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Original Article

Cell-free Supernatant from *Exiguobacterium acetylicum* Isolated from Water Cabbage (*Pistia stratiotes*) Roots Inhibits *Bacillus subtilis* and *Escherichia coli*

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ABSTRACT

Introduction: The study was carried out to isolate and identify potential antibiotic-producing bacteria associated with water cabbage (*Pistia stratiotes)* roots collected from Pampanga River, Pampanga, Philippines. Seven (7) bacterial colonies were randomly chosen at the 10^{-6} dilution factor. Antibiotic sensitivity test using agar well method revealed that only one isolate out of 7 selected colonies can inhibit the growth of the test organisms. Specifically, the isolate (namely T4) supernatant inhibited *E. coli* and *B. subtilis* but not *S. aureus*. T4's ability to inhibit *E. coli* was comparable with that of Tetracycline (positive control). Surprisingly, its inhibition of *B. subtilis* is significantly higher than that of Tetracycline. 16S rRNA gene sequence analysis using NCBI Basic Local Alignment Search Tool revealed 99% similarity of the isolate (T4) with *Exiguobacterium acetylicum*, a gram-positive, antibiotic-producing bacterium previously isolated from an apple orchard rhizosphere.

Key words: Cell-free supernatant, Antibiotic, *Exiguobacterium acetylicum*, *Pistia stratiotes*.

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INTRODUCTION

Available antibiotics generated by pharmaceutical companies nowadays are mostly natural products obtained from bacteria and other microorganisms.[1] These antibiotics are active substances which have the capability to kill pathogens by means of different mechanisms and strategies.[2] Some antibiotics such as beta lactam drugs like Penicillin, Cephalosporin, Carbapenems and Monobactams are known to disrupt bacterial cell wall[3] whereas Tetracycline and Aminoglycosides bind to the 30S ribosomal subunit[4] while Clindamycin, Macrolides and Chloramphenicol bind to 50S ribosomal subunit resulting in ribosomal activity disruption.[5] Furthermore, Sulfonamides disrupt folate synthesis,[6] quinolones disrupt DNA gyrase[7] and Rifampin targets the RNA polymerase.[8]

In some cases, active sites of compounds from natural sources are being identified and copied to be synthesized in the laboratory for mass production. Some antibiotics are being fused with another antibiotic or fragment of antibiotic to make them more effective and potent like in the case of Amoxiclav which is known for killing many infection-causing pathogens.[9]

Despite the effectives of antibiotics, many pathogens have developed antibiotic resistance making available antibiotics ineffective.[10] These pathogens evolved resistance because of over-prescription, under dosage, and misuse of antibiotics.[11] Bacterial pathogen versatilely combat antibiotic action with many strategies such as inhibition of drug uptake by modifying their membrane structure, creation of antibiotic pumps to release the antibiotics,[12] modifying the enzyme structure with the use of their enzymes and cleaving the antibiotics like in the activity of beta lactamases.[13]

Bacteria that produce antibiotics have gained selective advantage against competing microbes in their environments.[14] To out compete other microbes, these antibiotic-producing bacteria release chemicals that will kill other microbes by releasing substances that are potential antimicrobial agents[15] like in the case of Streptomycin produced by actinomycete *Streptomyces griseus* isolated in soil where competition of microbes is very stringent.[16]

As roots of many plants were reported as pools of potential antibiotic producing bacteria, root-associated bacteria from water plant must also be explored for antibiotic discovery. Water cabbage (*Pistia stratiotes*) which is an aquatic plant commonly found floating on lakes, streams and rivers is known to survive polluted areas like in Pampanga River.[17] However, there are no reported studies on its root-associated microbiome. In this study a root associated bacteria which was identified 99% *Exiguobacte-*

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rium acetylicum based on 16S rRNA gene sequencing was found to have a strong antibiotic potential.

MATERIALS AND METHODS

Research Design

In order to isolate and identify root-associated bacteria with a potential to inhibit the test strain organisms (*E. coli, B. subtilis and S. aureus*), isolation was undertaken using serial dilution, pour plate method, purification and broth culturing, preparation of cell free supernatant[18] followed by antibiotic sensitivity test[19] and molecular identification of 16S rRNA gene sequencing.[20]

Research Procedure

Collection and Plant Identification

The plant samples were collected from Danga River, a part of Pampanga River, using sterile tongs and then contained in a sterile bag. A separate plant specimen was submitted for identification and authentication at the Botany Division of the National Museum of Natural History, Manila, Philippines.[21]

Isolation of Root Associated Bacteria from Water Cabbage (*Pistia stratiotes*)

Sample Preparation

The ten (10) collected water cabbages were washed using 0.9% saline solution. Ten grams of roots were placed in a sterile 225ml flask. Sterile peptone water (HiMedia) with a pH of 7.0 was added and the flask was covered and agitated for 5 minutes. After homogenization of the sample, serial dilution was prepared to obtain countable colonies ranging from the 1:10 to 1:10^6.

