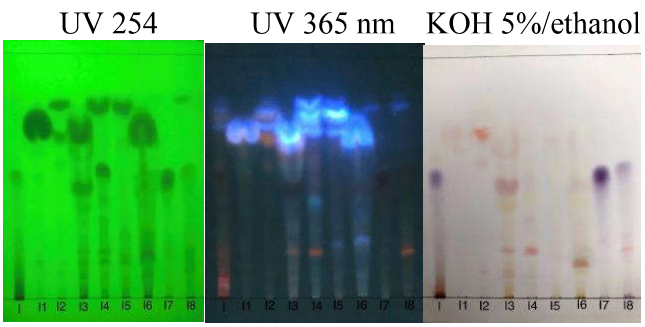

Figure S4. Chromatogram of fraction I1 – I8.

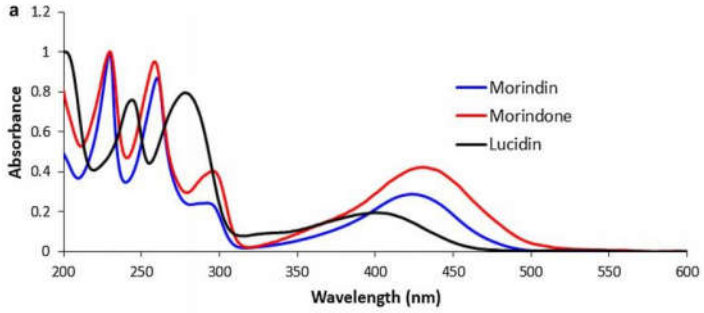

Figure S5. Overlay UV-Vis spectrum alizarin derivatives.

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ISOLATION AND IDENTIFICATION OF THE ENDOPHYTIC BACTERIA IN *HOUTTUYNIA CORDATA THUNB* AND THEIR ANTIBACTERIAL ACTIVITY AGAINST *STAPHYLOCOCCUS AUREUS* OF HUMAN FURUNCLES IN CAN THO CITY

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ABSTRACT

Background: *Endophytic bacteria from medicinal plants are sources of biologically active material for pharmaceutical industries to treat symptoms of numerous diseases. No history indicates that the Houttuynia cordata Thunb in these areas had ever been studied for endophytic bacteria previously.* ***Objectives:*** *To isolate and identify the endophytic bacteria from Houttuynia cordata Thunb and evaluate the antimicrobial activity to Staphylococcus aureus of human furuncle in Can Tho city.* ***Methods:*** *The entire Houttuynia cordata Thunb plant was used to isolate endophytic bacteria and their antibacterial ability against Staphylococcus aureus was done. Based on the sequence of 16SrRNA, some bacterial strains with antimicrobial activities were identified. Besides, comparing the sequence of endophytic bacteria strains with the sequence of bacterial strains found in the NCBI data bank.* ***Results:*** *The results showed that 60 endophytic bacteria strains in*

Houttuynia cordata Thunb were isolated on the PDA medium. Bacterial colonies were round or irregular, white or yellow color. All of the bacteria cells were rod shape, 34 per 60 strains were non-mobile and almost cells were gram-positive. Fourteen strains inhibited the growth of Staphylococcus aureus of human furuncle with the clear zone varied from 10 - 40 mm and three of them (PĐT2, BTT4 and NKT3) demonstrated the highest antibacterial activity. They were identified as Bacillus amyloliquefaciens strain CD2901 (98% of homology), Bacillus megaterium strain 22 (97% of homology) and Bacillus subtilis strain B237 (98% of homology). ***Conclusions:*** *The endophytic bacteria isolated from the stems of Houttuynia cordata Thunb belongs to Bacillus group which has been gaining importance because of its medicinal properties.*

Keywords: *antibacterial, Bacillus spp, endophytic bacteria, furuncle, Houttuynia cordata Thunb*

