IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
December 2020 Vol.:20, Issue:1

Antimicrobial Prospective of *Azadirachta indica* A. Juss (Neem) Leaf Extracts against Midgut Microbial Isolates from Dengue Vector - *Aedes aegypti*

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

RAMYA S[1], JAYAKUMARARAJ R*[2], NEETHIRAJAN K[1]

[1]Department of Zoology, Yadava College for Men (Autonomous), Madurai – 625 014, TN, India
[2]PG Department of Botany, Government Arts College, Melur, Madurai – 625106, TN, India

Submitted: 12 November 2020
Revised: 02 December 2020
Accepted: 22 December 2020

HUMAN JOURNALS
www.ijppr.humanjournals.com

Keywords: Neem; *Azadirachta indica*; Antibacterial Activity; Disc Diffusion Assay (DDA); *Aedes aegypti*; Mosquito; Midgut Microbes; Vector Born Disease (VBD)

ABSTRACT

Background and Objectives: *Azadirachta indica* A. Juss (Neem) a religious tree in India has been traditionally used in folklore medicine to combat various microbial diseases. This study evaluates the antibacterial activity of different solvent leaf extracts of Neem against the selected midgut microbial isolates from the dengue vector *Aedes aegypti*. **Methodology:** Antimicrobial activities of different solvent (Hexane, Petroleum ether, Chloroform, Ethyl acetate, Acetone, Ethanol, Methanol, and Aqueous) leaf extracts of *A. indica* were evaluated against selected midgut microbial isolates - *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa* and *Enterobacter aero genius* using disc diffusion method. **Result:** Different solvent-based leaf extracts of *A. indica* studied through Disc Diffusion Assay (DDA) exhibited significant antibacterial activity against all the tested midgut microbial isolates. Data indicate that the most significant solvent with maximum zone of inhibition (MZI) value was *B. subtilis* (Methanol extract - 21.96 ± 1.45) followed by *S. aureus* (Ethanol extract - 15.12 ± 1.47); *K. pneumonia* (Methanol extract - 24.98 ± 1.10); *E. coli* (Acetone extract - 23.64 ± 1.25); *P. aeruginosa* (Ethanol extract - 23.74 ± 1.00); *E. aerogenus* (Methanol extract - 22.10 ± 1.95) respectively. **Conclusion:** Results of DDA reveal that leaf extracts of Neem may be a prospective therapeutic agent to combat the growth of tested bacterial species. Screening of Neem plant against pathogenic microflora is expected to pave way for the development of plant-based natural products (PBNPs) to control Vector Born Disease (VBD) as less expensive organic bioactive molecules with better efficacy and safety with fewer or no side effect.

INTRODUCTION

Mosquitoes (Diptera: Culicidae) a small, two-winged, best-known group of insects remain a key threat for millions of humans and animals. Factually, mosquito-borne diseases remain a major health problem affecting at least one out of 6 persons throughout the world. However, only three genera namely *Aedes*, *Anopheles*, and *Culex* are of concern to public health as they transmit disease-causing pathogens including malaria, filariasis, and arboviral diseases such as Dengue, Yellow Fever, West Nile, Japanese encephalitis, Chikungunya, etc. Further, each of the disease-causing pathogen has its distinct life cycle, but encounter common events after being ingested and exposed to the mosquito's midgut environment. It has been established that the mosquito's midgut is the second largest organ and is the site where the disease-causing parasite, harmful bacteria, viruses, and toxins, as well as food and water, come in contact with the mosquito[1].

In 1987, the study of midgut microbiota in *Aedes* mosquitoes was started by Hung *et al.*[2] and reported a novel bacterial species *S. culicicola* from *Ae. sollicitans*. Later on, another bacterial species *S. taiwanense* (1988) and *S. diminutum* (1996) was also reported from *Culex*[3,4]. It has been proved that *S. taïwanense* significantly reduces the survival rate and flight capacity of adult *Ae. aegypti* and *An. stephensi*[5]. Furthermore, Yadav *et al.* reported that midgut bacteria significantly influence the parasitic life cycle in particular the vectorial capacity of the insects. Also, there is considerable interest in the characterization of endogenous microbiota in the mosquito midgut to identify potential bacteria for the prevention of disease[6]. All the existing control strategies for the management of VBDs seem to be insufficient to control the population of vectors and the microbial parasites within.

