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Identification of Secondary Metabolites Derived from *Aaptos spp*. and Their Antibacterial Activity

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ABSTRACT

Sponges have been recognized as productive producers of novel bioactive compounds, many of which exhibit antimicrobial properties. Among these sponges, *Aaptos* species have demonstrated antimicrobial activity. The escalating challenges posed by antimicrobial resistance and the prevalence of infections, particularly among immunocompromised populations, underscore the urgent need for alternative antimicrobial agents. Thus, exploring marine secondary metabolites for potential therapeutic applications holds significant promise. The present research investigates the bioactive compounds' profile derived from *Aaptos* sponge specimens collected from Raja Ampat Island, Indonesia. Extracts from these samples were obtained using ethanol and an ethyl acetate-methanol solvent. Subsequently, the antibacterial activity of these extracts was evaluated using a disc diffusion assay. To elucidate the chemical composition associated with antimicrobial activity, Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD), chemometric analysis, and metabolomic analysis were employed. The antibacterial assay revealed promising activity against both gram-positive bacteria (*Staphylococcus aureus* ATCC 13420 and *Bacillus subtilis* ATCC 6051) and gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 9027 and *Klebsiella pneumoniae* ATCC 1388). TLC analysis indicated the presence of potential alkaloid and terpenoid compounds in the extracts. However, chemometric analysis did not identify any extracts in the same quadrant as conventional antibiotics. Nevertheless, metabolomic analysis detected alkaloids resembling aaptamines. These findings offer new avenues for research, with the ultimate aim of contributing to developing novel and efficacious antimicrobial drugs. Moreover, this research adds to the bioprospecting efforts focused on marine sponges from Indonesia.

Keywords: *Aaptos,* Crude extract, PCA, HPLC-DAD, antibacterial

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INTRODUCTION

Marine biodiversity encompasses the multitude of organisms inhabiting oceanic ecosystems, representing an extensive reservoir of undiscovered life. With over 250,000 described species and boundless potential for discoveries, the marine environment remains a frontier of exploration. Thriving in environments characterized by extreme variations in pressure, luminosity, salinity, and temperature, marine organisms have evolved intricate mechanisms for survival, often relying on the production of secondary metabolites. Among these organisms, sponges face significant challenges due to threats such as rising sea surface temperatures, ocean acidification, eutrophication, and deteriorating water quality. (Simister *et al*., 2012). Sponges, acting as natural filters, play a crucial ecological role by extracting small particles, including bacteria and microorganisms, for sustenance (De Goeij *et al*., 2013). In response to the harsh marine conditions, sponges have developed unique defense mechanisms, producing secondary metabolites with diverse chemical compositions. These singular metabolites harbor a wealth of bioactive compounds, holding immense potential for advancements in human health. Exploration efforts have unveiled many bioactive marine natural products, numbering over 13,000, with a broad spectrum of biological activities. These activities span from antibacterial and antidiabetic to antifungal, anti-inflammatory, and anticancer properties (Choudhary *et al*., 2017; Corinaldesi *et al*., 2017; Blunt *et al*., 2018; Sipkema *et al*., 2005; Izzati *et al*., 2021).

The escalating global threat of antimicrobial resistance (AMR) underscores the pressing need to explore marine ecosystems for potential solutions. The emergence of AMR infections presents a severe threat to public health, with millions of cases reported annually and a significant number of fatalities attributed to antibiotic-resistant bacteria. The incidence of infections caused by antimicrobial-resistant bacteria has dramatically increased each year. According to data from the Centers for Disease Control and Prevention (CDC) in the United States, since 2013, there have been at least 2,868,700 cases of AMR infections, resulting in 35,900 deaths (CDC, 2019). Similarly, AMR surveillance data from Europe between 2020 and 2022 reported over 670,000 cases attributed to antibiotic-resistant bacteria, leading to approximately 33,000 fatalities (ECDC, 2022). The severity of the AMR crisis is further underscored by the staggering toll it exacts on human lives. In Southeast Asia alone, more than a million people succumbed to AMR infections in 2019 (Murray *et al*., 2022).

