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IDENTIFICATION OF SECONDARY METABOLITE COMPOUNDS IN TUNICATE (*POLYCARPA AURATA*) ASSOCIATED BACTERIA

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ABSTRACT

Background: Marine biodiversity in the last few decades has been explored and utilized as marine natural products. The secondary metabolites produced by marine organisms are utilized by humans in various aspects of life. One of the main source of secondary metabolites is marine invertebrates, such as tunicate. Several previous studies have shown that many metabolites have been identified in the last 40 years. **Objective:** This study aimed to identify the metabolite compounds produced by tunicate-associated bacteria. **Methods:** The two isolates of bacteria associated with tunicate *Polycarpa aurata*, *Bacillus wiedmannii* and *Virgibacillus salarius*, obtained from the culture collection of Tropical Marine Biotechnology laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang were used as material in this study. This study was carried out in Integrated Laboratory Diponegoro University from July to September 2020. Laboratory experiment was conducted by culturing these bacteria in Zobell Marine Broth 2216 at room temperature. The incubated culture was then added with ethyl acetate in a ratio of 1: 2 and then the supernatant was separated and evaporated. Analysis of the tunicate-associated bacteria methanol extract was carried out using Gas Chromatography-Mass Spectrophotometry (GC-MS). **Results:** 3-N-Hexyl-Delta-9-Tetrahydrocannabinol compound (100%) was discovered by GC-MS analysis from the tunicate-associated bacteria *B. wiedmannii* and *V. salarius* extract. **Conclusion:** 3-N-Hexyl-Delta-9-Tetrahydrocannabinol compound is an isomer compound of Delta-9-Tetrahydrocannabinol (Δ9-THC) which needs further research on its application in medical and non-medical aspects.

Keywords: *Ascidians; Isomere of Tetrahydrocannabinol; Secondary metabolite; Tunicate-associated bacteria*

INTRODUCTION

The pharmaceutical industry in the last few decades has done a lot of research on natural products, which one of them came from the marine environment. The total surface area of sea on the earth shows the extent of biodiversity in the marine environment, where there are millions of unique. species that can produce diverse natural products. [1, 2] Marine pharmacology is a discipline that explores the potential of pharmaceuticals in the marine environment and the development of new drugs. Previous studies have shown the existence of various pharmaceutical potentials from metabolite compounds produced by marine organisms, such as anti-cancer, anti-microbial, anti-fungal, and analgesic. [3] Marine Natural Products are defined as secondary metabolites produced by marine organisms, as a form of adaptation from their defense, food chain, or as communication signal to their environment. [4] Marine invertebrates are proven to be one of the main sources of bioactive compounds, where most marine invertebrates are usually sessile, soft, and prone to predators and microbial colonization. Therefore, marine invertebrates need a way to survive such potential dangers. [5]

One of the marine invertebrates that become a source of active compounds are tunicates. This species is encased in an extracellular or tunic sheath composed of cellulose. Tunicates consist of three classes, namely Ascidiacea, Thalacea, and Appendicularia, where Ascidians are the most widely known types of tunicate that produce various bioactive compounds. [6] In the past 40 years, around 1080 compounds from tunicates were explored, including those that have been used clinically, currently being tested in clinical trials, and are still in the early stages of exploration. [7, 8]

Several previous studies have shown that the origins of these active compounds came from microorganisms living on the invertebrates. These microorganisms form secondary metabolites which facilitate chemical defense to the invertebrates from some adverse conditions. It caused a large number of active compounds were produced from marine invertebrates and associated bacteria for the discovery of marine natural products. This interaction also provides a great opportunity for the discovery of new compounds without massive

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damage to marine ecosystems by exploring the associated bacteria. [9]

