Effect of Methanolic Extract of Simarouba gluca on Antibiotic-Resistant E. coli Isolated from Surface Waters of Killi River

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ABSTRACT

The emergence of antibiotic-resistant bacteria is posing a challenge and threat to the environment especially their prevalence in water bodies. One of the most prevalent bacteria in E. coli is a gut bacteria found in the gastrointestinal tract of animals. This is a pilot study carried out to identify the presence of such antibiotic-resistant E. coli in surface waters of a prominent river in Trivandrum city known as Killi river. The isolated bacteria were confirmed to be E. coli by biochemical and molecular analysis. We also wanted to look for the effect of methanolic extract of the paradise tree, Simarouba gluca on such multidrug-resistant bacteria. Our studies have analyzed the phytochemical composition of methanolic extract of S. gluca and confirmed it by TLC analysis. We also looked for the antibacterial effect of the extract using the agar diffusion method and show that the methanolic extract of S. gluca exhibits good antimicrobial activity against antibiotic-resistant E. coli. This study is the first of its kind to have demonstrated the effectiveness of the plant extract as a potent antimicrobial agent against environmental strains of E.coli isolated from Killi river that are resistant to some of the common antibiotics used for the treatment of a wide variety of infections in both humans and animals. This study would offer a better option for designing plant-based compounds while preparing drugs for combating these kinds of bacteria.

Keywords: S. gluca, Methanolic extract, E. coli, Killi river, Antibiotic resistance, Trivandrum

INTRODUCTION

Antibiotic Resistance is one of the major challenges and threats faced by the medical community these days with respect to public health. Antimicrobial resistance is considered one of the leading causes of death by enhancing the risk of infections especially nosocomial ones (Murray et al., 2019). The spread of antibiotic-resistant bacteria in water bodies is alarming and is usually contributed by unscientific anthropogenic activities like uncontrolled domestic sewage and industrial effluent disposal, dumping agricultural waste, and discharging hospital wastes. One of the major bacteria showing such antibiotic resistance is E. coli as shown in previous studies that such E. coli are prevalent in various water bodies around the globe (Ranjbar et al., 2016; Odonkor & Addo, 2018). E. coli which is generally considered an indicator of fecal contamination in water bodies harbors antibiotic resistance genes, especially β-lactamase resistance (bla) genes (Bordarczak & Piotrowska-Seget, 2019), and possibly transfer the same to other bacteria thus horizontally spreading antibiotic resistance and is considered as a key organism in harboring and transmitting antimicrobial resistance (Galindo-Méndez et al., 2020).

This calls for a search for natural compounds which effectively impede the growth of such antibiotic-resistant microorganisms (Dubreuil, 2020), and plants being rich in phytochemicals harbor compounds that could be developed into antimicrobial drugs. Our search for such a plant lead us to choose Simarouba gluca hailed as the paradise tree which is well known for its anticancer properties (Mathew et al., 2019). Besides this, S. gluca is also known to possess analgesic, antiviral, antimicrobial, and antioxidant properties (Santosh et al., 2016; Dahar & Rai, 2019). It is reported that the methanolic extract of S. gluca can act as an efficient ethnomedical agent and is a possible candidate for curing various agents due to its antimicrobial, antioxidant, and anticancer activities (Ramasamy et al., 2022). But the antimicrobial studies have been mostly conducted in standard strains of both gram-negative and gram-positive organisms also some studies show the antibacterial activity of S. gluca extract against multidrug-resistant S. typhi (Nagaraj et al., 2021). But the potential antimicrobial activity of S. gluca against beta-lactamase-resistant bacteria isolated from environmental sources has not been reported.

With such an aim in mind, we decided to conduct a pilot study by isolating and characterizing E.coli from surface water samples of a major river in the capital city of Kerala, Trivandrum namely, the Killi river plays a very important role in providing water supply to the citizens residing in many prominent locations in the city and also it supports the livelihood of many people who depend on the river for fishing, cattle rearing, washing, etc (e.g. Mannnamoola, the place where Killi river flows is named after washermen existing in this place who does washing for their livelihood; The name Mannnamoola comes from two words “mannan” and “moola” meaning the locality of...
mannans or the washermen). Thus Killi river has a great heritage associated with the history of Trivandrum city, but currently facing the threat of water pollution. Killi river. Killi river is regarded as highly polluted due to the discharge of wastes and sewage, domestic effluents, etc which are the offshoots of unregulated tourism and industrial pollution (Vijayan et al., 2018). Killi river is also found to degraded water quality with more coliform content, especially during the pre-monsoon season both of these are mainly caused due to pollution as a consequence of unscientific human activities (Jyothishakshmy et al., 2020). Not many studies have been conducted to characterize antibiotic-resistant bacteria present in this river, although a single study has identified certain Enterobacteria resistant to some antibiotics from the Karamana river in Trivandrum city (Sreeleekshmi et al., 2020). Thus this work is the first of its kind which reports the presence of antibiotic-resistant E.coli in surface waters collected from various locations of Killi river and discusses the possibility of using S. glauca extract as a possible antimicrobial agent against such bacteria.

