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Research

Identification of Endophytic Actinomycetes Isolated From *Hopea ferrea* and Its Antibacterial Activity Against Cariogenic Bacterium

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ABSTRAK

Dental caries affects around 36% of the world's population and results in the loss of primary teeth in about 530 million youngsters. It is described as the loss of the enamel layer of the tooth by acids generated by the activity of cariogenic bacterium such as *Streptococcus mutans* on carbohydrates. This study aims to identify bioactive endophytic actinomycetes as an antibacterial agent against *S. mutans* DSM 20523 and to identify the bioactive endophytic actinomycetes using the 16S rRNA sequencing approach. To determine the percentage zone of inhibition, three endophytes at 10-day-old culture have been streaked onto ISP2. Then, *S. mutans* was streaked perpendicular to the endophytes. Crude extracts from three endophytes have been prepared by using four different types of media namely Tryptic Soy broth, Kings B, International Streptomyces Project Medium No.1 (ISP1) and Starch Casein broth. These crude extracts proceeded to undergo disk diffusion and MIC assays against *S. mutans*. All three endophytes from streaked plates with the highest percentage zone of inhibition were identified using the 16S rRNA gene molecular approach for characterisation. Endophytic actinomycetes which were isolated from *Hopea ferrea* showed potent antibacterial activity against *S. mutans*. All endophytic actinomycetes isolates from streak plates showed good inhibitory activity against the *S. mutans*. The highest percentage of inhibition was shown by the PT9-13S2 isolate followed by PT9-8S2 and PT9-13W2. Furthermore, crude extracts of PT9-8S2 and PT9-13W2 (the highest ZOI) from TSB media showed inhibitory activities against *S. mutans,* from disk diffusion as well as minimum inhibitory assays. From the 16S rRNA molecular approach, endophytic actinomycetes PT9-8S2 was closely related to *Streptomyces collinus* while PT9-13S2 and PT9-13W2 were closely related to *Streptomyces malachitospinus*. From this study, endophytic actinomycetes showed a promising source as antibacterial agents against cariogenic bacterium *S. mutans* which is the main causative agent of dental caries.

Key words: Antibacterial, dental caries, endophytic actinomycetes, *Hopea ferrea*, *S. mutans*

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INTRODUCTION

For millennia, plants have acted as a store of therapeutic bioactive substances against a variety of ailments; nevertheless, the separation and purification of plant compounds in sufficient yield remains a key challenge. In addition to the low yield, plant exploitation for metabolite extraction relates to environmental problems, and novel technologies, such as the employment of endophytic microbes instead of plants, have provided compounds with great medicinal promise (Savi *et al*., 2019). The connection between the host plants and the endophytic population is governed by colonization and is influenced by genotype, growth stage, physiological status, plant tissue type, agricultural techniques, and environmental factors such as temperature, water supply, and nutrients (Santos *et al*., 2018). This evolutionary process results in a close association between the endophyte and its host, and this interaction is generated by cellular and molecular alterations that interfere with the plant's development. As a result of these relationships, mutualistic or symbiotic interactions

between plants and microbes result in positive selection (Santos *et al*., 2018).

In recent years, researchers have been actively experimenting with the advantages of using endophytes as the producers of active metabolites for antimicrobial agent production. Endophytes are microorganisms that interact with the host plant and produce no disease signs as a result of this connection, according to microscopic research (Savi *et al*., 2019). Endophytes deliver nutrients to the plant and can also aid in the acquisition of vital elements from the environment, such as nitrogen, phosphorus, and iron, as well as producing secondary metabolites that can suppress phytopathogen infection (Savi *et al*., 2019). Based on a journal article, *Streptomyces,* which is one of the genera in endophytic actinomycetes, is well-known for its ability to produce a wide range of natural compounds with enormous structural and biological diversity, many of which have applications in biotechnology, medicine, and agriculture (Shan *et al*., 2018). Apart from that, a strain isolated from root tissue of *Bosenbergia rotunda (*L) Mansf A (Mojicevic *et al*., 2019) which is identified as *Streptomyces* sp. has the potential antibacterial activity against Gram-positive bacteria such as *Staphylococcus aureus* ATCC 25932, *Bacillus cereus* ATCC 7064 and *Bacillus subtilis* ATCC 6633 with a minimum inhibitory concentration value of 0.5 µg/mL and minimum bactericidal concentration of 2–8 µg/mL (Taechowisan *et al*., 2017).

