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Antimicrobial activity of bacterial endophytes from Chirata (*Swertia chirata* Wall.) and Datura (*Datura stramonium* L.)

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Abstract

Background: Exploration of biocides in microbial system becomes increasingly interesting. Among the different microbial systems, the study of endophytes becomes an important field of research because of their agricultural as well as pharmaceutical importance. Bacterial endophytes from two medicinal plants from North Eastern states of India had been studied for their potential antibacterial and antifungal properties.

Results: Bacterial endophytes from two medicinal plant species abundantly available in North Eastern states of India, Chirata (*Swertia chirata*) and Datura (*Datura stramonium*), had been isolated and studied for their anti-bacterial and anti-fungal activities. Six bacterial endophytes were isolated from Chirata and 5 from Datura. All these bacterial isolates were initially isolated by observing their colony morphology, and then 9 morphologically distinct bacterial isolates were identified at molecular level by their 16s rRNA sequencing. Among the identified isolates, 5 were found to be belonged to genus *Staphylococcus*, 2 *Bacillus*, and one each belonged to *Rhodococcus* and *Cupriavidus*. Antibacterial study was carried out against 2 plant pathogenic bacteria, viz., *Xanthomonas oryzae* and *Ralstonia solanacearum*. Antifungal study was carried out against 2 plant pathogenic fungi, viz., *Fusarium oxysporum* and *F. solani*. Out of 6 bacterial isolates from Chirata, one showed antibacterial as well as antifungal properties, and another one showed antifungal properties only. Likewise, out of 5 bacterial isolates from Datura, one found to have both antibacterial and antifungal properties, and another one has only antifungal properties.

Conclusion: Few bacterial endophytes isolated from *S. chirata* and *D. stramonium* L., abundantly available in North Eastern part of India, had potential antibacterial as well as antifungal properties. This could be considered as the baseline information on potential antibacterial and antifungal agents from bacterial endophytes. Further investigations are required to assess their efficacy against several other bacterial and fungal diseases of economic importance.

Keywords: Endophytes, *Swertia chirata*, *Datura stramonium*, Antimicrobial activity

Background

A broad range of synthetic agents are widely used to balance the economic losses caused by pathogens in agriculture. Due to their effect on nature and the health risks, their application on a broader scale has been phasing out (Kim et al. 2017). Recently, there has been an

emerging attempt to explore nature-friendly compounds, which could substitute for chemically synthesized products. It is found that microorganisms associated with plants (endophytes) rather than plants themselves have proved to offer material and products with superior therapeutic potential (Guo et al. 2008). Endophytes are an increasingly important area of research in many fields because of their chemical diversity and their ability to produce many novel secondary metabolites that can be

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utilized for fuel, medicine, and agriculture. It is their chemical diversity that sparks profound interest in these microorganisms. Some of the antimicrobial compounds produced by endophytic microbes have the effectiveness against pathogens, which have developed resistances to antibiotics. Several strains from the *Ascomycota pestalotiopsis* have been shown to have a broad range of antimicrobial effects, even against methicillin-resistant *Staphylococcus aureus* (Rai et al. 2014). Many endophytes are members of common soil bacterial genera, such as *Pseudomonas*, *Burkholderia*, and *Bacillus* (Lodewyckx et al. 2002). These genera are well known for their diverse range of secondary metabolic products including antibiotics, anticancer compounds, volatile organic compounds, antifungal, antiviral, insecticidal, and immunosuppressant agents. While most of the research has been focused on fungal based production of antimicrobial products, a number of low-molecular-weight compounds found to be active at low concentrations against a range of human, animal, and plant pathogenic bacteria have been isolated from bacterial endophytes. One member of the plant-associated fluorescent *pseudomonads*, *P. viridiflava*, which was isolated on and within the tissues of many grass species (Miller et al. 1998) was found to produce 2 novel antimicrobial compounds called ecomycins.

