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Preliminary evaluation of the antifouling potential of Halimeda gracilis extracts through antibacterial screening of marine biofilm-forming bacteria

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ABSTRACT

This study evaluated the antibacterial and preliminary antifouling potential of extracts from the green seaweed *Halimeda gracilis* against marine biofilm-forming bacteria. Biofilm-forming bacteria were isolated from plastic panels submerged in the Vellar estuary, Tamil Nadu, India. Four biofilm-forming bacterial strains were identified through biochemical and morphological characterization: *Vibrio* sp., *Staphylococcus* sp., *Pseudomonas* sp., and *Escherichia coli*. Maximum inhibition was shown by the ethyl acetate extract, with inhibition zones of 22 mm against *Vibrio sp*., 24 mm against *E. coli*, 29 mm against *Staphylococcus sp*., and 24 mm against *Pseudomonas sp*. at 100 µg/ml concentration. Overall, results from this study provide evidence to suggest that *H. gracilis* possesses bioactive compounds with antifouling activity, specifically with activity demonstrated in the semi-polar fraction, which could be developed for use as a potential sustainable alternative to conventional toxic antifouling agents. These results are part of ongoing research into finding green solutions for marine biofouling.

1. Introduction

Marine biofouling, which is the undesirable deposition of microorganisms, flora, and fauna onto submerged man-made structures, is one of the most significant threats facing maritime industries globally (Demirel et al., 2022; Hadžić et al. 2022). This phenomenon results in considerable economic losses for shipping companies due to extended fuel consumption; increased engine stress; increased maintenance frequency. The transport of invasive species on ship hulls and ballast tanks also pose a major ecological problem. Bacterial adhesion occurs through structures like fimbriae, pili, and flagella, in addition to extracellular polymeric substances (EPS), and acts like a bridge between bacteria and the conditioning film(Bajire et al. 2023; Garibay-Valdez et al. 2023; Uc-Peraza, Castro, and Fillmann, 2022). Traditionally, antifouling surfaces have depended solely on toxic biocides, especially tributyltin (TBT)-based compounds that proved highly effective in repressing marine growth (Uc-Peraza et al., 2022). However, these compounds have been banned by the International Maritime Organization due to their severe environmental impacts, including shell thickening in oysters, endocrine disruption in various invertebrates, and biomagnification throughout marine food webs(Cima and Varello, 2022; Hossain, Hossain, and Jahan, 2022; Jasim et al. 2022; Tan, 2023). Post-TBT, copper-based paints supplemented with "booster" biocides became prevalent, but concerns regarding their environmental persistence and toxicity have also emerged. The search for environmentally benign antifouling solutions has led researchers to investigate natural products from various marine organisms, including sponges, corals, seagrasses, and seaweeds, which have evolved chemical defense mechanisms against epibiosis. Seaweeds represent particularly promising candidates due to their accessibility, diversity, and rich complement of secondary metabolites (Lomartire and Gonçalves, 2022; Manikandan and Vivek, 2024; Nwuzor et al. 2021). *Halimeda gracilis*, a green seaweed belonging to the Halimedaceae family, is characterized by segmented and calcified thalli composed of 60–80 % aragonite with non-calcified nodes separating the segments. It has shown a significant bioactive potential in earlier studies since those extracts exhibited antioxidant, antibacterial,

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antifungal and antidiabetic activities. However, its antifouling performance has been less investigated. In this study, we have investigated the antifouling potential of the *H. gracilis* extracts against marine fouling bacteria isolated from biofilms of contaminants on artificial substratum(Aziman et al. 2021; Jia et al. 2024; Sampaio et al. 2022; Vinothini et al. 2023). The aims of the present study were: (1) to isolate and identify biofilm-forming bacteria from immersed panels, and (2) to evaluate the antibacterial activity of various solvent extracts from *H. gracilis* against these isolates. These findings add to the growing body of knowledge on green antifouling strategies based on naturally derived compounds.

