ISSN 2063-5346

BIOACTIVE PRINCIPLES OF *AZADIRACHTA INDICA* LEAVES MEOH EXTRACT AND THEIR ANTIBACTERIAL PROPERTY AGAINST *VIBRIO CHOLERA* STRAINS

Guru Prasad V.[1], Lavanya L.[2], Sharangouda J.Patil[3], Khalid Imran[4] and Vaddi Damodara Reddy[5*]

[1]Department of Microbiology, School of Allied Health Sciences, REVA University, Bengaluru-560064, India

[2]Department of Biochemistry, School of Allied Health Sciences, REVA University, Bengaluru-560064, India

[3]Department of Zoology, NMKRV College for Women (Autonomous), Bengaluru-560011, Karnataka, India

[4]Department of Microbiology, Krupanidhi degree college, Bengaluru-560035, India

[5]Department of Biotechnology, School of Applied Sciences, REVA University, Bengaluru-560064, India

Article History: Received: 03.04.2023 **Revised:** 25.04.2023 **Accepted:** 15.05.2023

Abstract

The aim of the current study explore the properties of *Azadirachta indica* leaves (Family: Meliaceae) of Indian medicinal plant by phytochemical screening qualitatively, quantification their volatile compounds by gas chromatography and mass spectroscopy analysis (GCMS) and their antibacterial property against *Vibrio cholera* using MTCC strains *V. cholera* 3904 and 3906. In order to identify the potential biomolecules, methanolic (MeOH) extract was subjected to standard phytochemical methods. Qualitative analysis revealed the presence of alkaloids, total carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, saponins, and tannins. GCMS analysis carried out due to potential phytochemical principles in the plant extract, observed 32 volatile compounds resulted on their retention time and % area of concentration in mass spectrum. The antibacterial potential of methanol extract exhibited maximum zone of inhibition 16.5 ±0.3 mm in *V. cholera* MTCC3904 and 15.9 ±0.5 mm in *V. cholera* MTCC3906 strain at 100 µL concentration compared to that of positive control azithromycin 22.65 ±0.1 and 22.725 ±0.4 mm respectively in 30 µl/ml concentration. Total antibacterial activity found 23.46 ±0.696 mm and 22.03 ±0.12 mm respectively in studied strains of bacteria and it is almost nearer to standard azithromycin and it is observe due to wide range of phytochemicals present in the plant.

Keywords: *Azadirachta indica,* Phytochemicals, GCMS analysis, *Vibrio cholera,* Azithromycin.

Introduction

A tropical evergreen tree with the widespread name "neem tree," *Azadirachta indica* (*A. Indica*), is indigenous to the Indian subcontinent [1]. Neem has been valued for thousands of years for a variety of advantageous qualities, including those in traditional medicine and agriculture for treating a variety of common human maladies. Due to its potential as a non-toxic infection-control agent for use in farming, *A. indica* initially garnered attention on a global scale [2]. In fact, azadirachtin, one of the most prevalent substances in neem plants, is a biopesticide that is becoming ever more popular [3-5]. However, several components of the neem tree have been utilised in traditional Indian medicine for thousands of years for their alleged antacid, antipyretic, antiparasitic, antiviral, antibacterial, antidiabetic, antidermatitic, contraceptive, anticancer, antioxidant, anti-inflammatory, dental, antifungal, and other healing and protective properties [2, 6]. Every component of *A. indica*, including the roots, leaves, stem, bark, seeds, fruits, gum, flowers, etc., has been utilised as a common household remedy for medical conditions affecting people. In addition, neem twigs are a major source of chewing sticks for oral hygiene in millions of people worldwide [7, 8]. Along with the uses of *A. indica* in the disciplines of dentistry, oncology, endocrinology and dermatology, among others infectious disease and modern medicine researchers have more recently initiated to forfeit attention to the neem tree as a possible source for novel antimicrobials [9-15].

The demand for innovative therapies has been prompted by the rising rates of antibiotic resistance among bacterial pathogens; as a result, the antibacterial capabilities of neem have received the majority of contemporary consideration in studies on the plant's antimicrobial potential. Neem products have long been used for dental hygiene, and the plant has found success in the food industry, which lends credence to this field of study. The ability of pathogenic bacterial species to form biofilms has generated interest in understanding how these communities contribute to heightened tolerance to antibacterial agents in addition to typical antibiotic resistance. Although the significance of biofilm-associated pathogens in human disease is widely acknowledged, there are currently few innovative treatments that can remove biofilms in an efficient manner. However, intriguing studies indicate that neem is consistently superior to many other herbal extracts in preventing bacterial growth and in targeting biofilm-grown cells, making it worthwhile to pursue as a source for drug discovery [16, 17].

A severe form of diarrheal condition, cholera has a high potential for epidemic transmission. Cholera has been a significant public health problem for ages, causing significant sickness and mortality, especially in the world's least developed regions [18]. A gram-negative bacterium of the *Vibrionaceae* family called *V. cholerae* may exist freely in rivers, ponds, saltwater and lakes. *V. cholerae* is a motile, curved, gram-negative bacillus that ranges in length from 1.4 to 2.6 μm and has a single polar flagellum. At least 33 different kinds of curved bacilli make up the *vibrio*, and 12 of these have been associated to infections in people [19]. The principal source of cholera outbreaks that can wreak devastation in densely populated areas of Asia, Africa, and Latin America is the *V. cholerae* O1 and O139 serogroups that produce cholera toxin (CT) [20]. El Tor and classical biotypes of *V. cholerae* O1 are separated further. O1 El Tor biotype, which took the place of the O1 classical strains that sparked the previous six pandemics and is currently responsible for the continuing cholera pandemic that began in 1961, is the cause [21]. Cholera outbreaks are currently mostly brought on by El Tor variant strains that carry the classical type ctx genes in

many developing nations. *V. cholerae* strains that do not belong to the O1 or O139 serogroups are sometimes referred to as non-O1/non-O139. Some of these strains are toxic and can occasionally cause sporadic of diarrhea [22].

