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Simarouba gluca Leaf Extract as a Potential Antimicrobial against Antibiotic Resistant Environmental Isolates of *Escherichia coli*

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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 water bodies is *E. coli* which is usually found in the gastrointestinal tract of animals. This is a pilot study carried out from January 2022 to July 2022, whose major challenge was to identify the presence of such antibiotic resistant *E. coli* in surface waters of a polluted river namely Killi river, a major river in Trivandrum district. The isolated bacteria were confirmed to be *E. coli* by biochemical and molecular analysis. Then we were interested to look for a potential plant extract which can act as a natural remedy against such multi drug resistant environmental isolates. With this aim in mind, methanolic extract of the leaves of the paradise tree, *Simarouba gluca* was prepared, the qualitative phytochemical composition analysed and confirmed by TLC. The antibacterial effect of the extract was studied using the agar diffusion method and it showed that the methanolic extract of *S. gluca* exhibited 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 of *S. gluca* as a potent antimicrobial agent against environmental strains of *E. coli* that are resistant to some of the common antibiotics. The contribution of the study is that it 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 as one of the leading causes of death, majorily 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 waste. One of the major bacteria showing such antibiotic resistance is *E. coli*. As shown in previous studies, such *E.coli* are prevalent in various water bodies around the globe (Ranjbar *et al*., 2016; Odonkor and Addo 2018). *E. coli* which is generally considered an indicator of fecal contamination in water bodies harbors antibiotic resistance genes, especially the β-lactamase resistance (bla) ones (Bondarczuk and Piotrowska-Seget 2019), and possibly transfer the same to other bacteria thus horizontally spreading antibiotic resistance. So it 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 and 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 and few 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 antibiotic 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. This 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. Mannanmoola, the place where Killi river flows is named after washermen existing in this place who does washing for their livelihood; The name Mannanmoola 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 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) and hence surface waters collected from the same was chosen for isolation and characterization of antibiotic resistant bacteria. Killi river is also found have 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 (Jyothylakshmy *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 (Sreelekshmi *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 riverand discusses the possibility of using *S. gluca* extract as a possible antimicrobial agent against such bacteria.

MATERIALS AND METHODS

A. Isolation and biochemical characterization of E. coli from water samples

The surface water samples were collected from four different locations in the Trivandrum district in January 2022, where Killi river flows: Maruthankuzhi, Jagathy, Killipalam, Kalady south, and was transported aseptically to the lab. Water from these areas is considered to be highly polluted as many prominent hospitals, religious institutions, and residential areas are located on the banks of these places. The water samples were subjected to standard MPN tests to estimate the coliform content. To isolate *E. coli*, the samples from the MPN positive tubes were streaked onto EMB agar (TM media, India) and McConkey agar (TM media, India). The colonies obtained were subjected to standard biochemical tests for the identification of *E. coli*. The tests include gram staining, IMViC tests, Catalase tests, and monitoring the bacteria for its characteristic growth on TSI Agar and Blood agar (Lupindu, 2017).

B. Genomic DNA isolation and molecular characterization of the isolates

An overnight culture of possible bacterial isolates in LB broth (Himedia, India) were subjected to genomic DNA isolation using a bacterial genomic DNA isolation kit (Transiom Bacterial gDNA kit, Gujarat) as per manufacturers protocol and was quantified using UV-visible spectrophotometer. PCR amplification of the 100 ng of this isolated gDNA was carried out using primers specific for 16S rRNA in a thermocycler (Agilent Technologies, USA), to confirm that the isolates were *E. coli*. The primers used were- *E. coli* forward 5'AGAGTTTGATCCTGGCTCAG3' *E. coli* reverse- 5'CTTGTGCGGGCCCCCGTCAATTC3'. The cycling conditions were as follows- initial denaturation at 94°C for 4 minutes, final denaturation at 94°C for 30′, annealing at 55°C for 30′, extension for 72°C for 1' (Indira *et al*., 2021). The PCR products were visualized by agarose gel electrophoresis in 2% gel and compared using a 1 kb DNA ladder (Transiom genomics, India).

Antibiotic susceptibility: The susceptibility of characterized *E. coli* against various antibiotics was studied using the Kirby Bauer Disk diffusion susceptibility test (Hudzicki, 2009). The antibiotic discs used for the test were Ampicillin, Amoxicillin, Oxacillin, Ceftriaxone, Cefixime, Cefotaxime, Tetracycline, etc. After the incubation period, the zone diameter was measured and susceptibility interpreted using standards published by CLSI for each antibiotic, and results were classified as resistant, intermediate sensitive, or sensitive accordingly.

C. Plant collection

Leaves of *Simarouba glauca* were collected locally in January 2022, from Trivandrum and the leaves were dried using the shade dry protocol. The material was evaluated by Dr. Ajith Kumar P, Associate Professor and Head, Department of Botany, Govt Arts College., Trivandrum. Fresh leaves of *S. glauca* were washed thoroughly in running tap water followed by distilled water to remove dirt and other impurities. The excess water was blotted onto a blotting paper and the leaves were shade dried for about three weeks. The shadedried leaves were powdered using a mixer grinder to a fine powder.

