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Secondary metabolites from endophytic bacteria and evaluation of their antibacterial potential

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Abstract

The objective of this study was to isolate endophytic bacteria from *Cymbopogon citratus* (lemon grass) leaves and investigate their antibacterial activity against bacterial pathogens. Fresh leaves of *C. citratus* were collected from Madhavaram botanical garden. Isolation of endophytic bacteria was done by standard procedure. Identification of the bacterial isolates were performed by morphological and Gram's staining characteristics. A total of 7 bacterial isolates from the leaves of *C. citratus* were isolated. Based on the morphological and Gram staining characteristics, 3 bacterial strains were selected for the further studies, which is well differentiated with others. The chloroform extracts of selected 3 bacterial strains were screened for their antibacterial potential against three bacterial pathogens namely *Klebsiella pneumoniae* (MTCC3384), *Staphylococcus aureus* (MTCC3160) and *Escherichia coli* (MTCC443) by Kirby-Bauer disk diffusion method. The antibacterial study reveals that among the 3 selected bacterial strains, two strains namely *Streptomyces* sp. (EB2) and *Bacillus* sp. (EB3) showed potential antibacterial activity against all 3 tested pathogens with the zone of inhibition ranging from 11 to 15 mm; however, the bacterial strain *Vibrio* sp. (EB1) did not showed activity against all 3 tested pathogens. The positive control Amoxicillin showed zone of inhibition ranging from 12 to 18 mm and the negative control Chloroform did not express any zone of inhibition. In conclusion, the current study revealed that crude extracts of endophytic bacteria *Streptomyces* sp. (EB2) and *Bacillus* sp. (EB3) have potential antibacterial property

Keywords: Antibacterial activity; *Bacillus* sp.; *Cymbopogon citratus;* Endophytic bacteria; *Streptomyces* sp

1. Introduction

Antimicrobials including antibiotics, antivirals, antifungals and antiparasitic, which are used to prevent and treat infectious diseases in humans, animals and plants. Antimicrobial resistance (AMR) is one of the top global public health and development threats. It is estimated that bacterial AMR was directly responsible for 1.27 million global deaths in 2019. AMR occurs when bacteria, viruses, fungi and parasites no longer respond to antimicrobial medicines. As a result of drug resistance, antibiotics and other antimicrobial medicines become ineffective and infections become difficult or impossible to treat, increasing the risk of disease spread, severe illness, disability and death [1]. *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*) *and Staphylococcus aureus* (*S. aureus*) are the most common bacterial pathogens [2]. Increasing resistance to third generation cephalosporins amongst *E. coli* and *Klebsiella* sp. is predominantly due to the production of extended-spectrum beta lactamases (ESBLs). ESBL producers are associated with increased morbidity and mortality, especially amongst patients on intensive care and high-dependency units. Accurate laboratory detection is important to avoid clinical failure due to inappropriate antimicrobial therapy [3].

Natural sources of drugs have played important roles in medicine during the last decades. Since 1981 to 2006 nearly 70% of new drugs and chemical agents had natural sources. Over 22,000 natural products are isolated from microorganisms [4]. The bioactive compounds derived from natural sources exhibit tremendous structural and

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chemical diversity [5]. Endophytic microbes are considered natural reservoirs due to their ability to produce myriad bioactive compounds [6]. Valuable bioactive metabolites, such as alkaloids, steroids, terpenoids, lactones, quinines, lignans and phenols have been isolated from endophytic fungi and bacteria [7]. Endophytic bacteria isolated from medicinal plants are crucial for the production of antimicrobial agents since they are capable of possessing bioactive compounds with diverse structures and activities [8]. Endophytic bacteria offer a promising, sustainable alternative by promoting plant growth and enhancing nutrient acquisition [9]. Studies on the isolation of bioactive products from bacterial endophytes can help in the discovery of several new compounds that can also be developed as antimicrobial agents to manage antibiotic resistance.

Among the different types of bacteria, *Streptomyces* is the most prolific drug producing genus. *Streptomyces* species, filamentous Gram-positive bacteria, have shown an outstanding capacity for the production of secondary metabolites with diverse chemical structures and different biological activities such as: antibacterial, antifungal, anticancer, immune suppression, etc. Additionally, *Bacillus* is considered to be a promising source of bioactive secondary metabolites [10].