2. Materials and methods

2.1. Study area and panel deployment

The study was conducted in the Vellar estuary (Lat. 11°29'N; Long. 79°46'E) at Parangipettai, Tamil Nadu, India. This estuary has a permanent connection to the Bay of Bengal and experiences semi-diurnal tides with a maximum amplitude of approximately one meter. Plastic panels (polyethylene terephthalate) were deployed at a depth of one meter and retrieved at intervals of 24 h and weekly over a 4-week period following established protocols (Mishra et al., 2022). Retrieved panels were transported to the laboratory in sterile containers with water from the collection site maintained at ambient temperature.

2.2. Isolation and enumeration of biofilm bacteria

Biofilm material was collected from the panels using sterile cotton swabs and immediately transferred to tubes containing nutrient broth. After 24 h of incubation at room temperature (28 ± 2°C), the bacterial suspensions were serially diluted (10^{-1} to 10^{-6}) with sterile seawater. Aliquots (0.1 ml) from appropriate dilutions were spread in triplicate on Zobell Marine Agar (ZMA) plates and incubated at 28 ± 2°C for 24 h (Ismaila et al. 2025; Rehman, Vrouwenvelder, and Saikaly 2021; Sukrri et al. 2024; Waheed et al. 2022). Total heterotrophic bacterial counts were recorded as colony-forming units per milliliter (CFU/ml).

2.3. Identification of bacterial isolates

The colonies were characterized morphologically and selected for, and further sub-cultured onto selective and differential media such as Mannitol Salt Agar (MSA), Eosin Methylene Blue Agar (EMB), MacConkey Agar (MCA), Pseudomonas Isolation Agar (PIA), and Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS). The inoculated plates were kept at 37°C for 24–48 h. Bacterial isolates were identified by observing colony morphology, Gram reaction and biochemical characteristics according to conventional methods(Liu et al. 2025; Ruhal and Kataria, 2021). Although the bacterial isolates originated from a marine environment, incubation was carried out at 37°C following standard microbiological protocols to ensure robust and rapid growth on selective and differential media. This temperature was chosen for consistency in morphological and biochemical identification, as many marine heterotrophic bacteria exhibit facultative growth at a wide temperature range, including 37°C, even though the natural seawater temperature is lower.

2.4. Detection of biofilm-forming ability

Bacterial isolates were examined for their ability to form biofilm using the Congo Red Agar (CRA) method (Al-yozbakee, 2024). The composition of the medium was as follows: brain heart infusion broth (37 g/L), sucrose (50 g/L), agar (10 g/L), and Congo red stain (0.8 g/L). Congo red was prepared as a 0.8 g/100 ml, concentrated aqueous solution, autoclaved individually, and added to the agar when it cooled to 55°C. Test organisms were inoculated onto the plates, which were then incubated aerobically for 24–48 h at 37°C. Biofilm production was indicated by black colonies with a dry crystalline consistency, while non-producers remained pink (Abdulkadir et al. 2024). Intermediate biofilm producers displayed darkening of colonies without crisp dry crystalline features (Garibay-Valdez et al. 2023).

2.5. Collection and extraction of seaweed

Halimeda gracilis was collected from Vedalai, Palk Bay, Ramanathapuram district, Tamil Nadu, India *(9°13′10.44″N, 79°6′30.71″E)*. A voucher specimen of *Halimeda gracilis* has been deposited in the Herbarium of the Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu, India, under accession number CASMB–HG–2024–01. The seaweed was thoroughly washed with seawater to remove epiphytes and debris, followed by freshwater rinses and final washing with distilled water. The cleaned material was shade-dried for 7–10 days, ground to a fine powder, and stored in airtight containers until further use. The powdered seaweed (20 g) was extracted using a Soxhlet apparatus with 200 ml of either pure ethyl acetate or chloroform (1:10 w/v; seaweed powder to solvent) for 8 h at 40–50°C. The resulting extracts were concentrated under reduced pressure using a rotary evaporator (Buchi R-210, Switzerland) at 40°C, dried completely, and stored at 4°C in airtight containers until further use (Muzaki, 2022; Sukrri et al. 2024). The extraction yields were calculated as the weight of dried extract relative to the initial dry weight of seaweed powder. The yields obtained were 5.8 % (w/w) for the ethyl acetate extract and 3.1 % (w/w) for the chloroform extract. The solvents were chosen based on their polarity range: ethyl acetate (semi-polar) was expected to extract phenolics, terpenoids, and fatty acids, whereas chloroform (non-polar) primarily targets lipophilic compounds. This selection allowed comparative assessment of polarity-dependent extraction efficiency and bioactivity.

