Aqueous, Acetone and Ethanol extract of *Nerium indicum* L. as potential antibacterial agent against *Pseudomonas aeruginosa*.

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Abstract

Bacterial diseases are widespread all over the world. The problem of bacterial infections of *Pseudomonas aeruginosa* which effect environment as well as human causes nosocomial infections hence a result of it is ubiquitous nature, ability to survive in moist environments and resistance to many antibiotics and antiseptics. *Nerium indicum* which act as antibacterial potential, parts of plant are accounted as a curative agent and have been used in wisdom in a variety of ailments. The leaves are the important part of this plant which is rich in bioactive compounds such as glycoside, Oleandrin, flavonoid, tannin, neriiin, phytosterin and l-strophnathin, rosaginin and nerlin, volatile oil, fixed oil, neriodorin and neriodorein, which exhibited antimicrobial activities. In present study, *N. indicum* extracts were analysed for various bio-molecules and their antibacterial affectivity was studied. *N. indicum* extracts viz, acetone, ethanol and aqueous at different concentrations (200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml) were prepared and their effectiveness as an antibacterial were studied against *P. aeruginosa* by using disc diffusion method and MIC. Observations of study showed the effectiveness in all the extracts tested. It was also observed that acetone extract was most effective in comparison to ethanol and aqueous extracts.

**Keywords:** Antibacterial activity, Bioactive compounds, MIC.
INTRODUCTION

*N. indicum* Linn. commonly known as “kaner”, belongs to family Apocynaceae. It is widely distributed in Mediterranean region and Subtropical Asia but is native of Indian subcontinent and cultivated elsewhere. The plant originates from the Mediterranean region and is indigenous to Indo-Pakistan subcontinent (Patel, 2010). All parts of the plant are utilizing as curative agent and have been used in folklore in a variety of ailments. It is also an ornamental plant with leathery evergreen leaves and with clusters of red or pink or white flowers. The plant leaves and bark are used as heart tonic, diuretic, expectorant, diaphoretic and emetic (Patel *et al.*, 2010). Leaf juice in very small doses is given in snake and other venomous bites. Juice of young leaves are effective in ophthalmic with lacrimation infusion of the leaves is abortive. Root past is helpful in haemorrhoids, in various kinds of cancer like Ulceration and leprosy (Vinayagam *et al.*, 2011, Ahmed, 2006, Chauhan *et al.*, 2013, Sikkarwar *et al.*, 2003). The selected plant is also used in ayurveda for the treatment of various diseases.

Various phytochemical can be obtained from the plants which are very beneficial for mankind and medicinal plants have become the richest biological resource for such chemicals which are utilize in manufacturing of traditional drugs as well as in modern nutraceuticals, food supplements, medicines, folk medicines, raw material and pharmaceutical intermediates for synthetic drugs (Tumwine, 2011). The presence or absence of such compounds totally depends on the extent of accumulation, the amount of plant material used and the analytical method selected (Harborne, 1973).

Harmful bacteria that cause bacterial infections and diseases are called pathogenic bacteria. Bacterial diseases occur when pathogenic bacteria get into the body and begin to reproduce and cloud out healthy bacteria, or grow in tissues that are normally sterile. Bacteria may also give out toxins that damage the body. The control of bacterial infection has been remarkably effective so the discovery of antibacterial drugs is necessary (Rios and Recio, 2005). Infectious diseases remain the leading cause of death and account for one-quarter of all death in the world (WHO, 1998).

Antibiotics are one of our most important weapons in fighting bacterial infections and much benefited to the health-related quality of human life since their introduction. Hence, over the past few decades, these health profits are under the threat and many commonly used antibiotics have become less and less effective against certain illnesses, because many of them produce toxic reactions. This situation has forced researchers to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents, but the cost production of antibiotics are high and they produce adverse effects compared to plant derived drugs (Abiramasundari *et al.*, 2011). The existence of effective antibiotic resistant or multi-resistant strains are gently appearing, imposing the necessary need to search and development of new drugs (Silver, 1993).
Consequently, step must be taken to overcome this problem, for example, to control the excess use of antibiotic, investigation should be done to better understand the genetic mechanisms of resistance, and to continue studies to develop new drugs, either synthetic or natural. The ultimate aim is to offer appropriate and efficient antimicrobial drugs. Hence the current investigation aim to screen and evaluate certain antibacterial activity of *N. indicum*.