Streptococcus mutans, which has been chosen as the oral pathogen against endophytes for antimicrobial activity is the most common pathogen involved in dental plaque/biofilm. It is known as Gram-positive, facultatively anaerobic (round bacteria) which is widely found in the oral cavity of humans and is a major cause of tooth decay or dental caries. *S. mutans* mostly inhabit the mouth, pharynx, and gut. Several elements are present in dental caries, including adhesion to enamel surfaces, generation of acidic metabolites, the capability to build up glycogen reserves, and the ability to produce extracellular polysaccharides (Forssten *et al*., 2010). Plaque's antimicrobial medicines that suppress the formation of cariogenic oral infections can be used to prevent dental plaque; however, their short-term potency is a disadvantage. In addition, the body's normal bacterial flora is impeded by the regular use of broad-spectrum antimicrobial drugs, which are also capable of causing severe ill effects. Therefore, there is a global demand for new drugs that can target the virulence components of *S. mutans* that contribute to cariogenic biofilm development without decreasing the oral microbial inhabitants (Abdel-Aziz *et al*., 2020).

Based on a journal, findings revealed that the intracellular fatty acid arrays derived from endophytic *Arthrographis kalrae* could contribute to the biofilm-preventing alternatives, specifically *Streptococcus mutans* biofilms. It is found that the fatty acids at the concentration of 31.3 mg/mL completely inhibited *Streptococcus mutans* biofilm, and water-insoluble extracellular polysaccharide production in both polystyrene plates and tooth model assay using saliva-coated hydroxyapatite discs (Abdel-Aziz *et al*., 2020). Thus, by enhancing the production and exploring the potential of endophytes, the oral cavity might be decreased which at the same time can prevent the dental plaque/ biofilm. This will be a big concern as stated by WHO (World Health Organization), dental caries is an expensive disease to treat, consuming 5 to 10% of healthcare budgets in industrialized countries, and is among the main reasons for hospitalization of children in some high-income countries (WHO, 2017). As stated by researchers, endophytes promote the growth of their host plants and increase their resistance to pathogens and environmental stresses. In addition, they regulate the synthesis of secondary metabolites with important medicinal properties and produce a variety of biological effects. Therefore, endophytes are extremely promising biological resources that serve as an inexhaustible source of bioactive compounds for a variety of pharmaceutical and medical applications. Huge diversity in endophytic microorganisms suggests a wide variety of biologically active metabolites with various biological categories, including antitumor, anti-diabetic, immunomodulatory, herbicidal, antifungal, antiviral, and antibacterial (Abdel-Aziz *et al*., 2020)

MATERIALS AND METHODS

Materials

ISP2 agar media (Merck, Germany), Brain-Heart Infusion agar (Merck. Germany), Brain-Heart Infusion (BHI) broth (Merck. Germany), Tryptic Soy broth (TSB) media, ISP1 broth media, Starch Casein broth (SCB) media, King's B broth media, Streptococcus mutans DSM 20523, Dimethyl sulfoxide (DMSO), Chlorhexidine (0.2%), Petri dishes, 1 µ sterile loops, 96-well plates.

Methods

Growth and standardization of bacteria

ISP2 media powder was dissolved in 1L of ultra-pure water and the pH was adjusted to 7.2 ± 0.2. The ISP2 agar media has been used to grow endophytic actinomycetes isolates. All pure culture organisms were stored before use at -80 °C. Before culture preparation, organisms were revived from frozen stocks as follows: endophytic actinomycetes isolates were grown and maintained at 37 °C for 14 days. *S. mutans* DSM 20523 was grown and maintained on BHI medium at 37 °C.

Primary bacterial screening of endophytes against *S. mutans*

After 14 days of incubation for the endophyte cultures, a single streak of culture was streaked onto new ISP2 media by using a sterilized inoculating loop from a single colony of the endophyte culture. There were 56 plates of single-streaked endophytes altogether and placed inside a 37 °C incubator for 10 days.