Cymbopogon citratus (*C. citratus*), a plant native to Sri Lanka and southern India, is cultivated indoors or outdoors in South and Central America, in Savannah, tropical and subtropical regions [11]. Lemongrass (*Cymbopogon* spp.) is one of the most important crops that are widely appreciated in the pharmaceuticals, food preservation and cosmetic industries. *C. citratus* is considered to be one of the fast-growing crops with substantial biomass production in a wide range of soil types and climatic conditions [12]. *C. citratus* contains alcohols, ketones, esters, aldehydes, saponins, terpenoids, terpenes, neryl, nerol, myrcene, geraniol, citral, gernyl acetate and many flavonoids [13]. The plant contains carbohydrates, crude proteins, crude ash, fat and several polyphenolic substances, e.g., luteolin, apiginin, flavonoids, apigerol, caffeic acid, catechol and elimicin [14]. Lemongrass has anti-inflammatory and anticancer properties due to the presence of citral and geranial components [15]. Due to the presence of different secondary metabolites, lemongrass has historically been used to treat a variety of illnesses [16]. With this background, the current study is aimed to isolation and identification of bacterial endophytes from *C. citratus* leaves and screening of their bioactive secondary metabolites for antibacterial activity.

2. Materials and methods

The present work was carried out in the Department of Plant Biology and Plant Biotechnology, Thiruthangal Nadar College, Chennai, Tamil Nadu.

2.1. Sample collection

Fresh leaves of *C. citratus* were collected from Madhavaram botanical garden. A mature healthy plant leaves were collected. Samples were immediately brought to laboratory and were used within 24 hours and finally processed for isolation of endophytic bacteria.

2.2. Isolation of endophytic bacteria

The leaves were surface sterilized with 0.1% sodium hypochlorite for 5 minutes, 70% ethanol, followed by five times rinses in double distilled water for 5 minutes. Leaves were dried in laminar air flow [17]. To confirm that the surface of leaves were effectively sterilized, 1 ml of the sterile distilled water that was used in final rinse of surface sterilization procedures were plated on to nutrient agar media and incubated at 37°C for 24 hrs. Bacterial growths were observed after 24 hours.

2.3. Purification of endophytic bacteria

For purification of endophytic bacteria, subculturing was mainly done by streaking a loop full of broth media on the fresh pre-solidified nutrient agar plates and then incubated at 37°C for 24 hrs. The plates were incubated up to 5 days to find out antagonistic bacterial colony. The colonies which showed antagonism were picked up and streaked on nutrient agar plate separately in order to obtain pure isolated colonies. Pure culture was stored at room temperature for further studies.

2.4. Characterization of isolates

Bacterial isolates were initially identified based on color, culture morphology and Gram's staining test according to the Bergey's manual.

2.5. Gram Staining

Gram's staining was done where thin smears of obtained cultures were prepared on separate glass slides, air dried and heat fixed. Each smear was covered with crystal violet and allowed to stand for 1 minute followed by washing with distilled water. Further Gram's iodine was added and allowed to stand for 1 minute. Decolourized with 95% ethyl alcohol and the slide was rinsed with distilled water. Slide was flooded with safranin to counterstain and allowed to stand for 1 minute. Smear was rinsed with distilled water and dried with absorbent paper. Slides were then observed at 100X.

2.6. Tested pathogens

Bacterial cultures were obtained from Microbial Type Culture Collection (MTCC), Chandigarh. Subcultures were maintained by SRMU, Lucknow. One-gram positive culture- *S. aureus* (MTCC3160) and two-gram negative cultures- *E. coli* (MTCC443) and *K. pneumoniae* (MTCC3384) were used. These cultures were MDR (Multi-drug resistant) cultures which can survive in the presence of multiple types of antibiotics [18].

2.7. Fermentation and extraction of bioactive metabolites from bacteria

The endophytic bacterial strains were batch cultured for 72 hrs at 37°C containing 100 ml of Nutrient broth (NB) medium in 250 ml Erlenmeyer flasks. The extract was filtered through Whatman filter paper. The supernatant was used for the extraction of extracellular metabolites. The equal volumes of Chloroform added with supernatant (1:1) and vigorously agitated for 30 minutes. Then, the Chloroform phase was separated using separating funnel. The concentrated extracts were dissolved in solvent and stored for further studies.