I. INTRODUCTION

Vietnam has a very rich source of medicinal plants with antibacterial properties that have been used as medicine in the medical field and in people's life for a long time. They are often very familiar plants growing wild or being planted in the garden. The natural antibacterial active ingredients from plants and microorganisms are considered a promising source and an important contribution to the protection of human health. *Houttuynia cordata* Thunb is a herbaceous plant of Saururaceae family. It is perennial, used in medicine, and known for its antibacterial, anti-inflammatory, diuretic, antioxidant effects ... Some studies show that compounds from *Houttuynia cordata* Thunb such as essential oils or flavonoids show antibacterial, antiviral, anti-inflammatory and anti-allergic activity. Endophytic microorganisms in general and plant endophytic bacteria in particular have been known for their ability to stimulate the growth of plants through nitrogen fixation, dissolving inorganic phosphorus which is insoluble in soil and collecting plant hormones. In recent years, a lot of endophytic bacteria have been found to be able to collect natural antibiotic forms that can resist many of the drug-resistant bacteria [1]. This shows the promising potential for producing natural antibiotics from plant endophytic bacteria. Medicinal plants attract the attention of many scientists. Many research works are carried out in medicinal plants in general and *Houttuynia cordata* Thunb in particular. However, the research is mainly about the activity of compounds isolated from plants, there are a few studies on endophytic microorganisms with this medicinal plant. In the endophytic microbial system of medicinal plants, endophytic bacteria are known not only for supporting the plants to grow and develop well but also for producing metabolites with natural antibacterial activity. For the above reasons, the topic about the isolation of endophytic bacteria in *Houttuynia cordata* Thunb with the activity of fighting bacteria *Staphylococcus aureus* bacteria from furuncles in humans in Can Tho city was studied.

II. METHODS

2.1. Materials and methods

2.1.1. Time for carrying out the topic

The project had been carried out from January 2017 to January 2018 in the Microbiology Laboratory of the Biotechnology Research and Development Institute, Can Tho University and the laboratory of Can Tho University of Medicine and Pharmacy.

2.1.2. Location of collecting the research samples.

Houttuynia cordata Thunb samples were collected in the areas of Phong Dien (PD), Binh Thuy (BT) and Ninh Kieu (NK) Districts of Can Tho city.

Figure 1. *Houttuynia cordata* Thunb used in the experiment.

2.2. Research methods

2.2.1. Methods of collecting and processing samples

Staphylococcus aureus bacteria originating from people's furuncles were isolated and stored at the microbiology laboratory of Can Tho University of Medicine and Pharmacy Hospital.

Sample collection: Samples of *Houttuynia cordata* Thunb were collected by using a shovel to dig around the tree and dig deep with the size of 15 x 15 x 15 cm to collect the samples. Separate the roots and stems from the soil, place the plants and soil in separate plastic bags and clearly note the location and the time of collection. Then store the samples in a cooler and take them to laboratories.

Take the soil samples at the place of collecting the samples to measure pH. With the samples, clean them by tap water and then separate into individual organs (leaves, stems, roots) and conduct the following disinfection: For the leaf samples (L): Sterilize by immersion in 70% alcohol for 30 seconds; 3% H_2O_2 for 30 seconds and rinse with sterile water for 3-4 times for cleaning; For the stem samples (T): Cut into small fragments (about 2-3cm), sterilize in 70% alcohol for 1 minute, then followed by another immersion 3% H_2O_2 for 1 minute and rinse with disinfected distilled water for at least 3 times; and the root samples (R): Follow the same above process of the stem sample. All the samples were dried under sterile conditions.

2.2.2. Isolation of endophytic bacteria in *Houttuynia cordata* Thunb

Centrifuge the extract of separated parts of *Houttuynia cordata* Thunb at 2000 rpm at 4° C to remove residues. Aspirate the above clear fluid into the semi-solid medium and incubate 3-5 days at 30 ° C. Observe the appearance of pellicle rings, thin white film, apart 2-4 mm from the surface of the environment, this ring demonstrates the presence of endophytic bacteria. After that, absorb 50 µl of this area's solution to spread on PDA environment on the plate, then incubate at 30°C for bacteria to grow. Consequently, continue to implant and transfer bacteria until it is pure. The pure degree of bacteria was examined by observation under a microscope.

2.2.3. Observation of the morphology and the measurement of colony size

When implanting and transferring the bacteria on PDA environment plate, measuring the size and observing the forms of colonies including the targets: color, shape, buoyancy and colony cover type by usual eyes.

2.2.4. Observation of the shape and the movement ability of bacteria

After isolating the bacteria, conduct the observation of the shape and the movement of bacteria by means of squeezing method under an optical microscope at 400 times of magnification according [2].

2.2.5. Measurement of bacterial cell size and observation of the movement of bacteria

The motion of bacteria was observed by squeezing the method under an optical microscope at 400 times of magnification according to [2].

2.2.6. Gram coloring of bacteria

The bacteria were colored with Gram according to the description [3]. Observe the samples on the optical microscope at 400 times of magnification and record the Gram of bacteria. If the bacteria samples take the blue-purple of Crystal violet, they are Gram-positive samples. And others take the red-pink of Fushin are Gram-negative samples.