Plating of Sample

One (1) mL from each dilution prepared was seeded into sterile petri dishes. Subsequently pre-cooled nutrient agar (HiMedia) at 38°C to 40°C with a pH of 6.9 was poured in each of the plate. The plates were allowed to solidify for four (4) hours and were incubated in an inverted position at 35°C for 48 hours.

Colonies Selection and Purification

After incubation, the number of growing colonies were assessed. Plates with countable growths were used for colony selection. A total of six well-isolated colonies from the 10^{-6} dilution plate were picked individually and sub-cultured twice. Isolates were initially coded as T1 for the first colony, T2 to T7 for the subsequent colonies. Retention of the purified culture of the 7 isolates were also prepared for molecular identification after the antimicrobial screening.

Cell-free supernatant preparation, test strain preparation and antibacterial screening of the isolates

Cell Free Supernatant preparation

The seven isolated colonies were inoculated in 100ml nutrient broth (Hi-Media) in a flask and incubated at 35°C for 5 days with daily shaking intervention. After incubation, cell-free supernatant was prepared by obtaining 10ml of bacterial broth culture. Centrifugation at 2000 rpm for 5 minutes was done followed by membrane filtration using 0.45 µm nylon filter (Whattman) to ensure that there are no bacterial cells in the supernatant. The cell-free supernatants were stored at 4°C for 2 hours prior to antibiotic sensitivity testing.

Test strains preparation

Test organisms including *E. coli, B. subtilis*, and *S. aureus* were obtained from the Department of Public Health Medical Microbiology of the University of the Philippines, Manila. A loopful of each test organism was inoculated into 2.5 ml sterile peptone water contained in a small tube and compared to McFarland standard to give an approximately 1.5×10^8 cfu/ml of the test bacterium.

Positive and negative control

Tetracycline with a concentration of 100 µL per well was set as positive control and double distilled water as negative control.

Antibiotic sensitivity test

Prepared plates containing Muller Hinton Agar (HiMedia) with pH 7.2 was inoculated with 0.3mL of prepared inoculum of the test strains separately. Sterile 10mm cork-borer was used to make wells on each plate. Three (3) hole/ well were created on each plate representing 3 replications.

One hundred (100) µL of cell-free supernatant from each isolate were put on the nutrient agar well accordingly. Same procedure was done for the two controls. The plates were then incubated at 35°C for 24 hours. Zones of inhibition were measured after incubation and the measurement was expressed as millimeter (mm).

Molecular Identification of the isolate

The isolate exhibiting inhibition of test strains were subjected for molecular identification using 16S rRNA gene sequencing at the Philippine Genome Center (PGC) of the University of the Philippines Diliman. DNA extraction, 16S rRNA gene amplification, gel electrophoresis and capillary sequencing were done at the PGC.

DNA Sequence Analysis

Bioedit nucleotide sequence alignment software was used for cleaning and editing the data sequence. The sequence was compared with the publish sequences in GenBank using Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) to determine their identity.

Treatment of Data

Data were subjected to One Way Analysis of Variance (ANOVA). The difference among the means were further analyzed using HSD test. Statistical analyses made use of the GraphPad Prism software version 6.

RESULTS AND DISCUSSION

Culture selection

Out of 6 plates only the plate at 10^{-6} dilution contained countable colonies. The rest of the plate's colonies are reported as Too Numerous to Count (TNTC). Out of 40 colonies from highest plate (10^{-6} dilution), only 7 colonies (growing on the top layer of the agar) were randomly chosen.

Inhibitory potential cell free supernatant of seven isolates against *E. coli*

Figure 1 shows the zones of inhibition of the seven isolates against *Escherichia coli*. The fourth isolate (T4) and T+ (Tetracycline) inhibit *E.coli* with mean diameter of 24.6mm and 25. 33mm respectively. Mean comparison reveals that T4 and T+ are comparable, suggesting that the T4 cell free supernatant has same capability to inhibit *E. coli*. T0 (negative control) T1, T2, T3, T5, T6 and T7 did not inhibit *E. coli* with a mean diameter of zero (0 mm).