MATERIALS AND METHODS

Chemicals, Microbial Media and Reagents used for the study

Water, acetic acid, Mayer's reagent, sulfuric acid, Fehling's solution, zinc dust, biuret reagent, gallic acid solution, methanol, aluminum trichloride, potassium acetate, hydrochloric acid, Dragendroff's reagent, acidified alcohol, ferric chloride, Folin–Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), quercetin solution, Molisch's reagent, Luria Broth (LB), Mueller Hinton Agar (MHA) etc., were purchased from Himedia Laboratories Pvt. Ltd and Sigma-Aldrich .

Methanolic Extraction of *A. Indica (L)* Phytochemical

The Foundation for Revitalisation of Local Health Traditions Botany Department collected *A. indica* leaf fragments in April 2021 and identified them in the University of Trans-Disciplinary Health Sciences and Technology location (13°07'23.7"N 77°32'58.6"E). The plant samples were identified and verified by Dr. N.M. Ghanesh Babu, an associate professor who oversees the Center for Herbal Gardens at the University of Trans-Disciplinary Health Sciences and Technology. Before being ground into powder, the uniform, disease-free, and non-pale pieces were dried in an oven at 40 °C for four to five days (Fig. 1.). Leaf was extracted using the maceration method and the solvent methanol. Separate 25g of powdered leaf samples were extracted in 250 mL of methanol solvents for 12 hours at room temperature in a shaking incubator, and the mixture was then filtered using Whatman No. 1 filter paper. Following the incubation period, the solid particles were removed by filtering, and the filtrate was then spin at 10,000 rpm for 10 minutes. The supernatant was evaporated using an evaporator to create a dry pellet, which was then used for additional analysis. In order to prevent the active compounds from being harmed by heating, solvent evaporation was carried out under reduced pressure at 40 °C. extracts of *A. indica* leave thus obtained [23-24].

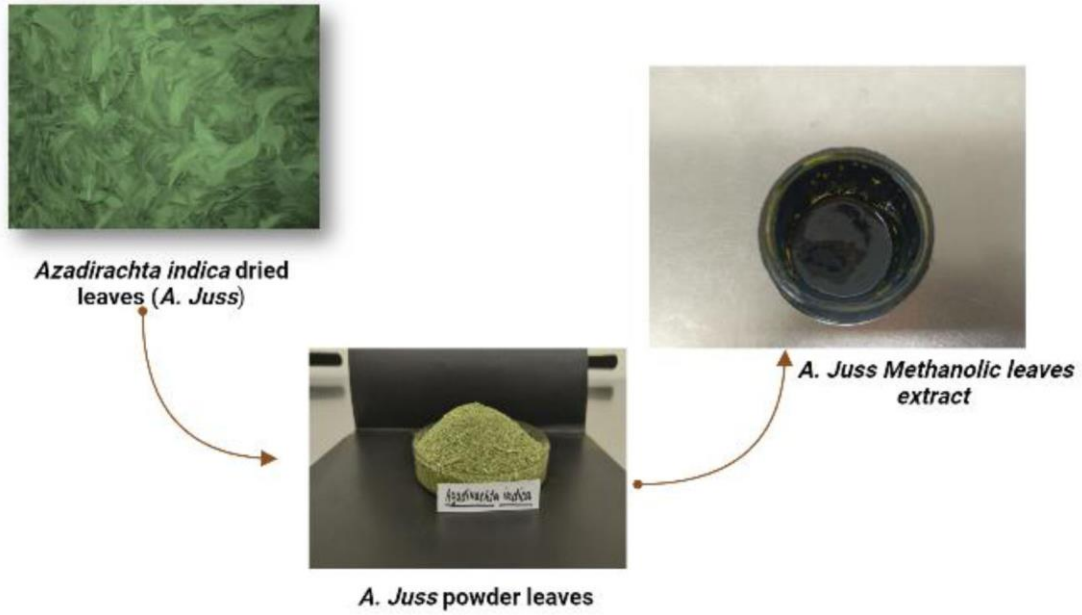

Figure 1: Collection of *A. indica* and drying (left) and ground by using a mixer (down) methanolic extract to (right).

Screening of Phytochemicals

Qualitative screening of phytochemicals of the methanol extract of *A. indica* leaves were conceded out in order to explore the principle metabolites present in the extract, which further continued it to finalize what are the active constituents are there by GCMS analysis and this study aims to identify the volatile compounds.

The methanol extract of *A. indica* leaves were subjected to standard phytochemical analysis as described by Sharangouda and Patil, [25], Harnborne, [26] and Fransworth, [27] to determine the presence or absence alkaloids, total carbohydrates, cardiac glycosides, flavonoids, glycosides, phenols, saponins, tannins, terpenoids and total proteins. Preparation of reagents for phytochemical assay followed standard protocol of Harnborne [28]. To make the concentration to obtain the proper solution methanol extract was re-dissolved in double distilled water and filtered.

Test for tannins

0.1 g of the purified extract was boiled in 2 ml of water/DMSO before being mixed with a few drops of 0.1% ferric chloride. A brownish green or blue-black colouring was then checked for [23, 24].

Test for alkaloids

0.1 g of the extract was mixed with 10 ml of acidified alcohol before being heated and filtered. Then, 1 ml of filtrate was added, along with 0.4 ml of diluted ammonia and 1 ml of chloroform, and was gently shaken. The chloroform layer was extracted using 2 ml of acetic acid. Then, this was separated into two sections, with one receiving Mayer's reagent and the other receiving Dragendroff's reagent. A test for alkaloids was considered successful if a cream or reddish-brown precipitate formed when using Mayer's or Dragendroff's reagent, respectively [24, 30].

Test for glycoside

0.2 g of the test substance was extracted using boiling/warming on a water bath along with 5 ml of each diluted sulfuric acid and water. Next, a 5% solution of sodium hydroxide was added to the acid extract before filtering and neutralizing it. Similar to how sodium hydroxide was used in the acid extract, water was added to the water extract in an amount equal to that of sodium hydroxide. The Fehling's solutions A and B were combined and heated in a water bath for two minutes after becoming alkaline. When red precipitate from acid extract is extracted in greater amounts than from water extract, glycoside may be present [24, 31].

Test for saponins

Saponins are present when an emulsion forms when three drops of olive oil are added to the froth that forms when 0.1 g of extract is added to 1 ml of distilled water [24, 32].