**MATERIALS AND METHODS**

**Collection and storage of plant material:** The leaves of plant were collected from Department of Botany, S.L.S., Khandari campus, Dr. B.R. Ambedkar University, Agra. The stored leaves were thoroughly washed with tap water and followed by distilled water. After cleaning the leaves were dried in shade and grinded into powdered form with the help of grinder.

**Test Organisms used:** Clinical pure culture of *Pseudomonas aeruginosa* were used in this study. This was obtained from the Department of Botany, Microbiology, S.L.S., Khandari campus, Dr. B.R. Ambedkar University, Agra. A 24 hour broth culture of the selected species was used for antibacterial screening.

**Extraction of Plant Materials:**

**Aqueous extract:** About 50gm of leaf powder was added to distilled water and boiled on slow heat for 4-5 hrs. It was then filtered through muslin cloth. The filtrate thus obtained was further stained through Whatman No. 1 filter paper. The extraction was carried out at room temperature.

**Organic extract:** Soxhlet apparatus was used (Okeke *et al.* 2001). About 50gm of dry powder material was uniformly packed into a thimble with 300ml solvent and runs into a soxhlet apparatus. Extract is store in airtight bottle at 4°C in a refrigerator for further use.

**Preliminary phytochemical analysis (Debela 2002):** The plant extracts (acetone, ethanol and aqueous) were subjected to phytochemical studies to qualitatively analyze the active components present in them. The compounds screened are alkaloids, tannin compounds, glycosides, flavonoids, steroids and triterpenoids, carbohydrates, proteins and amino acids.

**Antibacterial activity:** Disc diffusion method (Mukherjee *et al.*1995)

**Minimum Inhibitory Concentration (MIC):** Different concentrations of the *N.indicum* leaves were prepared. 25µl overnight broth culture of the test organism were inoculated into the dilutions with different concentrations and incubated at 37°C for 24 hours. The lowest concentration of the extract that inhibited the growth of the
test organism was recorded as the MIC.

**Minimum Bactericidal Concentration (MBC):** This was described by (Usman *et al.* 2007), the inhibited test tubes resulted from MIC was streaked using a sterile wire loop on to the agar plate free of bacteria and incubated at 37°C for 24 hours. The lowest concentration of extract which showed no bacterial growth was noted and recorded as the MBC.

**RESULTS**

**Preliminary phytochemical analysis:** Phytochemical constituents are secondary metabolites of plants and several other aromatic compounds are secondary metabolites of plants that act as a defence mechanism against the microorganisms, insects and other herbivores (Bonjar *et al.*, 2004) The antimicrobial activity may due to a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids that have antimicrobial activities (Crown, 1999). This may therefore explain the demonstration of antibacterial activity of *N. indicum*.

**Table-1:** Preliminary Phytochemical analysis of different leaf extracts of *Nerium indicum* leaves:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Phytochemical constituents</th>
<th>Name of the Test</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Alkaloids</td>
<td>Mayer’s Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.</td>
<td>Tannin compounds</td>
<td>Ferric chloride Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Glycosides</td>
<td>Borntrager’s Test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>Flavonoids</td>
<td>Alkaline Reagent Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5.</td>
<td>Steroids and Triterpenoids</td>
<td>Salkowski Test</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.</td>
<td>Carbohydrates</td>
<td>Benedict’s Test</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>Proteins</td>
<td>Ninhydrin Test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8.</td>
<td>Amino Acids</td>
<td>Biuret Test</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = Present; - = Absent

**Antibacterial activity of different leaf extracts of *Nerium indicum*:**

Different concentrations of *N. indicum* leaves extract was prepared and was evaluated by agar disc diffusion method. It was observed that all leaves extract of *N. indicum* which were prepared were effective against Gram negative bacteria and displayed
broad spectrum of activity. The disc diffusion method was mainly used for the
determination of antibacterial action. Among the different methods, disc diffusion has
been used more frequently to examine the antimicrobial activity of natural
antimicrobials (Kim & Kim, 2007; Mayachiew et al., 2010).

The results obtained are encouraging as the ethanol, acetone and aqueous extracts
showed considerable effectivity. It was observed that most active result was exhibited
in acetone extract and ethanol leaves extract had shown moderate result.
Comparatively in case of aqueous leaves extract starting three concentrations showed
moderate result in comparison to both two extracts and rest four concentrations
showed least activity with respective zone of inhibitions against the \textit{P. aeruginosa},
given in table-3.