2.6. Antibacterial activity assay

The antibacterial activity of the extracts was evaluated using the agar well diffusion method (Morgan et al. 2023; Quitério et al. 2022). Test bacteria were grown in nutrient broth for 24 h at 37 °C, and 100 µl of each culture (adjusted to 0.5 McFarland standard) was spread on Mueller-Hinton agar plates. Wells (6 mm diameter) were punched in the agar with a sterile cork borer, and 100 µl of each extract at various concentrations (25, 50, 75, and 100 µg/ml dissolved in 5 % DMSO) were placed in separate wells. Negative control consisted of DMSO (5 %). The plates were placed in 37°C incubator for 24 h and the zones of inhibition were measured in mm. The results were expressed as mean ± standard deviation, and all tests were conducted in triplicate.

2.7. Statistical analysis

Data were analyzed using IBM SPSS Statistics Version 26.0. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine significant differences between treatment groups. P-values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Total heterotrophic bacterial count

The density of total heterotrophic bacteria on the submerged panels ranged from 1.3×10^2 to 8.0×10^2 CFU/ml during the study period. The bacterial density showed a progressive increase over time, likely due to the maturation of the biofilm and increasing surface conditioning. Similar patterns of bacterial colonization on artificial substrates have been reported in previous studies (Salta et al., 2013; Satheesh et al., 2016), reflecting the primary stage of biofouling succession Figs. 1–4.

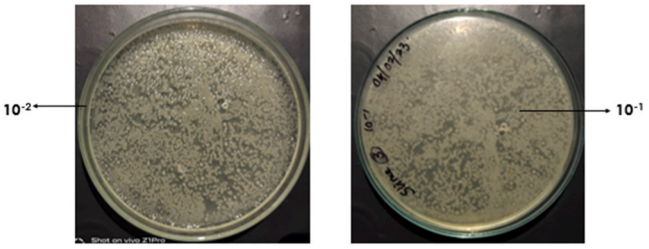

Fig. 1. Heterotrophic bacterial count showing colonies on agar plates with different dilutions. Left plate represents a 10^-2 dilution, while the right plate shows a 10^-1 dilution.

3.2. Identification of bacterial isolates

Based on growth characteristics on selective and differential media, morphological features, and biochemical tests (Table 1), four predominant bacterial isolates were identified: *Vibrio* sp., *Staphylococcus* sp., *Pseudomonas* sp., and *Escherichia coli*. These bacteria are commonly associated with marine biofilms and have been reported as primary colonizers in various marine environments (Dobretsov et al., 2013; Flemming and Wuertz, 2019).

3.3. Biofilm formation ability

The Congo Red Agar method identified two strong and two intermediate biofilm producers among the bacterial isolates (Table 2). *Staphylococcus* sp. and *Vibrio* sp. demonstrated strong biofilm-forming capabilities, characterized by black, dry crystalline colonies, while *Pseudomonas* sp. and *E. coli* showed intermediate biofilm formation, indicated by darkened colonies without the crystalline morphology. This variability in biofilm formation reflects differences in extracellular polymeric substance production among the isolates (Oliveira et al., 2010; Mizan et al., 2018).

3.4. Antibacterial activity of seaweed extracts

The ethyl acetate extract of *H. gracilis* exhibited significant antibacterial activity against all four biofilm-forming bacterial isolates, with inhibition zones increasing in a concentration-dependent manner. At the highest concentration tested (100 µg/ml), the extract showed maximum inhibition zones of 22 mm against *Vibrio* sp., 24 mm against *E. coli*, 29 mm against *Staphylococcus* sp., and 24 mm against *Pseudomonas* sp. In contrast, the chloroform extract showed no inhibitory activity against any of the test bacteria at all concentrations tested. The superior antibacterial activity of the ethyl acetate extract compared to the chloroform extract suggests that the bioactive compounds in *H. gracilis* responsible for antibacterial effects are predominantly semi-polar in nature. This observation aligns with findings from previous studies on other marine organisms, where semi-polar fractions demonstrated higher antimicrobial potency. The varying sensitivity towards ethyl acetate extract in bacterial isolates are *Staphylococcus sp*. highest sensitivity) can be explained by differences in cell wall structure and membrane composition between Gram-positive and Gram-negative bacteria(Cotas et al. 2024; Oliva et al. 2023; Romeu and Mergulhão, 2023). Statistical analysis showed that the antibacterial activity of various concentrations of the ethyl acetate extract was significantly different on all the bacterial isolates ($p < 0.05$). Post-hoc comparisons showed that all test bacteria had greater inhibition zones at 100 µg/ml than at lower concentrations ($P < 0.001$), thus verifying that the antibacterial effect is dose-dependent.