Test for cardiac glycosides

The presence of cardiac glycosides is indicated by the development of a brown ring at the interface following the addition of 2 ml of glacial acetic acid containing one drop of ferric chloride solution, followed by the addition of 1 ml of concentrated sulfuric acid to 0.5 mg of extract diluted with 5 ml of water [24, 30].

Test for flavonoids

The presence of cardiac glycosides is indicated by the development of a brown ring at the interface following the addition of 2 ml of glacial acetic acid containing one drop of ferric chloride solution, followed by the addition of 1 ml of concentrated sulfuric acid to 0.5 mg of extract diluted with 5 ml of water [24].

Test for terpenoids (Salkowski's test)

The presence of terpenoids is shown by the development of a reddish brown colouring at the interface following the addition of

strong sulfuric acid and 0.4 ml of chloroform to 0.1 g of the extract [24].

Test for proteins

When the biuret reagent (2 ml) is added to the test solution (2 ml), a violet color develops, signifying the presence of proteins [24].

Test for phenol

The presence of a dark green color after adding a few drops of a neutral 5% ferric chloride solution was regarded as positive for phenolic components. 50 mg of the extract was diluted in 5 ml of distilled water [24].

Gas Chromatography and Mass Spectroscopy Analysis

Preparation of Extract

10mg/ml aromatic medicinal plant methanolic extract of *A. indica* leaves were prepared in universal solvent methanol and for which 1µl plant extract was employed to quantify the volatile compounds by GCMS analysis.

Instruments and Chromatographic Conditions

GCMS analysis of *A. indica* extract was performed using a Thermo GCMS Clarus 500 (Perkin Elmer). For MS detection, the MS DSQ II electron ionization mode with ionization energy of 70 eV was used, with a mass range at m/z 50-650. 2B-XLB (Zebron capillary column) Column dimension: 30 meters length, 0.25mm (I.D), 0.25 film thickness(µm) 5% diphenylamine/95% dimethyl polysiloxane) was used for the analysis. The initial column temperature was programmed at 60°C/5min, respectively. The GC injector and MS transfer line temperatures were set at 230°C and 300°C respectively. GC was performed in the splitless mode. Helium (at flow rate=1.0 ml/min) was used as the carrier gas. A 2µL injection volume was used. The plant extract was dissolved in methanol and filtered with polymeric solid phase extraction (SPE) column and analyzed in GCMS for different constituents. Using computer searches on a NIST REFPROP Version 9.1 database and comparing the spectrum obtained through GCMS compounds present in the plants sample were identified.

Bacterial strain and inoculum preparation

The strains *V. cholerae* 3904 and 3906 strains were taken in the Microbial Type Culture Collection and Gene Bank (MTCC). The organism was subculture right away by transferring it into Mueller-Hinton broth aseptic circumstances, followed by an overnight incubation at 37 °C to verify the formation of bacterial colonies. The inoculated broth was transferred to slants and tested for their ability to inhibit bacterial growth using phytochemical.

Antimicrobial activity of *A. indica* leaves MeOH extract

Antibacterial activity of *A. indica* extract

A. indica leaves were examined for their antibacterial effectiveness against the *Vibrio cholera* bacteria MTCC 3904 and 3906 using the agar well diffusion method on Muller Hinton Agar (MHA) medium. In 1 mL of normal saline, three colonies with comparable characteristics were dissolved, and the turbidity was set at 0.5 McFarland. The isolates were then swabbed with a sterile swab on the MHA plate's surface. On MHA, 7 mm wells were aseptically made using a cork borer.100 µl of *A. indica* extract were put into media wells under sterile circumstances. Before being incubated at 37 °C for 24 hours, the plates were placed in the refrigerator for 1 hour to allow for extract diffusion. Methanol alone was employed as a negative control, and azithromycin was used as a positive control (30µg/ml). The

zone of inhibition was measured (in millimeters), and the mean was computed. The effectiveness of extracted *A. indica* against the examined species was tested in three duplicates. The data were then shown as mean and standard deviation [33].

MTCC 3904 and 3906 of the *Vibrio cholera* bacteria were used in the agar well diffusion method on Muller Hinton Agar (MHA) medium to test *A. indica* leaves for their antibacterial potency. Three colonies with similar features were dissolved in 1 mL of normal saline, and the turbidity was set at 0.5 McFarland. The MHA plate's surface was next swabbed by the isolates with a sterile swab. A cork borer was used to aseptically create 7 mm wells on MHA. Under sterile conditions, 100 µl of *A. indica* extract in various concentrations, including 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%, were added to media wells. The plates were chilled for 1 hour to facilitate extract diffusion before being incubated at 37 °C for 24 hours. Azithromycin (30 µg/ml) was utilised as a positive control and methanol alone as a negative control. The millimetres of the zone of inhibition were measured, and the mean was calculated. Three duplicate tests were conducted to determine the efficiency of the extracted *A. indica* against the targeted species. The mean and standard deviation of the data were then displayed [33, 34].

Results and Discussion

Phytochemicals Screening of *A. indica* MeOH Extract of Leaves

The results of qualitative analysis of phytochemicals of the methanolic extracts of *A. indica* are shown in Table 1. It was observed that leaves of plant extracts contained Alkaloids, Total Carbohydrates, Cardiac Glycosides, Flavonoids, Glycosides, Tannins, Saponins and Phenols are present in the leaves sample but Total Proteins and Terpenoids test show negative in *A. indica*.

Sl. No.	Parameters	*Azadirachta indica*
1	Alkaloids	+++
2	Total Carbohydrates	++
3	Cardiac Glycosides	+++
4	Flavonoids	++
5	Glycosides	+++
6	Tannins	++
7	Terpenoids	--
8	Saponins	+++
9	Phenols	+++
10	Total Proteins	--

Table: 1: Phytochemicals Screening of methanolic extract of *A. indica* leaves

Note: (++) medium quantity positive response was obtained for the chemical group in the extract. (+++) the positive response of greater quantity was obtained for the chemical group in the extract. (-) the negative response was obtained for that chemical group in the extract.

