

PRELIMINARY PHYTOCHEMICAL ANALYSIS AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF NEEM (*AZADIRACHTA INDICA*) LEAVES ON BACTERIAL PATHOGENS

Shiji Thomas

Assistant professor

EMEA college of arts and science, Kondotty, India

Abstract: Neem (*Azadirachta indica*) is considered as one of the most useful medicinal plants that possesses a wide spectrum of biological properties. The present study aims to detect the antibacterial efficacy of various extracts of fresh leaves of *Azadirachta indica* against different clinical strains. In the present study, biologically active components from the leaves of *Azadirachta indica* were extracted using four different solvents of increasing polarities (chloroform, methanol, ethanol and water). The antibacterial efficacy of these extracts was determined using agar well diffusion method. Further in this study, qualitative phytochemical screening for identification of various classes of active chemical constituents such as phenols, flavonoids, saponins, terpenoids, glycosides and alkaloids using the methanolic extract. The zones of inhibition observed in the case of gram positive and drug resistant strains were greater compared to the zones of gram-negative ones. The findings of the present antibacterial assay indicates that extract prepared using water exhibited less antibacterial activity compared to other extracts. This study provides scientific understanding to further explore other pharmacological properties of the plant.

Index terms: *Azadirachta indica*, antibacterial activity, bacterial strains, phytochemical analysis, HPLC

1. INTRODUCTION

Owing to the inevitable development of resistance that follows the introduction of antibiotics to the clinic, there is a constant need for newer antibacterial drugs (Walsh, 2003). The number of new antibacterial agents has decreased steadily in the United States over the last several decades (Flair, 2014). The deceleration in the discovery rate of newer antibiotics together with the limited lifespan of each drug once introduced to the clinic urged researchers around the world to turn to alternative approaches for fighting bacterial pathogens. Bioactive compounds of plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Antibacterial properties of several plants and their bioactive components are commercially exploited in various commercial products such as dental root sealers (Bal, 1989), antiseptics (Tyski *et al.*, 2013), food preservatives (Fatoki, 2013) and feed supplements. Bioactive compounds are chemically diverse group of compounds which include flavonoids, phenolic compounds, glycosides, terpenoids, alkaloids, tannins, quinones, lignins, proteins etc.

Azadirachta indica Juss. (Neem) (Meliaceae) is a popular evergreen tree found commonly in India, Africa and America. *A. indica* is one of the most useful traditional medicinal plants in India. Previous research on Neem demonstrated that it possesses bioactive substances with multiple medicinal properties. *Azadirachta indica* is traditionally used in folklore medicine for the treatment of diabetes. Neem leaves has antibacterial properties and is found to be effective in controlling airborne bacterial contamination in the residential premises (Saseed *et al.*, 2008). Azadirachtin, nimbin and nimbidine are considered to be the most important bioactive compounds found in the leaves of Neem (Mondall *et al.*, 2009). The paste of neem leaves is very effective to treat skin allergies, as well as healing of wound of small pox and chicken pox (Hla *et al.*, 2011).

The extract of Neem leaves has been shown to have antibacterial activity against *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *E. coli* (Koonan and Subbarao, 2011). The overuse of antibiotics in combating bacterial diseases often leads to the developments of multidrug resistant (MDR) bacteria. Extract of neem leaf is known to have antibacterial activity without drug resistant problem (Valarmathy *et al.*, 2010). Hence, neem leaf extract can be considered as an alternative to antibiotics to treat MDR bacterial diseases. The objective of the present research was to determine antibacterial efficacy of neem leaf extract against MDR pathogenic bacteria of poultry: *P. multocida*, *S. pullorum*, *S. gallinarum* and *E. coli*.

2. MATERIALS AND METHODS

2.1. Plant material collection and preparation of extracts

Fresh leaves of *Azadirachta indica* leaves free from disease were collected from Botanical Garden, Calicut University. The plant material was washed with water and rinsed with sterile distilled water, shade dried and powdered using mechanical grinder. The powdered material was then extracted using four different solvents (chloroform, methanol, ethanol and water). Dried extract was stored in air tight container and maintained at 4°C in a refrigerator.