2.3. Investigation of the antibacterial properties of endophytic bacteria strains with pathogenic bacteria strains

Antibacterial properties of bacterial strains were investigated by diffusion method through disk diffusion assay (Disk diffusion assay) with Staphylococcus aureus bacteria [4]. First, dilute and enumerate the bacterial density by dripping the counting method [5]. At the same time, count the density of the pathogenic bacteria strain. After determining the density of the pathogenic bacteria strains, survey the antibacterial activity of endophytic bacteria strains. The pathogenic bacteria strain was cultured in PDA environment and adjusted with the density of 10^8 CFU/mL. Arrange the experiment used to investigate the antibacterial activity of endophytic bacteria for Staphylococcus aureus bacteria.

The antibacterial ability of endophytic bacterial strains was assessed according to Galindo's convention (2004). The diameter of the antibacterial ring = total diameter-diameter of colonies.

The isolations selected were determined based on 16S rRNA gene sequencing analysis by (Lane, 1991) which are designed with the following sequence:

27F: 5'- AGAGTTTGATCMTGGCTC -3'

1492R: 5'- TACGGYTACCTTGTTACGACT-3'

The result compared the sequence of selected strains with the sequence of bacterial strains found in NCBI data bank.

III. RESULTS

3.1. Isolation and identification of *Staphylococcus aureus* bacteria

Staphylococcus aureus bacteria isolated from human furuncles are spherical, non-mobile, gram-positive, 0.5-1.5 μm in diameter, cells arranged into a shape of a bunch of grapes. Cell walls are resistant to lysozyme and sensitive to lysotaphine, *S. aureus* is aerobic or anaerobic bacteria depending on the adaptation. (Figure 2)

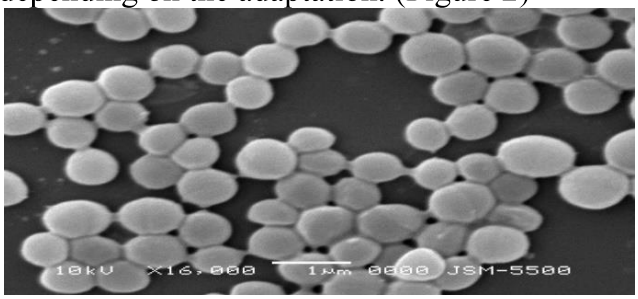

Figure 2. *Staphylococcus aureus* bacteria are taken by SEM.

3.2. Isolation and identification of endophytic bacteria of Houttuynia cordata Thunb

Sixty endophytic bacterial strains of *Houttuynia cordata* Thunb were isolated in PDA environment. Colonies of bacteria strains are round or irregular, white or yellow. All strains of bacteria are rod-shaped, gram- positive with some mobile strains. Bacteria are rod-shaped.

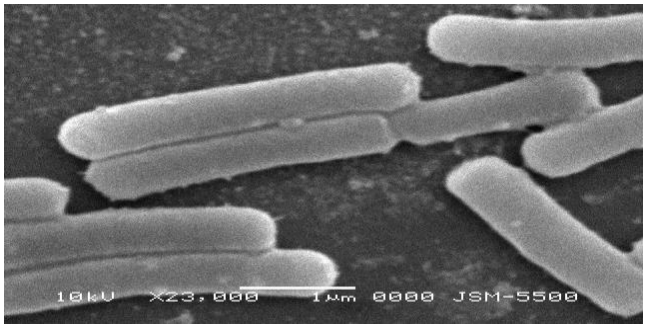

Figure 3. Endophytic bacteria of *Houttuynia cordata* Thunb are taken by SEM**.**

3.3. Investigation of antibacterial activity of endophytic bacteria of Houttuynia cordata Thunb with Staphylococcus aureus bacteria from furuncles in human.

From 60 endophytic bacterial strains of *Houttuynia cordata* Thunb, the investigation of antibacterial properties against *Staphylococcus aureus* collects 14 endophytic bacteria strains with antibacterial activity against *Staphylococcus aureus* bacteria from furuncles in human with 10-40mm sterile ring (Figure 4).

