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Antibacterial Activity of Endophytic Bacteria from Stem Bark of Dialium guineense (Wild).

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Abstract
Dialium guineense or African black velvet tamarind, is a common tree in West Africa whose parts have been established for its antimicrobial and therapeutic properties. In this study, the stem bark of the plant was accessed for endophytic bacteria and their activity against common clinical isolates were evaluated. Isolation of bacteria endophytes from the stem bark of the plant was achieved by surface sterilization using 70% ethanol and 2% sodium hypochlorite before aseptically cutting into small sizes of about 3.5 - 4.0 mm, plated on nutrient agar and then incubated for 24 hours. Pure isolates of the endophytes were obtained and identified macroscopically and molecularly by depositing 16SrRNA sequences of all the isolates on NCBI website. The endophytic bacteria isolates belong to the genera *Pseudomonas, Halopseudomonas, Burkholderia, Streptococcus* and *Bacillus.* Antibacterial activity carried out with the crude extracts of all endophytic bacteria isolates revealed that the bacteria endophyte mSB2 of the genera *Halospeudomonas,* had the most clearer zones and diameter of inhibition against all test isolates with zones ranging from 9.0 ± 0.35 mm to 35 ± 0.5 mm; hence, the extract from mSB2 was most active and posed effective antibacterial activities. This study established a fact that *Dialium guineesnse* harbors bacteria endophytes with active metabolites against common disease-causing organisms.
Keywords: *Dialium guineense,* Endophytic bacteria, Stem bark, Antibacterial activities, Metabolites.
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1. Introduction

Globally, infectious diseases have been a main cause of death and diverse kinds of disability which accounts for about 23% of worldwide diseases (Lomovskaya & Bostian, 2016) have suggested that improvement of the efficacy of available antibiotics might be a reasonable and sustainable option due to the challenge between the slow development of new drugs and the fast emergence of resistant strains. This may raise some hope rather than making the future management of infectious diseases look bleak (Ajiboye, Babatunde, Ajuwon & Odaibo, 2018). Endophytic organisms exist in healthy plant tissues without harming them or showing any symptoms of disease, but more research is needed to see whether they could serve as foundations of new natural products for use in industry, agriculture, and healthcare (Passari, Mishra, Saikia, Gupta & Singh, 2015). In a study, Zheng *et al.,* (2016) reported that endophytes were found in nearly every plant organ and have several ecological roles, such as influencing host populations by promoting plant growth as well as serving as agents of biological control. Interest in using endophytic organisms as potential metabolite sources for bioactivity has grown as a result of the hunt for novel antimicrobial drugs. According to Cruz, Notarte, Apurillo, Tarman and Bungihan (2020), about 25% of all human pharmaceuticals are obtained from endophytes.

The oldest form of health care known to mankind involves the use of plants and their parts for therapeutic purposes, a practice that has been embraced by all cultures throughout the world (Sofowora, 1993). The World Health Organization estimates that about 80% of the world's population uses herbal medicine for some aspect of their health care (WHO, 2013). Despite the popularity of modern medicine and the variety of drugs available for various ailments, it has been observed that 85% of patients combine herbal therapy with the medicines prescribed at hospitals or clinics (Amira & Okubadejo, 2017). This shows the level of confidence patients have in herbal recipes. It is thought that the therapeutic capabilities of many traditional medicinal plants come from the metabolites produced by their endophytic populations, making these plants an important resource in the quest for novel bioactive endophytic organisms (Kaul, Gupta, Ahmed & Dhar, 2012). For example, it was established that the plant *Indigofera suffruticosa* continues to be explored for its endophytic composition (Kaul *et al.,* 2012).

Due to the tight relationships that most plants have with numerous microorganisms, endophytic organisms have a chance to infiltrate plant tissue undetected and without harming the host (Schulz, Wanke, Draeger & Aust, 1993). Most endophytes are able to thrive in the diverse environments offered by plants as each of the over 300,000 plant species known may support one or more endophytes (Strobel, Daisy, Castillo & Harper, 2004). It has been discussed that majority of the bioactive chemicals or substances produced by different endophytic organisms have shown antibacterial and antimalarial action in addition to their capacity to serve as enzymes, making them promising candidates for use in agriculture, medicines, and the food industry. The relationship of