GCMS analysis of MeOH extract of *A. indica* leaves

The GCMS chromatogram of the extract is shown in Figure 2. GCMS analysis resulted in identification of 42 different metabolites. Volatile compound identification was done in comparison with the reference standards present in NIST and Wiley 9.1. Some of the bioactive were analyzed with their respective % of area present and retention time. The obtained peaks of the chromatograms, shown highest % of area by Sucrose (13.77), Diisooctyl phthalate (11.84), Phytol (4.16), Isosorbide Dinitrate (3.97), .alpha.-Tocopheryl acetate (3.43), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (2.91), Pentadecanoic acid (2.15), Pentasiloxane, dodecamethyl- (1.59), Columbin (1.336), Eugenol (1.19), Adenine, N4-

pentafluoropropionyl- (1.19), 1H-Cyclopenta[a]phenanthrene-7-carboxylic acid, 2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-10,13-dimethyl-3-oxo-17,2'- (1.18), 16-Allopregnen-3.beta.,7.alpha.-diol-20-one (1.12), Glycine, N-[(3.alpha.,5.beta.,7.alpha.,12.alpha.)-24-oxo-3,7,12-tris[(trimethylsilyl)oxy]cholan-24-yl]-, methyl ester (1.00) and rest of the 17 volatile compounds exhibited less than 1 % concentration of area as per the retention time. It has been identified many of these bioactive components are highly useful in biomolecules due to various biological activities (Table 2).

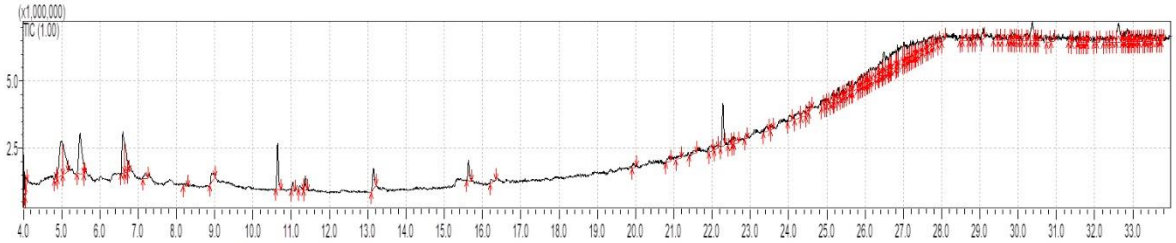

Figure 2: Spectrum of GCMS analysis and their volatile compounds representation of *A. indica* leaves

GCMS studies of *A. indica* leaves methanolic extract (Figure 2) exhibited total 32 volatile compounds as per the detection of graphical peaks, retention time and these are resulted in the analysis of thirty two volatile compounds that have pharmacological actions. These compounds identified by their characterization in differentiating retention time in each compounds along with their peak (%) in the studied plant extract in GCMS analysis. These characters classified by name of the individual molecules with their names as per the retention time and concentration of the peak area (%) in the spectrum (Table 2). The characterized results represented as per the peak and percent area of the volatile compounds, i.e., Sucrose (13.77), Diisooctyl phthalate (11.84), Phytol (4.16), Isosorbide Dinitrate (3.97), .alpha.-Tocopheryl acetate (3.43), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (2.91), Pentadecanoic acid (2.15), Pentasiloxane, dodecamethyl- (1.59), Columbin (1.336), Eugenol (1.19), Adenine, N4-pentafluoropropionyl- (1.19), 1H-Cyclopenta[a]phenanthrene-7-carboxylic acid, 2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-10,13-dimethyl-3-oxo-17,2'- (1.18), 16-Allopregnen-3.beta.,7.alpha.-diol-20-one (1.12), Glycine, N-[(3.alpha.,5.beta.,7.alpha.,12.alpha.)-24-oxo-3,7,12-tris[(trimethylsilyl)oxy]cholan-24-yl]-, methyl ester (1.00) and remaining 17 volatile compounds shown less than 1 % in the resulted compounds. Patil et al., [35] also reported similar compounds in GCMS studies in seeds of *Citrus medica* and various biological activities. Eramma and Patil, [36] revealed 41 distinct volatile compounds from crude and TLC fractions in *Flacourtia indica* root extract of methanol, GCMS analysis indicated the presence of, including Heneicosane (25.945), Squalene (20.51), Cholesterol (33.525), Cycloheptasiloxane, tetradecamethyl-(14.864), 2, 4-Di-tert-butylphenol-(16.032), Cycloheptasiloxane hexadecamethyl (16.848), Cyclononasiloxane octadecamethyl (20.733), and n-Hexadecanoic acid (22.092). Kolgi et al., [37, 38] reported the two metabolites such as alkaloid and flavonoid also revealed antioxidant and anticancer property in *Leucas aspera* leaves of chloroform and ethanol extracts. Similar compounds also reported for antioxidant property of *Simarouba glauca* seed extracts of petroleum and ethanol and revealed their qualitative and quantitative phytochemistry [39-40].

Table 2: Quantification of volatile compounds by GCMS analysis of MeOH extract of *A. indica* leaves based on retention time, peak area (%) and their names

Sl. No.	Compound Name	Retention Time	M/Z	Peak Area (%)
1	Isosorbide Dinitrate	4.031	40	3.97606
2	Eugenol	5.484	164	1.19678
3	Sucrose	6.595	57	13.77034
4	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	10.647	95	2.91033
5	9-Octadecen-1-ol, (Z)-	11.367	82	0.85243
6	Pentadecanoic acid	13.145	73	2.15868
7	Phytol	15.63	71	4.16048
8	Diisooctyl phthalate	22.281	149	11.84015
9	.alpha.-Tocopheryl acetate	30.367	165	3.43951
10	Succinic acid, monochloride 2-ethylbutyl ester	5.591	43	0.40207
1	Acetamide, N-(1-methylpropyl)-	8.27	44	0.70124
12	Dihydroartemisinin, 6-deshydro-5-deshydroxy-3-desoxy-	19.946	44	0.92796
13	17.alpha.-Hydroxypregnenolone	22.544	282	0.26562
14	Androst-11-en-17-one, 3-formyloxy-, (3.alpha.,5.alpha.)-	24.421	207	0.91673
15	33-Norgorgosta-5,24(28)-dien-3-ol, (3.beta.)-	24.958	415	0.64083
16	Glycine, N-[(3.alpha.,5.beta.,12.alpha.)-3,12-dihydroxy-24-oxocholan-24-yl]-	25.627	133	0.48805
17	3-Hydroxy-7,8-dihydro-.beta.-ionol	25.659	341	0.24647
18	Silane, diethylhexadecyloxy(2-methyl-4-methoxybutoxy)-	26.351	415	0.59425
19	Glycine, N-[(3.alpha.,5.beta.,7.alpha.,12.alpha.)-24-oxo-3,7,12-tris[(trimethylsilyl)oxy]cholan-24-yl]-, methyl ester	26.55	207	1.00275
20	Barbituric acid, 5-allyl-5-(cyclohex-2-en-1-yl)-	27.13	40	0.93705
21	Butanamide, 2,2,3,3,4,4,4-heptafluoro-N-[2-[(trimethylsilyl)oxy]-2-[4-[(trimethylsilyl)oxy]phenyl]ethyl]-	27.615	44	0.69468
22	Columbin	27.8	44	1.36164

