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Antibacterial activity and bioactive compounds of symbiotic bacterial isolates from *Polycarpa aurata* in Barrang Lompo Island, Makassar, Indonesia

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Abstract. *Herwin, Alam G, Sartini, Hasan N, Rahim A, Rante H, Yulianti R, Rahman L. 2025. Antibacterial activity and bioactive compounds of symbiotic bacterial isolates from* Polycarpa aurata *in Barrang Lompo Island, Makassar, Indonesia. Biodiversitas 26: 2339-2354. Polycarpa aurata* is a marine organism capable of producing secondary metabolites from its symbiont microorganism and has therapeutic potential, such as antitumor and antimicrobial. *P. aurata* isolated from the symbiont bacteria obtained the AQ2-1 isolate, and secondary metabolites were produced through fermentation to obtain the AQ2-1 fermented ethyl acetate extract. The ethyl acetate extract was a bioactive compound isolated using radial chromatography and preparative thin-layer chromatography to obtain isolate 1 and isolate 2. Using ultraviolet-visible spectroscopy analysis, one-dimensional and two-dimensional nuclear magnetic resonance, and mass spectroscopy, the structure of the compound showed an adenine riboside compound (isolate 1), which has a λ max of 242 nm, 10 carbon atoms, 13 hydrogen atoms, 5 nitrogen atoms, 4 oxygen atoms, and molecular formula of $C_{10}H_{13}N_5O_4$, m/z 267, and Bis(2-ethylhexyl) benzene-1,2-dicarboxylate (isolate 2), which has a λ max of 218 nm, 24 carbon atoms, 38 hydrogen atoms, 4 oxygen atoms, and a molecular formula of $C_{24}H_{38}O_4$, m/z 390. Based on an analysis of the biological activity of compounds 1 and 2 using bioautography thin-layer chromatography, these compounds were found to be active against *Escherichia coli* ATTC 25922, *Salmonella typhi* NCTC 786, *Shigella dysenteriae* ATCC 13313, *Pseudomonas aeruginosa* ATCC 27853, and *Vibrio cholerae* ATCC 25175 bacteria.

Keywords: Antibacterial, chemical compound, isolate AQ2-1 symbiont bacteria, *Polycarpa aurata*

INTRODUCTION

Tunicates are marine organisms that contain many bioactive compounds sourced from symbiotic microbes that may have applications as anticancer, antitumor, and antibacterial agents (Casertano et al. 2020). They are essential for maintaining the stability of marine ecosystems by offering fertile habitats for various marine organisms, contributing to the food chain and serving as prey for other marine species (Jabir et al. 2022). Tunicates have the most prevalent marine ecosystem, with more than 3,000 species inhabiting diverse habitats, ranging from shallow to deep seas. These organisms possess a wide range of bioactive and characteristic compounds (Casertano et al. 2020). Bioactive chemicals may be derived from the host organism or from symbiotic bacteria interacting with it, acting as precursors for therapeutic development. Microbes are continuously examined as a source of antibiotics, antivirals, antibacterials, antifungals, and antiprotozoals to discover novel bioactive molecules for disease treatment (Cragg and Newman 2013). The investigation of bioactive compounds is increasingly critical due to the diminishing efficacy of numerous antimicrobial medications and the loss of therapeutic advantages resulting from overuse or incorrect dosages (Furusawa et al. 2018; WHO 2019).

Natural microbes exhibit distinctive structures and a diverse array of biological activities that address various diseases, including the production of bioactive compounds utilized in antibiotics, diabetes medications, and cancer therapies. Isolating most microbes to obtain bioactive compounds for drug development can be challenging (Carrano and Marinelli 2015; Sun et al. 2019). Marine organisms,

such as the *Polycarpa aurata* (Quoy & Gaimard, 1834) type tunicate, characterized by their soft or semi-rigid bodies and various colors, including blue, yellow, white, and red, serve as potential sources for bioactive compounds. *P. aurata* exhibited antimicrobial properties in extracts of hexane, chloroform, and methanol. This treatment demonstrates notable efficacy against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* (Manoppo et al. 2019). The methanol extract from the water of Manado Bay, North Sulawesi, exhibits the highest activity against *E. coli* and *S. aureus* (Rompas et al. 2022). Chloroform fractions exhibited moderate activity against *Salmonella typhi* and *E. coli* (Kumayas et al. 2015). Endophytic bacteria from *P. aurata* exhibited both bacteriostatic and bactericidal antibacterial properties (Litaay et al. 2015).

The pursuit of metabolites as beneficial substances is motivated by the prevalence of pathogenic bacterial infectious illnesses in the community. This condition can be caused by several antibiotic-resistant bacteria that infiltrate the body through both systemic and topical routes. The disease is attributed to bacterial infections, including *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (Wimmerstedt and Kahlmeter 2008; Haghi et al. 2010; Boss et al. 2016). Addressing antibiotic resistance is a major challenge for society, as it requires urgent resolution. Antibiotic resistance arises when the bacteria are not susceptible to the drug's effects, preventing them from effectively killing or inhibiting them. Antibiotic resistance is increasing and endangering all parts of the world (O'Neill 2016; Murray et al. 2022). The World Health Organization (WHO) released a list of pathogenic bacteria that are resistant to antibiotic drugs; thus, research and development of new drugs are needed (WHO 2017). Antimicrobial resistance significantly threatens human health (Dhingra et al. 2020). Due to the problem of antibiotic resistance, it is necessary to search for novel bioactive compounds derived from marine biota, such as the *P. aurata* species (Schmidt et al. 2012).

Investigations have revealed that *P. aurata* contains novel alkaloids, such as policarpaurins, tetracyclic pyridoacridines, pentacyclic pyridoacridines, segoline A, tetrahydro-β-carbolines, N-methyl-β-carbolinium, cyclic peptides, depsipeptides, and various aromatic alkaloids (Menna et al. 2011; Dou and Dong 2019). The hydrophylic extract of *P. aurata* was identified as containing three new compounds: N-(4-methoxybenzoyl)-N'-methylguanidine (i), butyl 2-(4-methoxyphenyl)-2-oxoacetate (ii), and 2-(4-methoxyphenyl)-Nmethyl-2-oxoacetamide (iii) (Wessels et al. 2001). *P.aurata* was identified as the compound isatin originating from the symbiont bacteria *Pseudoalteromonas rubra* strain, which has antimicrobial potential against the Gram-positive bacteria MDR *Bacillus cereus*, *Micrococcus luteus*, and Gram-negative MDR *E. coli* (Ayuningrum et al. 2019a). Certain tunicates, such as the *Ecteinascidia turbinate* species, marketed as the cancer treatment Yondelis®, and the *Aplidium albicans* species, known as Aplidin®, have been developed into pharmaceutical applications (Watters 2018). Thus, the exploration of bioactive compounds from *P. aurata* is necessary development for the treatment of infectious diseases.

MATERIALS AND METHODS

Ethical approval

This study was reviewed and approved by the health research ethics committee to maintain and respect the dignity of living creatures (animals), which are the research subjects, with registration number UMI1022408586.

Sampling area and isolation of symbiont bacteria

Polycarpa aurata were collected from Barrang Lompo Island in Makassar City, South Sulawesi, Indonesia (Figure 1). The sampling site was positioned at a longitude of 119°19'50.12"E and a latitude of 5°3'0.26"S. The samples were collected through snorkeling and skin-diving techniques from subtidal environments at depths of 15-30 m.

The isolation of the symbiont bacteria from *P. aurata*, characterized by its white color, was performed using a Nutrient Agar (NA) medium prepared with seawater and distilled water. This process yielded seven isolates of symbiont bacteria, which were subsequently incubated at a temperature of 37℃ for a duration of 1-3 h. The isolated compound was purified and evaluated for its antibacterial activity against pathogenic bacteria (Hidayat et al. 2018). The active isolate was produced from secondary metabolites through fermentation in a Maltose Broth (MB) medium supplemented with yeast extract nutrients for 68 h. The fermented product was then extracted using ethyl acetate pa to obtain the ethyl acetate extract (Sipriyadi et al. 2020).

Isolation and purification of *Polycarpa aurata* symbiont bacteria

The surface contamination of the wet-sorted *P. aurata* sample was treated by applying 70% ethanol for 1 min, followed by 5.25% sodium hypochlorite for 1 min. The sample was then rinsed three times with sterile Aquadest for 1 min each time. The *P. aurata* specimen was dehydrated and finely chopped into small pieces, measuring around 1 cm. The symbiont bacteria were isolated using NA medium with seawater as a solvent and NA medium with distilled water as a solvent. The isolation of symbiont bacteria was carried out aseptically, in which small pieces of *P. aurata* were placed on top of the NA medium in a sterile petri dish and then incubated at a temperature of 37℃ for 1-3 days, depending on the growth rate of the symbiont bacteria (Krishnan et al. 2012; Hidayat et al. 2018).

The symbiotic bacteria were purified and isolated by aseptically taking 1 bacterial colony using a sterile wire, and then a part of the bacteria was spread over the surface of the NA medium in a sterile petri dish. Each isolate was purified using the quadrant grinding method, followed by incubation at a temperature of 37℃ for 24 h. The pure insulate was prepared by inoculating on the NA medium and transferring it into sterile reaction tubes as stock (Marzuki et al. 2018).