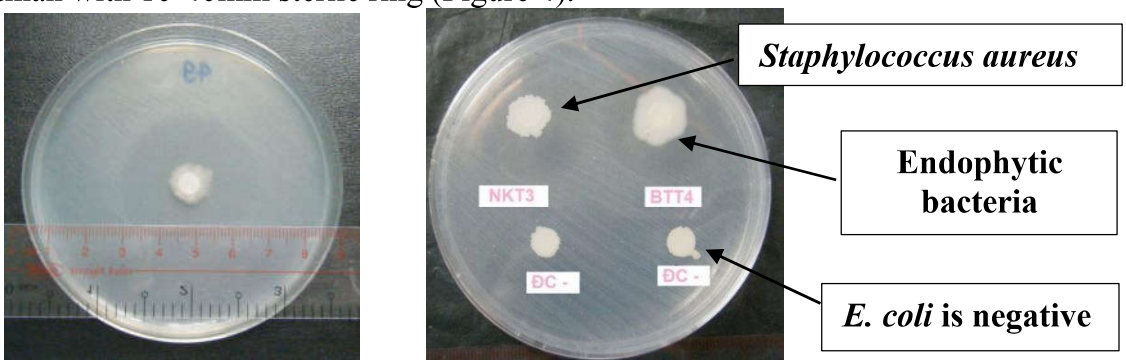

Figure 4. Sterile ring when investigating the antibacterial properties of endophytic bacteria strains.

3.4. Investigation result of antibacterial activity

The antibacterial ability of endophytic bacterial strains isolating with *Staphylococcus aureus* is shown through the formation of a light ring around the filter paper with the endophytic bacterial fluid of *Houttuynia cordata* Thunb and in the PDA environment spread with pathogenic Staphylococcus aureus bacterium. The formation of light ring was observed in 3 consecutive days. The experiments were repeated for 3 times and the antibacterial ring was measured for 3 times, the result showed that there were 14 strains in 60 endophytic bacterial strains of the *Houttuynia cordata* Thunb that resisted the *Staphylococcus aureus* in the investigation, zone of inhibition was from 10- 40 mm as shown in Figure 5.

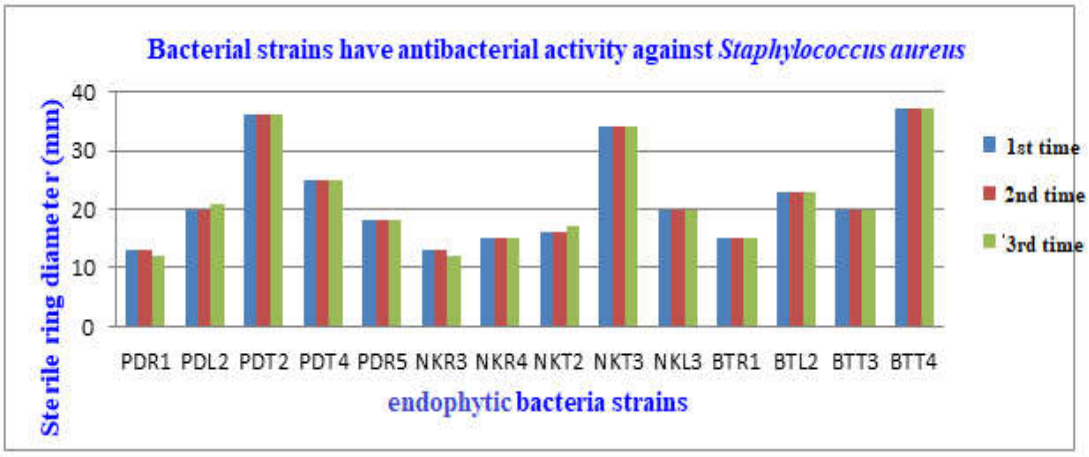

Figure 5. Antibacterial ability of 14 the endophytic bacteria strains of *Houttuynia cordata* Thunb for *Staphylococcus aureus.*

PD: Phong Dien; NK: Ninh Kieu; BT: Binh Thuy; R: Root; T: Stem; L: Leaf

Identification of some promising bacteria strains

From 14 endophytic bacterial strains of *Houttuynia cordata* Thunb with antibacterial activity against *Staphylococcus aureus* bacteria, select 3 strains with the highest antibacterial activity. They are PDT2, BTT4, NKT3, identified as *Bacillus amyloliquefaciens* strain CD2901, *Bacillus megaterium* strain 22 and *Bacillus subtilis* strain B237 (Table 1).

Table 1. Result of solving the sequence of 03 promising endophytic bacteria strains.