23	Adenine, N4-pentafluoropropionyl-	28.634	281	1.19689
24	Ethanethioic acid, S-[8-(diethylphosphono)octyl] ester	29.762	282	0.49885
25	17.alpha.-Hydroxyprogesterone, trimethylsilyl ether	29.859	73	0.78701
26	Vitamin E	30.367	165	3.43951
27	Dodecanoic acid, 1a,2,5,5a,6,9,10,10a-octahydro-5,5a-dihydroxy-4-(hydroxymethyl)-1,1,7,9-tetramethyl-11-oxo-1H-2,8a-methanocyc	30.498	381	0.39049
28	Pentasiloxane, dodecamethyl-	31.995	281	1.59299
29	N-Nitrososolasodine	32.319	44	0.27810
30	1H-Cyclopenta[a]phenanthrene-7-carboxylic acid, 2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-10,13-dimethyl-3-oxo-17,2'-	33.105	207	1.18412
31	16-Allopregnen-3.beta.,7.alpha.-diol-20-one	33.495	281	1.12747
32	Fucoxanthin	33.751	40	0.84538

Antimicrobial Activity of *A. indica* leaves MeOH extract

The in vitro Antibacterial activity was carried out against 24 h old cultures of two bacteria by Agar disk diffusion method using Muller-Hinton (MH) agar plates using azithromycin as positive and methanol as negative control. For the antibacterial susceptibility test of *A. indica* leaves extract against *Vibrio cholera* MTCC-3904 and 3906 strains were used by well diffusion method, maximum inhibitory zone on 100% methanol extract were 16.5 ±0.3 mm and 15.9 ±0.5 mm whereas minimum at 60% methanol extract were 10.8 ±0.7 mm and 11.15 ±0.4 mm respectively. The positive control of azithromycin (30 µg) shown maximum inhibitory zone on 22.65 ±0.1 mm and 22.725 ±0.4 mm at lover concentration 30 µl/ml on *Vibrio cholera* strain MTCC-3904 and 3906 respectively (Figure 3). Srivastava et al., 41] reported antibacterial activity of spices against Vibrio species isolated from pond water and observed results in Black pepper (*Piper nigrum*) found maximum antibacterial zone on 100% ethanol and methanol and minimum at 70% ethanol extract of spices. Coriander (*Coriandrum sativum*) found maximum antibacterial zone on 85% ethanol and minimum at 70% ethanol extract. Cinnamon (*Cinnamomum verum*) found maximum antibacterial zone was observed on 85% as well as 100% acetone and minimum at 70% ethanol extract. Green Cardamom (*Elettaria cardamomum*) also found a very significant effect against Vibrio species maximum inhibitory zone on 100% ethanol, minimum at 70% methanol and as well as acetone extract (Table 3 and 4).

Total antibacterial activity of *A. indica* leaves of MeOH extract

Based on the minimum inhibitory concentration at higher zone of inhibition on 100% methanol extract, further experimented with total antimicrobial

activity on the same organisms in 100% concentration in triplicates and found zone of inhibition 23.4667 ±0.696 mm and 22.0333 ±0.12 in *Vibrio cholera* MTCC-3904 and 3906 strains respectively whereas positive control shown 29.8 ±0.52 mm and 29.6±0.23 mm and it was significant increase in the methanol extract of plant *C. verum* leaves and found total antibacterial activity when compared to negative and positive control (Table 4). The data of antibacterial efficacy of some other individual components e.g. camphene, limonene, caryophyllene has already been established [42]). The study of Ragasa et al., [43] showed that the ethanolic extract from the sapwood of *Dracontomelon dao* exhibited antimicrobial activity against *Staphylococcus typhimurium*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans* and *Aspergillus niger* and also reported GC-MS analysis revealed that out of the 54 compounds found in the extract, 21 compounds have antimicrobial properties while 15 compounds have antioxidant properties. GCMS chromatogram of the chloroform extract of *Rhus semialata* seeds showed the presence of more than forty bioactive constituents with Tridecane, Decane, Anethole, (Z)6,(Z)9-Pentadecadein-1-ol, Squalene as the major constituents and also proven their antimicrobial properties in various organisms [44]. Several pharmacological studies conducted on *Dracontomelon dao* reported antimicrobial, antioxidant, anti-inflammatory, anti-diabetic, and anti-trypanosomal activities [45-48] and the isolation of the chemical constituents of its several parts suggest the possession of medicinal and therapeutic properties [49].