With its 17,500 islands and 81,000-kilometer coastline, Indonesia is a global hub of marine biodiversity. Indonesian sponges have played a pivotal role in enriching our understanding of chemical diversity and biological activity, leading to the discovery of 105 new compounds between 2007 and 2020 (Izzati *et al*., 2021). Through meticulous research, up to 2020, scientists had identified and characterized 62 distinct secondary metabolites originating from the *Aaptos* genus (He *et al*., 2020). These compounds were extracted from various species within the genus, including *Aaptos* (Schmidt, 1864), *Aaptos suberitoides* (Brøndsted, 1934), *Aaptos lobata* (Calcinai *et al*., 2017), *Aaptos ciliata* (Wilson, 1925), *Aaptos nigra* (Lévi, 1961), and several other unidentified *Aaptos* spp. In terms of their molecular structures, the predominant compounds derived from *Aaptos* species exhibit remarkable structural diversity, with the majority categorized as aaptamines, comprising 47 of the identified compounds, while the remaining 15 are classified as other compounds (He *et al*., 2020). Continued exploration and research in this domain promise to unveil additional compounds with diverse biological activities, presenting novel opportunities for the development of antibiotics or other therapeutic agents to combat antimicrobial resistance. This research emphasizes the critical importance of conserving marine biodiversity, particularly in regions like Raja Ampat, as a valuable source of potential biomedical advancements and solutions to pressing global health challenges.

Despite significant advances in marine natural product research, the untapped potential of marine sponges from the Raja Ampat Islands remains largely unexplored. Thus, this research aims to characterize the crude extract obtained from marine sponges identified as *Aaptos spp.* collected from Raja Ampat, Indonesia. The study's findings contribute to our understanding of the potential properties of marine sponge extracts, adding to the growing body of knowledge on bioactive compounds derived from marine organisms.

MATERIALS AND METHODS

Sample Collection

The research utilized marine sponge samples RA3 and RB1, collected from the Raja Ampat Islands situated at latitude 0° 13′ 60.00′′ N and longitude 130° 30′ 59.99′′ E. Sample collection was conducted via SCUBA diving in the Saonek Area of the Raja Ampat Islands. The identification of RA3 and RB1 specimens was based on morphological comparisons conducted in situ and corroborated by reference to previously published literature. Upon collection, three thumb-sized sponge specimens (each measuring 1x1 cm^3) were carefully placed in 50 mL disposable tubes (Falcon®, USA), containing 30 mL of absolute ethanol (Merck, Darmstadt, Germany). These tubes were then transported to the laboratory for further processing. Subsequently, the ethanol used for preservation was transferred to new disposable tubes and subjected to evaporation, producing the ethanolic extract, hereafter referred to as (E). Furthermore, the remaining sponge samples were frozen at -80 °C and stored under these conditions for subsequent extraction using ethyl acetate: methanol extraction, denoted as (EM).

Sponge extraction

The extraction of sponge samples was conducted following a previously published protocol, with adjustments made to the quantity of starting materials (Tsukamoto *et al*., 2010; Rohde *et al*., 2015; Indraningrat *et al*., 2022).

Extraction with ethanol: A sponge tissue sample, roughly the size of a thumb and weighing approximately five grams, was immersed in absolute ethanol. Subsequently, the ethanol solution was filtered using Whatman filter paper (Marlborough, USA), and the solvent was removed via rotary evaporation using a Buchi Rotavapor R-300 Series instrument (Flawil, Switzerland) operated at 100 rpm, with a chiller set at 4 °C and a heating bath at 30 °C. The resulting crude extracts were concentrated to approximately 1 mL using a vacuum concentrator (Concentrator plus, Eppendorf, Germany) before being transferred to 1.5 mL microtubes (Axygen™, Union City, CA). These dried extracts were then stored at -20°C until further use.