The discovery of bioactive compounds that have been isolated from tunicate-associated bacteria consists of various compounds from alkaloids, polypeptides, and polyketides, that have been successfully developed to become new drug candidates. A previous study discovered a cytotoxic compound to leukemia cells in mice called Sesbanimide A, which was isolated from the *Agrobacterium* bacteria associated with *E. turbinata* tunicate.[10] Didemnins A-C compounds isolated from *Tridemnum solidum* associated bacteria, *Tistrella mobilis* and *Tistrella bauzanensis*, which has a potential as a cytotoxic, and became the first marine natural product to be carried out in clinical trials. [11] In research on the tunicate *Polycarpa aurata*, an isatin compound from the associated bacterium *Pseudoalteromonas rubra*, had potential as an antimicrobial to gram-positive bacteria MDR *B. cereus* and *Micrococcus luteus*, also negative pathogenic bacteria MDR ESBL *E. coli* dan *E. Coli*.[12]

Although as many as 7% of the tunicate-associated bacteria have been known to produce tunicate metabolites, the sources of the other 93% metabolites are still unknown.[13, 14] Therefore, the objective of this study is to identify metabolite compounds from the tunicate *Polycarpa aurata* associated bacteria as a potential to be a discovery in the pharmaceutical field.

METHOD

This study used an experimental design to identify metabolite compounds from the tunicate Polycarpa aurata associated bacteria. The experiment began with the extraction of the tunicate-associated bacteria, then analyzed with the Gas Chromatography-Mass Spectrometry method to identify its bioactive compounds.

2.1 Extraction and sample preparation

The tunicate-associated bacteria, *Bacillus wiedmannii* and *Virgibacillus salarius*, were obtained from the Faculty of Marine and Fisheries Sciences, Diponegoro University, Semarang, which then carried out on liquid culture as a pre-culture. The pre-culture was made by making 30 ml of Zobell Marine Broth 2216 as a liquid medium using a 100 ml Erlenmeyer. Incubation was carried out for 3x24 hours at room temperature using a shaker that moved at a speed of 120rpm. A total of 5% pre-culture was then transferred to 600 ml of Zobell Marine Broth 2216 which was divided into four 250 ml Erlenmeyers, then incubated for 5x24 using a shaker that moved at a speed of 120 rpm at room temperature.

The incubated culture was then added with ethyl acetate in a ratio of 1: 2 and then the supernatant was separated using a separatory funnel. The extraction was then carried out using a rotary evaporator until the entire ethyl acetate solution was successfully evaporated. The extraction results were then dissolved in 10 ml methanol for GC-MS analysis.

2.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

GC-MS analysis was carried out at the Integrated Laboratory, Islamic University of Indonesia, Yogyakarta, using the GC-MS-QP2010 SE system (Shimadzu, Kyoto, Japan), which consists of a headspace sampler (AOC-20s) and autoinjector (AOC-20i). This system used FID and MS detectors, and the columns used are Rtx-5MS (5% diphenyl / 95% dimethyl polysiloxane) and Carbowax (Polyethylene glycol), with column size of 30mm x 0.25 mm (length x diameter) and a film thickness of 0.25 µm. The mobile phase used in the analysis was helium (> 99.99%) with a linear velocity of 40.5 cm/second. The initial temperature was set at 80^{o}C (3 minutes) and increased to 280^{o}C with a ramp rate of 10°C/min. The relative quantities of chemical compounds present in the extract of tunicate-associated bacteria were shown in the form of a percentage based on the peak area on the chromatogram.[15]

2.3 Identification of compounds

Analyzed bioactive compounds from the extract of tunicate-associated bacteria were identified based on the retention time of the Rtx-5MS column on Gas Chromatography and matched to the spectrum based on the Wiley Spectral Libraries database.

RESULTS

GC-MS analysis of the tunicate-associated bacteria *B. wiedmannii* and *V. salarius* were confirmed by the presence of metabolite compounds in the methanol extract (Figure 1 and 2). The compounds contained could be seen from the retention time (RT), molecular formula, molecular weight, and peak area (%), which were shown in Table 1-2. The compounds were analyzed based on

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Mass Spectrometry linked to Gas Chromatography. Metabolite compounds contained in the two methanol extracts of tunicate association bacteria are 3-N-Hexyl-Delta-9-Tetrahydrocannabinol (Figure 3) with a peak area of 100% in each of the tunicate-associated bacteria extracts.