The method described by Harbone (1998) was used with slight modification in this study. 10 gm powder of each dried plant material was taken and mixed with 100 ml of the solvent. It was mixed well and kept on an orbital shaker at 200 rpm for 48 hours at room temperature. The extract was then filtered using Whatman no.1 filter paper. The procedure was repeated for three times or until clear supernatant solvent was formed. The supernatant was collected and evaporated to dryness. The residue thus obtained was weighed and used for further analysis.

2.2. Screening of plant extracts for antimicrobial activity

Antimicrobial activity of plant extracts was analysed by agar well diffusion test and minimum inhibitory concentration/minimum bactericidal concentration assays.

2.3. Bacterial Strains

Ten clinical strains and four standard strains were used in the present study. The clinical strains were procured from Government Medical College, Kozhikode. These were *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella typhi*, *Shigella flexneri* and two strains of methicillin-resistant *Staphylococcus aureus* (MRSA-I and II). The identification and maintenance of cultures were performed by using classical diagnostic microbiology procedures (Pelczar,1993).

2.4. Antimicrobial activity screening

Agar well diffusion method: This method was employed for screening the antimicrobial activity of plant extracts against the test microorganisms. 0.01 ml from the standardized overnight bacterial cell suspension (10^7 CFU/ml) was spread evenly over the Mueller-Hinton agar plates (Arora and Jasleen, 1999). Wells were then bored into the agar medium with a sterile 6 mm cork borer. Fifty microliters of the extracts of plant materials at a concentration of 100 mg/ml was dispensed in to each well in the plate. After incubation at 37°C for 24 hours, the diameter of the zones of inhibition were measured. DMSO was used as the negative control.

2.5. Analysis of phytoconstituents in the plant extracts

2.5.1. Analysis of phytoconstituents by biochemical methods: The methanolic extract of *Azadirachta indica* (neem) was subjected to qualitative phytochemical screening for the identification of various classes of active chemical constituents such as phenols, flavonoids, saponins, terpenoids, glycosides and alkaloids. The phytochemical analysis was done according to standard methods (Vogel, 1956).

2.5.2. Analysis of bioconstituents by HPLC: For identifying the different phytoconstituents, methanol extract of the plant leaves was subjected to High performance liquid chromatography (HPLC) (Mahmoud *et al.*, 2011). Compounds were separated on a C18 reverse phased column maintained at room temperature. Mobile phase used was methanol and water and the flow rate was maintained at 1ml/minute. The mobile phase was filtered through a 0.45 µm membrane filter (Millipore) prior to use. The dried extract was dissolved in the mobile phase and appropriately diluted. After filtering through a filter paper and a 0.45 µm membrane filter (Millipore), 20 µl of the extract was injected directly into HPLC system.

3. RESULTS

3.1 Percentage yield of extract of the extracts

To carry out an elaborate antibacterial analysis, extracts were prepared using four different solvents - methanol, ethanol, water and chloroform. Percentage yield of the extracts were found to be different with different solvents and it is given in the table 1.

Table 1. Percentage yield of extracts of *Azadirachta indica*.

Solvent used	Dry mass (gm)	Percentage yield
Methanol	1.4	14
Ethanol	1.22	12
Water	1.0	1
Chloroform	1.09	1

3.2 The preliminary antibacterial screening

The preliminary screening results of ethanol, chloroform, methanol and water extracts are presented in Table 2. The ethanol and methanol extracts of leaves of *Azadirachta indica* expressed considerable antibacterial activity against most of the bacterial strains tested in this study. The methanol and ethanol extracts were quite efficient against drug resistant gram positive strains used in this study. Data from this study is in line with previous studies which suggested that the alcohol and chloroform extracts of Neem leaf has anti-staphylococcal properties [18]. Methanol extract possessed higher antibacterial efficiency against all the tested bacteria when compared to the other extracts. The chloroform extract showed good to moderate antibacterial activity. Negative control, DMSO had no effect on microbial growth.