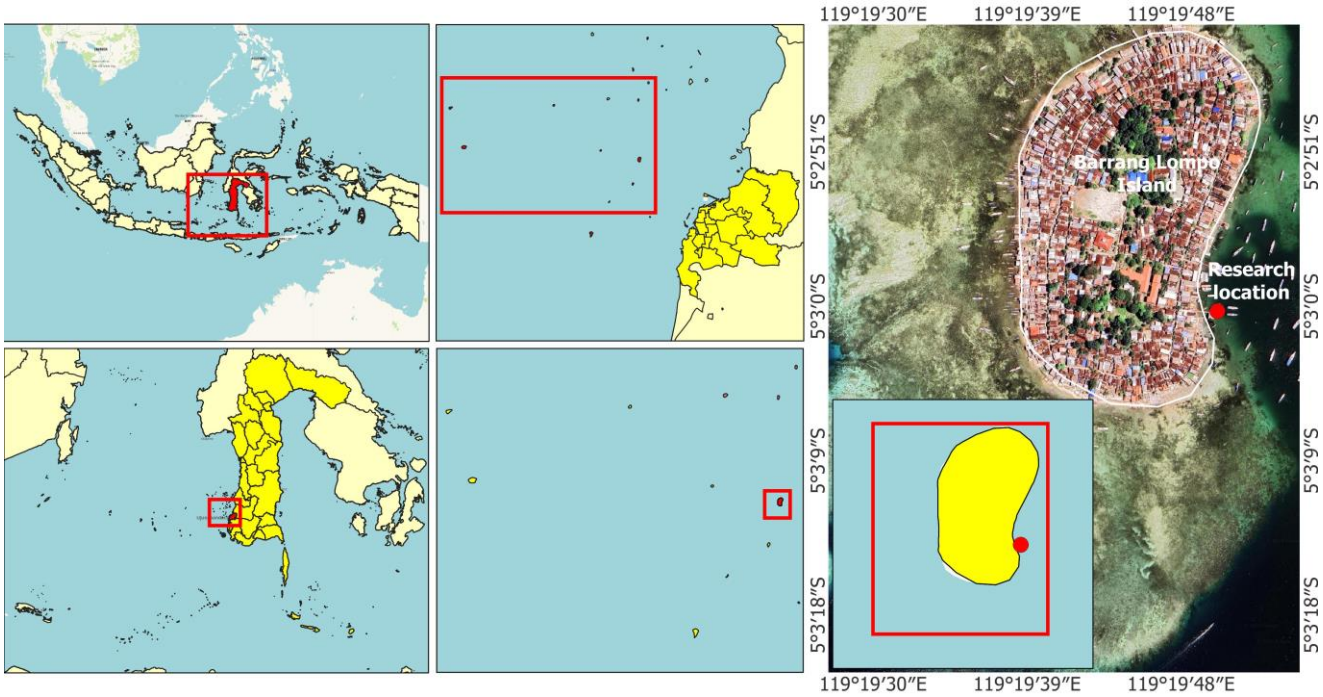


Figure 1. Location of *Polycarpa aurata* on Barrang Lompo Island (longitude of 119°19'50.12"E and latitude of 5°3'0.26"S), Makassar City, South Sulawesi, Indonesia

Antibacterial test of the symbiont *Polycarpa aurata* bacteria isolates

An antibacterial test of the symbiont bacteria isolates was conducted in a densely diluted manner in which all the isolates of *P. aurata* bacteria that grew on the NA medium were blocked by cutting them using sterile stainless cylinder shrinkers (6 mm). The pieces of the bacterial isolates were placed in a petri dish containing a sterile MHA medium with a suspension of the test bacteria (*E. coli* ATCC 25922, *S. typhi* NCTC 786, *V. cholerae* ATCC 25175, *S. dysenteriae* ATCC 13313, *P. aeruginosa* ATCC 27853, *S. epidermidis* ATCC 14990, *B. subtilis* ATCC 6633, *P. acnes* NCTC 737, *S. aureus* ATCC 25923, and *S. mutans* ATCC 25175) and then incubated at a temperature of 37°C for 18-24 h. Each isolation was observed by visually examining the formation of a lymphatic zone around the isolation piece and measuring the diameter of the barrier zone (Rante et al. 2022).

Production and extraction of secondary metabolites

The AQ2-1 bacterial isolate was cultured in a 10 mL MB medium and incubated at a temperature of 37°C for 68 h. The symbiont bacteria culture was fermented by transferring 10 mL into a 100 mL MB medium. The fermentation process was carried out using a shaker at a speed of 150 rpm for 68 h (optimal time of the AQ2-1 isolate growth) at 37°C. Secondary metabolites were produced in 25 repetitions to a fermentation medium volume of 2,500 mL in an NB medium. The fermentation medium was modified as a substrate source with variations in nitrogen, namely peptone, tryptone, meat, and yeast extract, at a concentration of 0.3% (v/v) to obtain the supernatant and mycelia. The supernatant was extracted with an ethyl acetate solvent to obtain the ethyl acetate extract, and the weight of the extract was calculated (Sipriyadi et al. 2020).

Fractionation and purification of isolates

The ethyl acetate extract of the symbiont bacteria AQ2-1 isolate from *P. aurata* was fractionated using VLC with a column diameter of 6 cm (using a stationary phase silica gel 60 GF_{254} with a silica weight of 10 g) and pa-grade solvents. The mobile phase used consisted of DCM and ethyl acetate at various ratios (9:1, 8:2, 7:3, 1:1, 4:6, and 2:8), followed by acetone (150 mL) and methanol (three repetitions). Fractions were analyzed using TLC measuring 1 cm × 7.5 cm with chloroform:methanol (9:1) as the mobile phase (Kurniawan and Ersam 2018; Farabi et al. 2021). The antibacterial activity of the fractions was assessed using bioautography-TLC, which facilitated the identification of the active fractions. The active fractions were utilized for the isolation and purification of active isolates (Kabir et al. 2021; Salsabila et al. 2022).

The active fractions derived from VLC underwent additional purification to isolate chemical compounds using RC with a plate size of 1/1 mm. This process utilized a mobile phase comprising n-hexane and ethyl acetate at varying ratios (4:1, 3:7, 2:8, and 1:9), along with acetone (100 mL, applied three times) and methanol (200 mL), culminating in the acquisition of subfractions (Tomou et al. 2020). The subfractions were systematically collected and observed using TLC measuring 1 cm × 7.5 cm through the mobile phase of chloroform:methanol at a ratio of 9:1. Pure isolates were meticulously isolated from the subfractions through the application of PTLC measuring 20 cm × 20 cm

using the mobile phase of chloroform:methanol pa (9:1) to obtain isolates 1 and 2. The purity of isolates 1 and 2 was tested using two-way TLC using the mobile phase of chloroform:methanol (9:1) and n-hexane:ethyl acetate (1:1) to obtain pure isolates (Khiralla et al. 2020; Credo et al. 2022).

Spectroscopic analysis

Pure isolates derived from symbiont bacteria (isolates 1 and 2) were identified through UV-vis spectroscopy using a diode array detector. The samples were analyzed by positioning them between the monochromator and the detector. The spectra produced were subsequently recorded on the device (Gultom 2016; Tejamukti et al. 2020). The pure isolates obtained from fractionation were identified through 1D and 2D-NMR spectroscopy using $CDCl_3$ as the solvent at a frequency of 500 MHz. The samples were positioned as films within glass tubes situated between two north and south coils and rotated vertically at speeds exceeding 25 Hz using air-driven spinners. The spectra were obtained using JOEL JNM ECA-500 spectrophotometer. Chemical shifts are expressed in parts per million (ppm) downfield from tetramethylsilane (TMS), which serves as the standard (Gultom 2016; Lu et al. 2021). The connectivity of ^1H atoms was established through COSY analysis, and ^1H-^{13}C two- and three-bond interactions were examined using HMBC and HSQC 2D spectroscopy with $CDCl_3$ as the solvent at 500 MHz (Cherfia et al. 2020; Vind et al. 2022).

MS was used to identify the pure isolates and ascertain their molecular weights. The mass spectra were obtained through liquid chromatography-electrospray ionization-time of flight-mass spectroscopy (LC-ESI-ToF-MS) using a SunFire column (4.6 nm × 150 mm) under isocratic conditions with a mobile phase of 0.1% formic acid in water:acetonitrile (45/55, v/v) at a flow rate of 0.7 mL/min. The injection volume was 10 µL, with a capillary voltage of 1800 V and a cone voltage of 60 V. The molecular weight of the symbiont bacterial isolate was ascertained from the resulting spectra (Casertano et al. 2019; Asmara et al. 2023).

Bioautography-TLC assay

Bioautography-TLC was used to evaluate the antibacterial activity of the ethyl acetate extract, subfractions, and pure isolates derived from the fermentate isolate AQ2-1 of symbiont bacteria sourced from *P. aurata*. Chromatograms were placed in petri dishes containing 10 mL of MHA medium, with 20 µL of test bacteria suspension added: *E. coli* ATCC 25922, *S. typhi* NCTC 786, *V. cholerae* ATCC 25175, and *S. dysenteriae* ATCC 13313 (McFarland standard at a concentration of 1×10^8 cells/mL) (Ambarwati et al. 2020; Prasetya et al. 2024). The mixture underwent homogenization and was allowed to stand for a duration of 60 min. The plates were subsequently removed and incubated for a duration of 24 h at a temperature of 37°C (Gulve and Deshmukh 2012; Shetty et al. 2014). The inhibitory zones were noted, and the Retention factor (Rf) values of the symbiont bacterial isolate were determined (Suhendar and Sogandi 2019; Syame et al. 2022).

RESULTS AND DISCUSSION

Sample preparation and determination

Polycarpa aurata are marine organisms classified as tunicates. In this study, they were collected from Barrang Lompo Island, Makassar City. The samples were retrieved using snorkeling and skin-diving methods from subtidal habitats at depths of 15-30 m. The specimens used in this study were white-colored *P. aurata* (Figure 2).

Following analysis, the determination number 038/ILK.BIO/PP.13/06/2023 identified the tunicate sample as belonging to the species *P. aurata*. The description shows that it has a vase-shaped body with two siphons (water channels), one located at the top and the other on the side. This tunicate can grow to a size of 5-15 cm, has a rigid body, and lives solitarily attached to substrates. This species has a white body with purple patterns in certain parts.