D. 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. The extract was then 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.

E. 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.

F. Thin layer chromatography (TLC) of the extract

Leaf extract (10µL of 1g/mL) was applied on the TLC plate (Sigma-Aldrich, Canada) and then separated using two different solvent systems namely chloroform: ethyl acetate: formic acid (Labogens, India) in the ratio 8:2:0.2, V/V/V and N-hexane: ethyl acetate: methanol: formic acid (Labogens, India) in the ratio 3:4:3:0.1, V/V/V/V. The eluted spotted plates were dried at room temperature and visualized under visible light, UV light (254 nm), and iodine vapor. The Rf values were calculated for the separated compounds (Mathew *et al*., 2019).

G. Antibacterial activity of plant extract

The study was carried out as mentioned in Gajbhiye and Koyande (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 negative control. The plates were observed after incubation at 37°C for 18-20 hours. The zone of inhibition, if present was measured.

RESULTS

A. 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*.

Bacteria isolated from four different stations of Killi river	Test	Observation	Result
	MPN	Gas production	Positive
	Gram staining	Appearance of pink colour	Gram negative
	Catalase	Production of gas bubbles	Positive
	Indole production	Appearance of red color band at the junction of medium and reagent	Positive
	Methyl red	Appearance of red colour	Positive
	Voges Proskauer	No colour change	Negative
	Simmon Citrate	No colour change	Negative
	TSI	Yellow slant and yellow butt with gas production	A/A

B. 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*. (Fig. 1).

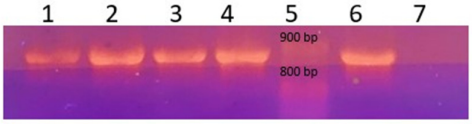

Fig. 1. Shows the agarose gel with PCR amplified product of 16S rRNA in various isolates of *E. coli*. Lanes 1- 4 represent the bands obtained from isolates 1A, 4C, 6C, and 7A respectively. Lane 5 shows the 1kB DNA ladder and lanes 6 and 7 shows positive and non template control.

C. 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 cefixime, 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 Fig. 2.

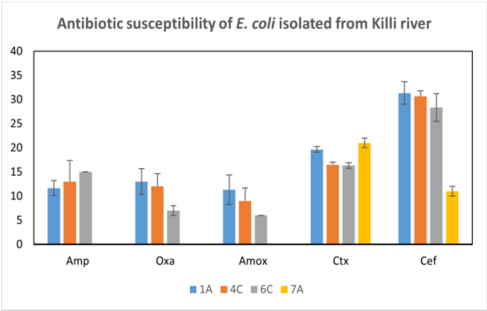

Fig. 2. Shows the graph of quantitative analysis of the antibiotic susceptible pattern of *E. coli* isolated from surface waters of four different locations Killi river represented as average ± standard deviation for 3 independent experiments.

D. Preparation of methanolic extract of S. gluca leaves

The methanolic extract from the dried and ground leaves of *S. gluca* was prepared and was found to be

dark green in color. The calculated yield percentage was 70.3.

E. 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 2: Shows the results of preliminary phytochemical analysis of methanolic extract of *S. gluca*.

Phytochemicals Tested	Methanolic extract of S. glauca test result	Observation
Alkaloids (Mayer's reagent test)	+ve	Formation of pale yellow precipitate.
Flavanoids	+ve	Presence of yellow precipitate
Terpenoids (Salkowski's test)	+ve	Presence of reddish brown ring.
Steroids	+ve	Upper layer of the solution is red and H_2SO_4 layer as yellow with green fluorescence.
Tannins (Breaemer's test)	+ve	Presence of blue-black colour
Phenols (Ferric chloride test)	+ve	Formation of violet colour
Glycosides (Keller-killiani test)	+ve	Formation of yellow colour.
Coumarins	+ve	Formation of yellow colour
Carbohydrates (Benedict test)	+ve	Green to brick red colour
Saponins	-ve	Absence of froth
Proteins	-ve	Absence of violet colour

F. 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 according to the studies using the extracts of *Syzygium* where the separated compounds showed similar Rf values and possessed antibacterial activity against *E. coli* (Famayuide *et al*., 2019). A total of five bands were developed when the *S. gluca* 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. These Rf values are similar to those studies which separated the methanolic extract of *S. glauca* and indicates the presence of phenols, alkaloids, terpenoids, etc. (Kumar *et al*., 2016; Mathew *et al*., 2019). Fig. 3 represents the image of silica gel with different bands visualized under different light conditions after TLC.