Despite the many known benefits conferred by endophytes, conventional agricultural practices continue to take priority. Current agriculture relies heavily on fungicides and high levels of chemical fertilizers. The use of fungicides has a negative effect on endophytic fungi, and the used fertilizers reduce a plant's dependence on its endophytic symbiont (Le Cocq et al. 2017). In addition to the aforementioned benefits, the interest in use of bioinsecticides derived from endophytes is a potential area for phase away from inorganic pesticide-based cultivation system. As we have become more aware of the damage that synthetic insecticides cause to the environment, genotoxicity, and harm to beneficial insects, biological insecticides may become more important to the agricultural industry (Strobel, 2003). It has already been established that the endophytes of medicinal plants participate in biochemical pathways and produce analogous or novel bioactive compounds (Zhao et al. 2011). In this study, bacterial endophytes from 2 abundantly available folklore medicinal plant species from North East India, *Chirata* (*S. chirata*), and *Datura* (*D. stramonium*) were tested to explore their antibacterial as well as antifungal properties against few economically important pathogenic bacteria, viz., *Xanthomonas oryzae* and *Ralstonia solanacearum*, and fungi, viz., *Fusarium oxysporum* and *F. solani*.

Methods

Sample collection and cultivation

Seeds of *S. chirata* and *D. stramonium* were collected from Gossaigaon, Kokrajhar (26.4371° N, 89.9767° E),

and Bijoyagar, Jorhat (26.7509° N, 94.2037° E) of Assam, respectively. Subsequently, they were grown in the experimental field for further study.

Isolation of endophytes

Bacterial endophytes were isolated at flowering stages of both the plant species. After proper surface sterilization as described by Devi et al. (2017), the shoots (5 g) and roots (1 g) of the both the plant samples were weighed and grinded using mortar and pestle, separately. The extracts were then centrifuged at 10,000 rpm for 10–20 min. The aliquots were then spread over nutrient agar plate amended with fungicide, cycloheximide. Nutrient agar plates prepared were incubated at 37 °C for 24–48 h. Colonies with different morpho-types were selected and purified for further study.

Mass multiplication of endophytic isolates

Pure cultures of endophytic bacteria were fermented at shake-flask level in 1 L Erlenmeyer flasks that contained 600 ml nutrient broth. Bacterial culture for each bacterial isolate was grown at 28 °C and 200 rpm in a mechanical shaker incubator, and growth curve was drawn to know the best time of harvest for secondary metabolites. The point just after entering the stationary phase was considered the best harvest time.

Molecular analysis of endophytic bacterial isolates

Genomic DNA isolation was done by using the modified method of Ligozzi and Fontana (2003). Amplification of 16S rRNA gene was performed using the 27 F-AGAG TTTGATCCTGGCTCAG forward and 1492 R-GGTT ACCTTGTTACGACTT reverse primers. The PCR was carried out with an initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 10 min. The amplified products of the isolates were sequenced using 454 automated Pyrosequencer (AgriGenome, Kerala, India). The sequenced 16S rRNA genes from both the ends were further pre-processed and assembled to a consensus sequence using Codon Code Aligner version 4.1(Codon Code Corporation, USA).

Phylogenetic analysis of 16S rRNA

Phylogenetic analysis of 16S rRNA gene sequences for each bacterial strain along with their closest relatives was performed using the Kimura 2-parameters method incorporated in MEGA version 5.1.0 by NJ method to assign each bacterial isolate in their respective taxonomic position (Tamura et al. 2007). The 16S rRNA gene sequences of the 9 different strains were submitted to GenBank of NCBI through sequence submission tool, Bank. It was where they were assigned GenBank accession numbers. In addition, the strains identified were

taken together for construction of phylogenetic tree using NJ method incorporated in MEGA.

Culture of plant pathogenic bacteria and fungi

Two bacterial plant pathogenic species, *Xanthomonas oryzae* and *Ralstonia solanacearum*, and two plant pathogenic fungal species, *Fusarium oxysporum* and *F. solani*, were collected from the Plant Pathology Department of Assam Agricultural University, Jorhat, Assam.