DISCUSSION

The compounds obtained from GC-MS analysis of the tunicate-associated bacteria *B. wiedmannii* and *V.salarius* methanol extracts were 3-N-Hexyl-Delta-9-Tetrahydrocannabinol, with the IUPAC name 3-hexyl-6a, 7,8,10a-tetrahydro-6, 6,9-trimethyl- 6H dibenzo [b, d] pyran- 1-ol. The identical compound with different retention times was analyzed because there was no target screening for specific compounds. Non-target screening has the risk of misassignment for compounds with no strong molecular signals produced and compounds with similar spectra, such as isomers. [16]. 3-N-Hexyl-Delta-9- Tetrahydrocannabinol compound was included in an isomeric group of Delta-9-Tetrahydrocannabinol (Δ9-THC) compound, known as parahexyl [17]. Parahexyl is a homolog of THC, which was first discovered in 1949 in an attempt to elucidate the structure of Δ9-THC, the active compound of cannabis. Parahexyl is similar in structure and activity to THC [18].

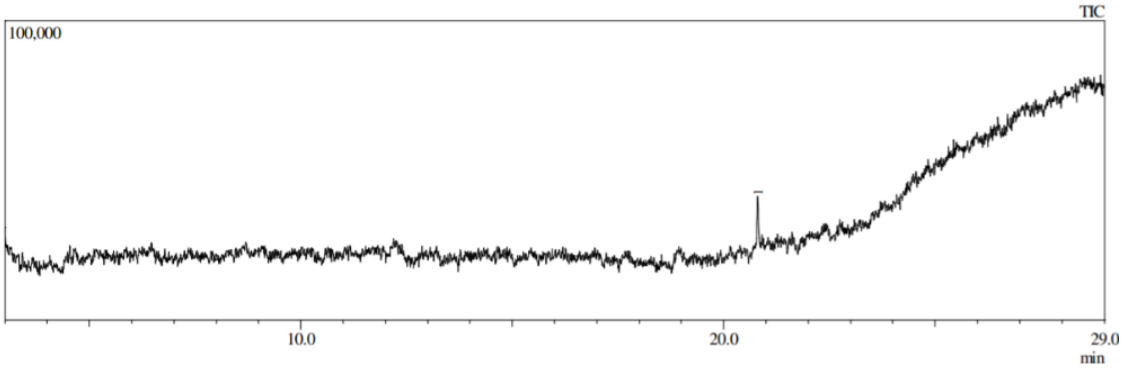

Figure 1. Gas Chromatography-Mass Spectrometry chromatogram of *B. wiedmannii* methanol extract

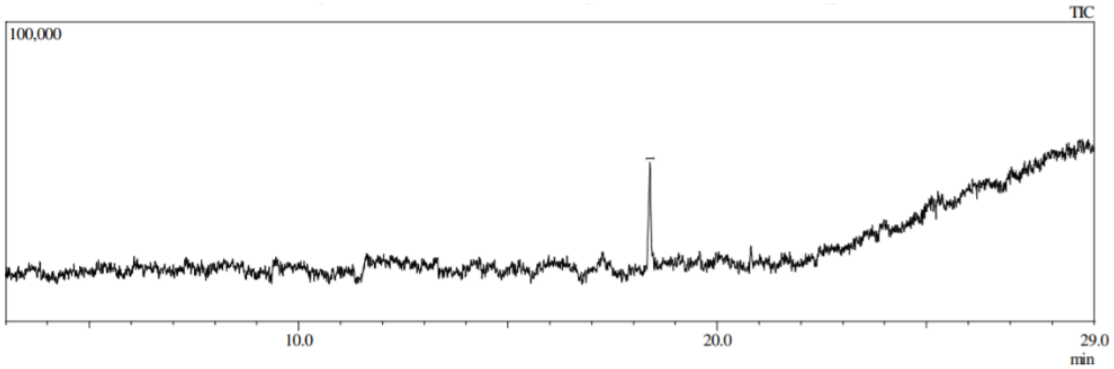

Figure 2. Gas Chromatography-Mass Spectrometry chromatogram of *V. Salarius* methanol extract