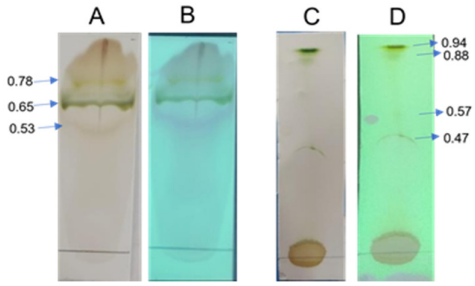

Fig. 3. Shows the results of TLC analysis of a methanolic extract of *S. gluca* using two different solvent systems. A and B shows the separated bands when the solvent system I was used and visualized under visible light and UV light respectively, whereas C and D shows the separated bands when solvent system II was used.

G. 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. All the four isolates under the study were sensitive to plant extract and the zone of inhibition increased as the concentration of plant extract increased as shown in quantitative analysis, Fig. 4.

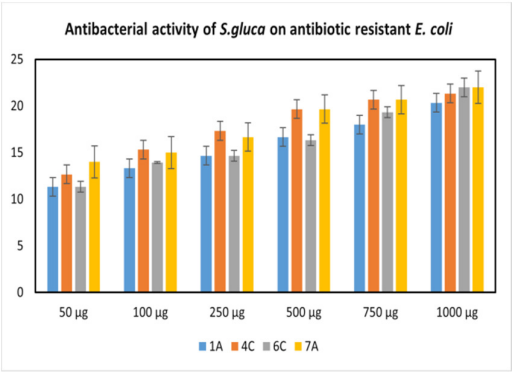

Fig. 4. Shows the graph of the quantitative analysis of the antimicrobial activity of plant extracts on the antibiotic resistant *E. coli* isolated from Killi river represented as average ± standard deviation of 3 independent experiments.

DISCUSSION

Presence of antibiotic resistant *E. coli* in water bodies is a potential hazard to public health and is an evidence of fecal pollution. On the other hand, presence of such *E. coli* can act as a disease risk assessment for water bodies (Odonkor and Mahami 2020). Our pilot study

also aimed to look for the presence of antibiotic resistant *E. coli* present in the surface waters of a major river flowing through Trivandrum city, the Killi river. Killi river is a major river supporting the lives and livelihood of a good size of population living on its banks, but is a highly polluted river due to anthropogenic activity (Vijayan *et al.,* 2018). Hence we chose the surface waters of this rivers from various locations to look for the presence of antibiotic reistant bacteria, especially *E. coli*. The chosen isolates from various locations of Killi river, as mentioned earlier, showed resistance against the major antibiotics used and some of the isolates were extremely resistant with completely no zone of 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.

Natural compounds, especially those derived from plants, either crude or isolated compounds have the potential to be used as drugs (Silva *et al*., 2013). For example, the hexane and ethanolic leaf extacts of *Acacia* (Rana *et al*., 2016) and methanolic extract of *Cinnamon*is effective against *E. coli, S. aureus* etc (Gajbhiya and Koyande 2022). 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). Studies similar to the one carried out by us which looks at bacterial resistance to currently used antibiotics necessitates the search for effective anti microbialtherapeutic agents (Buitimea, 2020). Thus, we wanted to see if there is any plant extract with potential effects on such antibiotic resistant bacteria, especially the *E. coli* which we isolated from polluted water. Our plant of choice was *Simarouba gluca* which is locally available and well grown in Trivandrum city. *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* (Mathew *et al*., 2019). Some studies have shown the anti bacterial properties of *S. gluca* extracts by disk diffusion assays on standard bacterial strains (Karthikeyan *et al*., 2019; Hussain *et al.*, 2020). 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). A similar study for preliminary assessment of methanolic extract of *S. gluca* leaf extract collected from Baglantaluk of Nashik showed presence of phenols, tannins, saponins etc (Vasait and Khandare 2017). 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. This observation was consistent with the previous studies as shown above. The methanolic extract of *S. gluca* showed antibacterial activity against the antibiotic resistant *E. coli* and this could be attributed to the various secondary metabolites present in plant extract especially phenols, alkaloids, terpenoids, etc. The study reports for the first time, a potential plant extract with antibacterial properties against the antibiotic resistant environmental strains of *E. coli*.

CONCLUSIONS AND FUTURE SCOPE

The pilot study has been successful in isolation and characterisation of antibiotic resistant environmental strains of *E. coli* from polluted water in a major river of Trivandrum city. In this study, the *E. coli* isolated, from the four stations of Killi river showed resistance mostly 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 antibioticresistant strains could still be present in these waters. This calls for more study and analysis of the surface waters in a major river to see the presence of such bacteria and further suitable intervention as it is highly dependent upon by the citizens. Methanolic extract of *S. gluca* was found to be highly effective against such environmental isolates of *E. coli* which is resistant to antibiotics. A major scope is developing the plant extract of *S. gluca* to a suitable drug formulation as it has shown effectiveness against environmental isolates of the gut bacteria, *E. coli*.

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Conflict of Interest. None.

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