Synthesis of *Cinnamum zeylanicum* and *Acacia nilotica* Extracts and Their Antibacterial Activity against *Staphylococcus aureus* and *Streptococcus pyogenes*

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Abstract: Different plants are used medically and these therapeutic plants have great importance for healing contagious wounds. This herbal treatment is actually also a substitute of different antibiotics and having less side effects on intestinal systems of animals. The foremost concern of this study was to observe the antibacterial activity of *Cinnamum zeylanicum* and *Acacia nilotica*. Pathogenic bacteria obtained from wound samples and later identified by biochemical and molecular characterization. Methanol (an organic solvent) was used to extract *Cinnamum zeylanicum* and *Acacia nilotica* to check their antimicrobial exertion by using agar diffusion method. Different antibiotics such as, ampicillin, oflaxocin, ticarcillin and cefexime, showed their susceptibility toward antibiotics. The zone of inhibitions for antibiotic and plant extracts’ antibacterial activity were measured. Pathogenic bacteria were identified as *Staphylococcus aureus* and *Streptococcus pyogenes* by molecular characterization. These bacteria showed susceptibility to antibiotics and also the plant extracts. Antibiotic oflaxocin showed maximum activity against these two pathogens (12.25 ± 0.44 and 12.375 ± 0.47) while antibiotic cefexime showed minimum effect (1.25 ± 0.28 and 0.625 ± 0.25). Plant extracts showed significant antibacterial activity with maximum activity (14 ± 0.9 by *Acacia nilotica* and 12 ± 0.5 by *Cinnamum zeylanicum*) in 100% solution. It can be concluded that methanolic extract of traditional therapeutic plants proved to be a promising source of antimicrobial agents against antibiotic resistant bacteria. *Cinnamum zeylanicum* and *Acacia nilotica* were observed to be competent as antibacterial tool against pathogenic bacterial strains.

Key words: therapeutic plants, pathogenic bacteria, antibiotic susceptibility, *Cinnamum zeylanicum*, *Acacia nilotica*

1 Introduction

For health requirements, about 80% population of the world depends on conventional drugs as claimed by World Health Organization (WHO). Use of herbal plants as assembly in Asia shows the wide record of human connection with around. Plants have a mandatory source of medicinal use of western types of pharmaceutical products and local medicine preparations. Plant’s extract in any form such as oil are becoming a substitute of different antibiotics used as herbal treatment which have less side effects on health condition. 700 species among 6000 species of flowering plants in Pakistan have a great therapeutic importance. 500 of these medicinal plants are recognized in our country and around the world for their active ingredients through studies and 250 to 300 different species introduced in our country’s herbal markets are recognized. Globally, at least 130 medicines contain all single chemical components derived from plants, or simply unnaturally modified. However, some of them are currently being developed for the purpose of unnaturally low cost.

Mostly used medicinal treatments such as Homeopathy, Ayurvedic and Unani in eastern countries is highly dependent over plants. Now a days, herbal treatment of disease is extremely accepted. An absolute chemical, pharmacological and biological study can be achieved by potential research products. Different components of plants and also plant’s extracts are whacking sources of different antibiotic agents especially for diseases induced by resistant micro-
organisms\(^7\). Due to continuous and regular use of same antimicrobial agent, microbes become resistant. Therefore, it is the need of time to develop antimicrobial agents with different and novel sources like plants\(^8\).

Including different things such as herbal medicines, Cinnamon is the oldest one and its history is also included in different Chinese books of about 4000 years back\(^9\). Cinnamon has been customarily cultivated in Asian countries and it is long lasting tree. Cinnamon belongs to family Lauraceae. Cinnamon have bark different beneficial properties such as antioxidant, antiulcerogenic, antiallergic and anti-pyretic. \textit{Acacia nilotica} is the member of family Mimosaceae and in India it is commonly known as “babool” and in Pakistan known as “kikar”. \textit{Acacia nilotica} forests are found in tropical areas of India and Pakistan and having great value for fuel, fodder, tannin and small timber. These plants are distributed in Australia, America and Asia and their beneficial aspects are confirmed for the cure of gonorrhoea, wounds, diarrhea and leucorrhoea\(^6,10\). The aim of the current research was to study the antibacterial activity of plant extracts against common pathogens especially \textit{Staphylococcus aureus} and \textit{Streptococcus pyogenes}. The purpose of this study was to explore biological ways of controlling bacterial infections rather than using antibiotics as bacterial pathogens are becoming resistant to commonly used antibiotics.