\textbf{Table-3:} The Antibacterial activity of different leaf extracts of \textit{Nerium indicum}
against \textit{P. aeruginosa}:

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{Bacterial \hspace{0.5cm} pathogen} & \textbf{Different \hspace{0.5cm} concentration (mg/ml)} & \textbf{Acetone} & \textbf{Ethanol} & \textbf{Aqueous} \\
& & \textbf{Mean} & \textbf{S.D.} & \textbf{Mean} & \textbf{S.D.} & \textbf{Mean} & \textbf{S.D.} \\
\hline
\textit{P. aeruginosa} & 200 mg/ml & 16.83 & 1.21 & 10.33 & 2.68 & 12.33 & 2.74 \\
& 100 mg/ml & 14.16 & 0.94 & 9.16 & 1.46 & 10.66 & 1.49 \\
& 50 mg/ml & 11.83 & 0.68 & 8.83 & 1.21 & 9.16 & 0.9 \\
& 25 mg/ml & 9.83 & 0.37 & 8.83 & 1.21 & 7.33 & 3.4 \\
& 12.5 mg/ml & 9.16 & 0.69 & 8.66 & 0.94 & 7.00 & 3.21 \\
& 6.25 mg/ml & 8.16 & 0.37 & 7.5 & 0.5 & 5.00 & 3.55 \\
& 3.125 mg/ml & 8.33 & 0.46 & 7.5 & 0.5 & 2.5 & 3.54 \\
\hline
\end{tabular}
\caption{The Antibacterial activity of different leaf extracts of \textit{Nerium indicum} against \textit{P. aeruginosa}.

S.D.= Standard Deviation

\textbf{ANNOVA TABLE:}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\textbf{Source of Variation} & \textbf{SS} & \textbf{DF} & \textbf{MS} & \textbf{F} & \textbf{P-value} & \textbf{at 1\%} \\
\hline
Between Column & 44.95 & 2 & 22.47 & 10.07 & 6.01 & \\
\hline
Between Raw & 110.46 & 6 & 18.41 & 8.25 & 4.46 & \\
\hline
Within Groups & 26.87 & 12 & 2.23 & & & \\
\hline
Total & 182.28 & 20 & 43.11 & & & \\
\hline
\end{tabular}
\caption{ANNOVA TABLE:}

\textbf{CD = 1.946 at 1\%; **Significant (P = 0.01)}
Figure 1: Graphical representation of antibacterial activity of different leaves extracts of *N. indicum* against *P. aeruginosa*:

**MIC and MBC of *N. indicum* leaf extracts against *P. aeruginosa***: Micro dilution method was used to determine the lowest concentration of the plant extracts that inhibiting the growth of the bacteria and found to be effective in the assessment of MIC.

The MIC value of acetone and ethanol leaves extract of *N. indicum* was found to be 25mg/ml against *P. aeruginosa*. The MIC value of aqueous leaves extract of *N. indicum* was found to be less potent 100mg/ml.

**Table 4**: The MIC and MBC of *Nerium indicum* leaf extracts against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Bacterial strains</th>
<th>Plant extracts</th>
<th>MIC (mg/ml)</th>
<th>MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td><em>P. aeruginosa</em></td>
<td>Acetone</td>
<td>25</td>
<td>No microbial death</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aqueous</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Many naturally occurring compounds present in plants have been shown to possess antimicrobial properties and perform as a source of antimicrobial agents against pathogens (Deans and Ritchie 1987; Kumar *et al.*, 2006). Higher plants are a very
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good source of medicines that made significant contribution for human health (Anesini and Perez, 1993, Adoum, 2009). The presence of antibacterial substances used for the treatment of many diseases in the higher plants is well originated. (Fridous et al., 1990, Didry et al., 1998).

The main aim of the present study was to evaluate the ability of the plant extract to inhibit the growth of pathogenic bacteria. Study conducted by Zaheer et al., 2010, in vitro antibacterial activity of P.alba against Gram positive bacteria Staphylococcus aureus, Bacillus subtilis, and Gram negative bacteria Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi was carried out and fruitful result was observed against Staphylococcus aureus and Bacillus subtilis.

Phytochemical analysis of the plant extracts:
Phytochemical compounds such as alkaloids, tannins, flavonoids, saponins, and several other aromatic compounds are secondary metabolites of plants that act as a defence mechanism against the microorganisms, insects and other herbivores (Bonjar et al., 2004). The present study investigation was carried out on the leaves extracts which revealed the presence of the medicinally active constituents.