Table 2: The Minimum Inhibitory Concentrations (MIC) of *A. Indica* extract leaf extract against *Vibrio cholerae*

Sample	**Zone of Inhibition(mm)**									
NS1	**10 µl/ml**	**20 µl/ml**	**30 µl/ml**	**40 µl/ml**	**50 µl/ml**	**60 µl/ml**	**70 µl/ml**	**80 µl/ml**	**90 µl/ml**	**100 µl/ml**
3904	**0**	**0**	**0**	**0**	**0**	10.8 ±0.7	12 ±0.5	13.5 ±1.0	13.8 ±05	16.5 ±0.3
3906	**0**	**0**	**0**	**0**	**0**	11.15 ±0.4	12 ±0.5	13.15 ±0.3	14.15 ±0.3	15.9 ±0.5
Positive Drug (Azithromycin 30 µl/ml)	**3904**	22.65 ±0.1								
	3906	22.725 ±0.4								
Negative (120µl/well Methanol)	**3904**	0								
	3906	0								

Table 3: *A. indica* methanolic extract's antimicrobial activity against *Vibrio cholera* was measured in terms of the diameter of the zone of inhibition (mean± S.D., n = 3)

Sample	3904	3906
	Zone of Inhibition(mm) Crude methanol extract 120µl/well	
NS1	23.4667 ±0.696	22.0333 ±0.12
Positive Drug (Azithromycin 30 µl/ml)	29.8 ±0.52	29.6±0.23
Negative (120µl/well Methanol)	0	0

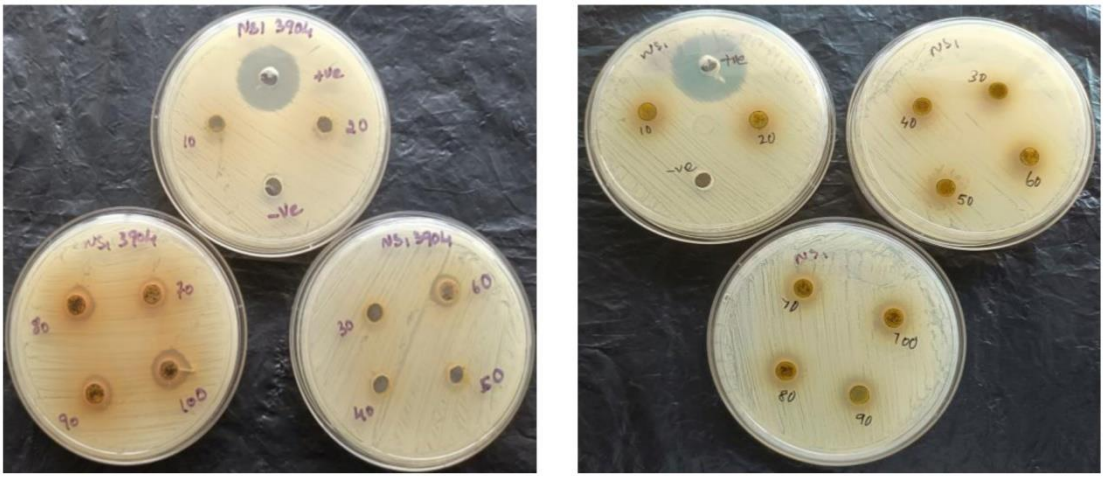

Figure 3: Determination of effect of *Azadirachta indica* leaf methanol extract (NS1) on *Vibrio cholerae* (MTCC-3904 and MTCC-3906) with different concentration by agar well diffusion assay method where organism was spread on Mueller Hinton Agar (MHA)

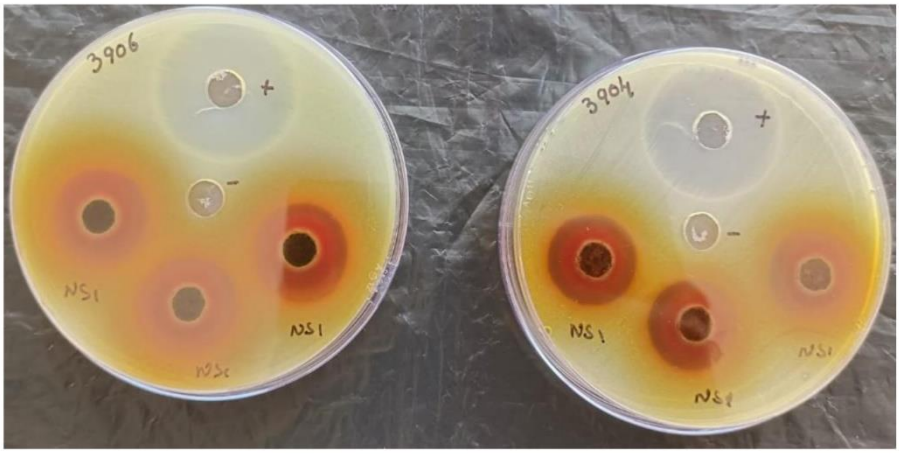

Figure 4: Determination of effect of *Azadirachta indica* leaf methanol extract (NS1) on *Vibrio cholerae* (MTCC-3904 and MTCC-3906) by agar well diffusion assay method where organism was spread on Mueller Hinton Agar (MHA

Conclusion

This study shown that *Azadirachta indica* leaf methanolic extract was a potent antibacterial against *Vibrio cholerae*, the harmful disease's causative agent. Neem extract was also discovered to have antihemorrhagic properties. The patient infected with *Vibrio cholerae* may be treated using the active extract discovered in this investigation. multiple medication resistance emerging A significant clinical issue in the control and management of the illness is *Vibrio cholerae*. Yet, diverse strains of multidrug-resistant *Vibrio cholerae* were susceptible to bactericidal action from the active leaf extract in this study. This would imply that the active *Azadirachta indica* leaf extract discovered in our research could be a source for the creation of novel antibacterial chemical medicines that could be used to treat cholera and diarrhoea patients. Neem has been used traditionally in India to cure cholera and diarrhoea, and the study's findings provide some scientific credence to such claims. Investigating the component of this extract that has these antibacterial properties against *Vibrio cholerae* should be the focus of further work.

ACKNOWLEDGEMENT

The authors are thankful to the School of Allied Health Sciences, REVA University, Bengaluru-560064, Karnataka, India for providing for necessary facilities for the research work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