2.8. Disc diffusion method

The antimicrobial activity of the crude bacterial extract was done by the Kirby-Bauer disc diffusion method [19]. The loopful cultures of the bacterial pathogens transferred into freshly prepared Nutrient Agar (NB) medium and incubated for 24 hrs at 37°C. The 100 µl of the pathogenic cultures were streaked on the top of the solidified media. The 60 µl of the crude extract was loaded in the sterile discs (6 mm) and placed on the surface of the solidified medium containing petri plates. Amoxicillin (10 µg/disc) was used as positive controls. A solvent chloroform served as a negative control. The bacterial pathogens containing plates were incubated at 37°C for 24 hours. After incubation, the zones of inhibition of crude extracts against tested pathogens were recorded.

2.9. Statistical analysis

The statistical analysis of all the experiments was carried out using Microsoft Excel.

3. Results

3.1. Sample collection

In the present study, the leaf sample of *C. citratus* was collected from Madhavaram botanical garden, Chennai, Tamil Nadu and transferred to the laboratory for bacteria isolation.

3.2. Isolation of endophytic bacteria

The leaf sample was washed thoroughly and surface sterilized with sodium hypochlorite, ethanol and sterile distilled water to prevent the surface bacteria to grow, then it is inoculated in nutrient agar medium incubated at 37°C for 24 hours. A total number of 7 pure bacterial cultures were isolated from the leaf samples EB1, EB2, EB3, EB4, EB5, EB6, EB7.

3.3. Characterization of bacterial isolates

All the 7 bacterial strains isolated from the leaf samples in the present work were initially differentiated based on the morphological and Gram-staining technique. Based on the morphological and Gram staining characteristics, 3 bacterial strains were selected. Among the 3 bacterial strains, two strains were white in colour and remaining one strain in cream colour. The consistency showed creamy, dusty and sticky for the isolate number of EB1, EB2 and EB3, respectively. The isolates EB1 and EB3 showed smooth margin and the isolate EB2 showed rough margin.

The Gram staining results of the selected 3 bacterial strains were provided in the Table-1. The Gram staining results showed, two Gram-positive and one Gram-negative bacterial strains. Based on the morphological and Gram-staining

technique, the selected 3 bacterial strains identified as *Vibrio* sp., *Streptomyces* sp. and *Bacillus* sp. with the isolate number of EB1, EB2 and EB3, respectively (Figure-1, Table-1).

