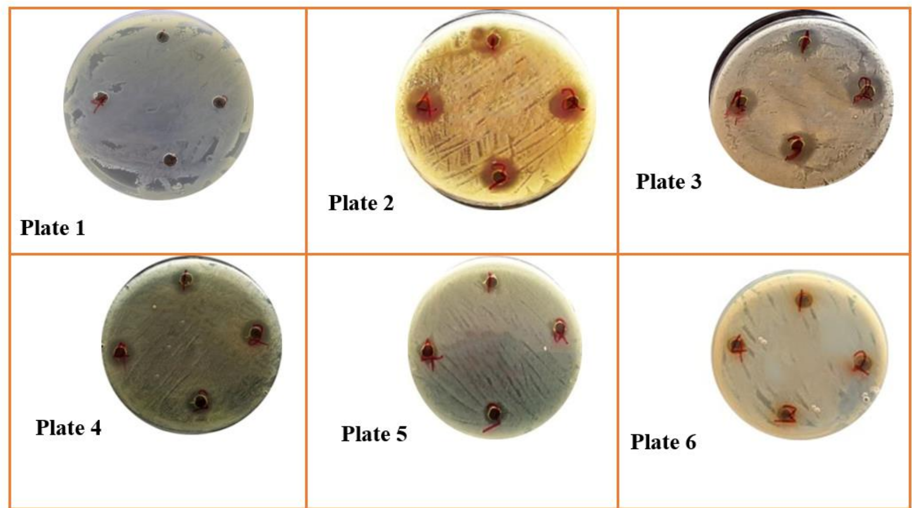
3. RESULTS AND DISCUSSION

3.1 Antibacterial Activity of Herbal Plant Extracts

The effect of *A. indica* leaves extracts on the bacterial pathogen *A. hydrophila* was studied by agar well diffusion method.

Table 1. Antibacterial zone of inhibition of *A. indica* extracts against fresh water fish Bacterial pathogen *A. hydrophila*

S. No.	Herbal plant extracts	Zone of inhibition (mm)			
		0.25 µl	0.5 µl	0.75 µl	1.0 µl
1	*A. indica* – Isopropyl alcohol	14.2 ± 0.2	14.7 ± 0.5	15.4 ± 0.3	15.9 ± 0.2
2	*A. indica* – Ethanol	14.0 ± 0.2	14.6 ± 0.5	15.2 ± 0.3	15.6 ± 0.2
3	*A. indica* – Ethyl acetate	10.0 ± 0.5	10.3 ± 0.3	10.8 ± 0.3	11.2 ± 0.2
4	*A. indica* – Acetone	8.3 ± 0.4	8.8 ± 0.3	9.4 ± 0.4	9.8 ± 0.2
5	*A. indica* – Hexane	8.0 ± 0.6	8.7 ± 0.5	9.2 ± 0.4	9.6 ± 0.2



Plates 1-6. Plates showing zone of inhibition against fresh water fish bacterial pathogen *A. hydrophila*

(Plate 1: Control, Plate 2: A. indica – Isopropyl alcohol, Plate 3: A. indica – Ethanol, Plate 4: A. indica – Ethyl acetate, Plate 5: A. indica – Acetone, Plate 6: A. indica – Hexane)

The isopropyl alcohol extract of *A. indica* leaves showed maximum zone of inhibition 14.2 ± 0.2 mm, 14.7 ± 0.5 mm, 15.4 ± 0.3 mm, and 15.9 ± 0.2 mm at 0.25 µl, 0.5 µl, 0.75 µl, and 1.0 µl concentrations respectively. The ethanol extract of *A. indica* also showed better inhibitory zone 14.0 ± 0.2 mm,14.6 ± 0.5 mm, 15.2 ± 0.3 mm, and 15.6 ± 0.2 mm at 0.25 µl, 0.5 µl, 0.75 µl, and 1.0 µl concentrations. The ethyl acetate, acetone and hexane extract showed lower inhibitory zone i.e., 10.0 ± 0.5 mm, 10.3 ± 0.3 mm, 10.8 ± 0.3 mm and 11.2 ± 0.2 mm; 8.3 ± 0.4 mm, 8.8 ± 0.3 mm, 9.4 ± 0.4 mm and9.8 ± 0.2 mm; 8.0 ± 0.6 mm, 8.7 ± 0.5 mm, 9.2 ± 0.4 mm and 9.6 ± 0.2 mm at 0.25 µl, 0.5 µl, 0.75 µl, and 1.0 µl concentrations respectively against fresh water fish pathogen *A. hyrdrophila* (Table 1 and Plates 1 to 6).

The largest inhibition zone against *Salmonella typhi* was observed with the ethanol extract of *Azadirachta indica*, followed by *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* in descending order of effectiveness [13]. The methanolic extracts from the *A. indica* exhibited antibacterial effects to *Pseudomonas aeruginosa,* while the ethanol and ethyl acetate extracts of *A. indica* exhibited antibacterial activity against *Staphylococcus aureus* [14-16]. The *A. indica* leaves were extracted with methanol and the extract exhibited activity against *K. pneumoniae* and *S. aureus* [17]. The phytochemical compounds extracted from *Azadirachta indica* A. Juss.(Meliaceae) showed various biological activities and including, antibacterial activity against multidrug resistant bacterial strains [18,19].