On the 10th day, the single-streaked endophyte plate cultures were observed and continued to streak 3 lines of *S. mutans* perpendicular to the endophyte by using the cross-streaked method. The cultures were once again placed at 37 °C for 2 days to observe the development of a zone of inhibition (ZOI).

$$\text{Zone of inhibition (\%)} = \frac{y-x}{x} \times 100$$

y= length of *S. mutans* streaked

x= length of *S. mutans* grow

Fermentation of endophytes for secondary metabolite production

For secondary metabolite production, 5 to 7 agar plugs with a heavy growth of endophytes with the highest percentage of inhibition zone from different plates (PT9-13S2, PT9-8S2, PT9-13W2), sized 3 mm × 3 mm have been cut and inoculated into a flask containing 300 mL of media broth. Four different media broth were used in this study which were King's B broth, Tryptic Soy broth, ISP3 broth, and ISP4 broth (pH 7.2). All flasks have been incubated in an incubator shaker at 30 °C for 10 days at 180 rpm to develop the pre-seed culture (Kumar & Jadeja, 2018).

On the 10th day, the broth cultures were filtered by using Whatman filter paper, and the filtrate of each sample was added with 80 mL of brine solution. The purpose of adding a brine solution called salting out is to make the extraction more efficient. The metabolites were then recovered from the supernatant by using liquid-liquid extraction with an equal volume of ethyl acetate (Sproule *et al*., 2019) (300 mL) by using a separating funnel and gently shaken. It was then left motionless for 1 hr before collecting the EA layer (Jayatilake & Munasinghe, 2020). The method was repeated two more times, and then all three fractions were combined, condensed, and dried by using a rotary evaporator (40 °C) and freeze-drying (-40 °C). Crude extracts were stored at -20 °C ± 2 °C till further use (Singh & Dubey, 2020).

Disk diffusion

Screening of antibacterial activity was done using crude extracts produced from four different media to determine their effect against *S. mutans*. 5 mg, 10 mg, and 20 mg of crude extracts were weighed and added with 100 µL of 5% DMSO. The prepared crude extracts were then tested against *S. mutans* by following Kirby Bauer's method. Chlorhexidine (0.2%) was used as positive control and 5% DMSO as growth control.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration (MBC)

NCCLS broth microdilution techniques were used to calculate the MICs of the crude extract. Dimethyl sulfoxide (DMSO) was used to dissolve the agents. Then, a 96-well microplate with 96 wells per side was used to inoculate 50 µL of the bacterial suspension (10^8 cells/mL), with each well containing a different concentration of the test agents. The test agents were diluted by using a two-fold serial dilution method. Chlorhexidine was used as a benchmark for antibacterial activity (positive control). All extracts were done in triplicates. The range of sample dilutions in BHI broth and a final concentration of the test agent that inhibited bacterial growth was identified, as evidenced by the absence of turbidity. As a

growth control, test agent-free broth with 5% DMSO was incubated. By inoculating 50 µL of medium from each of the wells of the MIC value well that showed no turbidity onto BHI agar plates, the minimum bactericidal concentration (MBC) was ascertained. The plates were incubated for 24 hr at 37 °C. The smallest concentration of the test agent at which no microbial growth was seen on the plates was called the minimum bactericidal concentration (MBC).

16S molecular approach

Single colonies of each endophyte grown on plates have been cut at 1 cm × 1 cm for characterization using a molecular approach. The method has been followed as in the manual.

The bacterial 16S rDNA, full-length 1.5 kb, was amplified using universal primers 27F(5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-TACGGYTACCTTGTTACGACTT). The total reaction volume of 25 µL contained gDNA purified using an in-house optimized protocol, 0.3 pmoL of each primer, deoxynucleotides triphosphates (dNTPs, 400 µM each), 0.5 U of thermostable DNA polymerase, supplied PCR buffer and water. The PCR was performed as follows: 1 cycle (94 °C for 2 min) for initial denaturation; 25 cycles (98 °C for 10 s; 53 °C for 30 s; 68 °C for 1 min) for denaturation, annealing, and extension. The PCR products were purified by the standard PCR clean-up method. The purified PCR products were subjected to bidirectional sequencing with universal primers 785F (5'-GGATTAGATACCCTGGTA) and 907R (5'-CCGTCAATTCMTTTRAGTTT) using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The DNA extraction kit used was the Bacterial Lysis Buffer of Bacterial DNA Barcoding Kit (1st BASE, KIT-1100-50).

