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IDENTIFICATION OF EPIPHYTIC BACTERIAL SPECIES FROM LONGEVITY SPINACH LEAVES

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Abstract

Gynura procumbens or longevity spinach plant is a type of medicinal plant that distributes widely in Southeast Asia including Malaysia. *G. procumbens* is commonly called as pokok Sambung Nyawa, Sabong Nyawa, Akar Sebiak, or Kecam Akar by Malaysians; and often consumed as *ulam* or salad. Thus, there might be several microorganisms particularly bacteria that still inhabiting the leaves. Intensive studies on the therapeutic potential of *G. procumbens* have indeed been carried out over time. However, studies focusing on the link between medicinal plants and bacteria that inhabit the plant specifically epiphytic bacteria on the leaf surface have yet to be fully appreciated. Besides, only one available study on the diversity of bacteria inhabiting the *phyllosphere* was available showing the lack of related studies. Thus, this study aimed to identify epiphytic bacteria of *G. procumbens* leaves based on morphology through Gram-staining and genetics using 16S ribosomal RNA gene sequencing; and to determine the possible contribution of the bacteria to the medicinal properties of *G. procumbens* by further readings. The findings from this research suggested the presence of five species of bacteria i.e. *Bacillus subtilis, Bacillus amyloliquefaciens, Pantoea agglomerans, Sphingomonas melonis,* and *Burkholderia cepacia*. From further readings, it is known that some of the bacterial species have the potentials towards medicinal applications. Also, it is hypothesised that the production of some secondary metabolites in the plant might be due to the presence of the bacteria.

Keywords*: G. procumbens*; medicinal herb; epiphytic bacteria; 16S rRNA gene; secondary metabolites

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IDENTIFIKASI KHASIAT BAKTERIA EPIFIT DARIPADA DAUN BAYAM PANJANG

Abstrak

Gynura procumbens atau tanaman *longevity spinach* adalah sejenis tanaman ubatan yang tersebar secara meluas di Asia Tenggara termasuk Malaysia. *G. procumbens* biasanya disebut sebagai pokok Sambung Nyawa, Sabong Nyawa, Akar Sebiak, atau Kecam Akar oleh penduduk Malaysia; dan sering dimakan sebagai ulam atau salad. Oleh itu, mungkin terdapat beberapa mikroorganisma terutamanya bakteria yang masih mendiami daun berkenaan. Kajian intensif mengenai potensi terapi *G. procumbens* telah dilakukan dari masa ke semasa. Walau bagaimanapun, kajian yang menumpukan kepada hubungan antara tumbuhan ubatan dan bakteria yang menghuni tumbuhan tersebut khususnya bakteria epifit pada permukaan daun masih belum dapat dihargai sepenuhnya. Selain itu, hanya terdapat satu kajian mengenai kepelbagaian bakteria yang menghuni *phyllosphere*, menunjukkan kekurangan kajian yang berkaitan. Oleh itu, kajian ini bertujuan untuk mengenal pasti bakteria epifit dari daun *G. procumbens* berdasarkan morfologi melalui pewarnaan Gram dan genetik menggunakan penjujukan gen RNA ribosom 16S; dan untuk menentukan kemungkinan sumbangan bakteria terhadap sifat perubatan *G. procumbens* dengan pembacaan selanjutnya. Hasil kajian menunjukkan bahawa terdapat lima spesies bakteria iaitu *Bacillus subtilis, Bacillus amyloliquefaciens, Pantoea agglomerans, Sphingomonas melonis,* dan *Burkholderia cepacia*. Dari bacaan lanjut, diketahui bahawa beberapa spesies bakteria berpotensi terhadap aplikasi perubatan. Selain itu, dihipotesiskan juga bahawa pengeluaran beberapa metabolit sekunder di kilang mungkin disebabkan oleh kehadiran bakteria.