Table 2. Phytochemical analysis of *A. indica* leaves extracts

S.No.	Compounds	Ethanol	Isopropyl alcohol
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Tannins	_	_
4	Terpenoids	+	+
5	Carbohydrates	_	_
6	Proteins	_	_
7	Carboxylic acid	_	_
8	Phenols	_	+
9	Glycosides	_	+
10	Quinones	+	+

(+) present (-) absent

3.2 Phytochemical Analysis

The phytochemical examination of ethanol extract from *A. indica* leaves revealed the existence of alkaloids, flavonoids, terpenoids, and quinones. Similarly, the isopropyl alcohol extracts of *A. indica* indicated the presence of alkaloids, flavonoids, terpenoids, phenols, glycosides, and quinones (Table 2). The ethanol and aqueous extracts of *A. indica* leaves revealed the presence of phytochemicals like tannins, alkaloids phenols, steroids, terpenoidsand saponins [20]. The antibacterial

activity of *A. indica* was reported previously. In a study, Malar et al. [17] extracted *A. indica* leaves using methanol and reported the presence of phenolic and steroid compounds.

3.3 GC-MS Analysis of *A. indica* Extract

The GC-MS examination of the isopropyl alcohol extract obtained from *A. indica* leaves unveiled the presence of one primary compound and two secondary compounds. The primary compound was identified as Diethyl phthalate, while the secondary compounds detected were (Methoxy acetic acid, pentyl ester), and (2-hexanol, 3-methyl-) (Table 3 and Fig. 1). The GC-MS assessment of the ethanol extract derived from *A. indica leaves* indicated the existence of one primary compound and one secondary compound. Diethyl phthalate was identified as the major compound, while (2-hexanol, 3-methyl-) was identified as the minor compound (Table 4 and Fig. 2). The bioactive diethyl phthalate exhibited antibacterial effect against both Gram-positive and Gram-negative bacteria [21]. The bioactive compound derived from *Azadirachta indica* extract exhibited potential antibacterial effects against a wide range of bacterial pathogens [22]. Gas chromatography-mass spectroscopy is one of the important analytical tools used to identify phytochemical compounds from the medicinal plants. GC-MS plays an important role in chemotaxonomic studies and the phytochemical analysis of secondary metabolites from medicinal plants [23-25]. In our study, GC-MS detected bioactive compounds from the isopropyl alcohol and ethanol extract.

Table 3. GC-MS analysis of bioactive compounds of *A. indica* – isopropyl alcohol extract

S. No	Name of the compound	RT	Area (%)	MW	Molecular formula	Molecular structure
1.	Diethyl phthalate	14.423	86.752	222	$C_{12}H_{14}O_4$	
2.	Methoxy acetic acid, pentyl ester	7.325	9.635	160	$C_8H_{16}O_3$	
3.	2-hexanol, 3-methyl-	8.761	3.613	116	$C_7H_{16}O$	

Table 4. GC-MS analysis of bioactive compounds of *A. indica* – ethanol extract

S. No	Name of the compound	RT	Area (%)	MW	Molecular formula	Molecular structure
1.	Diethyl phthalate	13.908	100	222	$C_{12}H_{14}O_4$	
2.	2-hexanol, 3-methyl-	6.250	3.213	116	$C_7H_{16}O$	