Morphology of endophytic actinomycetes

All three single colonies of endophytes grown on ISP2 agar have been streaked onto ISP3 agar (oatmeal agar). Micromorphology of cultures on agar has been studied by using the slide culture method and spore chain morphology. The presence of spore structures has been identified and observed by using the microscope (Kaewkla & Franco, 2013).

RESULTS AND DISCUSSION

From 56 plates of grown endophytes, only 15 plates showed the zone of inhibition. It was then narrowed down to 3 endophytes that showed the highest zone of inhibition, which were isolates PT9-13W2 (31.7%), PT9-13S2 (74.3%), and PT9-8S2 (65.12%). These three endophytes have been selected for further antimicrobial assays.

Based on the media broth used for the fermentation method, only crude extract of endophyte, PT9-8S2, and PT913W2 from media of TSB (Tryptic soy broth) showed positive antibacterial testing from the disc diffusion and minimum inhibitory concentration assays. Figure 1 below shows the zone of inhibition of PT9-13W2 (TSB), which is the highest ZOI against *S. mutans,* and Table 1 shows the antibacterial activity of endophytes from disc diffusion assay.

Table 1. Antibacterial activity of the crude extract of endophytic actinomycetes PT98S2, PT913S2, and PT913W2 against the cariogenic bacterium

Medium (broth)	Endophytes	Diameter of Inhibition (mm)
TSB	PT913S2	0.000 ± 0.000
	PT913W2	28.00 ± 3.969
	PT9 8S2	5.167 ± 8.520
ISP1	PT913S2	11.00 ± 2.646
	PT913W2	5.667 ± 4.933
	PT9 8S2	14.33 ± 2.082
SCB	PT913S2	0.000 ± 0.000
	PT913W2	3.000 ± 5.196
	PT9 8S2	3.000 ± 5.196
KING'S B	PT913S2	11.67 ± 1.155
	PT913W2	11.33 ± 0.5774
	PT9 8S2	16.33 ± 2.309

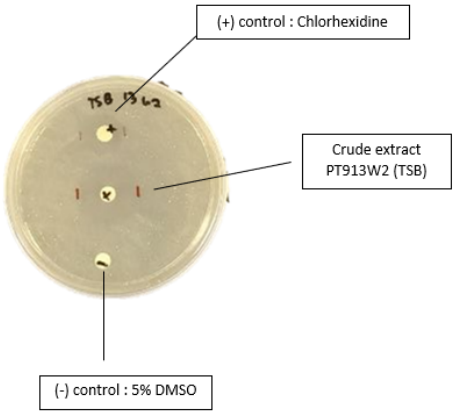


Fig. 1. Zone of inhibition by PT913W2 (TSB) with concentrations of 200mg/mL.

Subsequent experiments were performed to determine the MIC and MBC values of the crude extract against *S. mutans*. The MIC value of the crude extracts was tabulated in Table 2. The results revealed that ethyl acetate crude extract showed a bacteriostatic effect on *S. mutans* since the MIC value is lower than the MBC value. The MIC and MBC values obtained above determined that the endophyte extracts will only inhibit the growth of *S. mutans* but will not kill it completely.

Table 2. MIC and MBC results of the crude extract of endophytic actinomycetes PT98S2, PT913S2, and PT913W2 against the cariogenic bacterium

Medium (broth)	Endophytes	MIC value	MBC value
TSB	PT913S2	20.83 ± 3.608	166.7 ± 57.74
	PT913W2	8.333 ± 1.804	166.7 ± 28.87
	PT9 8S2	2.344 ± 0.0002887	2.604 ± 0.9021
ISP1	PT913S2	10.42 ± 1.804	166.7 ± 28.87
	PT913W2	32.33 ± 7.000	41.67 ± 7.217
	PT9 8S2	1.077 ± 0.09815	6.543 ± 0.7765
SCB	PT913S2	30.31 ± 10.50	25.13 ± 0.000
	PT913W2	27.92 ± 9.671	51.30 ± 0.000
	PT9 8S2	12.00 ± 5.196	48.02 ± 20.79
KING'S B	PT913S2	50.00 ± 0.000	150.0 ± 50.00
	PT913W2	33.33 ± 7.217	150.0 ± 50.00
	PT9 8S2	37.50 ± 0.000	133.3 ± 28.87