Extraction with a mixture of ethyl acetate and methanol: The frozen sponge samples underwent freeze-drying to eliminate excess water. Subsequently, 500 mg of dried sponge material was pulverized into a powder using a mortar and pestle, followed by homogenization. The powder was then subjected to three rounds of extraction, each utilizing a 1:1 mixture of ethyl acetate (Merck, Darmstadt, Germany) and methanol (Merck, Darmstadt, Germany) at a volume of 5 mL per extraction. The maceration process lasted 20 min and was repeated three times. After maceration, the mixture underwent centrifugation at 500 g for 10 min to separate the extract-containing supernatant from the sponge biomass. The resulting extracts were subsequently filtered. The solvent was then removed using rotary evaporation via a Buchi Rotavapor R-300 Series instrument (Flawil, Switzerland) operated at 100 rpm, with a chiller set at 4°C and a heating bath at 30 °C. The concentrated extracts, totaling approximately 1 mL, were transferred to 1.5 mL microtubes (Axygen™, Union City, CA) using a concentrator vacuum (Concentrator plus, Eppendorf, Germany). These dried extracts were stored at -20 °C until further use. The freeze-dry and rotary evaporation for the compound extraction was performed by using Layanan ELSA, BRIN.

Antibacterial Activity Screening

The antibacterial activity of the sponge crude extracts was screened using the Kirby-Bauer agar diffusion method (Hudzicki, 2009). The bacterial strains employed for antibacterial screening were sourced from the collection of the Research Group Microbial-Omics, Research Centre for Applied Microbiology, BRIN.

Bacterial strains

Two gram-positive bacteria, namely *Staphylococcus aureus* ATCC 13420 and *Bacillus subtilis* ATCC 6051, along with three gram-negative bacteria, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 1388, and *Escherichia coli* ATCC 9637, were selected as the test bacterial panel. Before the assay, these bacterial strains were cultured in Nutrient Broth (NB; Merck) at 37 °C for 16 hours. After measuring the optical density ($OD600_{nm}$) of the overnight cultures, each bacterial culture was diluted with sterile NB broth to achieve an absorbance of 0.02, corresponding to approximately 5 x 10^7 CFU/mL. Subsequently, these diluted cultures were inoculated into Nutrient Agar (NA; Merck) and thoroughly mixed before being plated onto petri dishes until solid. The preparation of bacterial strains for this study adhered to a published protocol (Warsito *et al*., 2022).

Sample preparations

The preparation of the extracts followed a published protocol (Indraningrat *et al*., 2022). Each dried RA3 and RB1 extract was dissolved in 1% DMSO to achieve a final concentration of 0.5 mg/mL. Subsequently, sterile paper discs (6 mm in diameter; GE Healthcare) were soaked with 5 µL of each extract solution and allowed to dry. For the positive controls, standard antibiotics were prepared at stock concentrations of 1000 ppm. The selection of antibiotics was based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2013). Specifically, vancomycin at a concentration of 20 µg/disk (Sigma Aldrich, Hamburg, Germany) served as the positive control for *S. aureus* ATCC 13420, kanamycin at a concentration of 15 µg/disk (PhytoTech Lab, USA) served as the positive control for *B. subtilis*ATCC 6051, *K. pneumoniae* ATCC 1388, and *P. aeruginosa* ATCC 9027, while ampicillin at a concentration of 5 µg/disk (Sigma Aldrich, Hamburg) served as the positive control for *E. coli* ATCC 9637. As negative controls, sterile paper discs were soaked with solvents, including ethanol, methanol, ethyl acetate, and DMSO.

Disc Diffusion assay

Using sterile forceps, the sterile paper discs containing the extracts, positive controls, or negative controls were placed onto the surface of the inoculated agar plates. Subsequently, the plates were incubated at 37 °C for 24 hours. Each sample was tested in duplicate, and the zone of inhibition was measured as a diameter in millimeters (DOI).

Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) was employed for phytochemical screening to profile compound classes present in the extracts. Initially, 3 µl of RA3 ethanol extract (RA3.E), RA3 ethyl acetate:methanol extract (RA3.EM), RB1 ethanol extract (RB1.E), and RB1 ethyl acetate:methanol extract (RB1.EM) were spotted onto TLC plates coated with silica gel 60 F254 (Sigma Aldrich, Hamburg, Germany). Different mobile phases were utilized to separate alkaloid and terpenoid profiles. Chloroform:methanol (8:2) (Merck, Darmstadt, Germany) was used for alkaloid separation, while ethyl acetate:hexane (3:97) (Merck, Darmstadt, Germany) was used for terpenoid separation. The samples were allowed to migrate until they reached a distance of 8 cm from the spot (Rosmiati *et al*., 2011). Visual detection of spots corresponding to alkaloid and terpenoid profiles was carried out using UV light with wavelengths of 254 nm and 365 nm. In addition, for alkaloid profile detection, dragendorff's reagent was applied, whereas for terpenoid profile detection, anisaldehyde reagent was sprayed onto the TLC plates. Following treatment with anisaldehyde reagent for terpenoid profile detection, the TLC plates were heated at 105 °C until spots became visible. Subsequently, to confirm the presence of terpenoids, the silica gel was again visualized under UV light with wavelengths of 254 nm and 365 nm. The phytochemical screening of extracts was conducted based on methods developed by Ebada *et al*. (2008) and modified by Rosmiati *et al*. (2011).

High-performance liquid chromatography with diode-array detection (HPLC-DAD)

The HPLC analysis was performed using an Agilent Technologies 1200 Series HPLC System equipped with a G1315D DAD detector and a 4.6 x 150 mm inside diameter, 2.7 m InfinityLab Poroshell 120 EC-C18 column. RA3 and RB1 samples were subjected to analysis employing a solvent gradient composed of water (H2O; Merck, Darmstadt, Germany) and acetonitrile (ACN; Merck, Darmstadt, Germany) in varying proportions (85:15; 50:50; 0:100; 0:100). The flow rate was maintained at 0.8 mL/min to ensure optimal separation. To enhance peak resolution, the solvent mixture of water and acetonitrile was supplemented with 0.1% Trifluoroacetic acid (TFA; Sigma Aldrich, Dorset, UK). A 5 µL injection volume at a concentration of 0.2 mg/mL was utilized, with detection wavelengths set between 200-400 nm. The analysis was performed within a 40-minute timeframe.

Chemometric analysis

The HPLC-DAD method generated chromatograms for each compound, depicting peaks and their corresponding retention times. The peak with the highest intensity was identified as the primary compound present in the sponge extract. To determine similarities between compounds, the chromatograms of RA3 were compared to those of RB1 based on retention time and peak characteristics. Furthermore, comparisons were made between the ethanol extracts and methanol:ethyl acetate extracts to discern potential differences in the compounds obtained from the two different extraction solvents. The data on peaks and retention times were then classified into various groups, with each

compound's percentage area considered an independent variable. This classification was accomplished using SIMCA 17 software's chemometrics Principal Component Analysis (PCA) method, as Riyadi *et al*. (2014) outlined. In this research, a total of 19 independent variables were employed in the data analysis.

Metabolomics analysis

The investigation utilized liquid chromatography coupled with high-resolution mass spectrometry, employing the Thermo Scientific™ Vanquish™ UHPLC Binary Pump and the Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ High Resolution Mass Spectrometer. The liquid chromatography was conducted using a Thermo Scientific™ Accucore™ Phenyl-Hexyl analytical column with dimensions of 100 mm × 2.1 mm ID × 2.6 µm. The mobile phases consisted of MS-grade water with 0.1% formic acid (A) and MS-grade methanol with 0.1% formic acid (B), employing a gradient technique at a flow rate of 0.3 mL/min. Initially, mobile phase B was set at 5% and increased to 90% over 16 min. Subsequently, it was maintained at 90% for 4 min before returning to the initial condition (5% B) by 25 min. The column temperature was set at 40 °C, and the injection volume was 3 µL. Untargeted screening was performed using the full MS/dd-MS2 acquisition mode in either positive or negative ionization polarities. Nitrogen was utilized for sheath, auxiliary, and sweep gases, with settings at 32, 8, and 4 arbitrary units (AU), respectively. The spray voltage was set at 3.30 kV, the capillary temperature was maintained at 320 °C, and the auxiliary gas heater temperature was set at 30 °C. The scan range spanned from 66.7 to 1000 m/z, with a resolution of 70,000 for full MS and 17,500 for dd-MS2 in both positive and negative ionization modes. Control of the system was managed using XCalibur 4.4 software from Thermo Scientific, Bremen, Germany. To ensure optimal and robust instrumental performance throughout the analysis, the instrument underwent tuning and calibration weekly in both ESI positive and negative modes. Thermo Scientific Pierce ESI ion calibration solutions from Waltham, MA, were utilized for calibration, ensuring compliance with mass accuracy (<5 ppm), ion transfer, ion isolation, and instrumental sensitivity requirements (Windarsih *et al*., 2022).