endophyes with their host plants is considered a symbiotic one; where endophytes provide benefits such as increased plant development, protecting against herbivores, and infection and production of secondary metabolites (Yim, Wang & Davies, 2007). Endophytic microbes are a prospective source for the development of new medications for pharmaceuticals, industrial, and agricultural uses because of their various adaptations in unique settings (Mapperson, Kotiw & Davis, 2014; Teiten *et al.,* 2013). Investigating the secondary metabolites generated by microorganisms in their environment has motivated many scientists to discover latest substances with medicinal potentials against some infections (Strobel & Daisy, 2003). The secondary metabolites produced by these microorganisms include quinones, lactones, phenols, isocoumarins, lignans, alkaloids, terpenoids, steroids, and phenylpropanoids which confer antibiotic and competitive abilities to the endophytes against invading organisms (Deshmukh, Verekar & Bhav, 2015). Every plant species investigated has been found to host bacterial endophytes. Therefore, a plant without endophytes is quite unusual in nature (Partida-Martinez & Heil, 2011). In fact, a plant's ability to fend off phytopathogens and endure stressful conditions depends on the presence of the related beneficial bacteria (Timmusk *et al.,* 2011). A plant's endophytic diversity may be determined by a number of variables.

Dialium guineense commonly known as African black velvet tamarind, is a large tree found in many parts of Africa such as West Africa, Central African Republic and the Chad. It belongs to the Fabaceae-caesalpinioidaea family and it is 30 meters high with a closely packed leafy crown head, but often shrubby (Osanyinlusi, Awoniyi, Me & Ogundare, 2022). The bark, leaf and fruit of the plant have been seen to be effective in many therapeutic functons and against many diseases. The stem bark extract has significant analgesic property; hence, it can be used to reduce menstrual pain. Some researchers have authenticated activities of the leaves and stem bark of *D. guineense* which include its antibacterial and analgesic activities (Orji, Alo, Anyim & Okonkwo, 2012), as well as antioxidant properties (Gideon, Joachim & John, 2013).

2. Materials and Methods

2.1 Materials

Fresh healthy stem bark samples of D. *guineense* were collected from a tree plant in a partially bushy area with a sterile machete along Warrake road, Auchi, Edo state, South- south of Nigeria and were transferred into well labeled clean plastic bags which were immediately transported to the laboratory for analysis. The plant samples were deposited and authenticated at the Plant Biology and Biotechnology Unit (Herbarium Curation Sub-Division), Department of Biological Science, Edo State University, Uzairue, Edo State. Nigeria.

2.2 Methods

2.2.1 Sterilization of stem bark surface of Dialium guineense Wild

This was done according to Renugadevi, Ayyappadas, Subhapriya, Floryshobana and Vivekanandhan (2021) with slight modifications. The plant material was thoroughly washed under slow running tap water for about 30 – 45minutes until it was clean from visible dirt. This was followed by surface sterilization to remove epiphytes and the plant materials were properly immersed in 70% ethanol for a minute and then in 2% sodium hypochlorite for another 3 minutes. They were then rinsed with distilled water and dried on filter paper.

2.2.2 Isolation and purification of endophytic bacteria

The plant material were cut into small pieces of 3.5- 4 mm with the use of sterile surgical blades and plated separately on already prepared solidified nutrient agar plates. The plates were incubated at 37 ^{o}C for 24 hours. Growth cultures were observed for morphologically different bacteria colonies which were selected and streaked on fresh nutrient agar plates to obtain a pure culture of distinct colonies.

2.2.3 Identification of bacteria isolates

Isolated bacteria isolates were identified using phenotypic and microscopic characterization up to genus level. Further characterization was done using molecular techniques to identify the isolates up to species level. Pure cultures of bacterial isolates were sent to Inqaba, South Africa for DNA extraction, PCR purification of products and sequencing. The primers used were 27F:5'(AGAGTTTGATCMTGGCTCAG)3' and 1492R: 5'(CGGTTACCTTGTTACGACTT)3'. The region of target during sequencing was 16srRNA gene region. The BLAST analysis on sequences was done on NCBI (National Centre for Biotechnology Information) website depending on the percentage similarity and identity, maximum score, total score and query cover.