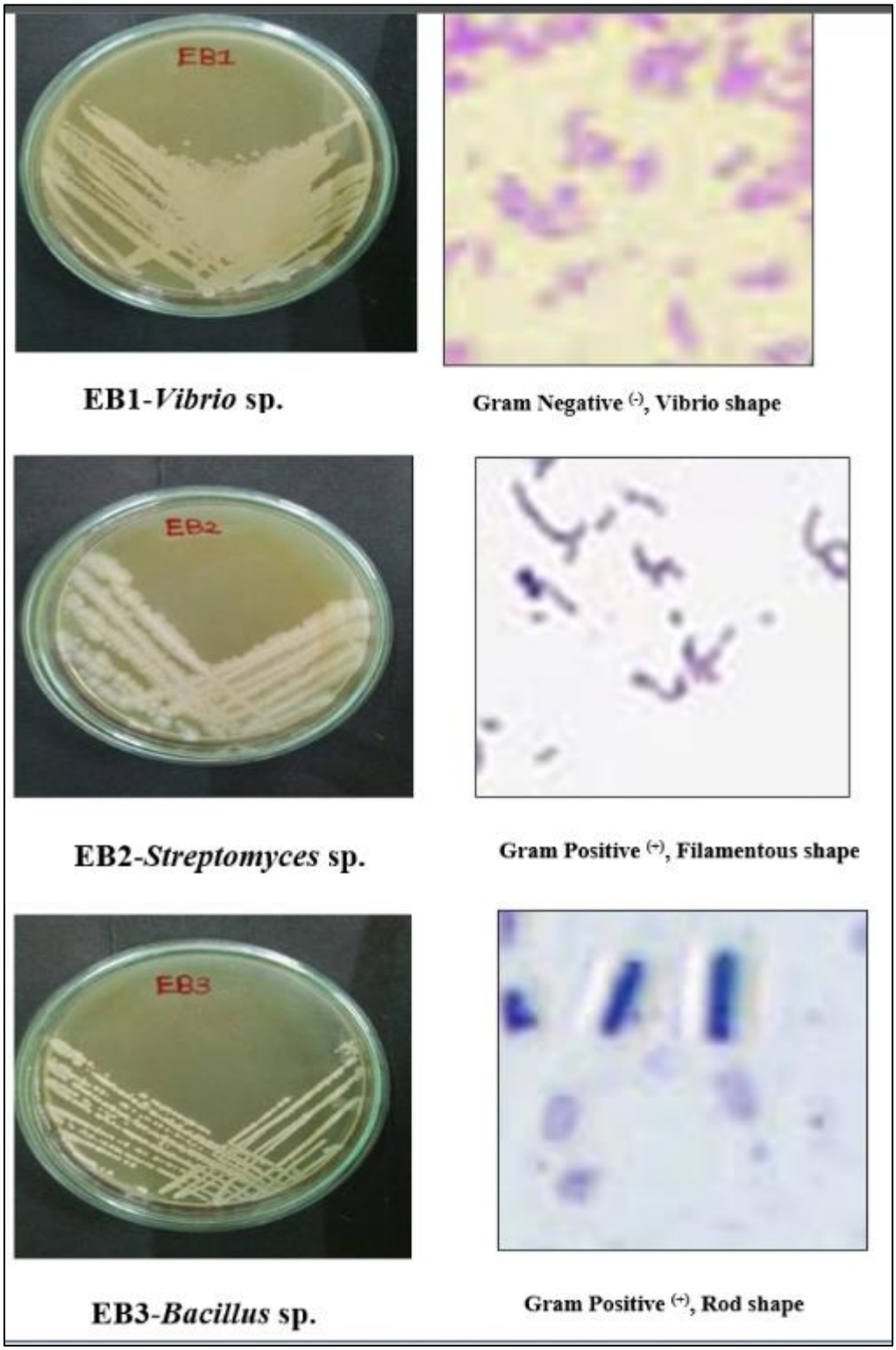

Figure 1 Morphological identification and gram straining of endophytic bacteria

Table 1 Gram staining results of endophytic bacteria

S.No	Isolate Number	Shape	Gram staining	Genus
1	EB1	Vibrio	Gram Negative (-)	*Vibrio* sp.
2	EB2	Filamentous	Gram Positive (+)	*Streptomyces* sp.
3	EB3	Rod	Gram Positive (+)	*Bacillus* sp.

3.4. Antibacterial activity of endophytic bacterial extracts

In the present study, the chloroform extracts of selected 3 bacterial strains were screened for their antibacterial potential against three bacterial pathogens by Kirby-Bauer disk diffusion method. The bacterial pathogens namely *K. pneumoniae* (MTCC3384), *S. aureus* (MTCC3160) and *E. coli* (MTCC443). The selected 3 bacterial pathogens were, one-gram positive culture- *S. aureus* and two-gram negative cultures- *E. coli* and *K. pneumoniae*.

The antibacterial study reveals that among the 3 selected bacterial strains, two strains namely *Streptomyces* sp. (EB2) and *Bacillus* sp. (EB3) showed potential antibacterial activity against all 3 tested pathogens, however the bacterial strain *Vibrio* sp. (EB1) did not showed activity against all 3 tested pathogens (Figure- 2). The *Streptomyces* sp. (EB2) showed antibacterial activity against all 3 tested pathogens with the zone of inhibition ranging from 11 to 14 mm. The maximum activity observed against *K. pneumoniae* (MTCC3384) and the minimum activity observed against *S. aureus* (MTCC3160). Similarly, the *Bacillus* sp. (EB3) showed antibacterial activity against all 3 tested pathogens with the zone of inhibition ranging from 12 to 15 mm. The maximum activity observed against *K. pneumoniae* (MTCC3384) and the minimum activity observed against *S. aureus* (MTCC3160). The positive control Amoxycilin showed zone of inhibition ranging from 12 to 18 mm and the negative control Chloroform did not express any zone of inhibition (Table- 2 & Figure - 3).