RESULTS AND DISCUSSION

Marine secondary metabolites, characterized by their diverse structures and multifaceted biological activities, represent important sources of unexploited drugs. As of the end of 2018, approximately 28,600 new compounds had been documented from various marine sources (Blunt *et al*., 2018). Notably, sponges from the phylum Porifera emerge as a prolific source, contributing to 30% of all known natural marine products (He *et al*., 2020). Compounds derived from sponges often serve as promising pharmaceutical candidates.

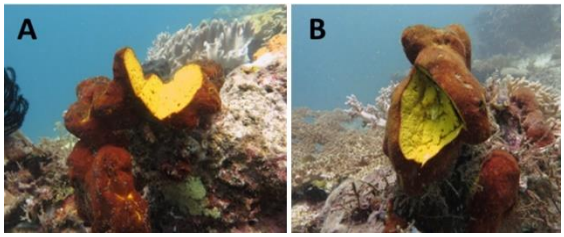
The genus *Aaptos* (Porifera, Demospongiae, Hadromerida, Suberitidae) thrives across diverse marine ecosystems, including the South China Sea, shallow waters in Japan, Indonesia, and the Caribbean. Around 20 species of *Aaptos* have been identified thus far (He *et al*., 2020). The intricate chemical compositions and diverse biological activities of *Aaptos*compounds, exemplified by the unique 1H-benzo[d,e][1,6]-naphthyridine alkaloids (aaptamines), contribute significantly to the expanding repository of marine-derived lead compounds with potential pharmaceutical applications. Global efforts in chemical investigations on *Aaptos* since the early 1980s underscore the ongoing exploration of these marine organisms, underscoring their importance in broadening the pharmaceutical landscape. For instance, a study by Arai *et al*. in 2014 unveiled aaptamine derivatives, such as 2-methoxy-3-oxoaaptamine, demonstrating anti-mycobacterial activity against *Mycobacterium smegmatis*, underscoring the potential of *Aaptos* compounds in combatting bacterial infections (Arai *et al*., 2014). More recently, aaptamine has exhibited significant biological effects, including anticancer, antiviral, antimicrobial, antifungal, antiretroviral, cytotoxic, antifouling, enzyme inhibiting, and antioxidant activities (Nadar *et al*., 2021).

Identification of Marine Sponge RA3 and RB1

The identification of sponges RA3 and RB1 was based on a comparison of their morphology with previously published journals. Both RA3 and RB1 exhibit similar external characteristics, displaying a brown-red exterior and a yellow interior. This morphology closely resembles that of sponge *Aaptos suberitoides* found in Ambon, Indonesia, and the coastal waters of Central

Vietnam. These sponges are recognized by their dark brown exterior and yellow interior (Mien *et al*., 2020; Pham *et al*., 2013) (Figureure 1).

Brøndsted's classification in 1934 identified *A. suberitoides* based on its characteristic globular or ball-shaped morphology (Van Soest, 1989). Descriptions indicate that these sponges appear as masses of globular osculiferous lobes, displaying an orange-brown coloration, possessing a tough yet compressible texture, and turning black upon exposure to alcohol (Van Soest, 1989). Furthermore, the type spicula of *Aaptos suberitoides* consists of long oxea megascleres, whereas the genus Aaptos typically features monoactinal oxeastyle spicules (Parama Cita *et al*., 2017; Setiawan *et al*., 2009). Considering their morphology, sponges RA3 and RB1 are likely to be classified within the genus *Aaptos spp*.