Kata kunci: *G. procumbens*; ramuan perubatan; bakteria epifit; Gen 16S rRNA; metabolit sekunder

1.0 INTRODUCTION

Gynura procumbens, commonly called as longevity spinach plant is a type of medicinal herb commonly eaten as *ulam* or salad by Malaysian people. *Ulam* is a type of dish where the plant parts such as leaves, stems, or shoots are eaten raw, or in fresh preparation. This plant has been reported widely distributed in Southeast Asia including Malaysia, Indonesia, Vietnam, and Philippines (Mou & Dash, 2016). *G. procumbens* is a fast growing-herbaceous shrub that can grow efficiently in well-draining fertile soil that is always moist overtime (Mou & Dash, 2016). As *G. procumbens* is mostly called *Pokok Sambung Nyawa* among Malaysians; in China, this plant is called *Bai Bing Ca* while in Thailand it is called as *Paetumpung* (Mou & Dash, 2016). In English, this climbing growth plant is called longevity spinach plant or longevity greens.

The previous study by Musthafa et al. (2017) concluded that the chemical constituents found in *G. procumbens* were flavonoid, saponin, tannin, terpenoids, kaempferol-3-o-rutinoside, kaempferol, astragalin, sterol glycosides, and rutin. It also contained pyrrolizidine alkaloids, spirostanol, coumarins, anthocyanins, quercetin, chlorogenic acid, sitosterol, stigmasterol, nucleic acid, plant defence proteins, and miraculin. Thus, the conducted study proves that *G. procumbens* have several phytochemical compounds and secondary metabolites that would contribute towards the medicinal properties of the plant.

Secondary metabolites in plants are phytochemicals that are evolved in plants for protection against herbivores and pathogens. Most microorganisms and herbivores have mechanisms that enhance the effects of plant metabolites, leading to evolutionary associations between specific groups of plants and other organisms. Moreover, microorganisms, particularly bacteria can also produce their secondary metabolites for growth and development. Thus, it is believed that *G. procumbens* which are served as *ulam* can also contain several species of bacteria that not only enhance the development of a plant but also contributing towards secondary metabolites in the plant.

Plant-associated bacteria are also terms as phytobacteria, while surface-colonising phytobacteria are called epiphytic bacteria. Unlike endophytic bacteria, the epiphytic bacteria group is yet not well studied by researchers but it is believed that bacteria inhabiting the phyllosphere contribute many benefits in terms of the contribution of secondary metabolites in plants. A previous study by Hashidoko (2005) showed that some epiphytic bacteria produced decarboxylate xenobiotic phenolic acids those accumulated in the plant tissues and surfaces as the major secondary metabolites. It is known that plant-associated bacteria not only help in producing real hormones of plants but also do produce an identical

chemical constituent compound that imitates the effect of the natural plant hormones called 'structural analogue' (Brader *et al.* 2014). A number of previous studies emphasised that secondary metabolites produced by plants could bring medicinal properties to humans.

16S rRNA gene sequences were further used in this study as a complementary approach in taxonomic identification of the bacteria from the leave surface of *G. procumbens*. 16S rRNA gene is a part of DNA that is most frequently used in taxonomic identification of bacteria and Archaea (Goodfellow *et al.* 2014). These gene sequences are valuable phylogenetic marker molecules for microorganisms because they are universally distributed in bacteria and Archaea, constant in function, and different positions of their sequences change at a very different rate (Weidner *et al.* 1996).

In this study, the GenBank database maintained by the National Center for Biotechnology Information (NCBI), U.S. National Library of Medicine was also used for bacterial identification. The Basic Local Alignment Search Tool (BLAST) provided by the GenBank was used to identify the bacteria from the surface leaves of *G. procumbens* up to species level by searching the highest percentage of similarities of biological sequences between bacterial strains found in this research and bacterial sequences deposited in the GenBank database (NCBI, 2019). Three DNA barcodes of bacteria isolated from this study were registered with the GenBank in order to get the accession numbers for publication purposes as well as genetic references for other users.