2 Materials and Methods

2.1 Bacterial sampling and isolation of bacteria

Bacterial sampling was done randomly from operation theaters of Jinnah hospital, Lahore during summer period of year 2020. 20 samples were collected from untreated wounds of patients by swabbing with the help of sterilized cotton swabs. Swabbing was done with the help of on duty doctors in hospital with prior permission from hospital authority and consent from patients. Research synopsis was approved by ethical committee/ BOS (Board of studies) of department of Zoology, GCU, Lahore. Samples were brought to Microbiological laboratory GCU, Lahore, by using saline solution of 0.85% in sterilized sample tubes. In laminar air cabinet, swabs were spread on agar plates with the help of glass spreader. The agar plates were incubated at 37°C for a day. Bacterial colonies can be differentiated by various shapes and symmetry after incubation process. The only isolated colony of bacteria was selected to isolate the pure culture and was selected with a sterilization loop and placed on new plate.

2.2 Characterization of bacterial isolates

2.2.1 Morphological, biochemical and molecular characterization of bacterial isolates

The selected bacterial isolates were investigated for morphological features like colony shape, size and color, gram’s staining and motility test. The isolates were further tested for biochemical characterization by applying various biochemical tests including IMViC tests. Differential and selective media were used to detect bacterial isolates\(^12\).

Optimum conditions for bacterial strains were determined on the basis of growth curve for different time intervals at 590 nm, different temperatures (37°C, 45°C, 55°C, 60°C, and 28°C) and pH (2, 4, 6, 8 and 10).

2.2.2 Antibiotic susceptibility of bacteria

The antibiotic susceptibility of the bacterial isolates was checked against ampicillin (AMP) ofloxacin (OF), cefexime (CFM) and ticarcillin (TI). The plates were incubated at 37°C for 24 hours. Different zones appeared after incubation depending on the sensitivity of the bacterial strains\(^13\). Inhibition zones were established around the bacterial strains which showed sensitivity towards relevant antibiotic (Fig. 1).

2.3 Collection of plant materials and solvent extraction

From Botanical Garden of Government College University, Lahore, leaves and branches of \textit{A. nilotica} were collected while Cinnamon bark was purchased from market. Leaves, branches of plants were thoroughly washed under tap water and once with sterile distilled water. After that, all parts of plant used in study were dried in shade of the wind. After drying, 25 g \textit{A. nilotica} in fine powdered form dissolved in 100 ml of methanol (1:4) and this solution was incubated for 1-7 days. After 7 days filtration and evaporation of the solution was done and extracts were dried in oven at 60°C. The concentrated extracts were finally soaked in methanol in the ratio 1:6\(^11\). Although methanol is considered a toxic solvent, but it is extremely useful for the extraction of certain phytochemicals due to its polarity. It is safe to use as it is highly volatile and is completely evaporated from extracts. As methanol is completely evap-
orated, so the antibacterial activity is done only by the plant extract.

2.4 Antibacterial activity of plant extracts

With the use of Kirby-Bauer disc diffusion method, antimicrobial activity of plant extract was checked. The paper discs were made of a sterile filter paper and soaked in plant extracts of different concentrations. Sterile filter paper discs were placed on the agar plate by using sterile forceps. For a control group, 100% methanol was used and plates were incubated at 37°C for 24 hours. By using millimeter scale, diameters of inhibition zones were measured (Figs. 2 and 3).

2.5 16S rRNA gene sequencing of bacterial strains

Phenol chloroform extraction method was used to isolate genomic DNA of bacterial isolates (Fig. 4). Afterwards, amplification was done using universal primers 16S-27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 16S-1522R (5’-AAGGAGGTGATCCAGCCGCA-3’) in a Techne thermal cycler (Progene). Polymerase chain reaction was done, under most favorable conditions, as shown in Table 1. The reaction mixture was prepared by mixing the following chemicals in measured quantities (Fermantas) in sterilized PCR tubes (Fig. 5). The genes of PCR product was purified by using GF-1 DNA recovery kit by Vivantis. After then, for

Table 1  Final volume and concentrations of reaction mixture for PCR.