The phytochemical analysis of N. indicum summarized in Tables before and the bioactive compounds of N. indicum may differ in their solubility depending on the extractive solvents used. This is supported from the research of De-Boer et al., 2005 that successful prediction of botanical components from plant material is largely dependent on the type of solvent used in the extraction procedure. Earlier study of Suganya et al., 2012 elaborated that the ethanol extract of N.oleander showed positive for carbohydrates, proteins, alkaloids, cardiac glycosidase, tannins and for acetone extract showed positive for alkaloids, cardiac glycosidase, and tannins, and these findings are similar to Santhi R et al., 2011 whereas Malik et al., 2015 exhibited that the highest activity of the N. oleander might be due the presence of terpenoids, saponins andamino acids.

Antibacterial Activity of the Plant Extracts:
In present study, all the extracts of N. indicum leaves were found to be active against the bacterial strain for their antibacterial activity. It was noted that acetone extract has greater effect in the inhibition from aqueous extract, which may be due to the fact that acetone is the best solvent for the active compounds extracted from the plant when compared with distilled water used in the case of aqueous extracts. (Jouda 2013)

It was observed that the acetone leaves extract of N. indicum was more active and showing inhibition effect up to dilution of 3.125mg/ml whereas Ates et al (2003), reported that the acetone extracts of Cinnamomum cassia bark extracts showed no
antibacterial activity against \textit{Y. enterocolitica}

The ethanolic leaves extract of \textit{N. indicum} was found to be moderate and showing inhibition effect up to dilution of 3.125mg/ml whereas Malik \textit{et al.}, 2015 ethanolic extract of \textit{Nerium oleander} showed biggest zone of inhibition 28mm at highest concentration (900mg/ml), whereas at lowest concentration (500mg/ml) of extract had the minimum zone of inhibition 18mm was observed against \textit{P.aeruginosa} also Malik \textit{et al.}, 2015, showed that the ethanolic extract of \textit{N. tabacum} showed the maximum zone of inhibition (26mm) against \textit{Staphylococcus aureus} whereas there was a less difference in zones were 20mm and 21mm against \textit{Escherichia coli} and \textit{P. aeruginosa} respectively.

The Aqueous leaves extract of \textit{N. indicum} was found to be least. Bakhiel \textit{et al.}(2008) showed that the aqueous extract of \textit{C. sativum} was active against \textit{S. aureus}, \textit{P. aeruginosa} and \textit{S. typhi}. Whereas, in result of Seddik \textit{et al.}, 2010 and Mohamed \textit{et al.}, 2010, which demonstrated that \textit{A. herba-alba} aqueous extracts had a weak antibacterial activity against \textit{E.coli}, but in the present study leaves aqueous extract of \textit{N. indicum} showed efficacy for both the bacterial strains.

\textbf{MIC of the plant extracts:}

The MIC value of acetone and ethanol leaves extract of \textit{N. indicum} against \textit{P. aeruginosa} was found to be 25 mg/ml and these extracts of \textit{N. indicum} gave the best antibacterial activity. Jouda 2013, reported that the MIC value of the methanol leaves extract of \textit{F. sycomorus} was efficiently active and exhibiting the highest potency with MIC from 6.25-3.125 mg/mL against \textit{S.aureus} and same for aqueous extract also, whereas the MIC values obtained showed that ethanol extract of \textit{E. camaldulensis} has the most potent effect against \textit{P. aeruginosa}.

The MIC value of aqueous leaves extract of \textit{N. indicum} was found to be less potent 100mg/ml against \textit{P. aeruginosa}. Present study was supported by the Britto \textit{et al.}, 2011, investigated that the methanol extracts for plants of Apocynaceae family had shown 12.5mg/ml concentration was found to be the minimum inhibitory concentration against most of the pathogens.

\textbf{CONCLUSION}

Many natural compounds, especially plants have been used for the treatment of several diseases and an impressive number of new medicines have been developed from them. Thus the plant kingdom is really a potential source of medicinal properties. Plant have their own chemical compounds, some of those chemical compounds also have curative values for us. In recent years, ethno-botanical and folklore uses of natural compounds, especially of plant origin received much attention.
as they are well tested for their efficacy and generally believed to be safe for human to use. Therefore, our results indicate that this plant extracts have inhibitory effect for growth of microorganism against the bacteria under studies, due to presence of some active compounds in this plant which act as natural antibiotics that can be use instead of antibiotics after experiments have performed on it. Hence, in addition to numerous other medicinal properties, the antibacterial activities of \textit{N. indicum} are also coming to light; effective steps are to be taken for its propagation so that this valuable tree does not face the danger of extinction.

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