2.0 MATERIALS AND METHODS

2.1 Study Site

A plant sample of *G. procumbens* was collected from Tanjung Malim, Perak. The leave of *G. procumbens* was chosen as this part is the most common part eaten by Malaysians as *ulam*. The surface region of *G. procumbens* leave is called phyllosphere (Hashidoko, 2005). All apparatus used in the laboratory were sterilised by using autoclave machine to minimise any contamination that will affect findings of the experiment.

2.2 Culture Media and Cultivation

Three methods were used to cultivate the bacteria from the leaf surface onto rich nutrient agar i.e. using dry cotton swab (method 1), by shaking the leaves in ultra-pure water (method 2) and by using damp cotton swab (method 3).

A rich medium agar containing peptone - 5 g/L, yeast extract - 3 g/L, and glucose - 1 g/L with pH 6.8 was used to isolate the bacteria from the leave surface of *G. procumbens* before undergoing incubation for 16-18 hours at 30°C (Kamarudin & Rehan, 2018). Microbial colonies with different features and colours were observed on the plates of rich medium agar and then were streaked again onto new rich medium agar to produce single colonies of bacteria. The process was done continuously using sterilised toothpick and cotton swab until a single colony of bacterium was observed on each agar plate without any mixture of two or more strains of bacteria. Then, the microbial culture collections were kept in the freezer for further use (Kamarudin & Rehan, 2018).

2.3 Bacterial species identification

Gram stain

Sterilised toothpicks were used to apply the bacterial colonies from bacteria-containing agar onto the surface of microscope slides containing droplets of distilled water as thin smears. Each thin smear was allowed to dry completely through heat fixation by quickly passing the slide through a flame for several times. For Gram staining process, four reagents were used namely crystal violet solution, Gram's iodine solution, alcohol (ethanol) and safranin. For primary staining, crystal violet solution was used before removing the stain by using indirect stream of distilled water. After that, Gram's iodine solution was used as a mordant to fix the dye forming an insoluble compound. The slides of fixed cultures were rinsed with ethanol and then indirect stream of distilled water in order to stop the decolorising process. Lastly, by using safranin reagent solution, a few drops of the solution were placed onto each slide and the slides were gently washed by using indirect stream of distilled water. The excess water was removed by allowing them to dry at room temperature. The prepared slides were examined under a compound microscope of 1000x magnification by using immersion oil. Then, the shapes and cellular structure of bacterial strains were identified by referring to previous journal articles and by comparing the results with the outcomes of genetic identification.

Total genomic DNA extraction for genetic identification

Total genomic DNA (tgDNA) extraction was done by using boiling method (10 min boiling at 98°C using PCR machine) and the supernatant containing tgDNA was used for further analyses. Another extraction method was also included by using FavorPrep™ Tissue Genomic DNA Extraction Mini Kit. Agarose gel electrophoresis was then used for determination of estimated yields of tgDNA,

the quantity and quality, on 1% agarose gel with FloroSafe DNA Stain (a non-carcinogenic alternative to ethidium bromide) as gel stain (Indriati *et al*., 2016).

Polymerase Chain Reaction (PCR)

The partial non-protein-coding 16S ribosomal RNA (rRNA) gene was amplified by using PCR machine. Table 1 shows the universal primers of the 16S rRNA gene used for the standard thermal cycle amplification i.e. PB36 forward primer and PB38 reverse primer (Foght *et al*., 2004). The expected length of each PCR product was approximately 1.5 kilo base pairs (kb) (Janda & Abbott, 2007).

Table 1: Universal primers primers of the 16S rRNA gene used for the standard thermal cycle amplification

Primers	Sequence (5'→ 3')
PB36 (20 bases)	AGR GTT TGA TCM TGG CTC AG
PB38 (18 bases)	GKT ACC TTG TTA CGA CTT

A volume of 25 µL reaction was prepared consisting of 12.5 µL of exTEN 2X PCR Master Mix, 0.5 µL of forward primer (PB36), 0.5 µL of reverse primer (PB38), 2.5 µL of DNA extract template and 9 µL of ultra-pure water. The cycle parameters of PCR was 4 min at 95°C for initial denaturation, 30 s at 95°C for denaturation, 30 s at an optimised temperature for annealing, 45 s at 72°C (60 s/kb; 29 cycles) for extension, 10 min at 72°C for final extension and then the temperature was held at 4°C.