Table 2: Antibacterial activities of various extracts of *Azadirachta indica* leaves against clinical strains using agar well diffusion method.

Microorganism	Zone of Inhibition of plant extract (in mm)				
	Methanol	Ethanol	Water	Chloroform	DMSO
MRSA I	20±0.57	20±0.57	-	20±0.57	-
MRSA II	20±0.57	20±0.57	-	22±1.15	-
<i>Escherichia coli</i>	12±0.57	8±0.57	-	-	-
<i>Klebsiella pneumoniae</i>	-	-	-	-	-
<i>Proteus vulgaris</i>	9±0.57	12±0.57	-	-	-
<i>Proteus mirabilis</i>	-	-	-	-	-
<i>Shigella flexneri</i>	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	9±0.57	-	-	15±0.57	-
<i>Salmonella typhi</i>	14±0.57	-	-	-	-
<i>Acinetobacter baumannii</i>	-	-	-	-	-

- : No zone of inhibition

3.3.1 Preliminary phytochemical analysis

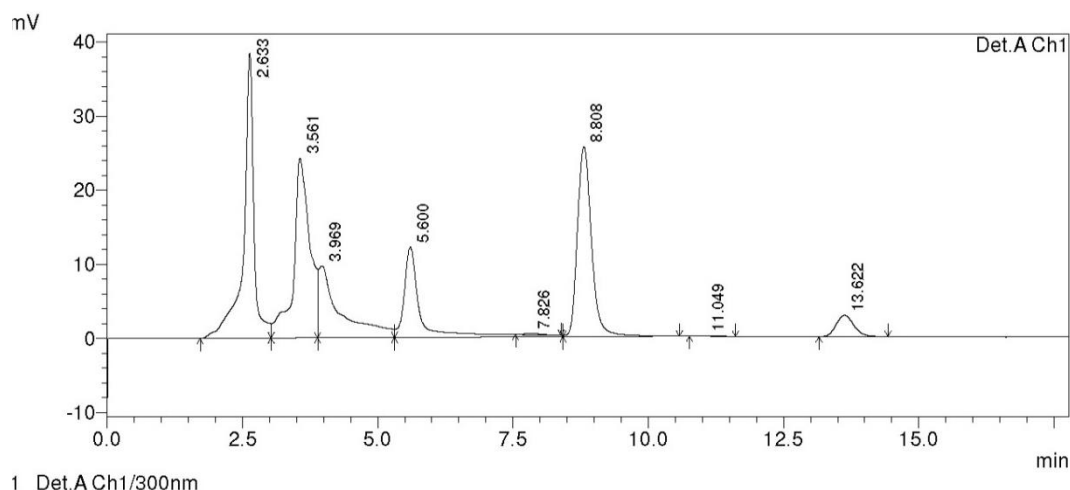
The results of the phytochemical analysis (Table 3) reveal the presence of various secondary metabolites viz. phenols, alkaloids and flavonoids in the leaf extract of *Azadirachta indica*. The antibacterial activity of the extract might be attributed to the presence of these compounds in the plant.

Table 3: Preliminary phytochemical analysis of methanol extract of *Azadirachta indica* leaves

Type of the extract	Phenols	Flavanoids	Terpenoids	Saponins	Glycosides	Alkaloids
Methanol	+	+	-	-	-	+

3.3.2. Analysis of bioconstituents by HPLC

Figure 1: HPLC profile of *Azadirachta indica*



1 Det.A Ch1/300nm

PeakTable

UV Detector Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.633	511233	38462	24.524	33.933
2	3.561	507064	24289	24.324	21.429
3	3.969	273548	9722	13.122	8.577
4	5.600	254263	12158	12.197	10.727
5	7.826	4542	228	0.218	0.201
6	8.808	466744	25557	22.390	22.548
7	11.049	1444	58	0.069	0.052
8	13.622	65801	2871	3.156	2.533
Total		2084640	113345	100.000	100.000

HPLC profile of *Azadirachta indica* exhibited a total of six different peaks at retention times 2.63, 3.56, 3.96, 5.6, 8.8 and 13.62 minutes. Similar results were obtained in previous studies. [19]. Further study for the isolation and purification of bioactive compounds responsible for the antimicrobial activity has to be conducted to ensure the antibacterial efficiency and clinical safety of these compounds.