Isolation and purification of *Polycarpa aurata* symbiont bacteria

The result of the isolation of symbiont bacteria from the white-colored *P. aurata* was performed using an NA medium prepared with seawater and distilled water. This process yielded seven isolates of symbiont bacteria, namely five pure isolates with seawater solvents (isolate coded AL2-1, AL2-2, AL2-3, AL2-4, and AL2-5) and two pure isolates with distilled water solvents (isolate coded AQ2-1 and AQ2-2). *P. aurata* is a marine biota that produce secondary metabolites from symbiont microorganisms with a variety of biological activities. Symbiont microorganisms found in tunicates group, such as *P. aurata*, *P. pomeria* (Savigny, 1816), and *Didemnum* sp., are *Bacillus tropicus* strains, *Virgibacillus massiliensis* strains, and *Vibrio natriegens* strains (Ayuningrum et al. 2019b). Other symbiont bacteria found in *P. aurata* are *Bacillus wiedmannii* strains and *Virgibacillus salaries* strains, with the secondary metabolite compound produced being 3-N-Hexyl-Delta-9-Tetrahydrocannabinol (Raisa et al. 2021).

Antibacterial Activity of Isolates Symbiont Bacteria

Antibacterial activity of symbiont bacteria isolates televated that isolates AL2-1, AL2-2 and AQ2-2 (did not provide antibacterial activity), isolates AL2-3 and AL2-4 were active against *P. acnes* NCTC 737 and *S. dysenteriae* ATCC 13313 bacteria (with inhibition zone diameters of 8.45 mm and 17.45 mm), and isolate AL2-5 was active against *S. dysenteriae* ATCC 13313 bacteria (with inhibition zone diameter 10.91 mm). Antibacterial testing revealed that isolate AQ2-1 demonstrated the highest inhibitory activity against 10 bacterial strains: *E. coli* ATCC 25922, *S. typhi* NCTC 786, *V. cholerae* ATCC 25175, *S. dysenteriae* ATCC 13313, *P. aeruginosa* ATCC 27853, *S. epidermidis* ATCC 14990, *B. subtilis* ATCC 6633, *P. acnes* NCTC 737, *S. aureus* ATCC 25923, and *S. mutans* ATCC 25175 (with the largest inhibition zone diameter against *V. cholerae* ATCC 25175 bacteria of 26.47 mm) (Table 1; Figure 3). Based on the grouping of the inhibition zona diameter of the antibacterial activity of the symbiont bacteria isolates *P. aurata*, it show that the inhibition zone diameter of the

AL2-3 isolate is include in the moderate category (between 5-10 mm), the AL2-4 and AL2-5 isolates are included in the strong category (between 10-20 mm) and the AQ2-1 isolate is included in very strong category (>20 mm) (Ariani and Niah 2019).

Production and extraction of secondary metabolites

The production of secondary metabolites of isolate AQ2-1 of *P. aurata* symbiont bacteria by fermentation using a variety of nutrients from nitrogen sources such as meat, peptone, tryptone, and yeast extract in a 100 mL fermenting media using shaker at a speed of 150 rpm for 68 h (optimal time of the AQ2-1 isolate growth) at 37℃, obtained the heaviest ethyl acetate extract weight with yeast yeast extract nutrient = 16 mg, then with peptone nutrients = 15.4 mg, meat = 14.7 mg and tryptone = 10.6 mg. The Bacterial growth there are strongly influenced by environmental factors, including physical, chemical, and biological factors. Physico-chemical factors include temperature, pH, aeration, agitation, and growth media composition. Meanwhile, biological factors are competition and contamination (Rocchetti et al. 2020).

Figure 2. White-colored *Polycarpa aurata* from Barrang Lompo Island, Makassar City

Table 1. Results of the antibacterial activity test of isolates symbiont bacteria using Agar Diffusion

Isolate code	Average diameter inhibition zone (mm)									
	BS	**SA**	**SE**	**Pac**	**SM**	**EC**	**SD**	**PA**	**VC**	**ST**
AL2-3	0,00	0,00	0,00	8,45	0,00	0,00	17,45	0,00	0,00	0,00
AL2-4	0,00	0,00	0,00	8,35	0,00	0,00	9,37	0,00	0,00	0,00
AL2-5	0,00	0,00	0,00	0,00	0,00	0,00	10,91	0,00	0,00	0,00
AQ2-1	20,38	18,91	17,88	21,93	22,23	19,52	25,39	22,19	26,47	18,94

Note: AL2-1: Isolate 1 white-coloured *Polycarpa aurata* symbiotic bacteria with seawater solvent; AQ2-1: Isolate 1 white-coloured *Polycarpa aurata* symbiotic bacteria with aquadest solvent; BS: *Bacillus subtilis* ATCC 6633; SA: *Staphylococcus aureus* ATCC 25923; SE: *Staphylicoccus epidermidis* ATCC 14990; PAc: *Propionibacterium acnes* NCTC 737; SM: *Streptococcus mutans* ATCC 25175; EC: *Eschericia coli* ATTC 25922; SD: *Shigella dysenteriae* ATCC 13313; PA: *Pseudomonas aeruginosa* ATCC 27853; VC: *Vibrio cholerae* ATCC 25175; ST: *Salmonella thypi* NCTC 786

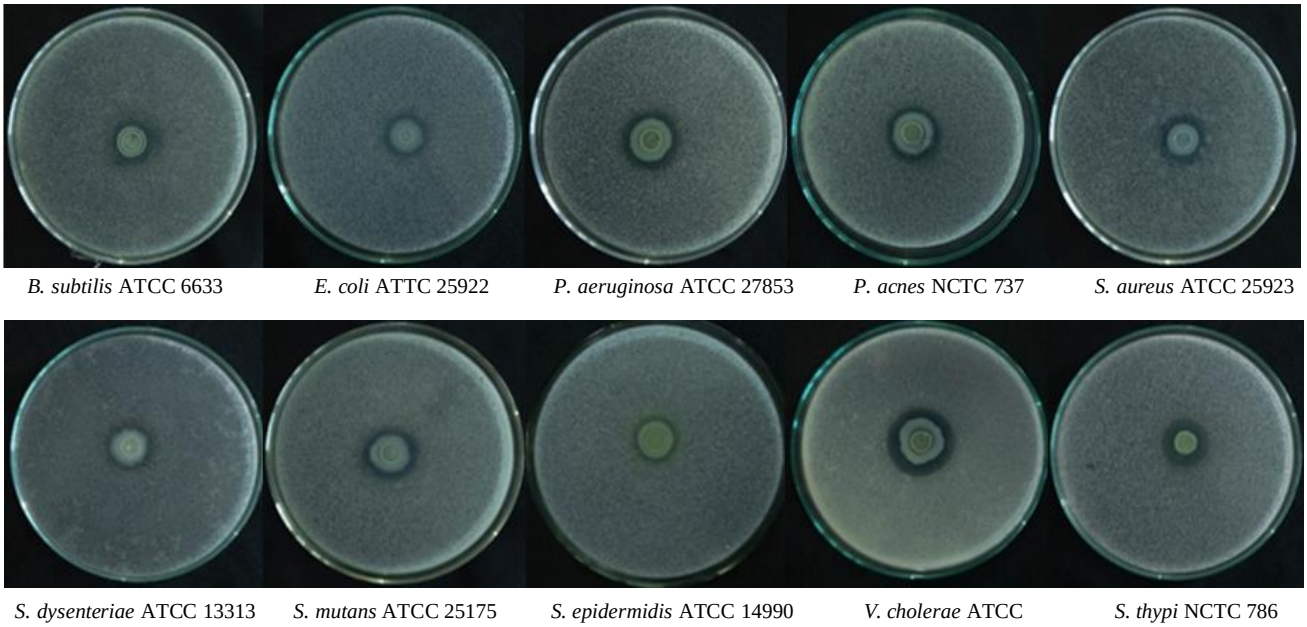


Figure 3. Antibaterial activity of AQ2-1 isolate symbiont bacteria against pathogenic bacteria

These environmental parameters have an interrelated relationship with each other. Brightness factors, depth, pH, temperature, salinity, time, and nutrient sources are factors that can influence the activity of bacterial. Bacterial require a pH value ranging from 6.5-7.5 generally acids have a bad influence on bacterial growth (Sidabutar et al. 2019; Sari et al. 2021). Temperature and salinity have a relationship with water depth, whereas the depth of a body of water increases, the salinity value increases, the salt content is high. Meanwhile, the temperature is also increasing (Al Tanto and Kusumah 2016; Sidabutar et al. 2019). The nutritional factors greatly influence the weight of secondary metabolites products by each isolate of the symbiont bacterial *P. aurata*. Based on nutritional variations in meat, peptone, tryptone and yeast extract, obtained different weights extract of each isolate.

Isolation of chemical compounds from ethyl acetate extract isolate AQ2-1

Fractionation results

The fractionation of 4.71 g of the ethyl acetate extract from the fermentate AQ2-1 isolate of *P. aurata* symbiont bacteria was performed using VLC with a column diameter of 6 cm and a stationary phase silica gel 60 GF_{254} (silica weight 10 g). DCM and ethyl acetate were combined at varying ratios (9:1, 8:2, 7:3, 5:5, 4:6, and 2:8) to prepare the mobile phase. Subsequently, 100% acetone and 100% methanol were used. A total of 12 fractions were obtained from this process. The fractions were subsequently aggregated into five primary categories according to their similarities: Fraction 1 (F1-F2 = 303 mg), fraction 2 (F3-F5 = 74.8 mg), fraction 3 (F6-F9 = 74.3 mg), fraction 4 (F10 = 102.3 mg), and fraction 5 (F11-F12 = 3.118 mg) (Table 2).