4. Discussion

Marine biofouling begins with the attachment and colonization of microorganisms on submerged surfaces, forming biofilms that facilitate the settlement of larger organisms. The bacterial isolates identified in this study, *Vibrio sp*., *Staphylococcus sp*., *Pseudomonas sp*., and *E. coli* are known as primary colonizers of marine environments and play a pivotal role in initiating fouling layers. Their confirmed ability to form biofilms, as demonstrated using the Congo Red Agar assay, supports their ecological relevance in the fouling process. (Budzałek et al. 2021; Guérin, Kulakauskas, and Chapot-Chartier, 2022; Suresh and Immanuel, 2023). The ethyl acetate extract of *Halimeda gracilis* exhibited strong antibacterial effects against all four biofilm-forming bacteria, suggesting the presence of bioactive secondary metabolites with antifouling potential. The absence of activity in the chloroform extract indicates that the active compounds are likely semi-polar. This observation aligns with prior studies where semi-polar fractions of seaweeds yielded higher antimicrobial potency due to the presence of compounds such as terpenoids, phenolics, and fatty acids. The higher yield and greater antibacterial activity observed in the ethyl acetate extract compared with the chloroform extract further support this polarity-driven extraction pattern, suggesting that most bioactive constituents in *Halimeda gracilis* are associated with the semi-polar fraction. Members of the *Halimeda* genus are particularly known for diterpenes and triterpenes, which may contribute to ecological defence mechanisms against microbial colonization and epibiosis.

Environmental factors, particularly temperature, can influence the antibacterial efficacy of seaweed extracts by affecting both bacterial metabolism and the stability of bioactive metabolites. The present study was conducted at a mean ambient temperature of $28 \pm 2°C$, which

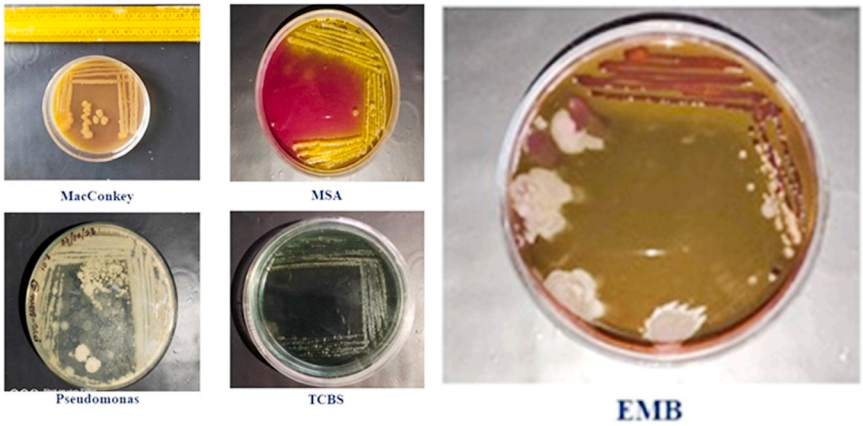

Fig. 2. Growth of bacterial isolates on selective media: MacConkey, MSA, Pseudomonas, TCBS, and EMB agar plates. Each plate shows distinct growth characteristics specific to different bacterial species.