2.2.4 Screening for antibacterial activities of endophytic bacteria

The method of Sharma and Mallubhotla (2022) was used with modifications for the screening of endophytic bacteria for antibacterial activities. Isolates of all pure bacteria cultures were inoculated in nutrient broth media for 96 hours and incubated at 37 ^{o}C to produce crude extracts. Screening of antibacterial activities was done with the crude extracts using agar well diffusion method. Each broth from endophtic bacteria was centrifuged at 1000 rpm for 10 min to obtain clear crude supernatant which was used to determine antibacterial activity. Six (6) bacteria isolates were procured from Irrua Specialist Teaching Hospital (ISTH), (Table 1) and used as target organisms. The test organisms were sub cultured on nutrient agar at 37 ^{o}C and after 24 hours, colonies from

these cultures were picked and inoculated into freshly prepared, well labeled Mueller- Hinton agar (MHA) plates with sterile cotton swabs. A cork borer (6 mm in diameter) which was sterilized with flames at intervals was used to bore into the middle of the plates and 10 µL of supernatant from the endophytic bacteria were then introduced into the wells in the plates and incubated at 37 °C for 24 hours. The inhibition of clear zones around the wells on the agar plate depicting the antibacterial activities of the endophytes were observed and calculated in diameter to the nearest millimeter (mm) inclusive of the well.

3. Results and Discussion

In this study, bacterial endophytes were identified to species level from the nineteen (19) samples of stem bark cultured on nutrient agar (table 1). Among the isolates, a total of five endophytic bacteria were identified as *Pseudomonas aeruginosa, Halopseudomonas xiamenensis, Burkholderia xiamenensis, Streptococcus pseudopneumoniae* and *Bacillus subtilis*; and designated as mSB1, mSB2, mSB3, mSB4 and mSB5 respectively.

Table 1: Selected test organisms for antibacterial assessment of crude extracts of endophytic bacteria from stem bark of D. *guineense*

Organism	Source
Staphylococcus aureus *Streptococcus pyogens* *Eschericia coli* *Klebsiella peumoniae* *Salmonella typhi* *Shigella flexneri*	All test organism are pure isolates procured from ISTH

ISTH - Irrua Specialist Teaching Hospital, Edo State, Nigeria.

All isolates were characterized with phenotypic, microscopic and molecular methods using sequencing for identification, targeting 16 srRNA gene region. They were classified as shown in table 2 below.

Table 2: Cultural, morphological characterization and molecular identification of bacteria endophytes isolated from stem bark of D. *guineensis*

Isolate	Cultural characteristics	Morphological characteristics	Molecular identity	Strain	Sequence ID
mSB1	Form a large opaque and flat colonies with irregular margins	Slender, rod shape	*Pseudomonas aeruginosa*	PAO1	NC002516
mSB2	Gram negative	Rod-shaped, length is 1.1-1.3 µm	*Halopseudomonas xiamenensis*	PX1NODE_4	GO010049
mSB3	Gram negative	Both round and irregular in shape, with irregular (undulate, fimbriate) margins,cream-colored	*Burkholderia xiamenensis*	ATCC32114	CP014842
mSB4	Gram positive	Lancet-shaped, arranged in chains and pairs (diplococci)	*Streptococcus pseudopneumoniae*	IS7493	NC015875
mSB5	Rod-shaped and Gram-positive, purple colour.	Rough, opaque, fuzzy white or slightly yellow with jagged edges	*Bacillus subtilis*	KCTC113613	MT377875

3.1 Screening of Endophytic bacteria for antibacterial activities

The crude metabolites of endophytic isolates was screened for antibacterial activities against *Staphylococcus aureus, Streptococcus pyogens, Escherichia coli, Klebsiella peumoniae, Salmonella typhi* and *Shigella flexneri* using agar well diffusion method. The zones of inhibition on the plates were measured to the nearest mm and included the diameter of the well (6 mm). Figures 1, 2 and 3 below showed that all crude metabolites of endophytic isolates demonstrated various levels of antibacterial activity against all test organisms (*Staphylococcus aureus, Streptococcus pyogens, Escherichia coli, Shigella flexneri, Klebsiella pneumonia* and *Salmonella typhi*) investigated in this study.