Isolation of compounds from Fraction 3 (F3)

Fraction 3 (F3), with a mass of 74.3 mg, underwent RC with a plate size of 1/1 mm to isolate bioactive compounds. The eluent system used comprised DCM and EA at varying ratios (7:3 and 6:4), followed by 100% acetone and 100% methanol. The process resulted in 15 subfractions, which were subsequently categorized into the following groups based on their similarities: Subfraction 1 (SF1-2): 11.2 mg, Subfraction 2 (SF3-6): 5.9 mg, Subfraction 3 (SF7-11): 33.5 mg, Subfraction 4 (SF12-13): 18.3 mg, and Subfraction 5 (SF14-15): 5.2 mg. RC was used to isolate compounds from SF3, yielding five subfractions. Subfractions 3 and 4 were merged for additional isolation to yield the target compounds. The five subfractions were Subfraction 1 (SF1-SF5): 9.8 mg, Subfraction 2 (SF6-SF7): 6.8 mg, Subfraction 3 (SF8-SF14): 5.2 mg, Subfraction 4 (SF17-SF18): 13.8 mg, and Subfraction 5 (SF19): 13.3 mg (Figure 4). Subsequent purification isolates through PTLC (20 cm × 20 cm) using the mobile phase of chloroform:methanol (9:1) and the results of the tested purity isolates through a two-way TLC using the mobile phase of chloroform : methanol (9:1) as direction 1 and n-hexane:ethyl acetate (1:1) as direction 2 effectively produced two target isolates, designated as isolate 1 and isolate 2.

Structural elucidation of isolates 1 and 2 from the isolate AQ2-1 symbiont bacteria of *Polycarpa aurata*

UV-Vis analysis

The pure isolates from the AQ2-1 *P. aurata* fermentate, namely isolates 1 and 2, were analyzed using UV-vis spectrophotometry at wavelengths of 200-400 nm and 200-500 nm (Figure 5).

According to the UV-vis spectroscopy results presented in Figure 4, isolates 1 and 2 exhibited two maximum absorption peaks at λ max 242.88 nm and 273.88 nm for isolate 1 and 218.88 nm and 250.88 nm for isolate 2, indicating the presence of a conjugated chromophore in these compounds.

NMR analysis

The isolated compounds (isolates 1 and 2) were solubilized in deuterated chloroform ($CDCl_3$) and subsequently subjected to analysis through ^{1}H-NMR and ^{13}C-NMR spectrophotometry at a frequency of 500 MHz. The objective of the analysis was to ascertain the quantity of protons and carbon-13 atoms present in isolate 1, as illustrated in Figures 6 and 7.

Based on the interpretation of the ^{1}H-NMR data ($CDCl_3$, 500 MHz) for isolates 1 and 2 derived from the AQ2-1 symbiont bacterial isolate of *P. aurata*, eight chemical shifts were identified for isolate 1 and 10 chemical shifts for isolate 2. For isolate 1, the observed chemical shifts included 3.83 (dd, ^3J = 3.55 Hz, ^3J = 12.9 Hz, 1H)b, 3.91 (dd, ^3J = 2.75 Hz, ^3J = 12.9 Hz, 1H)a, 4.29 (q, ^3J = 3.2 Hz, 1H), 4.43 (dd, ^3J = 3.35 Hz, ^3J = 5.25 Hz, 1H), 4.78 (t, ^3J = 5.55 Hz, 1H), 6.05 (d, ^3J = 6.20 Hz, 1H), 8.19 (s, 1H), and 8.31 (s, 1H), as shown in Figure 6a. For isolate 2, the chemical shifts observed were 0.82 (t, ^3J = 7.2 Hz, 3H), 0.86 (t, ^3J = 7.45 Hz, 3H), 1.23 (m, 2H), 1.25 (m, 2H), 1.28 (m, 1H), 1.35 (m, 2H), 1.61 (m, 2H), 4.15 (m, 2H), 7.45 (dd, ^4J = 3.3 Hz, 5.7 Hz, 1H), and 7.63 (dd, ^4J = 3.3 Hz, 5.7 Hz, 1H), as shown in Figure 6.B.

Table 2. Results of ethyl acetate extract fractionation using vacuum liquid chromatography

Sample name	Sample weight	Silica weight	KCV column diameter	Mobile phase	Fraction	Combined fractions
Ethyl acetate extract fermented white-colored *Polycarpa aurata* (Isolate code AQ2-1)	4.71 g	10 g	6 cm	1. DCM : EA (9:1) (150 mL)	F1	F1
				2. DCM : EA (8:2) (300 mL)	F2-F3	F2
				3. DCM : EA (7:3) (300 mL)	F4-F5	
				4. DCM : EA (5:5) (300 mL)	F6-F7	F3
				5. DCM : EA (4:6) (150 mL)	F8	
				6. DCM : EA (2:8) (150 mL)	F9	
				7. Acetone 100% (150 mL)	F10	F4
				8. Methanol 100% 1000 mL	F11-F12	F5

Note: DCM: Dichloromethane; EA: Ethyl Acetate; F: Fraction; VLC: Vacuum Liquid Chromatography

Based on the data interpretation ($CDCl_3$, 125 MHz), the analysis of the ^{13}C-NMR spectrum for isolate (1) revealed 10 chemical shifts at 64.27 (CH_2), 73.42 (CH), 76.43 (CH), 88.63 (CH), 91.07 (CH), 121.90 (C), 143.36 (CH), 151.20 (C), 155.30 (CH), and 158.43 (C) (Figure 7.A). For isolate (2), the analysis of the ^{13}C-NMR spectrum ($CDCl_3$, 500 MHz) showed 12 chemical shifts at 10.93 (CH_3), 14.01 (CH_3), 22.95 (CH_2), 23.72 (CH_2), 28.90 (CH_2), 30.34 (CH_2), 38.71 (CH), 68.12 (CH_2), 128.77 (CH), 130.84 (CH), 132.43 (C), and 167.72 (C, ester carbonyl) (Figure 7.B).

HSQC, COSY, and HMBC analyses

Adenine riboside (isolate 1) and bis(2-ethylhexyl) benzene-1,2-dicarboxylate (isolate 2) were chemical compounds confirmed using 1D and 2D NMR analyses (Figure 8).

The HMBC spectrum ($CDCl_3$, 500 MHz) of isolate 1 showed the exact location of each C and H atom in the compound. Based on the HMBC data, a long-range correlation was observed between 8.31 (s, 1H) and the C-2 and C-5 atoms and between 8.19 (s, 1H) and the C-3 and C-5 atoms, ensuring that -NH_2 was directly bonded to C-3, C-2, and C-5 as quaternary carbon. The overall HMBC correlations confirmed that adenine was the main structure in this molecule. The HMBC correlation of 6.05 (d, ^3J = 6.20 Hz, 1H) with C-1, C-5, C-2', and C-3' also revealed that ribose sugar is linked to the adenine structure by an N atom that is not part of C-1 or C-5. Therefore, the analysis concluded that isolate (1) is adenine ribose, consistent with the molecular weight analysis m/z 267 [M]+ ($C_{10}H_{13}N_5O_4$).

To determine the location of each C and H atom in isolate 2, the HMBC spectrum ($CDCl_3$, 500 MHz) was analyzed. The HMBC data revealed that the carbonyl C atom is directly attached to the aromatic ring via C-4. The aliphatic C chain in this compound is attached to the ester O atom, starting from C-1``,1``` (68.12 ppm; 4.15 ppm) and extending to C-6``,6````. There are two methyl groups (C-6``,6```` and C-8``,8```) and five methylene groups (C-1``,1```, C-3``,3```, C-4``,4```, C-5``,5``` and C-7``,7```). The methine group C-2``,2``` is a chiral center due to the ethyl group being attached to this C atom. Thus, the analysis concluded that isolate (2) is Bis(2-ethylhexyl) benzene-1,2-dicarboxylate, consistent with the molecular weight analysis m/z 390 [M]+ ($C_{24}H_{38}O_4$).

The structural analysis of isolate 1 was confirmed by 2D NMR spectra (Figure 8). The most important 1H-1H COSY correlations were observed among H-2' (δH 4.78, t, ^3J = 5.55 Hz, 1H), H2-3' (δH 4.43, dd, ^3J = 3.35 Hz, ^3J = 5.25 Hz, 1H), H3-4' (δH 4.29, q, ^3J = 3.2 Hz, 1H), H5'a-4' (δH 3.91, dd, ^3J = 2.75 Hz, ^3J = 12.9 Hz, 1H)a, and H5'b-4' (δH 3.83, dd, ^3J = 3.55 Hz, ^3J = 12.9 Hz, 1H)b. The major HMBC correlations included H3-2' to C-1' (δC 76.43), H4-2' to C-3' (δC 73.42), and H5'a to C-4' (δC 64.27). For isolate 2, the most important 1H-1H COSY correlations were among H-2" (δH 1.61, m, 2H), H2-7" (δH 1.35, m, 2H), and H7-8" (δH 0.86, t, ^3J = 7.45 Hz, 3H). The key HMBC correlations were H1-2' to C-2" (δC 76.43), H2-" to C-5" (δC 30.34), and H7-8" to C-8" (δC 10.93) (Figure 9).