Chemical compounds, insecticides, have been utilized for mosquito control as a part of an integrated strategy for many decades. This intensive use develops resistance in the target vector population besides significant negative impacts on non-target organisms[7]. To counter the effects of these compounds, researchers developed an alternative control method i.e., the introduction of plant-based insecticidal compounds (PBICs) that can sustain and induce less toxicity in the environment than synthetic insecticides[8]. Medicinal plants have been part and parcel of mankind since the dawn of civilization. Pharmacological studies have re-accredited the importance of biologically active PBNPs[9]. Even-though much work has been done on ethnomedicinal plants in India, interest in a large number of traditional products has been reported to possess antimicrobial and antifungal activity[10]. In particular, phytochemical

extracts from the Neem plant are potential sources of antiviral, antitumor, and antimicrobial [11]. All parts of the Neem plant (*Azadirachta indica* A. Juss) is endowed with medicinal property. During the past few decades, apart from studies on the chemistry of Neem compounds, considerable progress has been made in evaluating biological activity for biomedical applications. Today, Neem is considered a valuable source of unique natural products for the development of medicines against various diseases. Biological activities and medicinal properties of Neem have been extensively reviewed[12]. In the present study, we evaluate the antibacterial potential of *A. indica* towards selected microbial isolates from the midgut of *A. aegypti*.

MATERIALS AND METHODS

Study Area and Period

To study the efficacy of leaf extracts on selected microbial test organisms, the mature leaves of *A. indica* were collected from the college campus, Yadava College for Men (Autonomous), Madurai, TamilNadu, India during June 2019. The Flora of Presidency of Madras and The Flora of Tamil Nadu Carnatic were used for identification and authentication of the plants[13,14]. The collected material was washed thoroughly in running tap water, rinsed in distilled water, and shade dried in open air and ground to powder.

To isolate the test organisms (Bacteria), adult mosquito *A. aegypti* were collected from Tiruppalai, Madurai, India during December 2019. Isolation, identification of the midgut bacteria, and antibacterial tests were carried out at the PG Department of Zoology, Yadava College for Men (Autonomous), Madurai, Tamil Nadu, India.

Preparation of Phytochemical Extracts

Neem leaves were macerated in different solvents Hexane, Petroleum ether, Chloroform, Ethyl acetate, Acetone, Ethanol, Methanol, and Distilled water using the cold percolation method. The leaf extracts thus obtained were concentrated using a rotary evaporator (Buchi, Switzerland) and stored at 4°C until further use.

Test Organisms

Midgut of female individual mosquito *A. aegypti* was dissected out for the isolation of microbes. The guts were dissected aseptically in a laminar hood using sterile entomological

needles underneath a stereo microscope. The dissected midgut was transferred to 100 µl of sterile phosphate buffer solution (PBS) and ground to homogeneity.

Identification of midgut bacteria

Gut homogenates were serially diluted (10 folds) with PBS and 100 µl of each dilution was pour-plated on nutrient agar media (Distilled Water - 1 L; Beef Extract - 1 g; Yeast Extract - 1 g; Peptone - 2.5 g; pH - 7.4±0.2) (Hi-Media Laboratories, Mumbai, India) and incubated at 37 °C for 24 - 48 h. The last wash (PBS) was taken as a control. All the microbiological procedures were carried out in a sterile environment, strictly following aseptic laboratory practices and negative controls (PBS) were included throughout the experiment. Morphologically distinct bacterial colonies were sub-cultured on nutrient agar plates for isolation of pure colonies. Based on the morphological features and biochemical parameters the microbes were identified as *Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli, Pseudomonas aeruginosa,* and *Enterobacter aerogenus*. All bacterial cultures were maintained in NA slants, stored at 4°C, and sub-cultured periodically.

Antimicrobial Activity Test

Antimicrobial activity was tested using a disc diffusion assay (DDA) method as described by Ncube *et al.*[15]. Plant extracts were dissolved in 20% DMSO treated water. Inoculum for each of the microorganisms was prepared from broth cultures (105 CFU/ml). A loop of culture from the NA slant stock was cultured in LB medium overnight and spread with a sterile swab into Petri-plates. Sterile disc (6 mm dia, Himedia, Mumbai, India) impregnated with the plant extracts (5 mg/ml) was placed on the cultured plates and incubated for 24 h at 37°C. The solvent loaded disc without extracts served as control. The results were recorded by measuring the maximum zone of inhibition (MZI). A clear inhibition zone around discs indicated the presence of antibacterial activity. Streptomycin served as the standard reference.