MATERIALS AND METHODS

Isolation and biochemical characterization of E. coli from water samples

The surface water samples were collected from four different locations in the Trivandrum district where Killi river flows: Maruthankuzhi, Jagathy, Killipalam, Kalady south, and was transported aseptically to the lab. Water from these areas is currently facing the threat of water pollution. Killi river. Killi river is regarded as highly polluted due to the discharge of wastes and sewage, domestic effluents, etc which are the offshoots of unregulated tourism and industrial pollution (Vijayan et al., 2018). Killi river is also found to degraded water quality with more coliform content, especially during the pre-monsoon season both of these are mainly caused due to pollution as a consequence of unscientific human activities (Jyothishakshmy et al., 2020). Not many studies have been conducted to characterize antibiotic-resistant bacteria present in this river, although a single study has identified certain Enterobacteria resistant to some antibiotics from the Karamana river in Trivandrum city (Sreeleekshmi et al., 2020). Thus this work is the first of its kind which reports the presence of antibiotic-resistant E.coli in surface waters collected from various locations of Killi river and discusses the possibility of using S. glauca extract as a possible antimicrobial agent against such bacteria.

Preparation of leaf extract

10g of powdered Simarouba glauca leaf was weighed in a 250mL conical flask. To this 100mL methanol was added and the conical flask was placed in a rotary shaker at 28°C for 24 hours. Then the extract was filtered out using a Whatman No.1 filter paper. The yield percentage was calculated and the extract was stored at 4°C until further use. The yield percentage was calculated (Syahidah et al., 2017). The dried extract was dissolved in DMSO (Bio Balance Pharma Grade, Japan) and further diluted in 5% DMSO to obtain various concentrations viz 50 μg/mL, 100 μg/mL, 250 μg/mL, 500 μg/mL, 750 μg/mL, and 1000 μg/mL respectively for antimicrobial studies.

Preliminary phytochemical analysis

The extract was subjected to preliminary phytochemical analysis according to well-established protocols (Jose et al., 2020). The tests for carbohydrates, coumarins, flavonoids, glycosides, phenols, proteins, saponins, steroids, tannins, terpenoids, and alkaloids were carried out.

Antibacterial activity of plant extract

The study was carried out as mentioned in Ghabhajey and Khoyande, 2022. To the MH agar plates, grown overnight with the lawn culture of antibiotic-resistant E.coli, sterile wells were punctured and 100 μL of plant extracts were added in various concentrations. As mentioned earlier. DMSO was used as control. The plates were observed after incubation at 37°C for 20 hours. The zone of inhibition, if the present was measured.
RESULTS AND DISCUSSION

Isolation and characterization of E. coli
The standard MPN tests showed that the water samples collected from four different stations were turbid with high gas production and the calculated MPN value was 200, indicating the presence of coliforms. The confirmative tests showed the presence of small colonies with green metallic sheen on EMB agar (Himedia, India) and pink colonies on Mac Conkey agar (Himedia, India) indicating the presence of E. coli. The various biochemical tests performed indicated the presence of E. coli as shown in Table 1.

Table 1. Shows the summary of results of biochemical characterization of isolates, indicating the presence of E. coli.

<table>
<thead>
<tr>
<th>Test</th>
<th>Observation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPN</td>
<td>Gas production</td>
<td>Positive</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Appearance of pink</td>
<td>Gram negative</td>
</tr>
<tr>
<td>Catalase</td>
<td>Production of gas bubbles</td>
<td>Positive</td>
</tr>
<tr>
<td>Indole production</td>
<td>Appearance of red color band</td>
<td>Positive</td>
</tr>
<tr>
<td>Methyl red</td>
<td>Appearance of red</td>
<td>Positive</td>
</tr>
<tr>
<td>Voges Proskauer</td>
<td>No colour change</td>
<td>Negative</td>
</tr>
<tr>
<td>Simmon Citrate</td>
<td>No colour change</td>
<td>Negative</td>
</tr>
<tr>
<td>TSI</td>
<td>Yellow slant and yellow butt with gas production</td>
<td>A/A</td>
</tr>
</tbody>
</table>

Molecular characterization of the isolates
The PCR amplification of gDNA isolated from isolates using primers for 16S rRNA resulted in an amplified product of around 900 bp confirming the isolates to be E. coli. (Figure 1).