Agarose gel electrophoresis was then used for determination of estimated yields of PCR products, the quantity and quality, on 1% agarose gel with FloroSafe DNA Stain as gel stain. The PCR products were sent for DNA sequencing in suspension form. DNA sequencings were done at Apical Scientific Sdn. Bhd, Seri Kembangan, Selangor Darul Ehsan, Malaysia.

DNA sequence analysis

DNA sequences obtained from Apical Scientific Sdn. Bhd. were aligned and compared to the available data in the GenBank, NCBI, U.S. National Library of Medicine using Basic Local Alignment Search Tool (BLAST). The genus and species status of bacteria identified through the BLAST were compared with the results of the morphological identification via Gram staining.

2.4 GenBank submission

The results of the sequences obtained from BLAST analysis were edited and trimmed by using MEGA X software version 10.0.5 (BETA) (Kumar *et al*. 2018). BankIt, NCBI program was used to prepare the sequence data for GenBank submission.

3.0 RESULTS AND DISCUSSION

A number of 34 colonies of bacteria namely GS1 until GS34 were chosen randomly for further isolation. Positive PCR products were obtained for colonies of *GS3, GS9, GS11, GS20*, and *GS22*. Overall, the results suggested the presence of five different species of bacteria as summarised in Table 2 (morphological identification) and Table 3 (genetic identification). All the species were rod-shaped (bacilli) but of different Gram status. For example, there were three species of Gram-negative bacteria while the other two species were Gram-positive bacteria.

The findings from BLAST program show five species of bacteria *i.e. Bacillus subtilis, Bacillus amyloliquefaciens, Pantoea agglomerans, Sphingomonas melonis,* and *Burkholderia cepacia.* A number of three partial sequences of non-protein-coding 16S rRNA gene have been successfully registered with the GenBank, with Accession numbers of MN715778, MN715779, and MN715780 (Table 3). The other two sequences were not submitted to the GenBank due to their short gene sequence length.

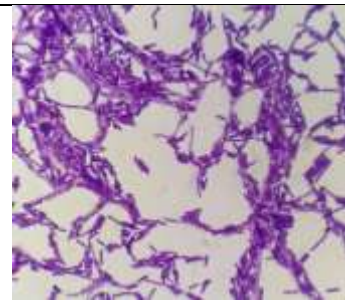
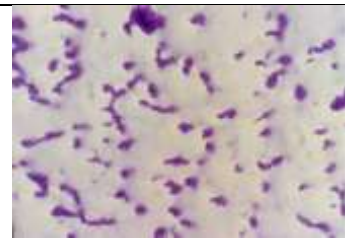
According to War *et al.* (2014), it was surprising that *B. subtilis* was found in the endophytic site of *Centella asiatica* showing varying potentials as it exhibited significant antagonistic activity. *Pantoea* sp. was also found in *C. asiatica* showing its ability to produce huge amount of soluble phosphate and acid phosphatase (War *et al*. 2014). Meanwhile, *S. melonis* have been found in both endophytic and phyllosphere site of *Arabidopsis thaliana* plant (Innerebner *et al*. 2011). This type of Alphaproteobacteria was commonly and abundantly found in phyllosphere sites of a plant, according to Vorholt (2012).