RESULTS AND DISCUSSION

In the present study, *A. indica* leaf extracts significantly inhibited the growth of all the bacterial strains tested. MZI for different solvent extracts against the selected bacterial strains isolated from the midgut of the dengue vector is given in Table 1. Data indicate that the inhibition response was not uniform towards the leaf solvent extracts. The variation in antimicrobial activity depended on plant parts used, solvent efficiency used, and the tested microbe. The most significant solvent with its MZI value for the isolates was *B. subtilis*

(methanol – 21.96 ± 1.45); *S. aureus* (ethanol – 15.12 ± 1.47); *K. pneumonia* (methanol – 24.98 ± 1.10); *E. coli* (acetone – 23.64 ± 1.25); *P. aeruginosa* (ethanol – 23.74 ± 1.00); *E. aerogenus* (methanol – 22.10 ± 1.95) respectively (Table 1). Overall order of MZI based on matrix analysis of different solvent extracts towards effectiveness was methanol > ethanol > acetone > ethyl acetate > chloroform > aqueous > petroleum ether > hexane.

The present data indicated that methanol and ethanol were the best solvents for antibacterial extraction as compared with other used solvents (Fig. 1). A similar study by Galeane *et al.* on *A. indica* showed that EA extract and butanol fraction presented greater activity against *S. mutans* and *S. mitis* presenting a MIC of 50 µg/ml for these strains and *E. faecalis* hydroethanolic extract and aqueous fraction were the most promising samples with a MIC of 50 µg/ml and 25 µg/ml, respectively[16]. Yerima *et al*. evaluated the antibacterial activity of bark, leaf, seed, and fruit extracts of *A. indica* on bacteria isolated from the adult mouth. Results revealed that bark and leaf extracts showed antibacterial activity against all the test bacteria used. Furthermore, seed and fruit extracts exhibited significant antibacterial activity only at higher concentrations[17].

Both culture-dependent and culture-independent techniques have been used to explore mosquito microbiota[18]. Bacterial isolates affect the life-span of mosquitoes. It has been observed that *S. marcescens* efficiently colonize midgut and determines survival and inhibition of sexual and asexual stages of *Plasmodium* through secretion of effector molecules[19,20]. Fouda *et al*. demonstrated that symbiotic bacteria influence the potential of reproduction (fertility and fecundity), pre-oviposition, and blood meal digestion in *Cx. pipiens*[21]. Further, *Bacillus* and *Staphylococcus* are essential for normal and high fecundity of *Cx. pipiens*. Gut microbial communities are the major components of mosquito innate immune responses and significantly influences *Plasmodium* infection in *An. gambiae*[22]. Studying the interaction between the gut microenvironment and vector competency might help control vector-borne diseases without disturbing the ecological balance.

CONCLUSION

Neem, the versatile traditional medicinal plant of India, is the versatile repository of bioactive compounds with diverse chemical structural motifs. As of now, little work has been done to characterize the biological activity and ponder plausible medical applications of the phytochemical compounds and hence extensive investigation is needed to exploit the

bioactive principles of Neem to cater to the wide array of therapeutic-biomedical applications. In the present study, the antibacterial activity of *A. indica* extracts towards drug-resistant/ clinically significant microbes isolated from the midgut of the dengue vector has been investigated. Phytochemical studies on active constituents of the Neem plant is expected to serve as the lead in the development of novel bioactive antimicrobial compounds and prevention of microbial disease in the days to come.

SIGNIFICANT STATEMENT

This study discovers that the medical importance of *A. indica* will help the researchers to discover cheap and new sources of antimicrobial substance against pathogenic microbes. The proposed concept note on the interaction between gut microenvironment and vector competency expected to advance efforts to control vector competency and disease spread locally and globally may be arrived at.

Author contribution: Dr. S. Ramya – Field Collection, Isolation and Characterisation of Microbes, Laboratory Experiments, Manuscript preparation; Dr. R. Jayakumararaj - Data Analysis, Manuscript preparation and Correction, Revision; Dr. K. Neethirajan Work-plan, Correction, Proofreading of MS.

Conflict of Interest: The Authors have no Conflict of Interest.