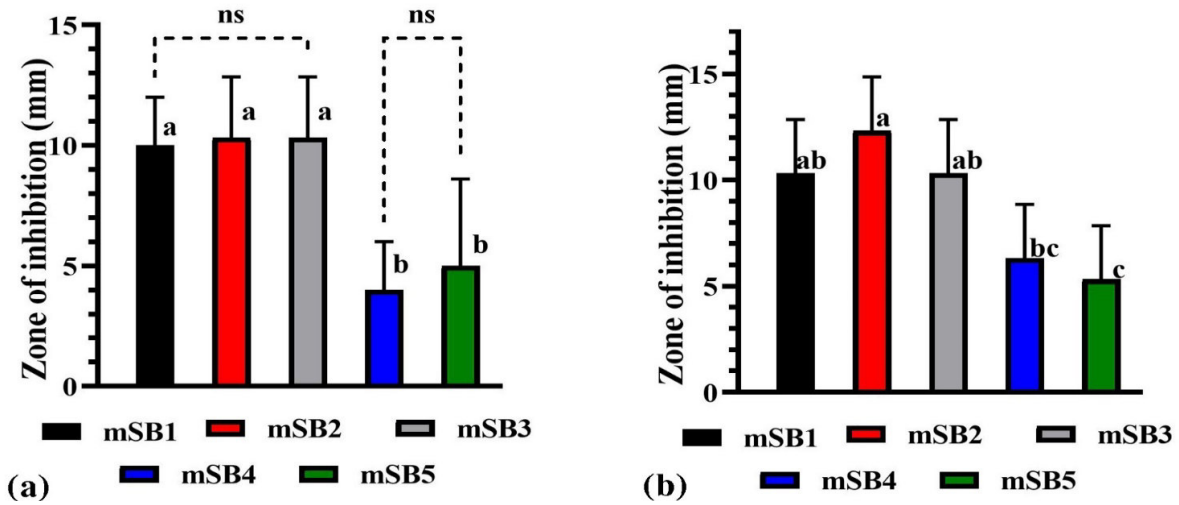

Figure 1: Antibacterial effect of crude metabolites of endophytic isolates on **(a)** *Staphylococcus aureus* and **(b)** *Streptococcus pyogens*. Zone of inhibitions are mean values ± standard deviation. Values with the same superscript are not significantly different ($p<0.05$). **Key: mSB1** - crude metabolites of *Pseudomonas aeruginosa*; **mSB2 –** crude metabolites of *Halopseudomonas xiamenensis*; **mSB3 –** crude metabolites of *Burkholderia xiamenensis*; **mSB4 -** crude metabolites of *Streptococcus pseudopneumoniae*; and **mSB5 -** crude metabolites of *Bacillus subtilis.*

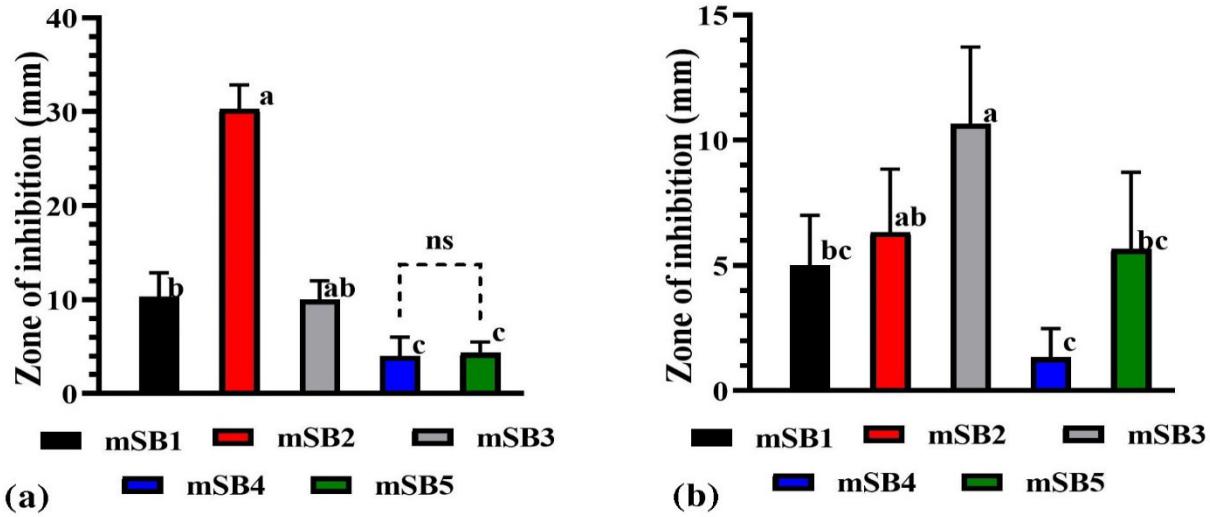

Figure 2: Antibacterial effect of crude metabolites of endophytic isolates on **(a)** *Escherichia coli* and **(b)** *Shigella flexneri*. Zone of inhibitions are mean values ± standard deviation. Values with the same superscript are not significantly different ($p<0.05$). **Key: mSB1** - crude metabolites of *Pseudomonas aeruginosa*; **mSB2 –** crude metabolites of *Halopseudomonas xiamenensis*; **mSB3 –** crude metabolites of *Burkholderia xiamenensis*; **mSB4 -** crude metabolites of *Streptococcus pseudopneumoniae*; and **mSB5 -** crude metabolites of *Bacillus subtilis.*