References

1. Noorul Aneesa G. (2016). Beneficial Effects of Neem Oil-An Updated Review. J. Pharm. Sci. Res. 8 (8), 756–758.
2. Govindachari T. R. (1992). Chemical and Biological Investigations on *Azadirachta indica* (The Neem Tree). Curr. Sci. 63 (3), 117–122.
3. Chaudhary S., Kanwar R. K., Sehgal A., Cahill D. M., Barrow C. J., Sehgal R., et al. (2017). Progress on *Azadirachta indica* Based Biopesticides in Replacing Synthetic Toxic Pesticides. Front. Plant Sci. 8, 610. 10.3389/fpls.2017.00610
4. Pasquoto-Stigliani T., Campos E. V. R., Oliveira J. L., Silva C. M. G., Bilesky-José N., Guilger M., et al. (2017). Nanocapsules Containing Neem (*Azadirachta indica*) Oil: Development, Characterization, and Toxicity Evaluation. Sci. Rep. 7 (1), 5929. 10.1038/s41598-017-06092-4.
5. Kilani-Morakchi S., Morakchi-Goudjil H., Sifi K. (2021). Azadirachtin-Based Insecticide: Overview, Risk Assessments, and Future Directions. Front. Agron. 3. 10.3389/fagro.2021.676208
6. Alzohairy M. A. (2016). Therapeutics Role of *Azadirachta indica* (Neem) and Their Active Constituents in Diseases Prevention and Treatment. Evid. Based Complement. Altern. Med. 2016, 7382506. 10.1155/2016/7382506
7. Brahmachari G. (2004). Neem-an Omnipotent Plant: a Retrospection. Chembiochem 5 (4), 408–421. 10.1002/cbic.200300749
8. Gupta S. C., Prasad S., Tyagi A. K., Kunnumakkara A. B., Aggarwal B. B. (2017). Neem (*Azadirachta indica*): An Indian Traditional Panacea with Modern Molecular Basis. Phytomedicine 34, 14–20. 10.1016/j.phymed.2017.07.001
9. Lakshmi T., Krishnan V., Rajendran R., Madhusudhanan N.

(2015). *Azadirachta indica*: A Herbal Panacea in Dentistry - an Update. Pharmacogn. Rev. 9 (17), 41–44. 10.4103/0973-7847.156337

10. Patel S. M., Nagulapalli Venkata K. C., Bhattacharyya P., Sethi G., Bishayee A. (2016). Potential of Neem (*Azadirachta indica* L.) for Prevention and Treatment of Oncologic Diseases. Semin. Cancer Biol. 40-41, 100–115. 10.1016/j.semcancer.2016.03.002
11. Aumeeruddy M. Z., Mahomoodally M. F. (2021). Ethnomedicinal Plants for the Management of Diabetes Worldwide: A Systematic Review. Curr. Med. Chem. 28 (23), 4670–4693. 10.2174/0929867328666210121123037
12. Iman M., Taheri M., Bahari Z. (2021). The Anti-cancer Properties of Neem (*Azadirachta indica*) through its Antioxidant Activity in the Liver; its Pharmaceutics and Toxic Dosage Forms; a Literature Review. J. Complement. Integr. Med. 10.1515/jcim-2021-0009
13. Patil, U. H., and Gaikwad, D. K. (2011). Phytochemical screening and microbicidal activity of stem bark of Pterocarpus marsupium. *Int J Pharma Sci Res*, *2*(1), 36-40.
14. Singh V., Roy M., Garg N., Kumar A., Arora S., Malik D. S. (2021). An Insight into the Dermatological Applications of Neem: A Review on Traditional and Modern Aspect. Recent Adv. Antiinfect Drug Discov. 16 (2), 94–121. 10.2174/
15. Yarmohammadi F., Mehri S., Najafi N., Salar Amoli S., Hosseinzadeh H. (2021). The Protective Effect of *Azadirachta indica* (Neem) against Metabolic Syndrome: A Review. Iran. J. Basic Med. Sci. 24 (3), 280–292. 10.22038/ijbms.2021.48965.11218
16. Noor A. A. (2011). Dose Response Curve of Plants Extracts against the Human Pathogens. Gomal Univ. J. Res. 27 (2).
17. Wylie, M. R., and Merrell, D. S. (2022). The Antimicrobial Potential of the Neem Tree *Azadirachta indica*. Frontiers in Pharmacology, 13.
18. De-Simone, S. G., Napoleão-Pêgo, P., Gonçalves, P. S., Lechuga, G. C., Cardoso, S. V., Provance, D. W., Jr, Morel, C. M., and da Silva, F. R. (2022). B-Cell Epitope Mapping of the *Vibrio cholera* Toxins A, B, and P and an ELISA Assay. International J. Mol. Sci. *24*(1), 531. https://doi.org/10.3390/ijms24010531
19. Ding, Y., Hao, J., Zeng, Z., and Jinbo Liu (2022). Identification and genomic analysis of a *Vibrio cholerae* strain isolated from a patient with bloodstream infection. Heliyon, *8*(11), e11572. https://doi.org/10.1016/j.heliyon.2022.e11572
20. Clemens, J. D., Nair, G. B., Ahmed, T., Qadri, F., and Holmgren, J. (2017). Cholera. *Lancet (London, England)*, *390*(10101), 1539–1549. https://doi.org/10.1016/S0140-6736(17)30559-7
21. Legros D. Global cholera epidemiology: opportunities to reduce the burden of cholera by 2030. J. Infect. Dis. 2018;218:S137–s140.
22. Jiang F., Bi R., Deng L., Kang H., Gu B., Ma P. Virulence-associated genes and molecular characteristics of non-O1/non-O139 *Vibrio cholerae* isolated from hepatitis B cirrhosis patients in China. Int. J. Infect. Dis. 2018;74:117–122.
23. Bakir Çilesizoğlu, N., Yalçin, E., Çavuşoğlu, K., and Sipahi Kuloğlu, S. (2022). Qualitative and quantitative phytochemical screening of *Nerium oleander* L. extracts associated with toxicity profile. Scientific Reports, *12*(1), 21421.
24. Pant, D. R., Pant, N. D., Yadav, U. N., and Khanal, D. P. (2017). Phytochemical screening and study of

antioxidant, antimicrobial, antidiabetic, anti-inflammatory and analgesic activities of extracts from stem wood of *Pterocarpus marsupium* Roxburgh. Journal of Intercultural Ethnopharmacology, *6*(2), 170.