REFERENCES

1. Brown, J.E., B.R. Evans, W. Zheng, V. Obas, L. Barrera- Martinez, A. Egizi, H. Zhao, A. Caccone, and J.R. Powell, 2014. Human impacts have shaped historical and recent evolution in *Aedes aegypti*, the dengue and yellow fever mosquito. Evolution, 68(2): 514-525
2. Hung, Shirley HY, T. A. Chen, R. F. Whitcomb, J. G. Tully, and Y. X. Chen. 1987. "*Spiroplasma culicicola* sp. nov. from the salt marsh mosquito *Aedes sollicitans*." International Journal of Systematic and Evolutionary Microbiology 37(4):365-370.
3. Abalain-Colloc, M. L., L. Rosen, J. G. Tully, JM BOV, C. Chastel, and D. L. Williamson. 1988. "*Spiroplasma taiwanense* sp. nov. from *Culex tritaeniorhynchus* mosquitoes collected in Taiwan." International Journal of Systematic and Evolutionary Microbiology 38(1):103-107.
4. Williamson, David L., Joseph G. Tully, Leon Rosen, David L. Rose, Robert F. Whitcomb, Marie-Louise Abalain-Colloc, Patricia Carle, Joseph M. Bove, and Janette Smyth. 1996. "*Spiroplasma diminutum* sp. nov., from *Culex annulus* mosquitoes collected in Taiwan." International Journal of Systematic and Evolutionary Microbiology 46(1): 229-233.
5. Humphery-Smith, Ian, Odile Grulet, Francette Le Goff, and Claude Chastel. 1991. "*Spiroplasma* (Mollicutes: Spiroplasmataceae) pathogenic for *Aedes aegypti* and *Anopheles stephensi* (Diptera: Culicidae)." Journal of medical entomology 28(2): 219-222
6. Yadav, Kamlesh K., Ajitabh Bora, Sibnarayan Datta, Kshitij Chandel, Hemant K. Gogoi, G. B. K. S. Prasad, and Vijay Veer. 2015 "Molecular characterization of midgut microbiota of *Aedes albopictus* and *Aedes aegypti* from Arunachal Pradesh, India." Parasites & vectors 8(1):641.

7. Araújo, Helena RC, Danilo O. Carvalho, Rafaella S. Ioshino, André L. Costa-da-Silva, and Margareth L. Capurro. 2015 "*Aedes aegypti* control strategies in Brazil: incorporation of new technologies to overcome the persistence of dengue epidemics." Insects 6(2):576-594.
8. Ghosh Anupam, Nandita Chowdhury, and Goutam Chandra 2012 "Plant extracts as potential mosquito larvicides." The Indian journal of medical research 135(5):581.
9. Ramya, S., K. Neethirajan, and R. Jayakumararaj 2012 "Profile of bioactive compounds in *Syzygium cumini*–a review." J. Pharm. Res 5(1): 4548-4553.
10. Ramya S, Kalaivani T, Rajasekaran C, Jepachanderamohan P, Alaguchamy N, Kalyanasundaram M, and Jayakumararaj R 2008 Antimicrobial Activity of Aqueous Extracts of Bark, Root, Leaves and Fruits of *Terminalia arjuna* Wight & Arn. Ethnobotanical Leaflets 12:1192-1197.
11. Rajasekaran, C., E. Meignanam, V. Vijayakumar, T. Kalaivani, S. Ramya, N. Premkumar, R. Siva, and R. Jayakumararaj 2008 Investigations on antibacterial activity of leaf extract of *Azadirachta indica* A. Juss (Meliaceae): a traditional medicinal plant of India, Ethnobotanical Leaflets, 12: 1213-1217.
12. Biswas, Kausik, Ishita Chattopadhyay, Ranajit K. Banerjee, and Uday Bandyopadhyay 2002 "Biological activities and medicinal properties of neem (*Azadirachta indica*)." Current Science 82(11):1336-1345.
13. Kumar, Venugopalan Santhosh, and Visweswaran Navaratnam 2013 "Neem (*Azadirachta indica*): prehistory to contemporary medicinal uses to humankind." Asian Pacific journal of tropical biomedicine 3(7): 505-514.
14. Gamble, J. S. 1935 "The flora of the Presidency of Madras, London: Adlard&son." Ltd. London.
15. Matthew, K. M. 1983 "The Flora of Tamil Nadu Carnatic (The Rapinat Herbarium, St. Joseph‟ s College, Tiruchirapalli, India)." Vol I-III.
16. Ncube, N. S., A. J. Afolayan, and A. I. Okoh 2008 "Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends." African Journal of Biotechnology 7(12):20-28
17. Galeane, Mariana C., Carlos HG Martins, Jaqueline Massuco, Tais M. Bauab, and Lu&is VS Sacramento. 2017 "Phytochemical screening of *Azadirachta indica* A. Juss for antimicrobial activity." African Journal of Microbiology Research 11(4):117-122.
18. Yerima, M. B., S. M. Jodi, K. Oyinbo, H. M. Maishanu, A. A. Farouq, A. U. Junaidu, M. N. Al-Mustapha, and A. L. Shinkafi 2012 "Effect of neem extracts (*Azadirachta indica*) on bacteria isolated from adult mouth." Nigerian Journal of Basic and Applied Sciences 20(1):64-67.
19. Dillon, Rod J., and V. M. Dillon 2004 "The gut bacteria of insects: nonpathogenic interactions." Annual Reviews in Entomology 49(1):71-92
20. Bahia, A C., Yuemei D., Benjamin, J. B., Godfree, M., Abhai, T., Omar, J., BenMarzouk, H., Ramesh, C. and George, D. 2014 "Exploring *Anopheles* gut bacteria for *Plasmodium* blocking activity." Environmental microbiology 16(9):2980-2994.
21. Tchioffo, M. T., Anne B, Luc A, Sandrine E. N., Albert N. B., Parfait H. Awono-Ambéné, Richard C., Geoffrey G., Isabelle, M. 2016 "Dynamics of bacterial community composition in the malaria mosquito's epithelia." Frontiers in microbiology 6 (2): 1500-1505.
22. Fouda, M. A., M. I. Hassan, A. G. Al-Daly, and K. M. Hammad. 2001"Effect of midgut bacteria of *Culex pipiens* L. on digestion and reproduction." Journal of the Egyptian Society of Parasitology31, no. 3(1): 767-780.
23. Boissière, A., Majoline T., Tchioffo, D. B., Luc A., Alexandra M., Sandrine E. N., Hamid R. S. 2012 "Midgut microbiota of the malaria mosquito vector *Anopheles gambiae* and interactions with *Plasmodium falciparum* infection." PLoS pathogens 8(5): e1002742