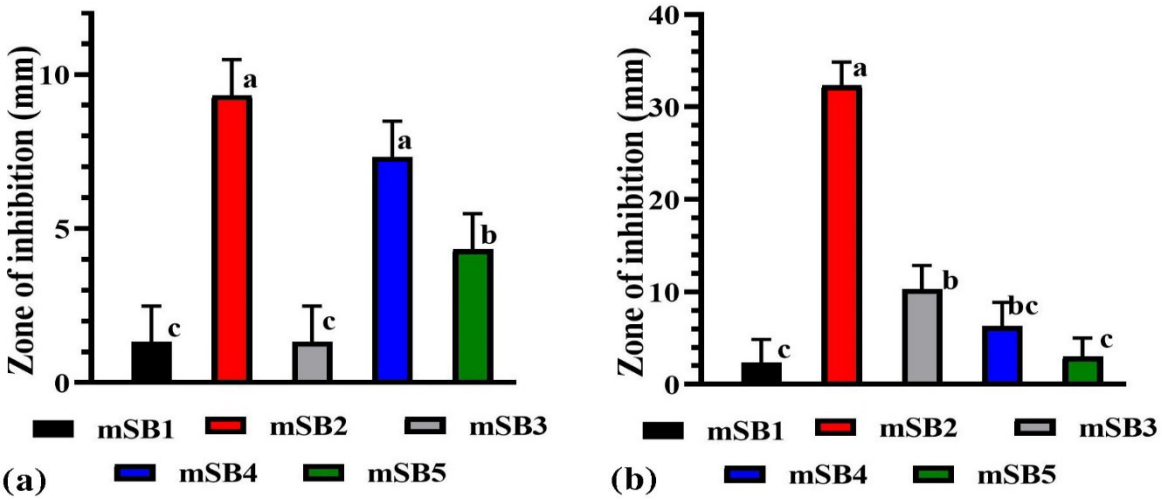

Figure 3: Antibacterial effect of crude metabolites of endophytic isolates on **(a)** *Salmonella typhi* and **(b)** *Klebsiella peumoniae.* Zone of inhibitions are mean values ± standard deviation. Values with the same superscript are not significantly different ($p<0.05$). **Key: mSB1** - crude metabolites of *Pseudomonas aeruginosa*; **mSB2 –** crude metabolites of *Halopseudomonas xiamenensis*; **mSB3 –** crude metabolites of *Burkholderia xiamenensis*; **mSB4 -** crude metabolites of *Streptococcus pseudopneumoniae*; and **mSB5 -** crude metabolites of *Bacillus subtilis.*

Overall, mSB2 (crude metabolites of *Halopseudomonas xiamenensis*) showed an average highest antibacterial activity against all test isolates. Thus, *H. xiamenensis* could have a greater potential as a broad spectrum antibiotic against gram positive and gram negative bacteria. Although, *B. xiamenensis* (mSB3) showed a closely related diameter zone of inhibition (between 5- 14 mm) against *S. aureus, S. pyogens, E. coli and S. flexneri*, a rather smaller zone of inhibition was observed against *S. typhi*. It was generally observed that all the endophytic bacteria had considerable biological activities that have medical potentials.

In order to combat the rising levels of medication resistance, newly identified antimicrobial metabolites from endophytes are emerging as viable substitutes (Taechowisan, Chanaphat, Ruensamran & Phutdhawong, 2012). Strains of bacteria species of *Bacillus*, *Streptomyces Pseudomonas, Acinetobacter, Serratia, Xanthomonas, Achromobacter, Agrobacterium, Micrococcus, Brevibacterium, Microbacterium, Pantoea stenotrophomon* and *Burkholderia* isolated from different parts of medicinal plants have been reported to show high levels of antimicrobial activity (Singh, Kumar, Singh & Pandey, 2017). Therefore, the endophytic bacteria from the stem bark of *D. guineense* could be employed as a potential antibacterial agent.

4. Conclusion

As discovered in this study, all the bacterial endophytes isolated from the stem bark of *Dialium guineense* possess active metabolites that can confer antibacterial activities. The presence of antibacterial compounds in the metabolites of all the bacteria endophytes is a pointer that they can be harnessed for future drug production against common diseases. Several bioactive compounds have been identified in many endophytic bacteria which are said to be responsible for a wide range of biological activities. For further research, it would be pertinent to evaluate the harmonization of specific standard techniques and measurement units in order to optimize the potentials of the metabolites of endophytic bacteria against drug resistant pathogens.

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