Moreover, *Sphingomonas* spp. exhibited a remarkable plant protective effect by suppressing disease symptoms and decreasing pathogen growth (Innerebner *et al*. 2011). *Pantoea* species and *Pseudomonas* species have also shown an ability to produce auxin indole acetic acid, which is the most common naturally occurring plant hormone (Rastogi *et al*. 2012). Furthermore, Eberl and Vandamme (2016) stated that the number of novel plant-associated *Burkholderia* species has increased significantly and it was recently shown in previous studies

by Sieber *et al*. (2015) and Carlier *et al*. (2016) that *Burkholderia* species live as symbionts with their host plant producing large amount of secondary metabolites, which appear to protect plants from herbivores.

B. amyloliquefaciens has been widely known as a stable leaf-colonizer that exhibits antimicrobial activity against many pathogenic organisms by producing biofilm on leaf surface (Nastro *et al.* 2013). *B amyloliquefaciens* also helps in preventing invaded pathogens such as cucumber mosaic virus, which has the reputation of having widest host range of any plant viruses (Lee & Ryu, 2016). *B. amyloliquefaciens* has been reported by Khan *et al*. (2017) as a bacterium that improves the phytoremediation potential of *Solanum lycopersicum* during copper stress and promotes growth, which has the same function as secondary metabolites in the plant.

Table 2: Description of isolated *Gynura procumbens*-associated bacteria based on Gram staining

Bacterial code name	Types of bacteria	Cell shape	Microscopic image (1000x)
GS3	**Gram positive**	**Rod-shaped (bacilli)**	
GS9	**Gram positive**	**Rod-shaped (bacilli)**	

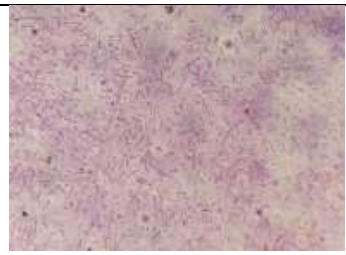
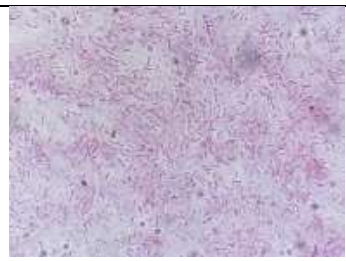
GS11	**Gram negative**	**Rod-shaped (bacilli)**	
GS20	**Gram negative**	**Rod-shaped (bacilli)**	
GS22	**Gram negative**	**Rod-shaped (bacilli)**	

Table 3: Description of isolated *Gynura procumbens*-associated bacteria based on genetic identification

Bacterial Code Name	Name of Species Suggested by BLAST	Percentage of Identity (%) from BLAST Analysis	GenBank Accession No.	PCR Fragment Length (Approximate bp)	Sequence Length (bp) from DNA Sequencing	Total Sequence (bp) for GenBank Submission
GS3	***Bacillus subtilis***	**98.73**	**MN715778**	**1500**	**1253**	**1247**
GS9	***Bacillus amyloliquefaciens***	**92.82**	**-**	**1500**	**608**	**-**
GS11	***Pantoea agglomerans***	**90.69**	**-**	**1500**	**734**	**-**
GS20	***Sphingomonas melonis***	**97.14**	**MN715779**	**1500**	**1284**	**1237**
GS22	***Burkholderia cepacia***	**98.35**	**MN715780**	**1500**	**791**	**785**

4.0 CONCLUSION

A number of five bacterial species i.e. *B. subtilis, B. amyloliquefaciens, P. agglomerans, S. melonis,* and *B. cepacia* were successfully isolated and identified in this study using morphological identification via Gram staining and genetic identification using partial 16S rRNA gene sequences. There were discoveries on these bacterial contributions towards other plants; promoting the growth as well as strengthen their resistance towards pathogenic microorganisms

and herbivores by aiding the production of secondary metabolites. The study of plant-related bacteria and their abilities to yield beneficial effects on plants are important to better understand their ecological role and their interaction with plants. It is also hypothesised that the production of some secondary metabolites in *G. procumbens* might be due to the presence of the bacteria. Finally, it is recommended for the next researchers to find out more about epiphytic bacteria that inhabit the leaf surface of *G. procumbens*.

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