Article Title: Antimicrobial Efficacy of Traditional Medicinal Plant Extracts Against the Antibiotic Resistant Isolates from Drinking Water Sources

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Abstract: Water is the second most essential factor for different life forms after oxygen. Most of the people obtain their drinking water from surface and underground sources. However both surface and ground water source could be contaminated by biological and chemical pollutants. In the rural area, majority of people directly use natural water sources like river water, bore well, lake water etc. for drinking purpose. In the present study, 12 different drinking water samples were collected from different villages of Pune, Maharashtra, India. The samples were analyzed bacteriologically and physico-chemically. The MPN positive samples were analyzed for antibiotic resistance by polydisc method. 27 bacterial isolates were reported to be resistant to antibiotic Imipenem (10mcg). Antimicrobial activity of Tulsi and Neem leaf extracts showed a great potential against these antibiotic resistant isolates. Thus, from the obtained results, it can be concluded that these plants can be used as natural therapeutic agents that may serve as lead for the development of new pharmaceuticals addressing the major therapeutic needs.

Keywords: Drinking water, Antimicrobial activity, bacteriological, MPN, Antibiotic resistant.

Introduction: Drinking water is one of the most important bacterial habitats on earth and known to be a major way of dissemination of microorganisms in nature. The water-bodies are recognized as a significant reservoir of antibiotic resistant bacteria (ARB). The development of multidrug resistant (MDR) bacteria in water supply systems is found to be a serious threat to the public health. The wastewater treatment plants are one of the major sources for continuous release of a variety of antibiotics into aquatic environment. Therefore, the load of ARBs in the aquatic environment has been increasing continuously. This serious issue is observed in developing countries like India due to the poor sanitization practices. The easy availability of antibiotics sometimes without a medical prescription has increased their widespread misuse. Apart from this, large quantities of antibiotics are being used in the clinical settings, agriculture, food industry and aquaculture etc. The global demand of antibiotics has been increased by 40% in the last decade. Therefore, due to heavy usage of antibiotics results into increasing numbers of ARBs. The continuous development of ARBs in an aquatic environment may cause a serious public health issue. Sewage is the most common source of drinking water pollution due to the leakage of water supplying pipelines and thus alters the physiochemical properties of drinking water. In addition to this, the sewage water also spreads several human pathogens via drinking water system. However, it was noticed that many other water sources are also found to be highly contaminated. It is reported that 37.7 million Indians are affected due to waterborne diseases and 15 million children are died due to diarrhea, annually. Therefore, there is a great need of an extensive water treatment before it is consumed. The microbiological safety of drinking water is of prime importance to protect public health. The protection of drinking water is generally considered as the primary strategy to make it safe for drinking purpose. From several years, it is well known that the herbs are commonly used in the preparation of many drugs that are used for the treatment of several human diseases. Thus, the anti-microbial property of these plants made them eligible as most safe and the efficient therapeutic agents against several bacterial pathogens. The plant of Neem has been used in the preparation of the traditional medicine as a source of many therapeutic agents in the Indian culture and grows well in the tropical countries. It is well known that this plant contains several bio-active molecules, having multiple medicinal properties. Similarly, Tulsi leaves also exhibited insecticidal and anti-bacterial activities and are employed in treatment of several diseases on the basis of the traditional experience. Previously, it was also reported...
that the Tulsi leaves (*Ocimum sanctum*) and Neem leaves (*Azadirachta indica*) have been used in purification of contaminated drinking water. Thus, the use of such plants for contaminated water treatment could be a safer, cost effective and an eco-friendly process. The side effects of antibiotics and quick development of bacterial resistance are the major problems. Therefore, several bioactive compounds of different plants species can be an alternative to these antibiotics. In the present study, anti-microbial activities of plant leaves extracts of Tulsi and Neem were tested against the antibiotic resistant microorganisms isolated from different drinking water sources of the villages in Pune district, Maharashtra, India.

**MATERIALS AND METHODS**

**Drinking water Sample collection**

12 samples water samples were collected from different drinking water sources (surface water and borewell water) of Dehugaon and Sudumbare villages of Pune, India. The water samples were collected in sterile screw capped sterile glass bottles. The bottles were labeled properly with details of source, date and time of collection. The bottles were carried in icebox and brought to the laboratory and stored in the refrigerator until further analysis.