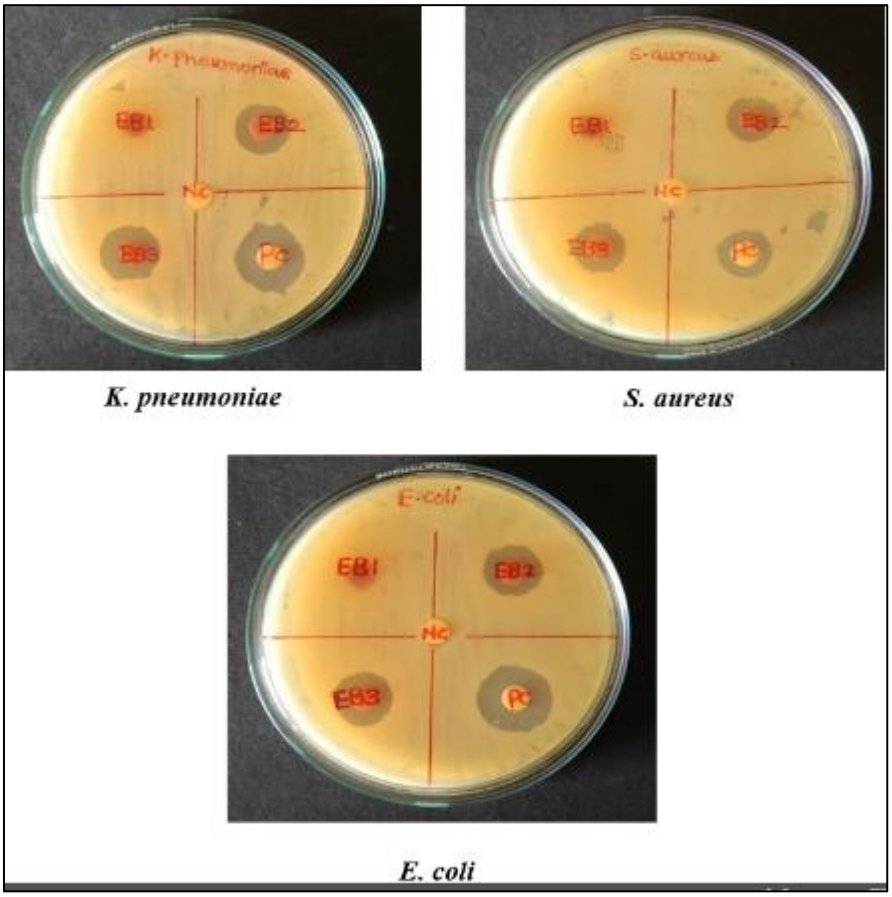

Figure 2 Antibacterial activity of endophytic bacterial extracts against tested pathogens

Table 2 Antibacterial activity of bacterial extracts

Isolate Number	*K. pneumoniae* (MTCC3384)	*S. aureus* (MTCC3160)	*E. coli* (MTCC443)
	Zone of inhibition (mm)		
EB1	-	-	-
EB2	14±0.14	11±0.14	13±0.21
EB3	15±0.28	12±0.07	13±0.17
Positive Control (Amoxicillin)	18±0.21	12±0.35	18±0.28
Negative Control (Solvent)	-	-	-