Table No. 1: Antibacterial activity of solvent based leaf extracts of *Azadirachta indica* against isolates from the midgut of *Ae. aegypti*

Test Organisms	Zone of Inhibition (Diameter in mm)								
	Hexane	Petroleum ether	Chloroform	Ethyl acetate	Acetone	Ethanol	Methanol	Aqueous	Streptomycin
B. subtilis	4.59 ± 0.23	5.12 ± 0.12	11.11 ± 1.02	15.06 ± 1.20	20.89 ± 1.11	21.57 ± 1.20	21.96 ± 1.45	12.66 ± 0.21	19.20 ± 0.25
S. aureus	3.22 ± 0.55	3.58 ± 0.65	10.02 ± 1.22	9.41 ± 1.06	12.45 ± 1.64	15.12 ± 1.47	13.55 ± 1.06	3.44 ± 0.11	14.50 ± 0.92
K. pneumonia	5.69 ± 0.74	3.98 ± 0.25	16.78 ± 1.63	14.02 ± 1.54	20.30 ± 1.95	20.69 ± 1.09	24.98 ± 1.10	10.12 ± 0.96	18.10 ± 2.82
E. coli	1.59 ± 0.42	1.98 ± 0.36	14.22 ± 1.08	15.64 ± 1.40	23.64 ± 1.25	20.51 ± 1.31	23.45 ± 1.40	10.25 ± 0.46	25.40 ± 1.52
P aeruginosa	2.88 ± 0.69	5.64 ± 0.41	12.99 ± 1.04	13.99 ± 1.09	20.01 ± 1.33	23.74 ± 1.00	19.36 ± 1.55	11.23 ± 0.32	22.10 ± 1.37
E. aerogenus	1.65 ± 0.19	3.54 ± 0.61	11.12 ± 1.44	12.35 ± 1.07	21.12 ± 1.50	21.98 ± 1.51	22.10 ± 1.95	9.11 ± 0.46	21.60 ± 1.49

Note: Data represents mean of triplicate with SD

	HE	PE	CE	EAE	AE	EE	ME	AQE	STR
B. subtilis	5	5	11	15	21	22	22	13	20
S. aureus	3	4	10	9	12	15	14	3	15
K. pneumonia	6	4	17	14	20	20	25	10	18
E. coli	2	2	14	15	24	21	23	10	25
P aeruginosa	3	6	13	14	20	24	19	11	22
E. aerogenus	2	4	11	12	21	22	22	9	22

Figure No. 1: Colour coded matrix based on antibacterial activity of leaf extracts of *A. indica* against midgut isolates (Green – Maximum inhibition (25); Red – Minimum or No inhibition (2) **Abbreviations**: HE - Hexane Extract; PE - Petroleum ether Extract; CE - Chloroform Extract; EAE - Ethyl acetate Extract; AE - Acetone Extract; EE - Ethanol Extract; ME - Methanol Extract; AQE - Aqueous Extract; STR - Streptomycin).