Screening of bacterial endophytes for their antimicrobial activities

The isolated bacterial endophytes were screened for their antimicrobial activity using agar well diffusion method with few modifications (Schillinger and Lücke, 1989) against plant pathogenic bacteria and with modified dual culture method (Gkarmiri et al. 2015) against fungal plant pathogens. For antimicrobial assay, 3 replications were taken and mean value was reported. The bacterial phytopathogens used were *X. oryzae* and *R. solanacearum*, and the fungal phytopathogens were *F. oxysporum* and *F. solani*. The bacterial endophyte isolates were grown in NA broth for 48 h, at 28 °C and 200 rpm in a mechanical shaker incubator, and then centrifuged at 10,000 rpm for 10 min. The supernatant obtained was filtered through micro-filter (0.22 µm pore size). The filtrate obtained was used for the determination of antimicrobial activity. To assay antibacterial activity, 500 µl of active broth cultures (overnight grown) of phytopathogenic bacteria, *X. oryzae*, and *R. solanacearum* were transferred to the NA agar Petri plates, separately and spread uniformly with a glass spreader. Four wells with 7-mm diameter were made in each of these plates using sterile gel puncher. Out of the 4, 2 wells were used for test samples, one was used as a positive control, and the other was used as a negative control. An aliquot of 200 µl of the sample was loaded into the wells of the plates. In the case of positive control, instead of the sample, 200 µl streptomycin was used (50 µg/ml), and in the negative control, the same amount of sterile water was used. All the plates were then kept in incubation at 30 ± 2 °C. After 24 h of incubation, plates were observed for formation of clear zone of inhibition around the well and measured. To evaluate antifungal activity, a loop-full of active fungal culture was placed on one edge of the sterile plates containing PDA media. One well with 7-mm diameter was made in the center of the plate using sterile gel puncher. An aliquot of 200 µl of the sample was loaded into the well of the plate. In place of test sample, 200 µl cycloheximide (50 µg/ml) was used in the positive control and the same amount of sterile water was used in the negative control. Plates were then incubated at 30 ± 2 °C and observed for formation of zones of inhibition every 24 h for 6 days.

Results

Isolation of endophytes from the plant samples

In the present study, a total of 11 endophytic bacterial isolates was isolated, 6 from Chirata and 5 from Datura at their flowering stages, and studied for their antimicrobial activity. Out of the 6 bacterial isolates obtained from Chirata, 2 were from roots, coded as CS1 and CS2, and 4 were from shoots coded as CS3, CS4, CS5, and CS6. The rest 5 bacterial endophytes were isolated from Datura. Among the 5 bacterial endophytes, 3 were from shoots, coded as DS1, DS2, and DS3, and 2 were from roots, coded as DS4 and DS5. Colony morphology, especially the shape and color of the colonies of all the bacterial isolates, was studied. Some bacterial colonies were found to be white, round, and smooth, while some others were creamy white and circular (Table 1). In both plant systems, more numbers of bacterial endophytes were found in shoots than in roots.

Identification of the endophytic isolates

Analysis of total base nucleotide of 16S rRNA gene sequences was done by multiple alignments using neighbor-joining method of MEGA 6 tool with 1000 bootstrap value. From the BLAST analysis of the sequence reads, the nine isolates were found to be from four different groups based on sequences similarities (Fig. 1).

Out of the 9 sequenced isolates, 5 were identified as belonging to *Staphylococcus* species (98% identity), 2 to *Bacillus* species (99% identity), one to *Rhodococcus*, and the rest one showed similarity with *Cupriavidas* as highly similar sequences tend to lie in the same group (Table 2).

In the present study, the *Staphylococcus* spp. were placed in 2 different groups, which indicated that they belong to the same species, but may be different in strain level. Similarly, *B. mycoides* and *B. subtilis* were placed at the same group, due to their sequence similarities. The isolates *R. jialingiae* and *C. metalidurans* were

Table 1 Morphological characterization of isolated endophytic bacteria

Code no.	Source of isolation	Colony morphology
CS1	Chirata, root	Whitish, circular
CS2	Chirata, root	Irregular, creamy white
CS3	Chirata, shoot	Large colonies, opaque, round
CS4	Chirata, shoot	Curled, round
CS5	Chirata, shoot	Small colonies, round
CS6	Chirata, shoot	Irregular, creamy, opaque
DS1	Datura, shoot	Yellowish in color, opaque
DS2	Datura, shoot	Very small colonies, transparent
DS3	Datura, shoot	Yellowish, circular
DS4	Datura, root	Creamish, irregular in shape
DS5	Datura, root	Small, circular