As described by (Taufiq & Darah, 2019), the fungal crude extracts used *Lasiodiplodia pseudotheobromae* IBRL OS-64, an endophytic fungus isolated from *Ocimum sanctum* leaf proved a good antibacterial activity toward *S .mutans* as well and produced an inhibition zone ranging from 16.0 to 21.2 mm with concentration of 1 mg/mL. Furthermore, the MIC and MBC values of the endophytic fungus crude extract mentioned above toward *S. mutans* were determined and the results showed that the MIC and MBC values were in the range of 125–500 µg/mL. For comparison, the endophytic fungus extract isolated from the *Ocimum sanctum* leaf requires less concentration than the endophytic bacteria extract isolated from the stembark of *Hopea ferrea* in producing the inhibition zone, MIC, and MBC altogether. Figure 2 shows the minimum inhibitory concentration on a 96-well plate.

DNA and RNA extractions have been done to determine the class of genus of actinomycetes of the endophytes strains. It was found that PT9-8S2 was highly similar to *Streptomyces collinus* strain NBRC 12759. In addition, PT9-13S2 was highly similar to *Streptomyces malachitospinus* strain NBRC 101004 while PT913W2 showed high similarity to *Streptomyces malachitospinus* strain NBRC 101004, which is the same as PT913S2. Figure 3 shows the results of gel electrophoresis of DNA extraction of all three endophytes and Figures 4, 5, and 6 show the phlegmatic tree diagram of the endophytes.

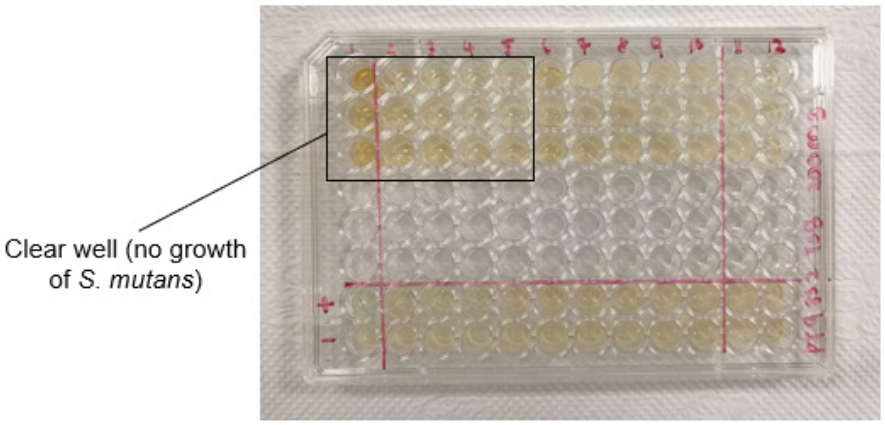


Fig. 2. MIC was performed on a 96-well plate with range values of 200 mg/mL to 3.125 mg/mL.

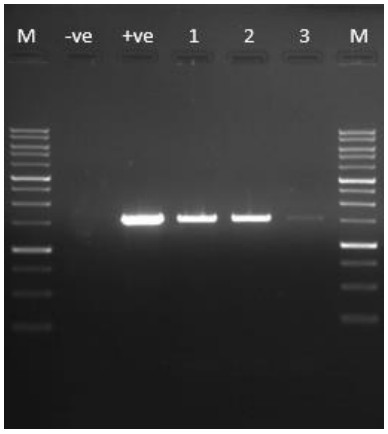


Fig. 3. Image of gel electrophoresis in which aliquots of 1 µL PCR were run on 1% TAE agarose gel at 100 V for 60 min.