Table 1. Compound identified in the *B. wiedmannii* methanol extract by GC-MS peak report

Peak	Compound name	RT	Area %	Formula	Molecular weight
1	3-N-Hexyl-Delta-9-Tetrahydrocannabinol	20.804	20.767	C22H32O2	328

Table 2. Compound identified in the *V. salarius* methanol extract by GC-MS peak report

Peak	Compound name	RT	Area %	Formula	Molecular weight
1	3-N-Hexyl-Delta-9-Tetrahydrocannabinol	18.391	18.333	C22H32O2	328

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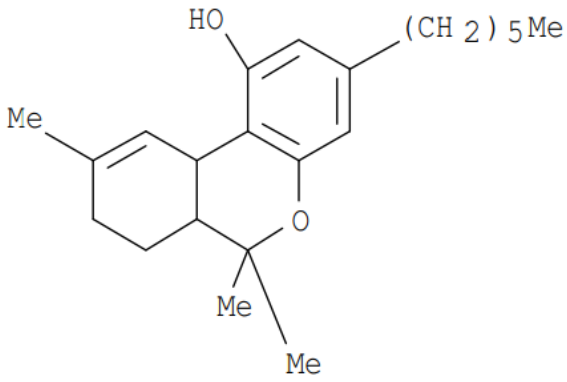

Figure 3. Molecular structure of 3-N-Hexyl-Delta-9-Tetrahydrocannabinol

Parahexyl compounds with the IUPAC name 3-hexyl-6a, 7,8,10a-tetrahydro- 6,6,9-trimethyl-6H dibenzo [b, d] pyran-1-ol has not been specifically studied before. Parahexyl group compounds in previous studies have shown several potential applications in the medical field. The compound parahexyl 3(1,2-dimethyl heptyl) has been tested on epilepsy patients for 3-7 weeks and has shown successful anti-epilepsy treatment results [19]**.** Another parahexyl class compound, namely 1-hydroxy- 3-n-hexyl- 6-6-9-trimethyl7-8-9-10-tetrahydro-6-dibenzopyran, has shown potential as a strong anti-depressant agent in 36 of 50 patients with depression. This parahexyl compound had a specific action in the thalamus and cortex by providing a euphoric effect, increasing feelings of joy and confidence, and decreasing anxiety [20]. Other isomeric compounds of cannabinoid have also been listed by the World Health Organization (WHO), where Delta-8-THC and Delta-9(11)-THC compounds had shown psychotropic effects similar to Δ9-THC [21]. The synthetic compound of Δ9-THC, known as Dronabinol, had been approved by the Food and Drug Administration (FDA) as a treatment for anorexia caused by HIV/AIDS and chemotherapy-induced nausea and vomiting (CINV) in patients who could not respond to conventional anti-emetics [22–24].

CONCLUSION

The results of GC-MS analysis of the tunicate *P. aurata* associated bacteria, *B.wiedmannii* and *V. salarius*, showed the presence of the 3-N-Hexyl-Delta-9-Tetrahydrocannabinol, which was an isomer compound of Δ9-Tetrahydrocannabinol (Δ9-THC). There has been no further research on these compounds, and we hope further research will be carried out on the benefits of the 3-N-Hexyl-Delta-9 Tetrahydrocannabinol compound for medical applications.

Ethical Approval

Ethical approval was obtained from the Medical Research Ethical Committee of Kariadi. The Ethical Clearance of this research was No. 56/EC/H/FK-UNDIP/VI/2020.

Conflicts of Interest

The authors declare no conflict of interest.

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Author Contributions

Conceptualization, S.R., A.W.U., and E.M..; Formal analysis, S.R.; Funding acquisition, S.R.; Investigation, S.R.; Methodology, S.R., A.W.U., R.H., and E.M.; Resources, S.R.; Supervision, A.W.U., R.H., and E.M.; Visualization, S.R; Writing—original draft, S.R.; Writing—review & editing, A.W.U., R.H., and E.M.

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