<table>
<thead>
<tr>
<th>Components</th>
<th>Final concentrations</th>
<th>Vol./ reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection water</td>
<td>–</td>
<td>27.5 µL</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1.5 Mm</td>
<td>4 µL</td>
</tr>
<tr>
<td>PCR Buffer 10X</td>
<td>1 X</td>
<td>5 µL</td>
</tr>
<tr>
<td>dNTPs</td>
<td>200 µM (0.2 mM)</td>
<td>5 µL</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>2.5 U</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Isolated DNA</td>
<td>1 µg</td>
<td>4 µL</td>
</tr>
<tr>
<td>16SF (AGAGTTTGATCCTGGCTCAG)</td>
<td>50 µM</td>
<td>2 µL</td>
</tr>
<tr>
<td>16SR (AAGGAGGTGATCCAGCCGCA)</td>
<td>50 µM</td>
<td>2 µL</td>
</tr>
<tr>
<td>TOTAL VOLUME</td>
<td>–</td>
<td>50 µL</td>
</tr>
</tbody>
</table>
ribotyping, purified genes of partial sequence of 16S rRNA was carried to MOLECULAR BIOLOGICAL PRODUCTS, Karachi, Pakistan. Sequences were matched with sequences deposited in NCBI and on the basis of homology, bacterial pathogens were identified.

3 Results

In current study, 25 samples of post-operative wounds of patients taken from Jinnah Hospital, Lahore, were used. Out of these 25 samples 10 strains were separated out in order to perform more biochemical tests to check their antimicrobial susceptibility. Gram positive coccus GC1 was found in the form of clusters, as a result of gram staining, GC1 at genus level after executing different biochemical and artificial media tests was identified as Staphylococcus species which was non-mobile in moving medium. β haemolysis was shown by GC1 on blood agar medium. This strain was identified at molecular level by ribotyping (Fig. 6). Gram positive coccusGC2 was visible in the form of chains as a result of gram staining. After incubation, the strain was observed non-motile in motility agar. After carried out various biochemical and microbial tests, GC2 was recognized as Streptococcus species. β haemolysis was shown by GC2 on blood agar medium. The bacteria was identified by molecular characterization (Fig. 7). Antibiotic resistant strains were also subjected to the plant extract having antimicrobial qualities and check their sensitivity towards antibiotic extract of plant. The antibiotic susceptibility of bacterial strains is shown in Table 2. Bacterial strains showed sensitivity against Cinnamum zeylanicum and Acacia nilotica as shown in Table 3. Both of these strains showed minimum to maximum sensitivity to antibiotics and to the plant extracts. Antibiotic ofloxacin showed maximum antibacterial effect against these two pathogens (12.25 ± 0.44 and 12.375 ± 0.47) while antibiotic Cefixime showed minimum effect (1.25 ± 0.28 and 0.625 ± 0.25) to S. aureus and S. pyogenes, respectively. Both bacteria showed sensitivity to 50% and 100% solutions of plant extracts.

3.1 Staphylococcus aureus (Partial sequence of 16s rRNA gene)

ATAATGGTGCCCCTTTTCTTTGAATTATTTTCAATTATTAATAGAAGGTGTCAAAGCATAGAGTTGGAGGT
AATAGAATGAGGTGAAAGGTGCTCAAAATGGTAAAGTAAAGGCTTCATATGCTTACATTTGAAGGTATTAAT
AATATTAATGATATTGAGCATTTAAAAGGGAGTTC
TATTTATCAAGAGGGGAGGCTGATCATGAAGATATCGTAC
TTGAGGAAAATGAAATTTTTATTATTCAGATATTAGA
GGTGACAGTTTTTTGATGATCATGAAAGCATATCGTAC
TTGATTCCTTATATTGCTGATGTGAAAATAAAAAATTATCATCACGCCAA

Fig. 5 PCR product of bacterial strains after agarose gel electrophoresis.
1 = Staphylococcus aureus
2 = Streptococcus pyogenes

Fig. 6 Phylegenetics tree of S. aureus.
Table 2  Antibiotic resistance of bacterial strains.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>STRAINS</th>
<th>OFLAXOCIN (OF) 5 µg</th>
<th>CEFEXIME (CFM) 5 µg</th>
<th>AMPICILLIN (AMP) 10 µg</th>
<th>TICARCILLIN (TI) 75 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zone of inhibition (mm)</td>
<td>Zone of inhibition (mm)</td>
<td>Zone of inhibition (mm)</td>
<td>Zone of inhibition (mm)</td>
</tr>
<tr>
<td>1</td>
<td>S. aureus</td>
<td>S (12.25) ± 0.44</td>
<td>S (1.25) ± 0.28</td>
<td>S (1.75) ± 0.64</td>
<td>S (3.0) ± 0.40</td>
</tr>
<tr>
<td>2</td>
<td>S. pyogenes</td>
<td>S (12.375) ± 0.47</td>
<td>S (0.625) ± 0.25</td>
<td>S (0.75) ± 0.28</td>
<td>S (2.25) ± 0.28</td>
</tr>
</tbody>
</table>