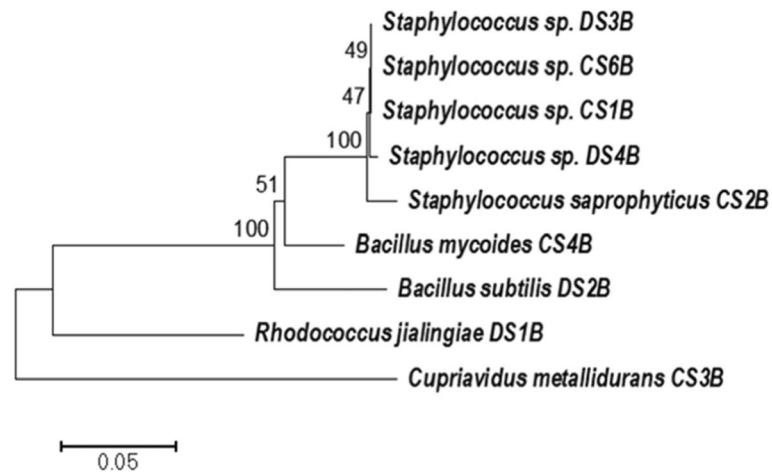


Fig. 1 Phylogenetic tree based on 16S rRNA sequences of endophytic bacteria

distantly related to the genera *Staphylococcus* and *Bacillus* and were placed in different groups (Fig. 1).

Anti-bacterial activity of bacterial endophytes

In this study, the antibacterial activity of bacterial endophytes against 2 plant pathogenic-bacterial species, i.e., *X. oryzae* and *R. solanacearum*, was evaluated. Antibacterial activity of the bacterial isolates was investigated by agar well diffusion method. Among the 6 bacterial isolates, isolated from Chirata, the CS1 (*Staphylococcus* sp.) and the CS4 (*Bacillus* sp.) inhibited the growth of *X. oryzae* and *R. solanacearum*, respectively (Fig. 2a, b). Similarly, the 2 bacterial isolates from Datura, the DS5 (unidentified) and the DS1 (*Rhodococcus jialingiae*), showed antibacterial activity against *X. oryzae* and *R. solanacearum*, respectively (Fig. 2c, d).

Antifungal activity of bacterial endophytes

In this study, the antifungal activity of bacterial endophytes was evaluated against 2 plant pathogenic-fungal species, i.e., *F. oxysporum* and *F. solani*. Antifungal activity of the bacterial isolates was investigated by modified

dual culture method. Among the 6 bacterial isolates from Chirata, only CS1 (*Staphylococcus* sp.) inhibited the growth of *F. oxysporum* (Fig. 3a). Similarly, only one bacterial isolate from Datura, DS5 (unidentified), showed antifungal against *F. solani* (Fig. 3b).

Discussion

The objective of the present investigation was to isolate and evaluate the antibacterial activities of endophytic bacteria from 2 important medicinal plants species, Chirata (*S. chirata*) and Datura (*D. stramonium*). All total of 11 bacterial endophytes were isolated from the shoots and roots of both plant species at their flowering stages, and their morphological as well as molecular characterization were carried out. In both the plant systems, more numbers of bacterial endophytes were found in shoots as compared to roots, which indicated that shoots may provide more favorable environment to endophytic bacteria than root. Flowering stage was considered because, reportedly, the root rhizospheric endophytes entered into the plant at vegetative stage, and their population is going to be increased and get

Table 2 Nucleotide nBLAST results of the endophytic isolates

Sl. no.	Sample	Result	Identification (%)	Accession no.
1.	CS1	<i>Staphylococcus</i> sp.	98	KT922162
2.	CS2	<i>S. saprophyticus</i>	98	KT922167
3.	CS3	<i>Cupriavidus metallidurans</i>	96	MH348792
4.	CS4	<i>Bacillus mycoides</i>	99	MG205997
5.	CS6	<i>Staphylococcus</i> sp.	98	MK120439
6.	DS1	<i>Rhodococcus jialingiae</i>	98	LT160790
7.	DS2	<i>B. subtilis</i>	99	KM008606
8.	DS3	<i>Staphylococcus</i> sp.	99	MK256328
9.	DS4	<i>Staphylococcus</i> sp.	97	MK256328