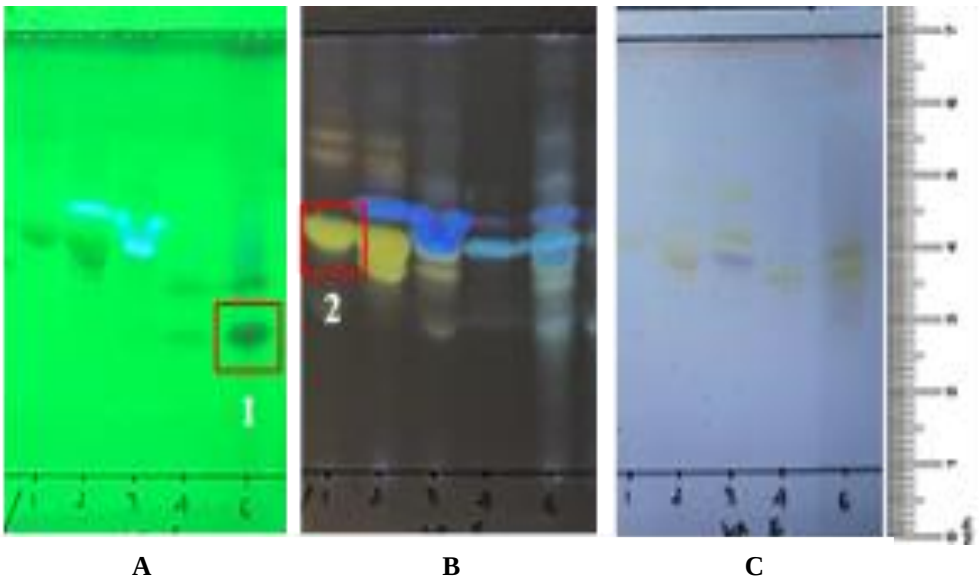


Figure 4. RC chromatogram of target isolates 1 and 2 with the mobile phase of chloroform:methanol (9:1). A: UV 254 nm; B: UV 366 nm; C: H_2SO_4 10%

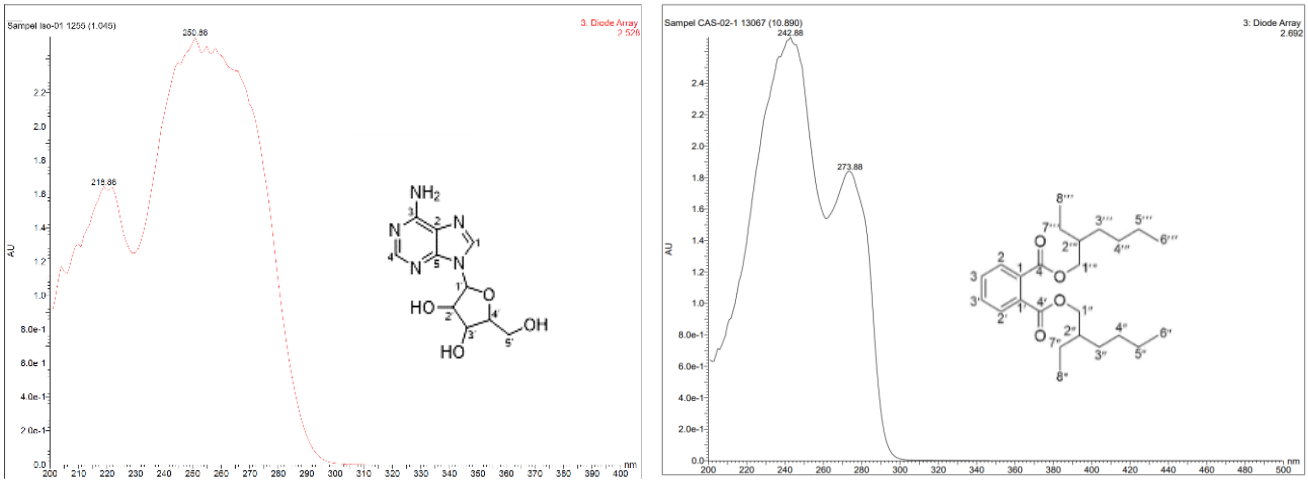
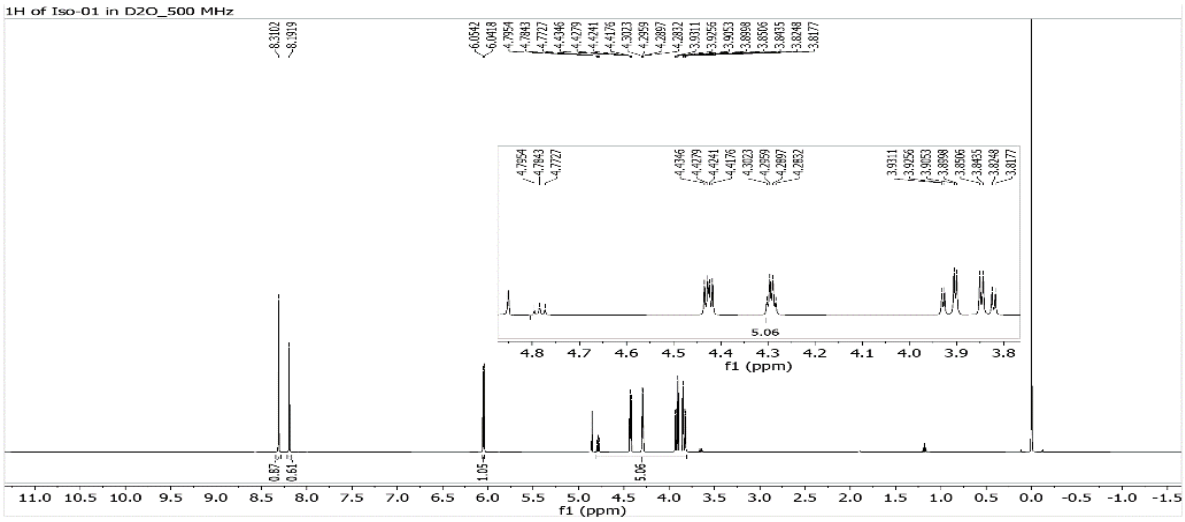
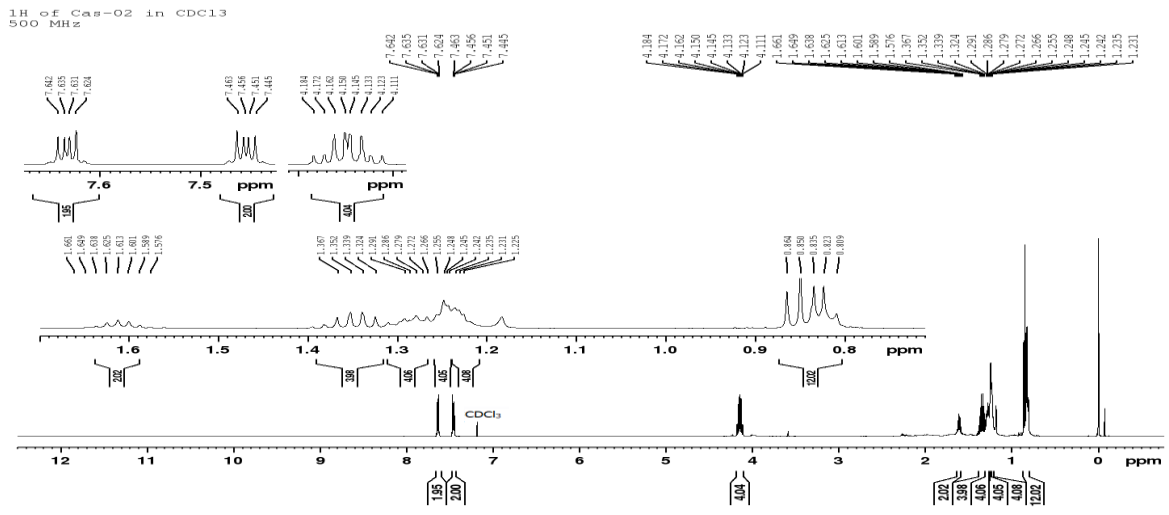


Figure 5. UV-Vis spectrum of isolates 1 and 2

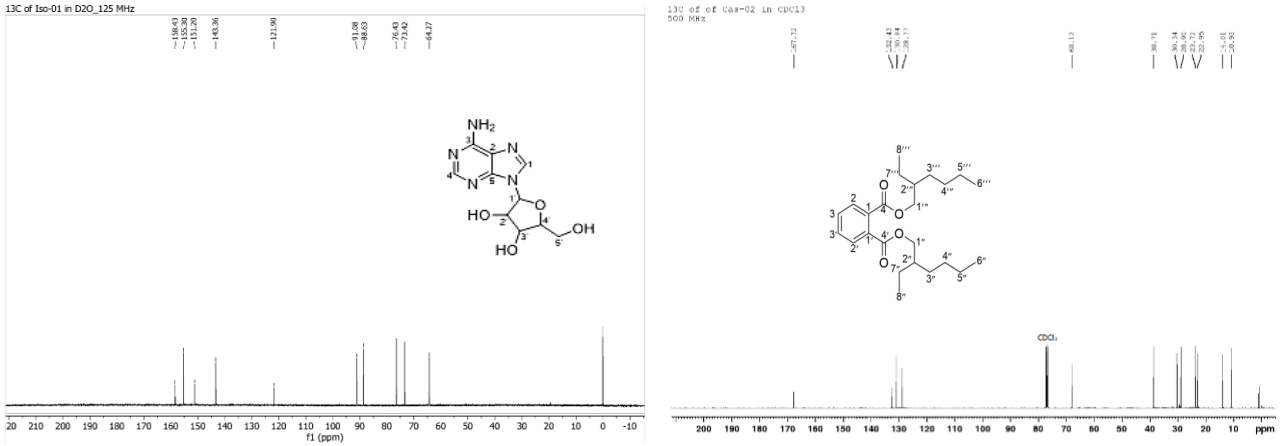


6.A. Isolate 1



6.B. Isolate 2

Figure 6. ^{1}H-NMR spectrum. 6.A: Isolate 1; and 6.B: Isolate 2



7.A. Isolate 1

7.B. Isolate 2

Figure 7. ^{13}C-NMR spectrum. 7.A: Isolate 1; and 7.B: Isolate 2

MS analysis

The pure isolates of isolates 1 and 2 were analyzed using MS, obtaining molecular weights of m/z 267 (isolate 1) as the adenine riboside compound with fragment compounds of m/z 296.01, m/z 290, m/z 270, m/z 269.19 $(M+H_2)^+$, m/z 268.04 $(M+H)^+$, m/z 172.87, and m/z 135.88 and molecular weights of m/z 413 (isolate 2) as Bis(2-ethylhexyl) benzene-1,2-dicarboxylate compound with fragment compounds of m/z 447.22, m/z 429.08 $(M+K)^+$, m/z 414.08, m/z 413.14 $(M+Na)^+$, m/z 391.15 $(M+H)^+$, m/z 279.09, m/z 275.19, and m/z 274.11 (Figure 10).