The significant comparable result on the inhibition of *E.coli* by T4 supernatant and Tetracycline denotes the strong antibiotic potential against

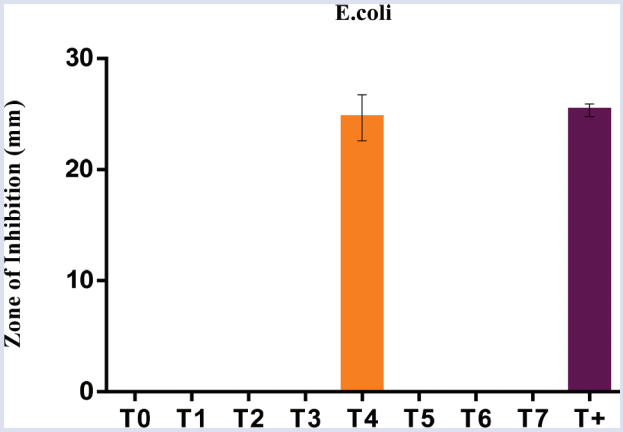

Figure 1: Zone of inhibition against *E. coli* by Bacterial Cell free Supernatant (T1, T2, T3, T4,T5, T6, T7) Negative Control(T0) and Positive Control (T+)

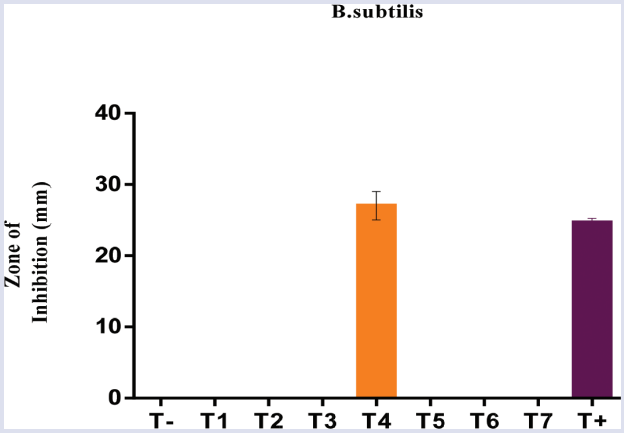

Figure 2: Zone of inhibition against *B. subtilis* by Bacterial Cell-free Supernatant (T1, T2, T3, T4, T5, T6, T7) Negative Control (T0) and Positive Control (T+)

gram negative bacteria. Also, it suggests that the cell free supernatant contains compound or peptide which is excreted by bacteria extracellularly.

Inhibitory potential cell free supernatant of seven isolates against B. subtilis

Figure 2 shows the zone of inhibition of seven isolates against *B. subtilis*. T4 and T+ (positive control) inhibit *B. subtilis* with mean diameter of 27mm and 24.66mm respectively. Mean comparison reveals that T4 is more effective than T+ in inhibiting *B. subtilis*, indicating the T4 supernatant contains a compound or antimicrobial peptide that is effective in inhibiting *B. subtilis*. T0(negative control) T1, T2, T3, T5, T6 and T7 did not inhibit *B. subtilis* with a mean diameter of zero(0 mm).[18]

The significantly higher potential of T4 to inhibit *B. subtilis* compared to the positive control (Tetracycline) signifies a potentially effective antimicrobial compound/peptide produced by the root-associated bacteria (T4).

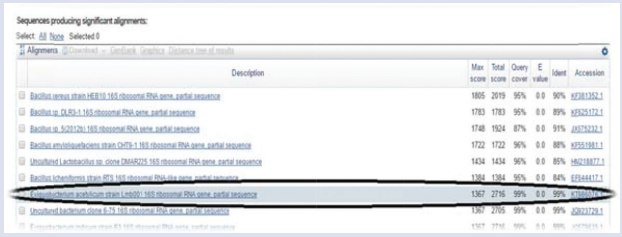

Figure 3: Molecular Identity of the isolated bacteria (T4) base of 16s RNA sequence

Figure 4: Amplicon of 16S rRNA genes sequence(T4)

Inhibitory potential of cell free supernatant of seven isolates against *S. aureus*.

S. aureus was not inhibited by the cell free-supernatant of T4 and as expected was inhibited by positive control with the mean diameter of 22mm. The non-inhibition against *S. aureus* suggests that the compound in T4 supernatant possesses specificity in terms of killing bacteria.

Molecular Identity of T4 bacteria based on 16S rRNA sequencing

Figure 4 presents the amplified 16S rRNA gene of the isolated bacteria with antibiotic potential. Sequence alignment (shown in figure 4) results from BLAST nucleotide of NCBI revealed T4 bacteria has 99% similarity with *Exiguobacterium acetylicum*.

DISCUSSION

The antibiotic producing bacteria isolated from the root of water cabbage (*Pistia stratioties*) in this study was identified as *Exiguobacterium acetylicum* with 99% homology on the sequence available in the public domain. *Exiguobacterium acetylicum* is a gram- positive, yellow bacterium which was found to produce volatile compound (Kumar, 2008) as it showed inhibitory activity against *E. coli* and *B. subtilis*.

CONCLUSION

In this study, a potential antibiotic-producing root-associated bacteria was isolated from water cabbage, *Exiguobacterium acetylicum*. The cell-free supernatant from the isolate was found to inhibit the growth of *E. coli and B. subtilis* but not *S. aureus*. Since the used supernatant is cell-free it can be concluded that a substance is secreted by *Exiguobacterium acetylicum* on its extracellular environment.

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