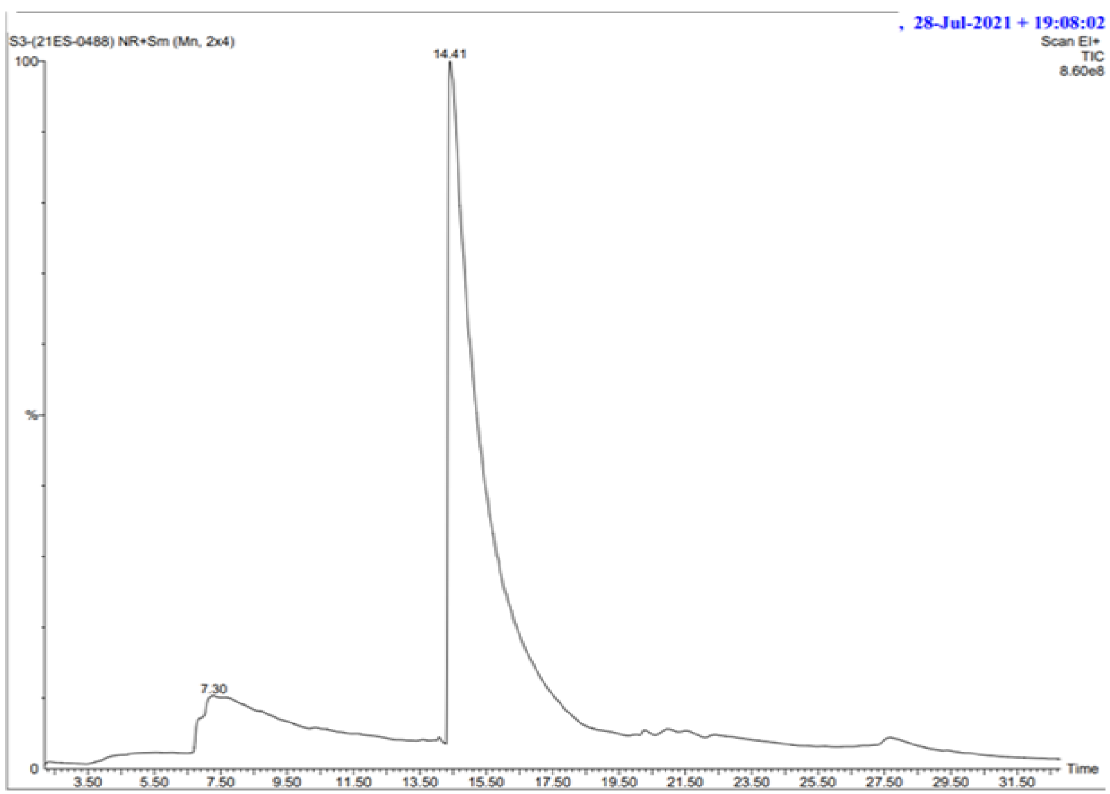


Fig. 1. Chromatography of isopropyl alcohol extract of *a. Indica*

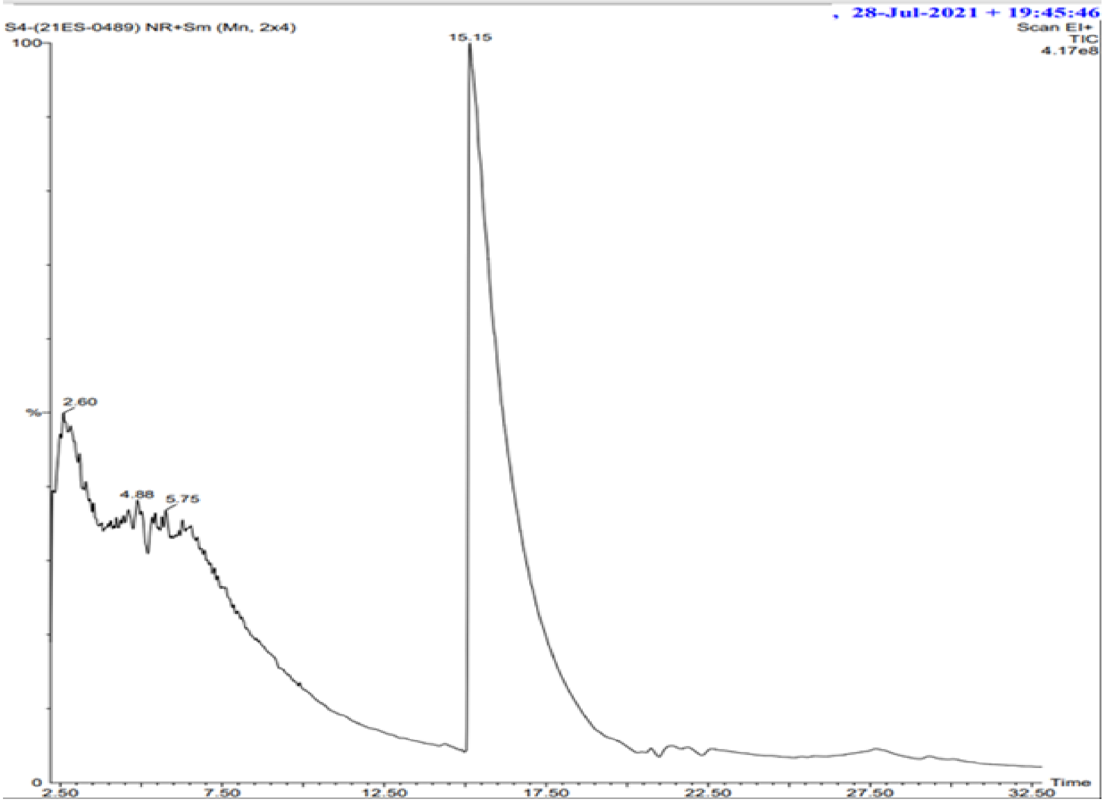


Fig. 2. Chromatography of ethanol extract of *A. indica*

4. CONCLUSION

The antimicrobial properties found in *Azadirachta indica*, commonly known as Neem, hold significant promise for combatting various pathogenic diseases in aquaculture. Notably, the bioactive compounds present in Neem leaves an alternative to synthetic drugs, potentially mitigating concerns regarding chemical residues and environmental impact. By harnessing the power of Neem and other herbal plants with strong antibacterial capabilities, not only the health of aquatic organisms be safeguarded, but also the overall ecosystem balance can be maintained. This research underscores the importance of exploring natural remedies and highlights the potential of *Azadirachta indica* as a valuable asset in the realm of aquatic disease management and environmental protection.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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