3.4 Statistical analysis

Means and standard deviations of the assays were calculated using conventional statistical methods. Each treatment was performed in three replicates. The data were statistically analysed with statistical package for social sciences (SPSS) software program (version 19) using one way analysis of variance (ANOVA) and t test to determine differences in means at the level of $p < 0.05$.

REFERENCES

- [1] Walsh, C. 2003. Where will new antibiotics come from?. *Nature Reviews Microbiology*, 1(1), 65-70.
- [2] Fair, R.J. and Tor, Y. 2014. Antibiotics and bacterial resistance in the 21st century. *Perspectives in medicinal chemistry*, 6, pp.PMC-S14459.
- [3] Bal, C.S., Sikri, V.K. and Agrawal, R. 1990. Efficacy of various eugenol and non-eugenol root canal sealers in the treatment of teeth with periapical radiolucent area--a clinical and radiological study. *Indian journal of dental research: official publication of Indian Society for Dental Research*, 2(1), pp.133-139.
- [4] Tyski, S., Bocian, E., Mikucka, A. and Grzybowska, W. 2013. Antibacterial activity of selected commercial products for mouth washing and disinfection, assessed in accordance with PN-EN 1040. *Medical science monitor: international medical journal of experimental and clinical research*, 19, p.458.
- [5] Regnier, T., Combrinck, S. and Du Plooy, W., 2012. Essential oils and other plant extracts as food preservatives. *Progress in food preservation*, pp.539-579.
- [6] Khan, S.A. and Aslam, J., 2008. Study on the effect of Neem (*Azadirachta indica*) leaves smoke in controlling airborne bacteria in residential premises. *Current research in Bacteriology*, 1(2), pp.64-66.

- [7] Mondall, N.K., Mojumdar, A., Chatterje, S.K., Banerjee, A., Datta, J.K. and Gupta, S., 2009. Antifungal activities and chemical characterization of Neem leaf extracts on the growth of some selected fungal species in vitro culture medium.
- [8] Hla, K.K., Aye, M.M. and Ngwe, M.H., 2011. Some chemical analyses and determination of antioxidant property of neem leaf (*Azadirachita indica* A. Juss). *Uni Res J*, 4, pp.227-235.
- [9] Francine, U., Jeannette, U. and Pierre, R.J., 2015. Assessment of antibacterial activity of neem plant (*Azadirachta indica*) on *Staphylococcus aureus* and *Escherichia coli*. *J Med Plants Stud*, 3(4), pp.85-91.
- [10] Valarmathy, K., Gokulakrishnan, M., Kausar, M.S. and Paul, K., 2010. A study of antimicrobial activity of ethanolic extracts of various plant leaves against selected microbial species. *International Journal of Pharma Sciences and Research*, 1(8), pp.293-295.
- [11] Harborne, J.B., 1989. *Methods in plant biochemistry*. Volume 1. Plant phenolics.
- [12] Pelczar Junior, M.J., Chan, E.C., Krieg, N.R., Edwards, D.D., Pelczar, M.F., Yamada, S.F., Nakamura, T.U., Dias Filho, B.P. and Lima, L.S., 1997. *Microbiologia: conceitos e aplicações*. In *Microbiologia: conceitos e aplicações* (pp. xxix-524).
- [13] Arora, D.S. and Kaur, J., 1999. Antimicrobial activity of spices. *International journal of antimicrobial agents*, 12(3), pp.257-262.
- [14] Vogel, A.I., 1956. *Practical organic chemistry*. Longmans, 2, pp.676-681.
- [15] Mahmoud, D.A., Hassanein, N.M., Youssef, K.A. and Abou Zeid, M.A., 2011. Antifungal activity of different neem leaf extracts and the nimonol against some important human pathogens. *Brazilian Journal of Microbiology*, 42(3), pp.1007-1016.