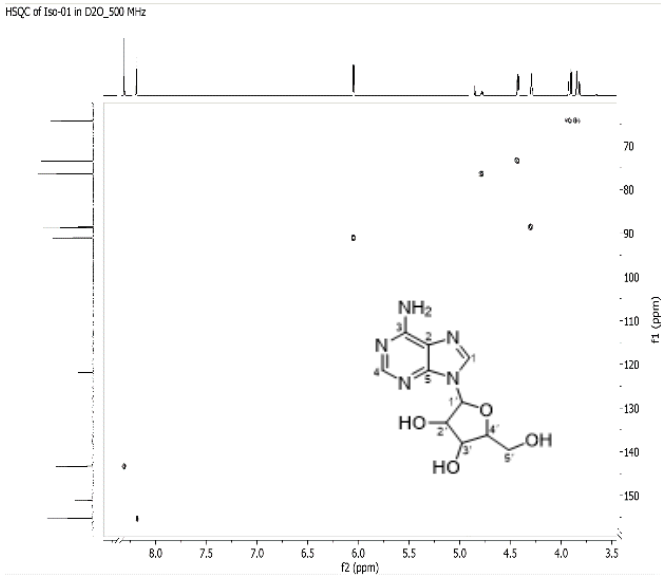
The spectroscopic tests showed that isolates 1 and 2 originated from the symbiotic bacteria of *P. aurata*, specifically the isolate code AQ2-1. The compounds were designated as adenine riboside (isolate 1) and bis(2-ethylhexyl) benzene-1,2-dicarboxylate (isolate 2). The elucidation of the structure of bioactive compounds from the symbiotic bacteria AQ2-1 isolate *P. aurata* resulted in the identification of isolates 1 and 2. The structures were analyzed using 1D and 2D-NMR spectra, encompassing HSQC, HMBC, and COSY (Tables 3 and 4).

Isolate 1

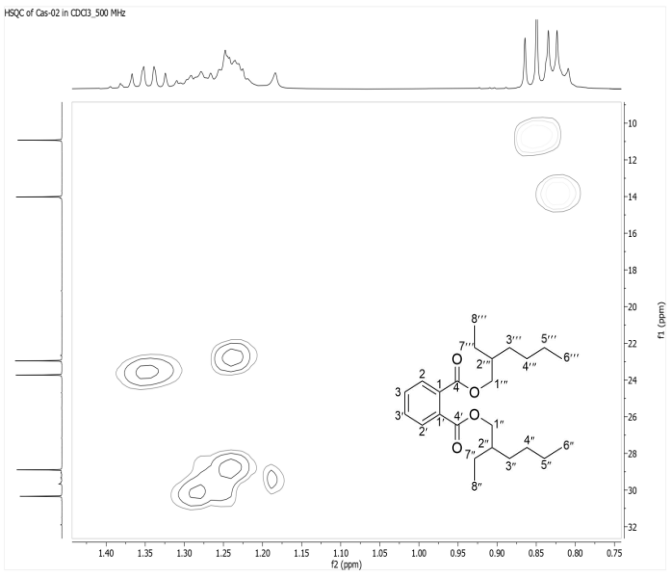
Adenine riboside was obtained in the form of a white powder that fluoresces brown when irradiated with UV light at 245 nm and fluoresces white under UV light at 366 nm. The spectroscopic analysis of adenine riboside showed a molecular formula of $C_{10}H_{13}N_5O_4$, m/z 267 $[M+H]^+$ (Figure 10.A). The 1H-NMR spectrum (Table 3 and Figure 6.A) showed resonances with the characteristic chemical shifts of 3.83 (dd, 3J = 3.55 Hz, 3J = 12.9 Hz, 1H)b, 3.91 (dd, 3J = 2.75 Hz, 3J = 12.9 Hz, 1H)a, 4.29 (q, 3J = 3.2 Hz, 1H), 4.43 (dd, 3J = 3.35 Hz, 3J = 5.25 Hz, 1H), 4.78 (t, 3J = 5.55 Hz, 1H), 6.05 (d, 3J = 6.20 Hz, 1H), and 8.19 (s, 1H) and 8.31 (s, 1H). The 13C-NMR spectrum (Table 3 and Figure 7.A) showed resonances with the characteristic chemical shifts of 64.27 (CH_2), 73.42 (CH), 76.43 (CH), 88.63 (CH), 91.07 (CH), 121.90 (C), 143.36 (CH), 151.20 (C), 155.30 (CH), and 158.43 (C). Isolate (1) consists of 10 carbon atoms, 13 hydrogen atoms, 5 nitrogen atoms, and 4 oxygen atoms. The aromatic region contains five carbon atoms, one carbon atom attached to -NH_2 (C-3, 158.43 ppm), and two methine carbon atoms (C-1, 155.30 ppm) and (C-4, 143.36 ppm); the rest are quaternary carbon atoms. The aromatic ring in this compound belongs to the adenine group. Furthermore, the five carbon atoms in the chemical shift range of 50-100 ppm form a ribose sugar (C-1', 91.08 ppm), (C-2', 76.43 ppm), (C-3', 73.42 ppm), (C-4', 88.63 ppm), and (C-5', 64.27 ppm).

Isolate 2

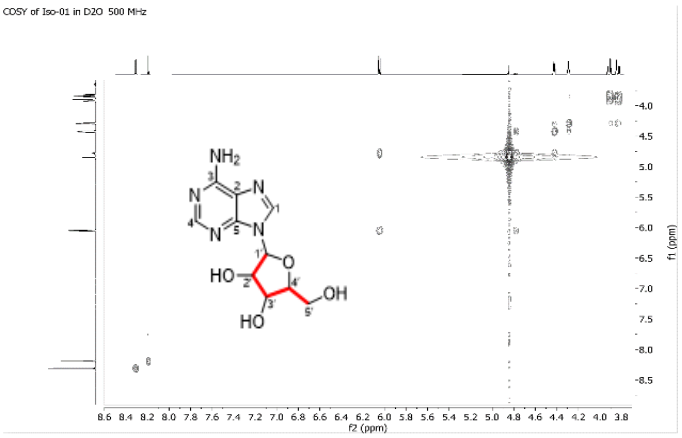
Bis(2-ethylhexyl) benzene-1,2-dicarboxylate is obtained in the form of a yellow oil that fluoresces brown when irradiated with UV light at 254 nm and fluoresces yellow under UV light at 366 nm. The spectroscopic analysis of Bis(2-ethylhexyl) benzene-1,2-dicarboxylate showed a molecular formula of $C_{24}H_{38}O_4$, m/z 390 $[M+Na]^+$ (Figure 10.B). The 1H-NMR spectrum (Table 4 and Figure 6.B) showed resonances with the characteristic chemical shifts of 0.82 (t, 3J = 7.2 Hz, 3H), 0.86 (t, 3J = 7.45 Hz, 3H), 1.23 (m, 2H), 1.25 (m, 2H), 1.28 (m, 1H), 1.35 (m, 2H), 1.61 (m, 2H), 4.15 (m, 2H), 7.45 (dd, 4J = 3.3, 5.7Hz, 1H), and 7.63 (dd, 4J = 3.3, 5.7Hz, 1H). The 13C-NMR spectrum (Table 4 and Figure 7.B) showed resonances with characteristic chemical shifts of 10.93 (CH3), 14.01 (CH_3), 22.95 (CH_2), 23.72 (CH_2), 28.90 (CH_2), 30.34 (CH_2), 38.71 (CH), 68.12 (CH_2), 128.77 (CH), 130.84 (CH), 132.43 (C), and 167.72 (C, ester carbonyl). Isolate (2) consists of 24 carbon atoms, 38 hydrogen atoms, and 4 oxygen atoms. The aromatic region contains three carbon atoms and one ester carbonyl carbon atom (C-4,4', 167.72 ppm), while the rest are non-cyclic aliphatic carbon atoms. The aromatic ring in this compound belongs to the benzene type, which consists of two symmetric quaternary carbon atoms (C-1,1 132.43 ppm) and two symmetric methine carbons (C-2,2, 128.77; 7.63 ppm and C-3,3`, 130.84; 7.45 ppm), in which both methine carbons are in a meta position to each other (J = 3.5, 5.5 Hz).