**Evaluation of bacterial load**

Enumeration of bacterial colonies was performed by using spread plate technique. The water samples were serially diluted with sterile distilled water. The different dilutions (0.1 ml) were spread on sterile MacConkeys agar plates and incubated at 37°C. The bacterial colonies formed on sterile MacConkeys agar plates were counted. The obtained colonies were also tested for antibiotic resistance study.

**Antibiotic resistance study**

40 isolated colonies were tested against eight different antibiotics on Mueller Hinton Agar by Kirby-Bauer disk

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**Table 1: Antibiotic Resistance of different isolates**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Antibiotic</th>
<th>Imipenem (10mcg)</th>
<th>Meropenem (10mcg)</th>
<th>Ciprofloxacin (5mcg)</th>
<th>Tobramycin (10mcg)</th>
<th>Moxifloxacin (5mcg)</th>
<th>Ofloxacin (5mcg)</th>
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diffusion method reported earlier. Briefly, overnight
grown bacterial cultures in broth were spread separately on
sterile Mueller Hinton agar plates and antibiotic
impregnated octadisk was placed on it. All plates were
incubated overnight at 37°C and observed for a clear zone
of growth inhibition. The obtained results were interpreted
using a reference table. The bacterial isolates that showed
resistance for two or more antibiotics (multidrug resistant)
were further characterized and identified. Antibiotics of
different classes used in this study were: Imipenem (10
mcg), Meropenem (10 mcg), Ciprofloxacin (5 mcg),
Tobramycin (10 mcg), Moxifloxacin (10 mcg), Ofloxacin
(5 mcg), Ofloxacin (5 mcg) and Levofloxacin (5 mcg).
Characterization of antibiotic resistant isolates
The antibiotic resistant bacterial isolates were
characterized morphologically and biochemically. The
colonial morphology, Gram staining, spore staining,
IMViC test, Catalase test, starch and casein hydrolysis
were carried out to characterize the obtained isolates.
Bergey’s manual of systematic bacteriology and the
manual of identification of medical bacteria were used for
partial identification of antibiotic resistant isolates.

Plant material collection
Fresh leaves of O. sanctum (Tulsi) and A. indica (Neem)
were collected from the local area of Pune, Maharashtra,
India. The collected plant leaves were washed with the
sterile distilled water thoroughly, air dried and then
homogenized to make a fine powder and stored in airtight
bottles for further use.

Preparation of plant leaves extracts
Aqueous extraction
The dried powder (5 g) of Neem or Tulasi leaves was
added separately into 200 ml of distilled water and boiled
on slow heat for 2 h. After boiling, the extract was filtered
through a muslin cloth and centrifuged at 5000 rpm for 10
minutes. The obtained supernatant was collected and
concentrated by boiling to make one-fourth of the original
volume.

Solvent extraction

<table>
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<tr>
<th>Sr. No.</th>
<th>Sample &amp; Isolate No.</th>
<th>Genus Name</th>
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<tr>
<td>1</td>
<td>DSI isolate 1</td>
<td>Salmonella</td>
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<td>2</td>
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<td>DSII isolate 2</td>
<td>Hafnia</td>
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<td>6</td>
<td>DSIII isolate 1</td>
<td>Cedecea</td>
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<td>7</td>
<td>DSIV isolate 1</td>
<td>Erwinia</td>
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<tr>
<td>8</td>
<td>DSIV isolate 2</td>
<td>Citrobacter</td>
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<tr>
<td>9</td>
<td>DS V isolate 4</td>
<td>Vibrio</td>
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<td>10</td>
<td>DSV isolate 3</td>
<td>Vibrio</td>
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<tr>
<td>11</td>
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<td>14</td>
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<td>Cedecea</td>
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<tr>
<td>15</td>
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<td>Edwardsiella</td>
</tr>
</tbody>
</table>

The anti-bacterial activity of different plant species
(aqueous and solvent extract) was evaluated by agar well
diffusion method. The test organisms were grown
overnight in the nutrient broth. The cell suspension of
0.1ml was inoculated into the molten Mueller Hinton agar
media and after proper homogenization it was poured into
100 mm Petri dishes. The wells were prepared in the
seeded plates with the help of a cork-borer (8 mm). Then
0.1ml of the plant leaves extracts was added into each well
separately and allowed to diffuse at 4°C for 20 min. The
plates were then incubated at 37°C for 24 h. The antimi-