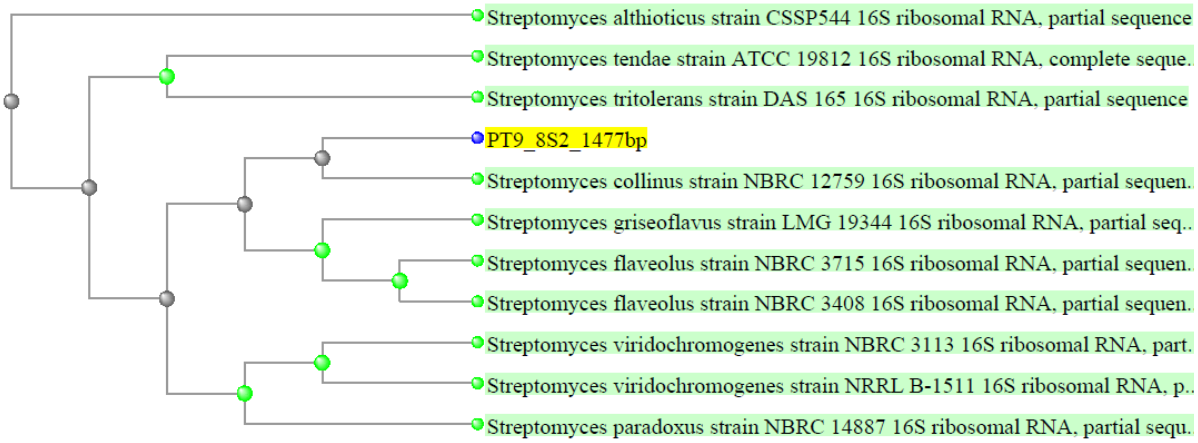


Fig. 4. Phylogenetic tree diagram of PT98S2

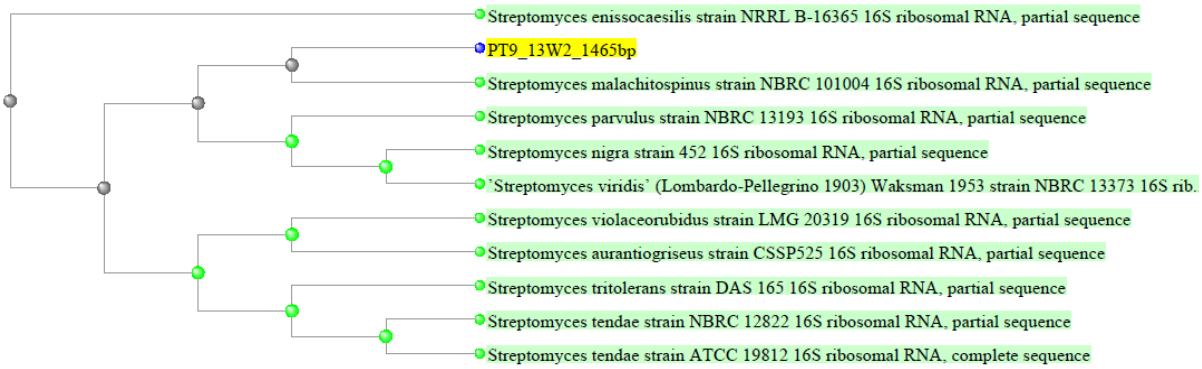


Fig. 5. Phylogenetic tree diagram of PT13W2

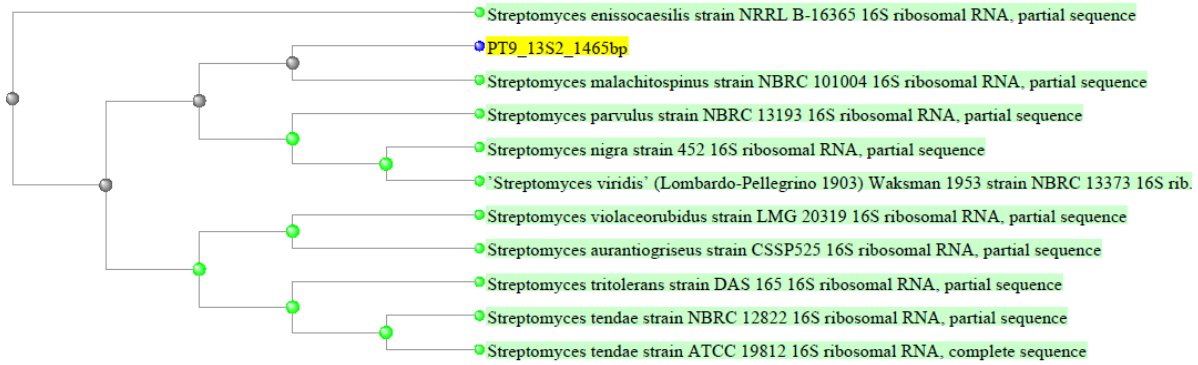
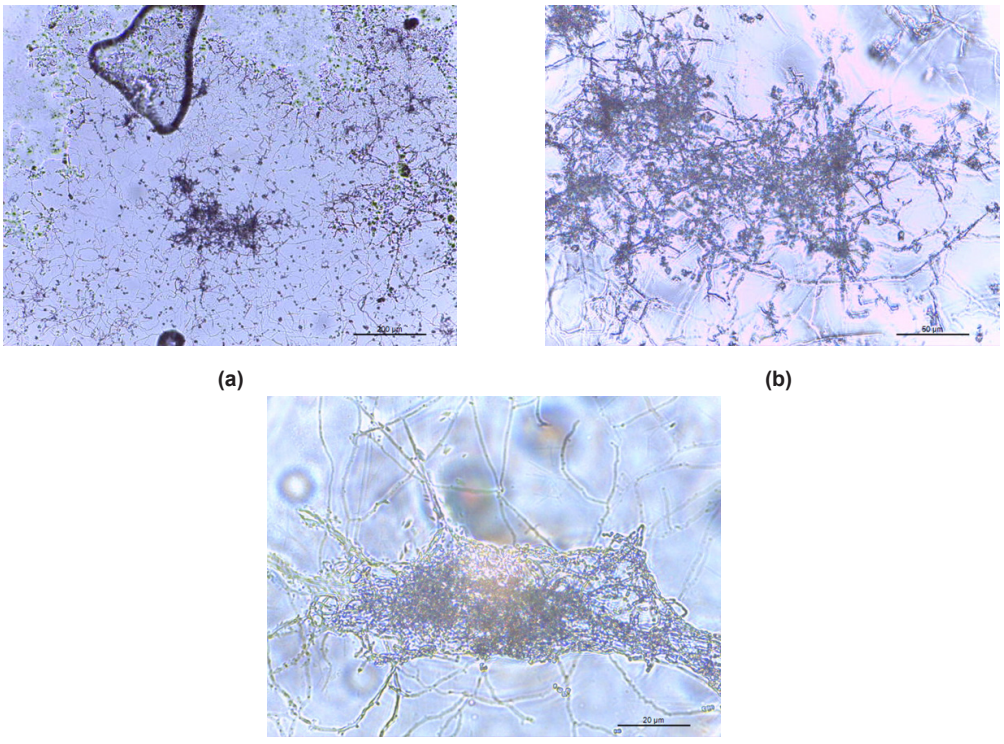


Fig. 6. Phylogenetic tree diagram of PT913SS2

The spore morphology of all three endophytes that have been grown on ISP3 (oatmeal agar media) was observed under the microscope. Figure 7a, 7b, 7c, (PT9-8S2); 9a, 9b, 9c, (PT9-13S2); 10a, 10b, 10c, (PT9-13W2) show the image of spore of each endophyte using 10x, 40x and 100x microscopic lens. The spores' characteristics are described in Table 3.



(a) **(b)**

(c)

Fig. 7. (a) PT98S2 on 10x microscopic lens. (b) PT98S2 on 40x microscopic lens. (c) PT98S2 on 100x microscopic lens