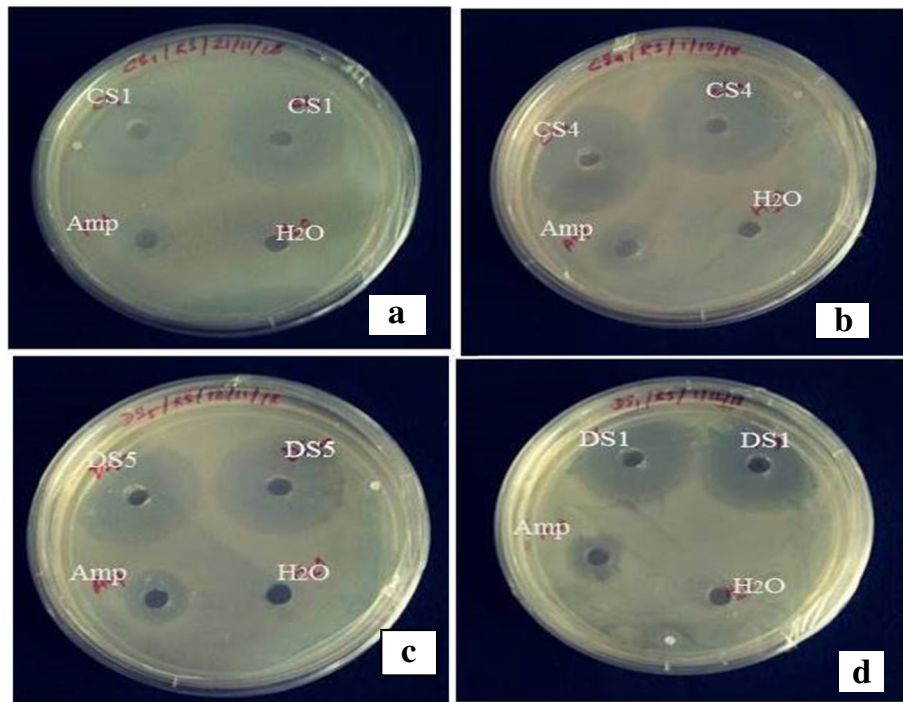


Fig. 2 Agar well diffusion assay of bacterial endophytes against plant pathogenic bacteria. **a** CS1 isolated from Chirata against bacterial pathogen (*X. oryzae*). **b** CS4 isolated from Chirata against bacterial pathogen (*R. solanacearum*). **c** DS5 isolated from Datura against bacterial pathogen (*X. oryzae*). **d** DS1 isolated from Datura against bacterial pathogen (*R. solanacearum*)

maximum number at flowering stage, and again started declined at maturity stage, partially due to factors like nutrient deficiency, water stress, and free radical formation (Marag and Suman 2018). Colony morphology, especially the shape and color of the colonies of all the bacterial isolates, was studied. Some bacterial colonies were found to be white, round, and smooth, while some colonies were creamy white and circular, which indicated their morphological diversities. From the molecular characterization results, a high species-specific

homogeneity was observed when 16S rRNA from different strains and different species were compared. Most of the sequenced isolates were identified as *Staphylococcus* species while; few of them identified as *Bacillus Rhodococcus* and *Cupriavidas* species. A similar study was undertaken by Bredow et al. (2015) on in silico analysis of 16S rRNA gene of endophytic bacteria isolated from the aerial parts and seeds of agricultural crops, and most of the isolates they isolated were identified to be genus, *Bacillus*. Another study was carried out by Liaqat and

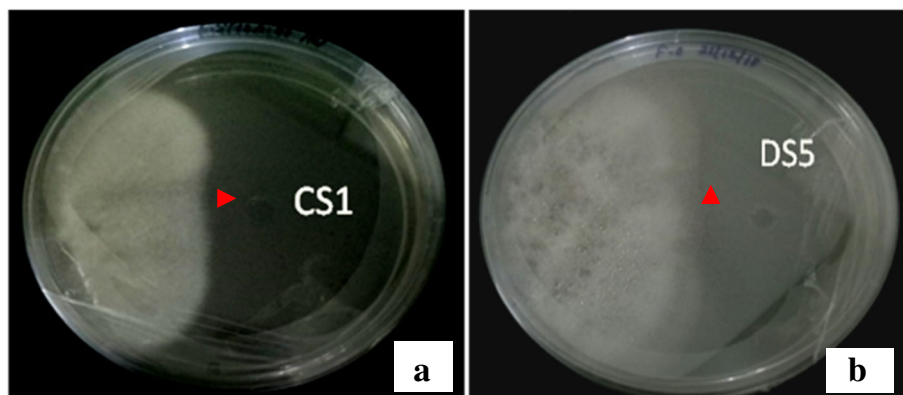


Fig. 3 Dual culture assay of antifungal activity of bacterial endophytes (arrows indicate wells). **a** CS1 isolated from Chirata against fungal pathogen (*F. oxysporum*). **b** DS5 isolated from Datura against fungal pathogen (*F. solani*)