![Figure 1](image)

Figure 1. Shows the agarose gel with PCR amplified product of 16S rRNA in various isolates of E. coli. Lanes 1-4 represent the bands obtained from 1A, 4C, 6C, and 7A respectively. Lanes 5 show the 1kB DNA ladder and lanes 6 and 7 have positive control and non-template control respectively.

Antibiotic susceptibility of the isolates
The antibiotic susceptibility test indicated the isolates were showing resistance to most of the tested antibiotics with a few exceptions, the first three isolates from three different locations were sensitive to cephalaxime, and the fourth one was resistant to the same. These first three isolates were highly resistant to penicillin-type antibiotics when compared to cephalosporins.

The isolate from the Jagathy station was showing resistance to all the antibiotics tested including cefpodoxime and tetracycline (data not shown). The graph showing the antibiotic susceptibility pattern of E. coli is shown in Figure 2.

![Figure 2](image)

Figure 2. Shows the graph of quantitative analysis of the antibiotic susceptible pattern of E. coli isolated from surface waters of four different locations Killi river represented as average ± standard deviation for 3 independent experiments.

Preparation of methanolic extract of S. glauca leaves
The methanolic extract from the dried and ground leaves of S. glauca was prepared and was found to be dark green in color. The calculated yield percentage was 70.3.

Qualitative phytochemical screening
The preliminary phytochemical screening of S. glauca leaf methanolic extract showed the presence of alkaloids, flavonoids, terpenoids, steroids, tannins, phenols, glycosides, coumarins, and carbohydrates. Saponins and proteins were not detected in the methanolic extract of S. glauca. This would be due to the solvent used for extraction used in this study and is similar to the findings in some previous studies (Lakshmi et al., 2014). The presence of these phytochemicals especially alkaloids, flavonoids, tannins, phenols, etc. is reported to be responsible for the antimicrobial, insecticidal and pharmacological properties of the plant (Kumar et al., 2016). The table showing the results of the phytochemical analysis is given (Table 2).

<table>
<thead>
<tr>
<th>Bacteria isolated from four different stations of Killi river</th>
<th>Gram staining</th>
<th>Catalase</th>
<th>Indole production</th>
<th>Methyl red</th>
<th>Voges Proskauer</th>
<th>Simmon Citrate</th>
<th>TSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. glauca</td>
<td>Gram negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
<td>A/A</td>
</tr>
</tbody>
</table>

Thin layer chromatography
When the plant extract was subjected to TLC using the solvent system I, a total of four bands were developed when observed under both visible and UV light. The calculated Rf values were 0.53, 0.65, 0.72 and 0.78. The solvent system was chosen as the components had intermediate polarity as it is reported that the extracts of Syzygium when separated using TLC under both visible, UV, and iodine vapor solvent system showed compounds with similar Rf values and possessed antibacterial activity against E. coli (Fatmyaide et al., 2019). A total of five bands were developed when the S. glauca methanolic extract was separated by TLC using the solvent system II, when visualized under visible, UV, and iodine vapor and the calculated Rf values were 0.47, 0.57, 0.88, 0.9, 0.94. The Rf values are similar to those obtained in the separation of methanolic extract of S. glauca could indicate the presence of phenols, alkaloids, terpenoids, etc. (Kumar et al., 2016; Mathew et al., 2019). Figure 3 represents the image of silica gel visualized under different light conditions under TLC.
Table 2. Showing the results of preliminary phytochemical analysis of a methanolic extract of S. glauca against antibiotic-resistant E. coli isolated from environmental samples.