KEY: mm = millimetre, S = sensitive, R = resistant, ( ) = zone of inhibition, ± = SD

Table 3  Plant extract susceptibility against bacterial isolates.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Strain</th>
<th>Acacia nilotica</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>1</td>
<td>S. aureus</td>
<td>(1.7) ± 0.6</td>
<td>(13) ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>S. pyogenes</td>
<td>(1.5) ± 0.6</td>
<td>(14) ± 0.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Strain</th>
<th>Cinnamum zeylanicum</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>50%</td>
</tr>
<tr>
<td>1</td>
<td>S. aureus</td>
<td>(1.2) ± 0.05</td>
<td>(12) ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>S. pyogenes</td>
<td>(1.4) ± 0.09</td>
<td>(13) ± 0.6</td>
</tr>
</tbody>
</table>

KEY: mm = millimetre
( ) = zone of inhibition
± = SD
TGGAGGATTGTTGGAATTAATGAAAAATGTAGATTATTA-ACCTACTTTCCCGAAATGTTGAGTTGTTTTA
ATCATTTCAATTTGAAACGTTGCC

3.2 Streptococcus pyogenes (Partial sequence of 16s rRNA gene)
GAAATGTAGTACAGATGAACTTTTAAGTGGAAAATT-
GTCACACGCAAAGGCTGCAAGGAAGATGGCCG
GTGTTGCTGTCAAGTTTTTCGTAGGAAGCCTTTTA-
AAAAAGCTCACAATTGGTTTTATAGTGATAA
AGATCGGGTTGTTCAAGAAGTGCAACAATGTTTACGC-
GTAAACAAAAGCATTTCATATTCAACATT
AAGACAGATGATCATATATATGTATTTGAAAAAGTA-
CAAGGATACACTTTAAAGTCTCAAAGAACAATC
AAAGGGATTTTCAAAGAAAGGAGAATTTTATACCATCA-
GATATGCGTGAGCCGTTTTATGAAAAAGGTGTT
CCCTATGGAGATGGAAAATCCTGAGCCAGGT-
GCTAATGATGTTTGGATAGTGAAACGCCAAGGAG
GTCAACACGCAAGGCCTGCAAGGAGCTTTTA-
AAAAAGCTCACAATTGGTTTTATAGTGATAA
AGATCGGGTTGTTCAAGAAGTGCAACAATGTTTACGC-
GTAAACAAAAGCATTTCATATTCAACATT
AAGACAGATGATCATATATATGTATTTGAAAAAGTA-
CAAGGATACACTTTAAAGTCTCAAAGAACAATC
AAAGGGATTTTCAAAGAAAGGAGAATTTTATACCATCA-
GATATGCGTGAGCCGTTTTATGAAAAAGGTGTT
CCCTATGGAGATGGAAAATCCTGAGCCAGGT-
GCTAATGATGTTTGGATAGTGAAACGCCAAGGAG
GTCAACACGCAAGGCCTGCAAGGAGCTTTTA-
AAAAAGCTCACAATTGGTTTTATAGTGATAA
AGATCGGGTTGTTCAAGAAGTGCAACAATGTTTACGC-
GTAAACAAAAGCATTTCATATTCAACATT
AAGACAGATGATCATATATATGTATTTGAAAAAGTA-
CAAGGATACACTTTAAAGTCTCAAAGAACAATC
AAAGGGATTTTCAAAGAAAGGAGAATTTTATACCATCA-
GATATGCGTGAGCCGTTTTATGAAAAAGGTGTT
CCCTATGGAGATGGAAAATCCTGAGCCAGGT-
GCTAATGATGTTTGGATAGTGAAACGCCAAGGAG
GTCAACACGCAAGGCCTGCAAGGAGCTTTTA-
AAAAAGCTCACAATTGGTTTTATAGTGATAA
AGATCGGGTTGTTCAAGAAGTGCAACAATGTTTACGC-
GTAAACAAAAGCATTTCATATTCAACATT