Eltem (2016) on identification and characterization of endophytic bacteria isolated from in vitro cultures of peach and pear rootstocks, and they reported that most of isolates were belonged to the genus, *Staphylococcus*. Although the 16S rRNA sequenced analysis was considered very good at identifying microorganisms at genus and species level, however, it was unable to differentiate at the sub species and strain level, and was therefore not an appropriate method to measure intra-species relationships (Stackebrandt and Goebel 1994). However, a large number of endophyte has been reported to be remaining unidentified due to their uncultivable nature (Schulz and Boyle 2005).

Isolated bacterial endophytes were finally screened for their antibacterial as well as antifungal activities, against few plant pathogenic bacteria and fungi, separately. Their antibacterial activity was studied against *X. oryzae* and *R. solanacearum* by agar well diffusion method. Out of the 11 bacterial isolates, only 2 bacteria isolates viz., CS1 (*Staphylococcus* sp.) and DS5 (unidentified), showed antibacterial activity against *X. oryzae*, while another 2 bacterial isolates viz., CS4 (*Bacillus* sp.) and DS1 (*Rhodococcus jialingiae*), showed antibacterial activity against *R. solanacearum*. The antifungal activity of bacterial endophytes was evaluated against 2 plant pathogenic-fungal species, i.e., *F. oxysporum* and *F. solani*, by modified dual culture method. Out of the 11 bacterial isolate, only 2 isolates showed antifungal activity. One isolate, CS1 (*Staphylococcus* sp.) showed antifungal activity against *F. oxysporum*, and the other, DS5 (unidentified) showed antifungal activity against *F. solani*. From both antibacterial and antifungal study, it was observed that the isolate, CS1 (*Staphylococcus* sp.) and DS5 (unidentified), had both antibacterial as well as antifungal properties. So, the results indicated the strain-specific nature of the endophytic bacterial isolates for their antimicrobial activity against these 4 plant pathogens. The endophytic bacterial isolates showing antimicrobial activity might have the metabolite(s) having antibiotic properties. Chen et al. (2019) isolated *Bacillus* endophyte from peanut root and showed that it produced antimicrobial compounds with inhibitory effects against *Aspergillus flavus* and *R. solanacearum*. In another study, Morare et al. (2018) isolated bacterial endophytes from the traditional medicinal plant *Crinum macowanii* Baker belonging to genera *Staphylococcus*, *Bacillus*, and *Acinetobacter* exhibited antibacterial activity against 5 bacterial pathogens viz., *Escherichia coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *S. aureus*, and *B. cereus*. Beiranvand et al. (2017) also reported that bacterial endophytes isolated from medicinal plants exhibited antimicrobial activity against some bacterial pathogens. Similar research works had been reported where endophytic microbial strains were isolated from medicinal plants and assayed for antimicrobial activities (Khan et al. 2017).

Conclusion

From this study, it was found that the bacterial endophytes isolated from the 2 medicinal plants, *S. chirata* and *D. stramonium*, could suppress the growth of bacterial as well as fungal phytopathogens. In order to understand the symbiotic role of these endophytes in the host plants, further studies are necessary to identify the active compounds produced by them inside the host systems, using analytical chemistry. Such compounds can be used against fungal phytopathogens as biocontrol agents.

Abbreviations

NJ: Neighbor joining; MEGA: Molecular evolutionary genetics analysis; NA: Nutrient agar; PDA: Potato dextrose agar

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Authors' contributions

All authors contributed to this research and preparation of manuscript. TN, BKS, and RD conceptualized and designed this research. The research was carried out by RD. TN, RD, and RRB analyzed the data and result. RD drafted the first version of the manuscript. RD, TN, RR B, BD, PKN, and PD revised and finalized the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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