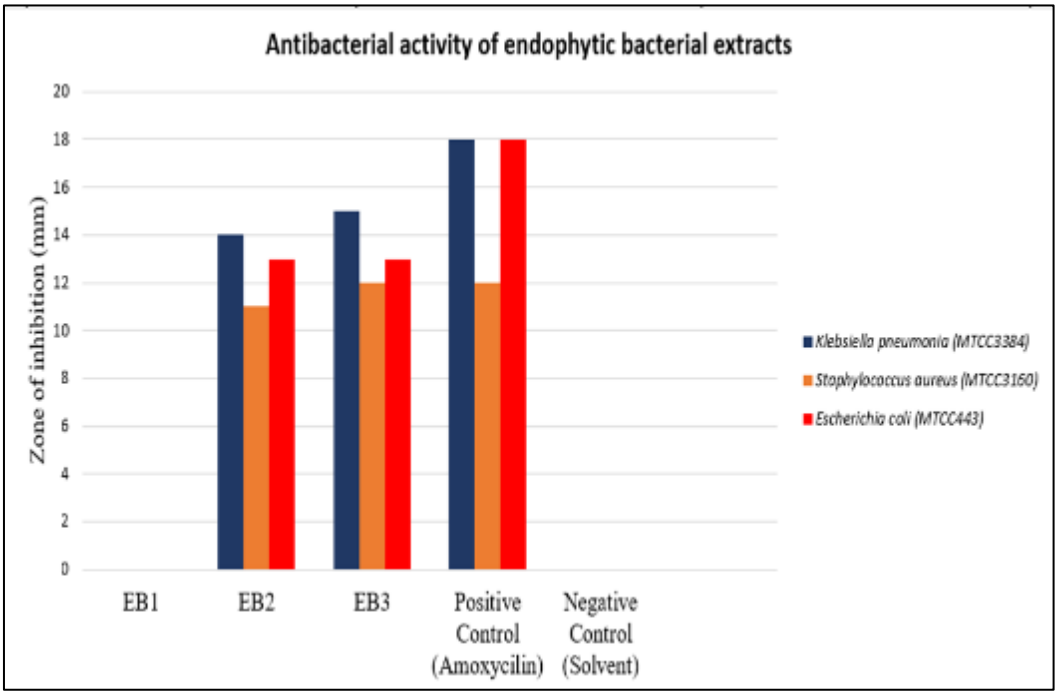

Figure 3 Antibacterial activity of endophytic bacterial extracts

4. Discussion

In the present study, the bacterial strains isolated from the leaf sample was initially differentiated based on the morphological and Gram staining characteristics. Similar observations were made by Alam, [20] isolated endophytic bacteria from roots, stems and leaves of *C. citratus* collected from the botanical garden of KSD's Model College. The isolates were differentiated using colony morphology and Gram staining characteristics. Likewise, Alam, [20] reported the isolation of 15 endophytic bacterial strains from *C. citratus* and colonies characteristics differentiated based on color, shape, margin and elevation followed by Gram staining technique revealed nine Gram-negative and six Gram-positive isolates.

Out of 3 species, *Streptomyces sp* and *Bacillus sp. showed good antibacterial activity against all 3 tested pathogens namely E. coli, K. pneumonia and S. aureus, while Vibrio sp. does not show antibacterial activity.* Alam, [20] reported that endophytic *Bacillus* from *C. citratus* exhibited strong inhibition of Gram-positive and Gram-negative bacteria, including *S. aureus* and *E. coli*, which is similar to the present findings. Kewat *et al*., [21] isolated endophytic *Streptomyces* from *C. citratus* leaves and reported significant antibacterial activity against *E. coli*, *K. pneumoniae*, and *S. aureus. Streptomyces* isolates from lemongrass leaves demonstrated broad-spectrum activity against Gram-positive and Gram-negative pathogens [21]. *Bacillus* endophytes were reported to produce lipopeptides such as surfactin and bacillomycin that

effectively inhibited *E. coli* and *S. aureus* [20][22]. In contrast, *Vibrio* spp. have been consistently described as metabolically diverse but poor producers of antibacterial metabolites, with limited or no activity against clinical pathogens [23].

Yohannan and Geetha, [24] activity observed chloroform extracts of *C. citratus* endophytes *Streptomyces* sp. and *Bacillus* sp. showed significant inhibition against *E. coli*, *K. pneumoniae*, and *S. aureus*. Interestingly, *Vibrio* sp. from *C. citratus* did not exhibit measurable inhibition, which is closely similar to the current study. In the present study, *Bacillus* sp. and *Streptomyces* sp., showed good antibacterial activity against *S. aureus* (MTCC3160), with the zone of inhibition ranging from 11 to 12 mm. Alam, [20] demonstrated that endophytic bacterial strains isolated from *C. citratus* leaves inhibited *S. aureus* with inhibition zones between 12–14 mm, highlighting their potential as sources of bioactive metabolites. Recently, several studies [25], [26] reported that endophytic bacterial isolates from *C. citratus* continued to show inhibition zones of 12–15 mm against *S. aureus.*

In this study, *Bacillus* sp. and *Streptomyces* sp., showed good antibacterial activity against *E. coli* (MTCC443), with the zone of inhibition 13 mm. Alam, [20] demonstrated that endophytic bacterial strains from *C. citratus* leaves inhibited *E. coli* with zones ranging from 12–15 mm. Additionally, Ramasamy et al., [27] reported that some *Streptomyces macrosporus* strains have shown significantly higher inhibition zones (up to 20 mm) against *E. coli.* In the present study, *Bacillus* sp. and *Streptomyces* sp., showed good antibacterial activity against *K. pneumoniae* (MTCC3384), with the zones of inhibition ranging from 14 to 15 mm. Similarly, a study by Radhakrishnan et al., [28] reported *Bacillus amyloliquefaciens* LPB-18 showed antibacterial activity against *K. pneumoniae* with a zone of inhibition of approximately 15 mm. Additionally, Hassan et al., [29] reported that *Streptomyces* sp. AS5 exhibited antimicrobial activity against *K. pneumoniae*, with inhibition zones reaching up to 18 mm.

5. Conclusion

In conclusion, the current study revealed that crude extracts of endophytic bacteria *Streptomyces* sp. (EB2) and *Bacillus* sp. (EB3) have potential antibacterial property. The bioactive compounds isolated from these bacterial strains would be useful for development of novel antibiotics. The future research will be focused on the identification of endophytic bacteria by 16S rRNA analysis and separation of the potential bioactive compounds from the selected bacterial extracts using chromatographic techniques and evaluation their potential against the bacterial pathogens.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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