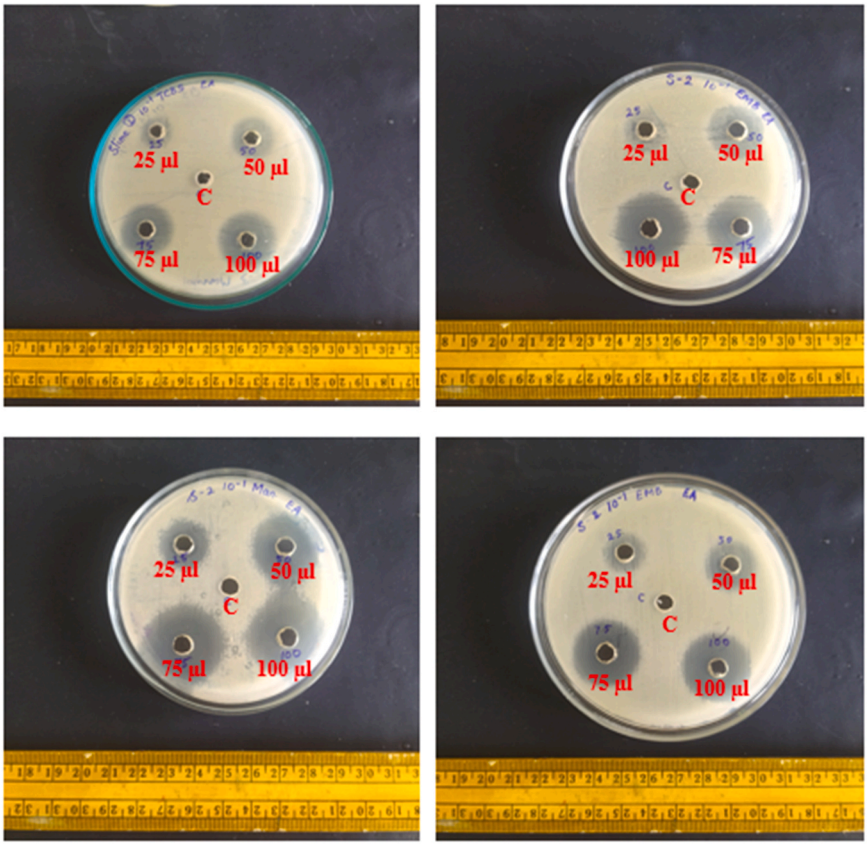

Fig. 3. Antifouling activities of the ethyl acetate extract of *Halimeda gracilis* against fouling bacteria. Different concentrations (25 µL, 50 µL, 75 µL, 100 µL) were tested on bacterial growth inhibition.

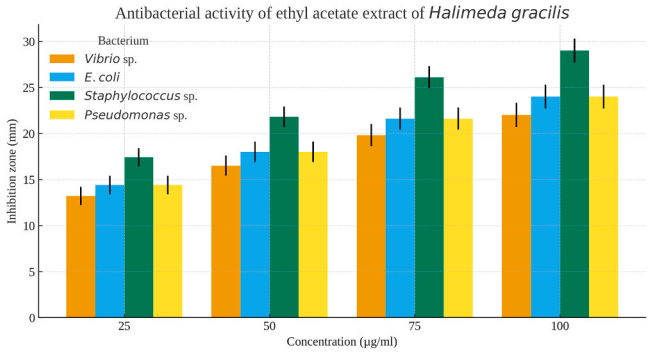

Fig. 4. Antibacterial activity of ethyl acetate extract of *Halimeda gracilis* against marine biofilm-forming bacteria. Data represent mean ± SD (n = 3). Significant differences between concentrations were determined by one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$, $p < 0.001$).

closely represents the natural conditions of the Palk Bay region (Lat. 11°29′N; Long. 79°46′E). Previous studies have shown that elevated temperatures may enhance the diffusion rate and reaction kinetics of secondary metabolites, thereby modulating antibacterial potency (Cotas et al., 2024). Therefore, the observed activity of *Halimeda gracilis* under these conditions likely reflects its ecological adaptation to tropical marine environments.

The significantly higher sensitivity observed in *Staphylococcus sp.* compared with the Gram-negative bacteria corresponds with structural differences in the bacterial cell envelope. Gram-positive bacteria, lacking an outer membrane, are generally more permeable to lipophilic and semi-polar compounds. This selective susceptibility pattern is consistent with other marine-derived natural extracts and reinforces the potential role of *H. gracilis* metabolites as natural antifouling agents targeting early bacterial colonizers.