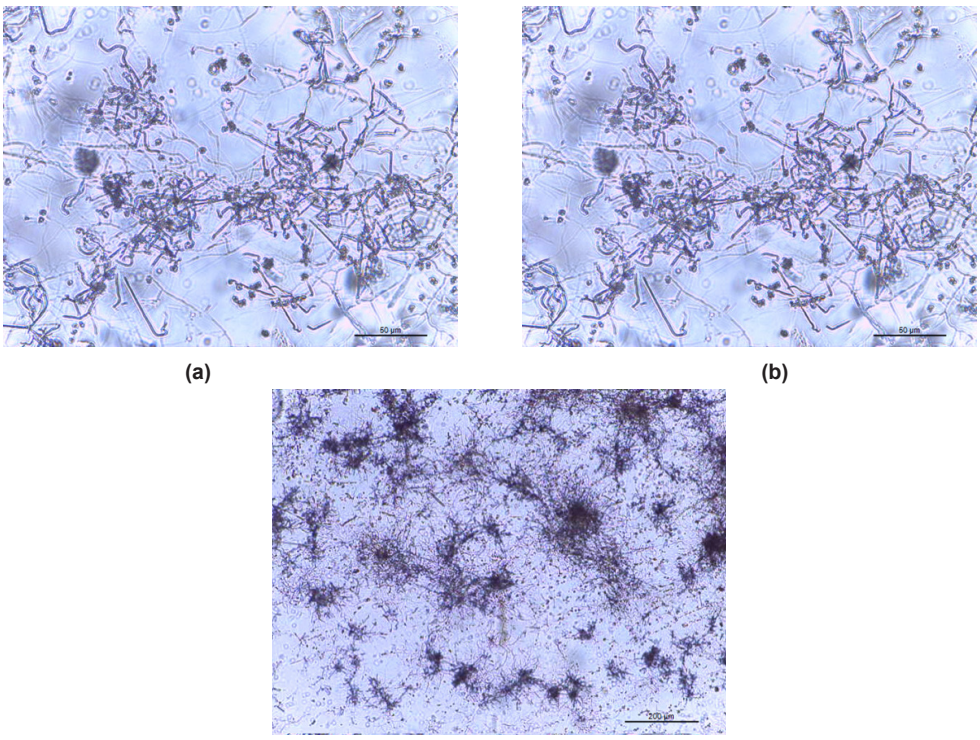


Fig. 8. (a) PT913S2 on 10x microscopic lens. (b) PT913S2 on 40x microscopic lens. (c) PT913S2 on 100x microscopic lens

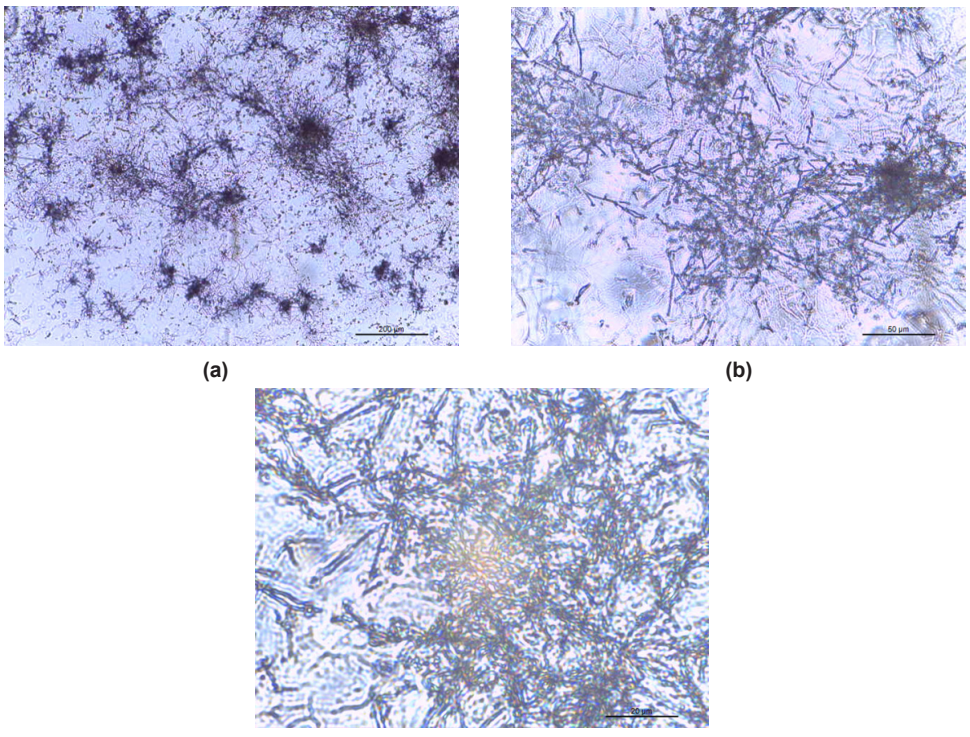


Fig. 9. (a) PT913W2 on 10x microscopic lens. (b) PT91W2 on 40x microscopic lens. (c) PT913W2 on 100x microscopic lens

Table 3. Characteristics of each endophyte

Endophytes	Characteristics
PT98S2	Rectiflexibiles
PT913S2	Spira
PT913W2	Verticillati

(Li *et al*., 2016)

CONCLUSION

In summary, the present study confirms the antibacterial potential of the crude extract of the endophytes isolated from *Hopea ferrea*. The crude ethyl acetate extract of the endophytes showed antibacterial activity against the cariogenic oral bacterium, *S. mutans*. Furthermore, it may have the potential to be incorporated into dental caries' treatment although this use will require additional investigation. However, it is important to point out that crude extract such as this needs to be further purified to obtain pure active substances and its antibacterial activity analysis can be suggested based on the present study.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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