4 Discussion

The development of antibiotic resistance has been recognized since the early stages of antibiotic treatment have expanded to some extent in recent years. This resistance can lead to increased mortality and the cost of health care. In current study, antibiotic resistant strains were selected and then tested for the antibacterial effect of medicinal plants. The bacterial pathogens were identified by biochemical and molecular characterization. Using the technique of amplification of 16s rRNA gene, the sequences were compared with deposited sequences in NCBI for identification of these pathogenic bacteria. Ribotyping has become very effective technique for identification of unknown bacteria. With the passage of time, microbial growth in wound increase and microbes modified themselves which causes more pain and patient feels more discomfort and then it requires more time for healing of wound. Apart from streptococcus, the most common pathogens of wounds are Staphylococcus aureus. Disease caused by infected wound is multifactorial and consists of wireless factors of local and systemic host immune mechanisms of microbes. Most of the studies showed that the major reasons of microbes resistance towards antimicrobial drugs: overuse and misuse of antimicrobial agents and its contamination, improvement in pathogens and transmission of multi-resistant microbes among humans. Because of antimicrobial resistance in microbes, there is an increase in death rate and cost of health care treatment. It has recently been described as a threat to global power and national security. The development of antibiotic resistance has been recognized from an early age, although antibiotic therapy has increased in the past few years.

Mostly, all serious infections and wounds are contaminated by microbes, but it’s also a fact that many microbes are beneficial for healing wounds. Most of the contaminants which are beneficial for healing wounds come out from local microflora of infected area. Staphylococcus aureus, Beta-hemolytic Streptococcus (S. pyogenes, S. agalactiae), Klebsiella, anaerobes, Proteus, Acinetobacter, E. coli, and Pseudomonas are usually present in an infected wound. Mostly people in under-developed countries are preferred herbal treatment and use of conventional drugs due to their low income. As, conventional healers claims that their drugs, medicines are available at reasonable price and having little or no side effect in contrast to unnatural medicines. In previous ten years a study was carried out in order to test the antimicrobial effect of Cinnamum zeylanicum against multi drug resistant strains (MDR) and these strains were found to be sensitive against plant extract. Goni and his coworkers in 2009 carried out a study with a combination of clove essential oil and cinnamon essential oil against multi drug resistant Acinetobacter and these strains were found to be sensitive against plant extract. Arshad and his coworkers in 2017 during their work with the methanolic extract of Acacia nilotica found, this extract was influential for all microbes involve in this study. Different microorganism such as P. aeruginosa, S. typhimurium, E. coli and K. pneumoniae showed sensitivity to Acacia nilotica extract. A study was carried out by Deshpande and Kadam in 2013 in which extraction of Acacia nilotica was done with ethanol and petroleum ether and resulted that both extractions hampered the development of all microbes involved in study. However, microbes showed sensitivity towards ethanol extraction than that of petroleum ether. They also checked antimicrobial exertion of Acacia nilotica against S. mutans and microbes showed 31 mm inhibition zone with methanol extraction. It is concluded, that Acacia nilotica extract with ethanol have a great inhibitory effect against S. mutans.

5 Conclusions

This study together with earlier reports concluded that the methanolic extract of traditional therapeutic plants proved to be a promising source of antimicrobial agents against antibiotic resistant bacteria. Methanolic plant ex-
Plant Extracts as Antibacterial Tools

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tracts are safe to use because not only well dissolved in methanol but also become methanol free completely. Methanol is highly volatile, so after thorough process of drying, the extracts become methanol free and safe to use. As the microbial resistance increases due to unnecessary and over prescribed antibiotics, these new sources of antimicrobial agents are effective against human pathogens. Throughout the world studies have found a thousand of phytochemicals and other active components in plants that inhibit growth of pathogenic bacteria. The medicinal plants used in this research are commonly found and proved effective than antibiotics used. Moreover, further research could help identify the nature of active chemical components found in medicinal plants.

Conflict of Interest
There is no conflict of interest among authors.

Author Contributions
NMA designed and supervised the study. HMT carryout the observations and calculations. MKK and KK performed experimental work. BM and KUK wrote first and final draft of manuscript. MC contributed in study design and experimental work. MD contributed in experimental work and data analysis. All authors approved final draft of manuscript.

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