The concentration-dependent antibacterial response observed in this study indicates that the extract maintains efficacy even at lower concentrations, suggesting its suitability for incorporation into coating formulations. When integrated within controlled-release antifouling systems, such natural products could minimize environmental toxicity while maintaining long-term bioactivity. Compared to synthetic booster biocides, seaweed-derived metabolites typically degrade faster and exhibit minimal bioaccumulation, supporting their use as sustainable alternatives in marine coatings.

Although this study focused primarily on antibacterial screening, the results provide a strong foundation for comprehensive antifouling investigations. Future research will explore the inhibition of biofilm maturation and extracellular polymeric substance (EPS) production, field-based assays assessing fouling settlement, and the stability of *H. gracilis* extracts within paint matrices. Chemical characterization (GC-MS, LC-MS, and FTIR analyses) and isolation of the active constituents will also help elucidate their mechanisms of action and enable potential structural optimization for enhanced stability and potency. Incorporating microscopic visualization techniques such as CLSM and SEM to confirm the disruption and surface detachment of bacterial biofilms following treatment with *Halimeda gracilis* extracts will strengthen the findings. Although the current study was limited to antibacterial screening, subsequent research will incorporate macrofouler assays, including barnacle cyprid settlement, mussel attachment inhibition, and Ulva spore germination tests, to validate the broad-spectrum antifouling potential of *H. gracilis* under realistic marine conditions.

Table 1
Morphological and biochemical characteristics of biofilm-forming bacterial isolates obtained from submerged panels.

Isolate code	Identified species	Colony morphology on ZMA	Gram reaction	Catalase	Oxidase	Indole	Citrate	Biofilm formation (CRA)
P1	*Vibrio sp.*	Cream, circular, convex colonies	–	+	+	+	+	Strong (black, dry crystalline colonies)
P2	*Staphylococcus sp.*	Golden yellow, circular, smooth	+	+	–	–	+	Strong (black, dry crystalline colonies)
P3	*Pseudomonas sp.*	Pale green, irregular, spreading colonies	–	+	+	–	+	Intermediate (darkened colonies without crystalline surface)
P4	*Escherichia coli*	Off-white, round, raised colonies	–	+	–	+	–	Intermediate (darkened, non-crystalline colonies)

Table 2
Biofilm formation and colony morphology of bacterial isolates. Strong biofilm formation was observed with dry black colonies (*Staphylococcus sp., Vibrio sp.*), intermediate with smooth black colonies (*Pseudomonas sp., E. coli*), and no biofilm with red/pink colonies (no isolates identified).

Biofilm Formation	Colony Morphology	Number of Isolates	Identified Species
Strong	Dry black	2	*Staphylococcus* sp., *Vibrio* sp.
Intermediate	Smooth black	2	*Pseudomonas* sp., *E. coli*
Negative	Red/Pink	0	None

5. Conclusion

The ethyl acetate extract of *Halimeda gracilis* demonstrated significant antibacterial activity against key marine biofilm-forming bacteria, including Staphylococcus sp., Pseudomonas sp., Vibrio sp., and Escherichia coli. The inhibition was concentration dependent, with the greatest effect observed at 100 μg/ml. The lack of activity in the chloroform extract indicates that the active metabolites are predominantly semi-polar in nature.

These results highlight the potential of *H. gracilis* as a promising source of environmentally benign antifouling agents capable of targeting the early stages of biofilm development. By inhibiting the colonization of pioneer bacteria, such extracts could reduce subsequent macrofouling and serve as sustainable alternatives to conventional toxic biocides currently used in marine coatings.

Future studies should extend this preliminary screening by incorporating biofilm inhibition and EPS reduction assays, followed by macrofouler settlement experiments and field trials under natural conditions. Detailed phytochemical analysis and structural characterization of active compounds will also be essential to understand their mechanisms of action and optimize their integration into marine antifouling formulations.

The findings from this work contribute to the growing evidence that marine seaweeds, particularly *Halimeda* species, represent a valuable natural resource for the development of next-generation, eco-friendly antifouling solutions that support both maritime efficiency and marine environmental health.

CRediT authorship contribution statement

Yuvaraj Dinakarkumar: Writing – original draft, Validation. **Shamitha Jayakumar:** Investigation, Data curation. **S. Bragadeeswaran:** Supervision, Conceptualization. **Panneerselvam Theivendren:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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