No.	Bacteria strain	Identification result	Similarities (%)
1	PDT2	*Bacillus amyloliquefaciens* strain CD2901	98
2	BTT4	*Bacillus megaterium* strain 22	97
3	NKT3	*Bacillus subtilis* strain B237	98

IV. DISCUSSIONS

Houttuynia cordata Thunb shows an inhibitory effect against *Staphylococcus aureus*. However, the most effective activity showed by the endophytic bacteria in the stem extract. And this result is consistent in all 3 areas that collected the samples. After screening 3 strains selected PDT2, BTT3, NKT4, the group of Bacillus bacteria was identified with very high similarities. They are all Gram-positive but at different locations, different strains of Bacillus were obtained. Antibiotics produced by the Bacillus species are more effective for Gram-positive bacteria.

The DNA fragment of PDT2 strain is 1230 bp long and has a 98% homogenous ratio with DNA sequence of the *Bacillus amyloliquefaciens* strain CD2901. *B. amyloliquefaciens* are soil bacteria, Gram-positive, rod-shaped. Like other Bacillus strains, *B. amyloliquefaciens* produces endosperm to survive for a long period of time in extreme weather conditions [6]. The result indicates that 4 strains of *B. amyloliquefaciens* (S1, S5, S13, S10) are resistant to *E. coli* and there is one *B. amyloliquefaciens* S1 strain resistant to *S. aureus*. *B. amyloliquefaciens* LBM 5006 is also resistant to Gram-positive bacteria such

as *B. cereus, B. subtilis, S. aureus, Listeria monocytogenes, L. inoccua, Leuconostoc mesenteroides* and *Corynebacterium fimi. B. amyloliquefaciens* strain is also resistant to Gram-negative bacteria such as *E. coli, Proteus vulgaris, Pseudomonas flourescens, Salmonella cholerasuis, Salmonella gallinarium, Serratia marcescens,* Trinh Thanh Trung et al reported that the strain of *B. amyloliquefaciens subsp. plantarum* SP 1901 was resistant to Gram-positive and Gram-negative bacteria causing human diseases such as *S. aureus, E. coli, P. aeruginosa* and *Shigella sp* [7]. *B. amyloliquefaciens* R3 is also resistant to *E. coli* by producing compounds such as biosurfactins [8]. This was tested the antibacterial properties of crude extracts by different solvents of *B. amyloliquefaciens*, and the results showed that the extract from ethyl acetate solvent was resistant to *B. cereus, S. aureus, E. coli*; extract from diethyl ether was resistant to *B. cereus, S. aureus* and E. coli; extract from chloroform solvent was resistant to *S. aureus.* According to the extract from the organic solvent of *B. amyloliquefaciens* VJ-1 was resistant to *B. subtilis, Enterococcus cloacae, S. aureus, S. epidermidis.*

The DNA segment of BTT4 strain is 1290 bp long and has a 97% homogenous ratio with the DNA sequence of *Bacillus megaterium* strain 22. *B. megaterium* is a rod-shaped, Gram-positive with spores. This is considered an aerobic bacterium, but it can still grow under anaerobic conditions when necessary, and it is one of the bacteria with the largest size found in sand, so it is called "Mega". Which was reported the types of lipopeptides that *B. megaterium* emitted for fighting the bacteria such as surfacin, linchenysin, iturin, bacillomycins, mycosubtilin, subtulene, agrastatin1 [5]. Among the Bacillus strains isolating from the soil, there are six *B. megaterium* strains producing bacteriocin, megacin and being able to resist *E. coli, Yersinia enterocolitica, S. aureus, Micrococcus flavus.* Which was isolated B. megaterium 22 strain from the soil in Alexandria, Egypt synthesized bacteriocin capable of inhibiting *Salmonella typhimurium, S. aureus, E. coli*, and *K. pneumoniae* bacterial strains [9].

The DNA fragment of the NKT3 strain is 1120 bp and has a 98% homogenous ratio with DNA sequence of *Bacillus subtilis* strain B237. *B. subtilis* bacterium has the potential to produce commercial products for use in medicine, agriculture and food industry. Bacillus sensu lato bacterium is capable of producing antibacterial compounds, including peptide and lipopeptide and bacteriocin antibiotics [6]. Which was tested the antibacterial activity of concentrated bacterial cell fluid and centrifuging fluid without bacteria of *B. subtilis* RLID strain. The result shows that centrifuging fluid without bacteria of *B. subtilis* RLID strain has the ability to fight the Gram-negative bacteria such as *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Acinetobacter baumannii* and *Yersinia aldovae,* Gram-positive bacteria such as *Staphylococcus aureus, Streptococcus pyogenes and Enterococcus faecalis.* Concentrated bacterial cell fluid of *B. subtilis* RLID 12.1 strain is resistant to 28 bacterial strains studied. That was reported that B. subtilis MIR 15 strain could resist *P. aeruginosa, E. coli* and *M. luteus*. Aslim et al found *B. subtilis* which was resistant to *E. coli* and *Y. enterocolitica* [5]. Research results of Rowaida Khalil showed that *B. subtilis* ATCC 6633 was resistant to *S. aureus* [9]. A study of Fernandes et al on antibacterial activity in 11 Bacillus strains isolated from a fermented seed in Cameroon showed that 1 strain of *B. subtilis* S12 was resistant to *S. aureus* and 3 strains of *B. subtilis* S16, S18 and SY was resistant to *E. coli* with an antibacterial ring diameter of 6-8 mm [10]. Which was reported that the B15 strain was able to resist *S. aureus* A12, E. coli A13; B7