RESULTS
Isolation of antibiotic resistant bacteria from different
water sources
Altogether, six Dehugaon samples showed presence of 22
different colony forming units and that of Sudumbre
showed 18. The water samples collected from Dehugaon
region were labeled as DS I-VI and the samples from
Sudumbre were labeled as SS I-VI. Each of these isolate
was tested for its antibiotic resistance against eight
different antibiotics by Kirby-Bauer disk diffusion
method. Zone of inhibition thus obtained can be seen in the
Table 1. It was seen that forty percent isolates were resistant
to the Imipenem antibiotic. Isolates such as DS II 1, DS V
3 and SS V 1 were resistant to two antibiotics of the eight
from the octadisk. DS II 2 was found to be resistant to
Imipenem and Meropenem, DS V 3 showed resistance to
Ciprofloxacin and Sparfloxacin while SS V 1 exhibited
resistance to Imipenem and Levofloxacin.
Characterization of antibiotic resistant isolates
The Bergey’s manual of systematic bacteriology and the
manual of identification of medical bacteria have been
referred for the partial identification of bacterial isolates
and is shown in Table 2. All the identified isolates
belonged to Enterobacteriaceae family reflecting that
drinking water source was fecally contaminated.

Antibacterial activity of plant leaves extracts
The antibacterial activity of different plant species was
evaluated by agar well diffusion method for both aqueous
and solvent leaves extracts. The aqueous extract of Neem
and Tulsi showed no antibacterial activity against the
bacterial isolates. However, the concentrated organic
extracts of Neem and Tulsi leaves extracts showed a
prominent antibacterial activity against almost all the
resistant isolates. It was observed that when the leaves
extracts were 1:1 diluted with DMSO, the extracts found
to be only active against the isolates, Escherichia, Erwinia
and *Citrobacter*. The antimicrobial activity of the extracts is shown in the representative figure 1 and the diameter of the zone of inhibition measured is presented in table 3.

**DISCUSSION**

Forty different isolates were obtained from 12 different drinking water sources. The antibiotic resistance of different isolates was tested against eight broad spectrum antibiotics having activity against both Gram-positive and Gram-negative bacteria. It was observed that almost 40% isolates were found to be resistant to the Imipenem antibiotic. The isolates DS II 2, DS V 3 and SS V 1 were resistant to two different antibiotics from the octadisk. The antibiotic, imipenem is a particularly active against *Pseudomonas* and *Enterococcus* species. The Gram negative bacteria are found to be resistant to imipenem to varying degrees was reported earlier\(^{14}\). Important clinical infections like urinary tract infection are caused due to *Enterococcus*. The meropenem is similar to Imipenem in its action and spectrum. However, the antibiotic meropenem is more active against *Enterobacteriaceae* and less active against the Gram positive bacteria. It is administered to adults with complicated UTI. The identified antibiotic resistant bacteria from the drinking water sources in the present study belong to *Enterobacteriaceae* family which is well known to cause urinary tract infection or gastrointestinal diseases. The presence of co-resistance and MDR signifies that there might be continuous heavy usage of antibiotics in the community. The non-human use of highly-important antibiotics also contributes to the drug resistance against a range of antibiotics\(^{15,16}\). The relatively cheap and commonly prescribed drugs commonly favor a high co-resistance is reported previously\(^{17,18}\). In rural communities, the high level of bacterial contamination is reported in drinking water sources due to poor sanitation practices and thus results into lack of safe-water supply for domestic purposes\(^{19}\). Studies illustrate that surface water
CONCLUSION

The results of the study indicate the poor sanitary conditions of the sampling sites and unhygienic practices of people leading to the presence of organisms belonging to the Enterobacteriaceae family. Furthermore, it also indicates the overuse and / misuse of antibiotics as antibiotic resistant bacteria were isolated from the drinking water samples. The study suggests the additional measures (hygienic and sanitary practices) are required by the individual to avoid post contamination of drinking water. From the above studies, it can also be concluded that the traditional plants are new sources of antimicrobials that can establish a scientific base for the use of modern medicine.

ACKNOWLEDGMENT

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