25. Patil S. M., Shirahatti P. S., Ramu R. (2021). *Azadirachta indica* A. *Indica* (Neem) against Diabetes Mellitus: a Critical Review on its Phytochemistry, Pharmacology, and Toxicology. J. Pharm. Pharmacol. 10.1093/jpp/rgab098
26. Sharangouda and Patil, S.B. (2007). Phytochemical screening and antifertility activity of various extracts of *Citrus medica* (Lemon) seeds in albino rats. Advances in Pharmacology and Toxicology, 8(2):71-4.
27. Harborne, J.B. (1998). Phytochemical Methods. Chapman and Hall, London, pp. 60-63.
28. Farnsworth, N.R. (1966). Biological and phytochemical screening of plants. 55(3): 225-276.
29. Harborne, J.B. (1973). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis, Chapman and Hall, London, UK.
30. Archana, P., Samatha, T., Mahitha, B. and Chamundeswari, N. R. Preliminary phytochemical scrTeening from leaf and seed extracts of *Senna alata* L. Roxb-an ethno medicinal plant. Int. J. Pharm. Biol. Res. **3**, 82–89 (2012).
31. Sati, S. C. and Kumar, P. Assessment of Himalayan juniper, *Juniperus squamata* Buch-Ham ex D. Don for phytochemical screening and antimicrobial potential against some infection causing pathogens. World J. Pharm. Res. 23, 998–1011 (2015).
32. Savithramma, N., Rao, M. L. and Suhrulatha, D. Screening of medicinal plants for secondary metabolites. Middle East J. Sci. Res. 8(3), 579–584 (2011).
33. Neglo, D., Adzaho, F., Agbo, I. A., Arthur, R., Sedohia, D., Tettey, C. O., and Waikhom, S. D. (2022). Antibiofilm Activity of *Azadirachta indica* and *Catharanthus roseus* and their Synergistic Effects in Combination with Antimicrobial Agents against Fluconazole-Resistant Candida albicans Strains and MRSA. Evid. Based Complem. Alternat. Med. 2022.
34. Altayb, H. N., Yassin, N. F., Hosawi, S., and Kazmi, I. (2022). In-vitro and in-silico antibacterial activity of *Azadirachta indica* (Neem), methanolic extract, and identification of Beta. d-Mannofuranoside as a promising antibacterial agent. BMC Plant Biol. 22(1), 1-14.
35. Patil, S.J., Venkatesh, S., Vishwanatha, T., Banagar, S.R., Banagar, R.J. and Patil, S.B. (2014). GCMS analysis of bioactive constituents from the petroleum ether extracts of *Citrus medica* seeds. *World Pharmacy and Pharmaceutical Sciences,* 3: 1239-1249.
36. Eramma, N. and Patil, S.J. (2023). Exploration of the biomolecules in roots of *Flacourtia indica* (Burm. F) Merr. methanol extract by chromatography approach. Letters in Applied NanoBioscience, 12(4): 166-177.
37. Kolgi, R.R., Haleshappa, R., Sajeeda, N., Keshamma, E., Karigar, C.S. and Patil SJ. (2021). Antioxidant studies, *in vitro* cytotoxic and cell viability assay of flavonoids and alkaloids of *Leucas aspera* (Wild.) Linn leaves. Asian Journal of Biological and Life Sciences, 10(1): 165-171.
38. Kolgi, R.R., Patil, S.J. and Karigar, C.S. (2020). Antioxidant and anticancer properties of chloroform extract of *Leucas aspera* from Tumkur district. Proceedings of National Conference National Web Conference on Sustainability in the Commerce,

Scientific, Technological, Linguistic and Environmental Sectors. pp.106-107.

39. Haleshappa, R., Patil, S.J., Usha, T. and Murthy SM. (2020). Phytochemicals, antioxidant profile and GCMS analysis of ethanol extract of *Simarouba glauca* seeds. Asian Journal of Biological and Life Sciences, 9(3): 379-385.
40. Haleshappa, R., Keshamma, E., Girija, C.R., Thanmayi, M., Nagesh, C.G., Fahmeen, L.G.H. et al., (2020). Phytochemical Study and Antioxidant Properties of Ethanolic Extracts of *Euphorbia milii.* Asian Journal of Biological Sciences, 13(1): 77-82.
41. Srivastava, A., Singh, A.N. and Singh M. (2016). Antibacterial activity of spices against Vibrio species isolated from pond water. European Journal of Experimental Biology, 6(2):21-25.
42. Rahaman, A., S.A. Siddiqui, F.O. Altuntas, S.Okay, F. Gul and I. Demirtas, 2019. Phenolic profile, essential oil composition and bioactivity of *Lasia spinosa* (L.) Thwaites. Brazilian Archives of Biology and Technology. Vol.62: e19170757.
43. Ragasa, C.Y., T.C. Batarra, J.L.A. Vivar, M.M. De Los Reyes and C.C. Shen, 2017. Chemical constituents of *Dracontomelon dao* (Blanco) Merr. et Rolfe. Pharmacogn. J., 9: 654-656.
44. Shruthy, S., Gothe, A., Aier, K, Kirankumar, S., Kalva P.K. and Patil S.J. (2019). Bioactive molecules and antimicrobial studies of Indian traditional medicinal plant *Rhus semialata* seeds. Research Journal of Medicinal Plants, 13: 10-17.
45. Dela Pena, J.F., Dapar, M.L.G., Aranas, A.T., Mindo, R.A.R. Cabrido, C.K., *et al*., (2019). Assessment of antimicrobial, antioxidant and cytotoxic properties of the ethanolic extract from *Dracontomelon dao* (Blanco) Merr. and Rolfe. Pharmacophore, 10: 18-29.
46. Khan, M.R. and Omoloso, A.D. (2002). Antibacterial and antifungal activities of *Dracontomelon dao*. Fitoterapia, 73: 327-330.
47. Mazura, M.P., M.H.S.N. Aisyah and B. Idayu, 2016. Assessing Malaysian forest species for lipogenase inhibitory activity. Proceedings of the 14th Seminar on Medicinal and Aromatic Plants, October 11-12, 2016, Malaysia, pp: 236-239
48. Yusro, F., Ohtani, K. and Kubota, S. (2016). Inhibition of α-glucosidase by methanol extracts from wood bark of *Anacardiaceae*, *Fabaceae*, *Malvace ae* and *Phyllanthaceae* plants family in West Kalimantan, Indonesia. Kuroshio Science, 9: 108-122.
49. Su, X.F., Liang, Z.Y. and Zhang, Y.X. (2008). Study on the chemical constituents of essential oil from the skins of stem of *Dracontomelon dao* (Blanco) Merr. et Rolfe. Lishizhen Med. Materia Medica Research, 19: 1640-1641.