strain was resistant to *S. aureus* A12, *E. coli* A13. Tanmayee R. Dasari found the antibacterial ability of lipopeptide produced by B. subtilis R14 against Gram-positive cocci such as *Enterococcus faecalis* and *S. aureus* stronger than Gram-negative bacillus such as *Pseudomonas aeruginosa* and *E. coli* [11]. That was isolated Bacillus strains from the conjunctiva of the eye and tested the ability to resist the bacteria causing eye diseases, the result was that *B. subtilis* PCA 11.2 was able to fight *S. aureus* SDA 40.2 and *S. aureus*. SDA 48. That was isolated *B. subtilis* LFB112 strain from herbs in China capable of producing antibacterial compounds identified as bacteriocins, which were resistant to both Gram-negative and Gram-positive bacteria such as *E. coli, Salmonella pullorum, Pseudomonas aeruginosa, Pasteurella multocida, Clostridium perfringens, Micrococcus luteus, Streptoccocus bovis* and *Staphylococcus aureus.*

V. CONCLUSION

Sixty endophytic bacterial strains of *Houttuynia cordata* Thunb were isolated from leaves, stems and roots collected in Phong Dien, Binh Thuy and Ninh Kieu of Can Tho city. Fourteen strains are resistant to *Staphyloccocus aureus* bacteria from furuncles in humans with a 10-40 mm sterile ring. Three endophytic bacterial strains of *Houttuynia cordata* Thunb with high antibacterial activity PDT2, BTT4 and NKT3 are identified in order of *Bacillus amyloliquefaciens* strain CD2901, *Bacillus megaterium* strain 22 and *Bacillus subtilis* strain B237. This study is a contribution and recommendation for future studies of endophytic bacteria in *Houttuynia cordata* Thunb as well as endophytic bacteria from other medicinal plants that have antibacterial activity against pathogenic bacteria in human.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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BIOACTIVE COMPOUNDS OF *BACILLUS SUBTILIS* STRAIN B237 ISOLATED FROM THE ENDOPHYTIC BACTERIA IN *HOUTTUYNIA CORDATA THUNB* BY GAS CHROMATOGRAPHY-MASS SPECTROSCOPY

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ABSTRACT

Background: *The study of the endophytic bacteria group capable of providing antimicrobial active compounds in Houttuynia (Houttuynia cordata Thunb) has not been studied much, so we examine this topic.* ***Objective:*** *To identify some compounds from the endophytic bacteria of Houttuynia cordata Thunb from Bacillus subtilis strain B237 having antibacterial activity against Staphylococcus aureus from people's furuncles in Can Tho city.* ***Subjects and methods:*** *Houttuynia cordata Thunb collected in Can Tho city bacteria are isolated in PDA medium. Investigate the antibacterial ability of endophytic bacteria of Houttuynia cordata Thunb with Staphylococcus aureus by diffusion method by filter paper ring and identify by 16S rRNA gene sequencing method of bacteria strains, then compare the sequence of bacterial strains with sequences of bacterial strains in NCBI data bank.* ***Results****: It shows that Bacillus subtilis strain B237 is intracellular bacteria in Houttuynia cordata Thunb, the strain inhibits the growth of Staphylococcus aureus of human furuncle with the clear zone varied from 10 - 40 mm. The biologically active compounds have been identified in hexane-acetone and acetone-methanol organic solvents. The determination of bioactive chemical compounds is based on peak area, retention time, molecular weight and molecular formula.* ***Conclusion****: GC-MS analysis of Bacillus subtilis strain B237 has compounds such as Octadecanoic acid, Pyrrolo[1,2-a]pyrazine-1,4-dione,*