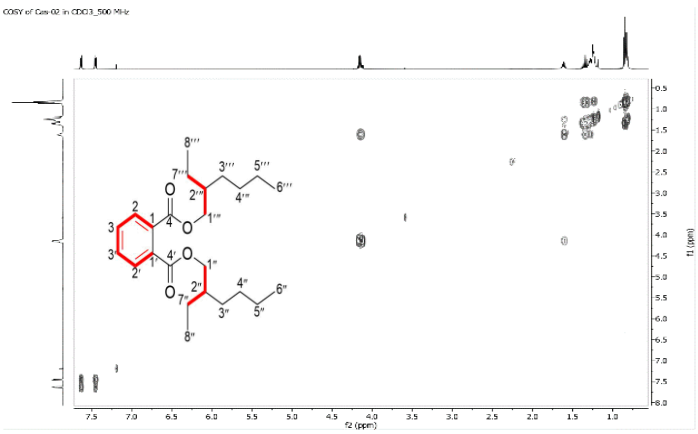
8.A. HSQC analysis of isolate 1



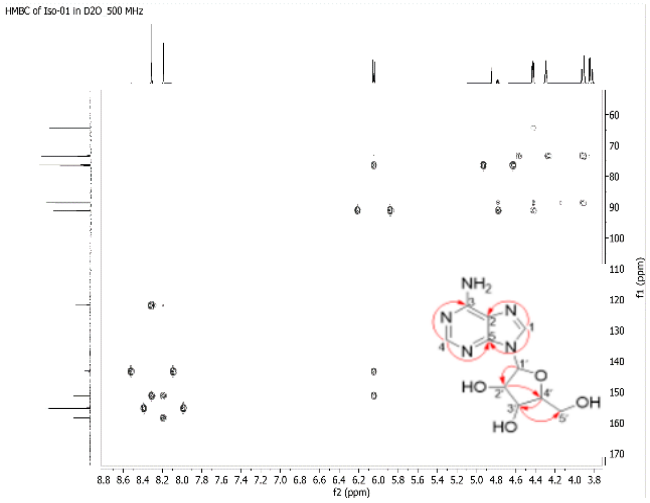
8.B. HSQC analysis of isolate 2



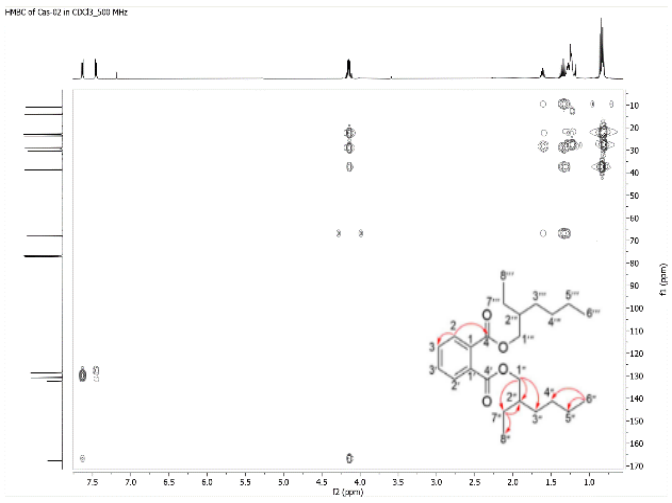
8.C COSY analysis of isolate 1



8.D COSY analysis of isolate 2



8.E. HMBC analysis of isolate 1



8.F HMBC analysis of isolate 2

Figure 8. 8.A-8.B Spectrum: HSQC; 8.C-8.D: COSY; and 8.E-8.F: HMBC isolates 1 and 2

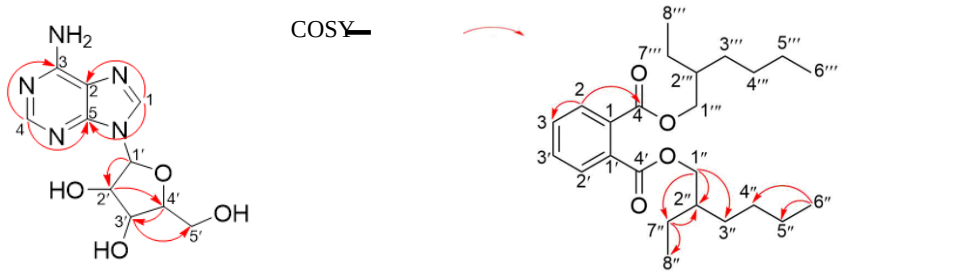
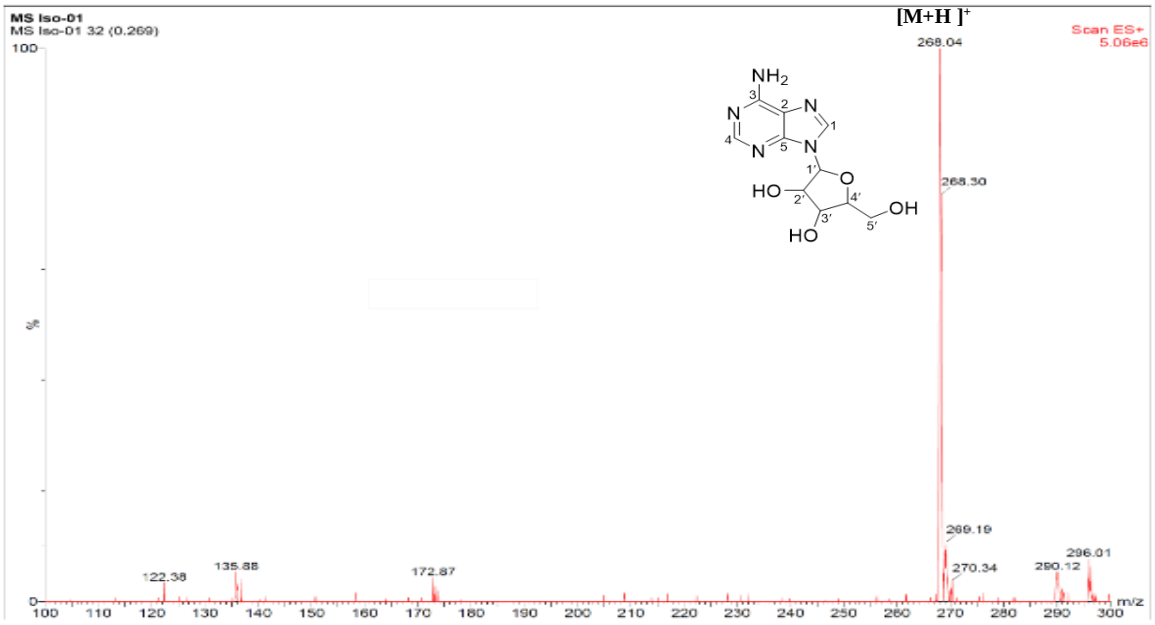
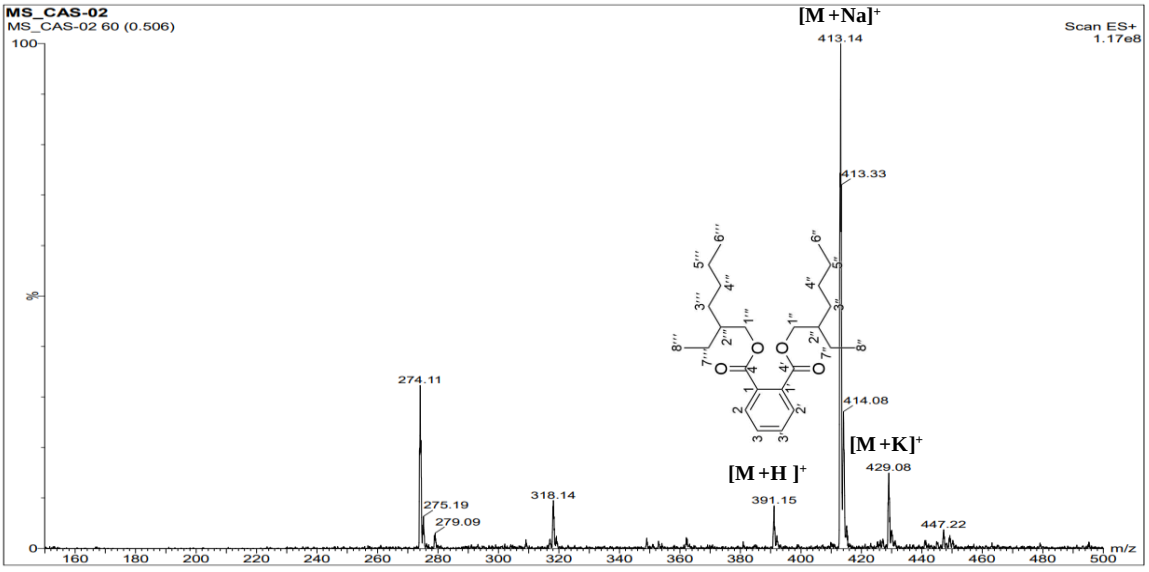


Figure 9. Correlation key between 1H-1H COSY and HMBC in isolates 1 and 2



10.A. Isolate 1



10.B. Isolate 2

Figure 10. MS spectrum. 10.A: Isolate 1; and 10.B: Isolate 2

Table 3. Structural data of isolate 1 from the symbiont bacteria AQ2-1 isolate of *Polycarpa aurata*

Position	^{13}C (ppm)	Type of C	HSQC/δH (*mult., J* in Hz, ΣH)	HMBC	COSY
1	155.30	CH	8.31 (*s*, 1H)	C-2, C-5	-
2	121.90	C	-	-	-
3	158.43	C	-	-	-
4	143.36	CH	8.19 (*s*, 1H)	C-3, C-5	-
5	151.20	C	-	-	-
1'	91.08	CH	6.05 (*d*, ^3J = 6.20 Hz, 1H)	C-1, C-5, C-2', C-3'	H-2'
2'	76.43	CH	4.78 (*t*, ^3J = 5.55 Hz, 1H)	C-1', C-4'	H-1', H-3'
3'	73.42	CH	4.43 (dd, ^3J = 3.35 Hz, ^3J = 5.25 Hz, 1H)	C-1', C-4', C-5'	H-2', H-4'
4'	88.63	CH	4.29 (*q*, ^3J = 3.2 Hz, 1H)	C-3'	H-3', H-5'
5'	64.27	CH_2	3.91 (*dd*, ^3J = 2.75 Hz, ^3J = 12.9 Hz, 1H)a	C-3', C-4’	H-4’
			3.83 (*dd*, ^3J = 3.55 Hz, ^3J = 12.9 Hz, 1H)b	C-4'	H-4'

Note: 1H-NMR and 13C-NMR chemical shift analysis in CDCL3; proton and carbon connected based on HSQC, HMBC and COSY analyses

Table 4. Structural data of isolate 2 from the symbiont bacteria AQ2-1 isolate of *Polycarpa aurata*