<table>
<thead>
<tr>
<th>Phytochemicals Tested</th>
<th>Methanolic extract of S. glauca test result</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids (Mayer’s reagent test)</td>
<td>+ve</td>
<td>Formation of pale yellow precipitate.</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>+ve</td>
<td>Presence of yellow precipitate</td>
</tr>
<tr>
<td>Terpenoids (Salkowski’s test)</td>
<td>+ve</td>
<td>Presence of reddish brown ring</td>
</tr>
<tr>
<td>Steroids</td>
<td>+ve</td>
<td>Upper layer of the solution is red and H2SO4 layer as yellow with green fluorescence</td>
</tr>
<tr>
<td>Tannins (Breaemer’s test)</td>
<td>+ve</td>
<td>Presence of blue-black colour</td>
</tr>
<tr>
<td>Phenols (Ferric chloride test)</td>
<td>+ve</td>
<td>Formation of violet colour</td>
</tr>
<tr>
<td>Glycosides (Keller-kiliani test)</td>
<td>+ve</td>
<td>Formation of yellow colour</td>
</tr>
<tr>
<td>Coumarins</td>
<td>+ve</td>
<td>Formation of yellow colour</td>
</tr>
<tr>
<td>Carbohydrates (Benedict test)</td>
<td>+ve</td>
<td>Green to brick red colour</td>
</tr>
<tr>
<td>Saponins</td>
<td>-ve</td>
<td>Absence of froth</td>
</tr>
<tr>
<td>Proteins</td>
<td>-ve</td>
<td>Absence of violet colour</td>
</tr>
</tbody>
</table>

Figure 3. shows the results of TLC analysis of a methanolic extract of S. glauca using two different solvent systems. A and B shows the separated bands when the solvent system I was used and visualized under visible light and UV light respectively.

Antimicrobial activity of the extract
Antibacterial activity of different concentrations of methanolic extracts of S. glauca on antibiotic-resistant E. coli was studied using the agar well diffusion method. The extract showed antibacterial activity against the antibiotic-resistant E. coli isolated from all four stations, in a concentration-dependent manner. The bacteria were sensitive to plant extract and the zone of inhibition increased as the concentration of plant extract increased as can be observed in quantitative analysis of the same as shown in Figure 4.

Figure 4. Shows the graph of the quantitative analysis of the antimicrobial activity of plant extracts on the antibiotic-resistant E. coli isolated from Killi river represented as average ± standard deviation of 3 independent experiments.

CONCLUSION
This pilot study aimed to look for the presence of antibiotic-resistant E. coli present in the surface waters of a major, yet highly polluted river flowing through Trivandrum city, the Killi river. Being a pilot study, out of the various E.coli isolated from surface waters from various locations, we chose only four isolates, each obtained from one particular location for this study. The chosen isolates showed resistance against the major antibiotics used and some of the isolates were extremely resistant with completely no inhibition around the antibiotic in disk diffusion assay. Although previous studies were showing the presence of E.coli resistant to tetracyclin, gentamycin, and chloramphenicol among other isolated bacteria from the Karamana river basin in the Trivandrum district (Sreelekshmi et al., 2020), this study is the first of its kind which has tried to isolate and characterize E.coli from surface water of various stations across Killi river with an aim to study its antibiotic susceptibility. Incidentally the E.coli isolated, from the four stations of Killi river showed resistance to only penicillin and cephalosporin antibiotics, but it could be a possibility that this study has not identified other bacteria which are resistant to other cephalosporin or carbapenem antibiotics and such antibiotic-resistant strains could still be present in these waters. This calls for caution while using this water to support life. Natural compounds, specially derived from plants either crude extracts or isolated compounds have the potential to be used as drugs (Silva et al., 2013). Bacterial resistance to currently used antibiotics necessitates the search for effective therapeutic agents. Plant extracts are a major source of therapeutic agents including antimicrobial agents with potential therapeutic effects against antibiotic-resistant bacteria (Alvarez-Martinez et al., 2021). S. glauca is found to be rich in alkaloids and methanolic extracts are shown to possess cytotoxic and antitumor properties in vitro and in vivo studies (Mathew et al., 2019). Phytochemical analysis and TLC of methanolic extracts of S. glauca leaves collected from UP in North India were found to possess various secondary metabolites like phenols, flavonoids, alkaloids, steroids, glycosides, etc. (Kumar et al., 2016). In the present study, we also screened the methanolic extract of S. glauca leaves for the presence of various
phytochemicals like alkaloids, phenols, terpenoids, etc. which was confirmed by standard qualitative tests and TLC using two different solvent systems. The methanolic extract of *S. glauca* showed antibacterial activity and this could be attributed to the various secondary metabolites present in plant extract especially phenols, alkaloids, terpenoids, etc. The study reports a potential plant extract with antibacterial properties against the antibiotic-resistant environmental strains of *E. coli*.

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ETHICS STATEMENT: None

REFERENCES


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