Position	^{13}C (ppm)	Type of C	HSQC/δH (*mult., J* in Hz, ΣH)	HMBC	COSY
1,1'	132.43	C	-	-	-
2,2'	128.77	CH	7.63 (*dd*, ^4J = 3.3, 5.7Hz, 1H)	C-3,3’, C-4,4'	H-3,3'
3,3'	130.84	CH	7.45 (*dd*, ^4J = 3.3, 5.7Hz, 1H)		H-2,2'
4,4'	167.72	C	-	-	-
1'',1'''	68.12	CH_2	4.15 (*m*, 2H)	C-2'',2’‘’, C-3'',3’‘’, C-7'',7'''	H-2'',2'''
2'',2'''	38.71	CH	1.61 (*m*, 2H)		H-1'',1’‘’, H-3'',3’‘’, H-7'',7'''
3'',3'''	30.34	CH_2	1.28 (*m*, 1H)		H-2'',2’‘’, H-4'',4'''
4'',4'''	22.94	CH_2	1.25 (*m*, 2H)		H-3'',3’‘’, H-5'',5'''
5'',5'''	28.90	CH_2	1.23 (*m*, 2H)		H-4'',4’‘’, H-6'',6'''
6'',6'''	14.01	CH_3	0.82 (*t*, ^3J = 7.2 Hz, 3H)	C-4'',4’‘’, C-5'',5'''	H-5'',5'''
7'',7'''	23.72	CH_2	1.35 (*m*, 2H)	C-2'',2’‘’, C-8'',8'''	H-2'',2’‘’, H-8'',8'''
8'',8'''	10.93	CH_3	0.86 (*t*, ^3J = 7.45 Hz, 3H)		H-7'',7'''

Note: 1H-NMR and 13C-NMR chemical shift analysis in CDCL3; proton and carbon connected based on HSQC, HMBC, and COSY analyses

Bioautography-TLC

The antibacterial activity test of the ethyl acetate extract, fractions, and pure compounds from the AQ2-1 isolate fermentate was carried out using bioautography-TLC against Gram-negative pathogenic bacteria, namely *E. coli* ATCC 25922, *S. typhi* NCTC 786, *V. cholerae* ATCC 25175, *Pseudomonas aeruginosa* ATCC 27853, and *S. dysenteriae* ATCC 13313 (Tables 5, 6, and 7).

The results of bioautography-TLC demonstrated that the chromatograms of pure isolates, fractions, and ethyl acetate extracts from the AQ2-1 isolates of symbiont bacterial of *P. aurata* exhibited antibacterial properties against pathogenic bacteria of Gram-negative and Gram-positive, with Rf values of 0.32-0.90. The ethanol extract of *P. aurata* has shown antibacterial properties against Gram-negative bacteria *E. coli* and *S. typhi* (Selviati et al. 2024). Alkaloid compounds are chemical compounds that act as antibacterials. They inhibit harmful bacteria by disrupting the synthesis of peptidoglycan within bacterial cells. Cell death occurs because of the disruption of the integrity of the cell wall layer (Pham et al. 2013).

Polycarpa aurata are invertebrate animals that contain many steroid compounds, alkaloids, flavonoids, and other compounds (Aulia 2011). These chemical compounds have a mechanism of action as antimicrobials, such as flavonoid compounds containing phenolic compound derivates, which have the ability to denature proteins and damage cell membranes (Rahayu 2000). There are around 300 alkaloid compounds in ascidians (tunicates), and they have various types of structures with pharmacological activity (Menna et al. 2011). The alkaloid compound, namely Bis(2-Ethylhexyl) Benzene-1,2-Dicarboxylate, has broad-spectrum antibacterial activity and has great potential as a drug for treating diarrhea (Dawa et al. 2023). This compound can also be produced from the symbiont bacteria Lactiplantibacillus plantarum as a larvicide bioactive compound (Javed et al. 2022). Alkaloid compounds have an antibacterial mechanism by interfering with the peptidoglycan components in the bacterial cell, so the bacterial cell wall layer does not form completely and causes the cell to die. The alkaloid compounds in *P. aurata*, namely polikarpatiamina A and B (compounds 1 and 2, respectively), have cytotoxic activity against lymphoma murine L5178Y cells (IC_{50} 0.41 μM) in compound 1 (Pham et al. 2013).

Table 5. Results of the antibacterial activity test of ethyl acetate extract using bioautography-TLC

Sample name	Rf value	Spot color			Active against test bacteria
		UV 254 nm	UV 366 nm	H_2SO_4 10 %	
Ethyl acetate extract AQ2-1	0.90	Brown	-	-	*Escherichia coli* ATCC 25922, *Vibrio cholerae* ATCC 25175, *Pseudomonas aeruginosa* ATCC 27853, and *Shigella dysenteriae* ATCC 13313
	0.87	-	Yellow fluorescence	Brown	*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Shigella dysenteriae* ATCC 13313
	0.80	-	-	Brown	*Shigella dysenteriae* ATCC 13313 and *Pseudomonas aeruginosa* ATCC 27853
	0.67	Blue	-	-	*Shigella dysenteriae* ATCC 13313 and *Pseudomonas aeruginosa* ATCC 27853
	0.61	-	-	Yellow	*Vibrio cholerae* ATCC 25175, *Pseudomonas aeruginosa* ATCC 27853, and *Shigella dysenteriae* ATCC 13313
	0.55	Brown	Blue fluorescence	Yellow	*Salmonella typhi* NCTC 786
	0.52	Brown	Blue fluorescence	Yellow	*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Shigella dysenteriae* ATCC 13313
	0.50	Yellow	Blue	Brown	*Escherichia coli* ATCC 25922, *Vibrio cholerae* ATCC 25175, and *Salmonella typhi* NCTC 786
	0.45	Brown	Blue fluorescence	-	*Escherichia coli* ATCC 25922, *Shigella dysenteriae* ATCC 13313, and *Salmonella typhi* NCTC 786
	0.41	Brown	Glows yellow	Yellow	*Escherichia coli* ATCC 25922, *Shigella dysenteriae* ATCC 13313, *Pseudomonas aeruginosa* ATCC 27853, *Vibrio cholerae* ATCC 25175, and *Salmonella typhi* NCTC 786
	0.32	Brown	Glows blue	Yellow	*Escherichia coli* ATCC 25922, *Shigella dysenteriae* ATCC 13313, *Vibrio cholerae* ATCC 25175, and *Salmonella typhi* NCTC 786

Note: AQ2-1: Isolate of symbiont bacteria from white-colored *Polycarpa aurata*; Mobile phase: Chloroform : Methanol (9:1)

Table 6. Results of the antibacterial activity test of fractions using bioautography-TLC

Sample name	Sub-fraction	Rf value	Spot color			Active against test bacteria
			UV 254 nm	UV 366 nm	H_2SO_4 10 %	
Sub-Fraction Isolate AQ2-1	1	0.86	-	Yellow	-	*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *and Vibrio cholerae* ATCC 25175
		0.80	-	Yellow	-	*Vibrio cholerae* ATCC 25175 and *Pseudomonas aeruginosa* ATCC 27853
		0.75	-	Yellow	Yellow	*Vibrio cholerae* ATCC 25175 and *Pseudomonas aeruginosa* ATCC 27853
		0.61	Brown	Blue fluorescence	Yellow	*Escherichia coli* ATCC 2592, *Pseudomonas aeruginosa* ATCC 27853, *and Shigella dysenteriae* ATCC 13313
		0.47	-	Yellow	-	*Shigella dysenteriae* ATCC 13313 and *Vibrio cholerae* ATCC 25175
	5	0.92	-	Gray	-	-
		0.80	-	Blue fluorescence	-	*Vibrio cholerae* ATCC 25175, *Pseudomonas aeruginosa* ATCC 27853, *and Salmonella typhi* NCTC 786
		0.71	-	Blue fluorescence	-	*Vibrio cholerae* ATCC 25175
		0.64	-	Blue fluorescence	-	-
		0.60	Brown	Glows yellow	Yellow	*Shigella dysenteriae* ATCC 1331 and *Vibrio cholerae* ATCC 25175
		0.55	Brown	Glows yellow	Yellow	-
		0.51	Brown	Blue fluorescence	-	-
		0.41	Brown	Blue fluorescence	-	*Shigella dysenteriae* ATCC 13313, *Salmonella typhi* NCTC 786, and *Vibrio cholerae* ATCC 25175

Note: AQ2-1: Isolate of symbiont bacteria from white-colored *Polycarpa aurata*; Mobile phase: Chloroform : Methanol (9:1)

Table 7. Results of the antibacterial activity test of a pure isolate using bioautography-TLC

Sample name	Compound name	Rf value	Spot color			Active against test bacteria
			UV 254 nm	UV 366 nm	H_2SO_4 10 %	
Pure isolates from the symbiotic bacterial isolate AQ2-1	Adenine riboside	0.41	Brown	Blue fluorescence	-	*Escherichia coli* ATCC 25922, *Shigella dysenteriae* ATCC 13313, *Salmonella typhi* NCTC 786, *Pseudomonas aeruginosa* ATCC 27853, and *Vibrio cholerae* ATCC 25175
	Bis(2-ethylhexyl) benzene-1,2-dicarboxylate	0.61	Brown	Glows yellow	Yellow	*Escherichia coli* ATCC 25922, *Shigella dysenteriae* ATCC 13313, *Salmonella typhi* NCTC 786, *Pseudomonas aeruginosa* ATCC 27853, and *Vibrio cholerae* ATCC 25175

Note: AQ2-1: isolate of symbiont bacteria from white-colored *Polycarpa aurata*, Mobile phase: Chloroform : Methanol (9:1)

In conclusion, the molecular formulas of isolates 1 and 2 from the fermentate isolate AQ2-1 of the symbiont bacteria *Polycarpa aurata* revealed molecular formulas of $C_{10}H_{13}N_5O_4$, m/z 267 $[M+H]^+$ and $C_{24}H_{38}O_4$, m/z 390 $[M+Na]^+$ and name structures of adenine riboside and bis(2-ethylhexyl) benzene-1,2-dicarboxylate, respectively. These isolates were found to be effective against pathogenic bacteria, including *E. coli* ATCC 25922, *S. typhi* NCTC 786, *S. dysenteriae* ATCC 13313, *P. aeruginosa* ATCC 27853, and *V. cholerae* ATCC 25175.

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