Figureure 1. The sponge *Aaptos* RA3 (A) and RB1 (B) taken underwater at Raja Ampat Islands, Photos courtesy of © Aji Nugroho

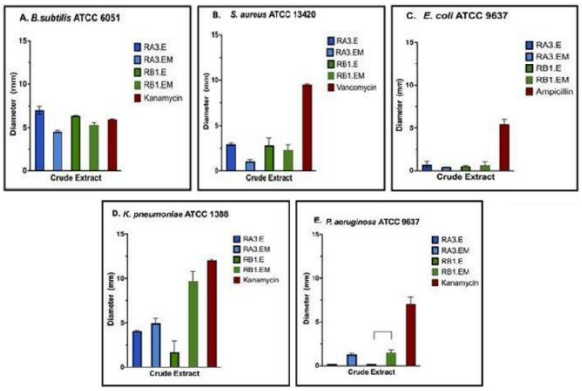
Extraction of Marine Sponge Aaptos Sp.

The maceration technique is the most commonly utilized method for isolating active compounds from multiple extracts with diverse bioactivities. The efficacy of the extraction process hinges on the solubility of the target compound in the chosen solvent, a factor heavily influenced by its polarity (Sarker & Nahar, 2012). Notably, the choice of extraction solvent significantly impacts the extraction of bioactive compounds from marine sponges (Ebada *et al*., 2008). Ethanol is frequently employed to extract polar secondary metabolites, owing to its effectiveness in solubilizing such compounds. Conversely, semi-polar secondary metabolites are often extracted using ethyl acetate, a solvent with intermediate polarity. Methanol, similar to ethanol, possesses polar characteristics and offers enhanced solubilization capabilities. Combining ethyl acetate and methanol solvents, as Ebada *et al*. (2008) suggested, facilitates the extraction of a diverse array of secondary metabolites. The variation in yield extracts observed between ethanol and methanol can be attributed to the polar nature of many compounds present in these sponges. Consequently, the yield of ethyl acetate extract tends to be higher in ethanol than in methanol due to the increased solubilization power of ethanol for polar compounds.

Antibacterial Activity Screening

The disc diffusion assay revealed that the crude extracts obtained from RA3 and RB1 exhibited antibacterial activity against both gram-positive and gram-negative bacteria tested. This antibacterial activity was confirmed to be specific to the sponge extracts, as the negative controls comprising the extraction solvents and the DMSO used for extract dilution did not exhibit any antibacterial effects.

The current research identified potential bioactivity in the ethanolic extracts of the sponge, indicating the presence of active compounds with antibacterial properties within the crude sponge extracts. Notably, ethanol absolute was employed as a common solvent for sponge preservation. To the best of our knowledge, this research is the first instance where the antibacterial potential of a sponge preservation solvent has been demonstrated.



Figureure 2. Diameters of the zone of inhibition of crude extracts RA3 and RB1 against: (a). *B. subtilis* ATCC 6051; (b). *S. aureus* ATCC 13420; (c*). E. coli* ATCC 9637; (d) *K. pneumoniae* ATCC 1388; (d). *P. aeruginosa* ATCC 9637. RA3.E and RB1.E indicate the ethanol (E) extracts, while RA3EM and RB1EM indicate the ethyl acetate: methanol (EM) extracts. Error bars indicate the standard error of mean over duplicate experiments (n=2)

Table I Thin layer chromatography (TLC) profile of *Aaptos* sp.

Compound		Alkaloid						Terpenoid						
Sample	Rf (cm)	Before sprayed Dragendorff			After sprayed Dragendorff		Rf (cm)	Before sprayed anisaldehyde			After sprayed anisaldehyde			
		Visible	UV 254 nm	UV 365 nm	Visible	Results		Visible	UV 254 nm	UV 365 nm	Visible	UV 254 nm	UV 365 nm	Results
	0.575	Invisible	Absorbs	Fluorescent	Orange	Positive								
RA3.E	0.388	Yellow	Absorbs	Absorbs	Orange	Positive	-	Invisible	Invisible	Invisible	Invisible	Invisible	Invisible	Negative
	0.238	Orange	Absorbs	Absorbs	Orange	Positive								
	0.55	Invisible	Absorbs	Fluorescent	Orange	Positive								
RB1.E	0.363	Yellow	Absorbs	Absorbs	Orange	Positive	-	Invisible	Invisible	Invisible	Invisible	Invisible	Invisible	Negative
	0.225	Orange	Absorbs	Absorbs	Orange	Positive								
	0.513	Invisible	Absorbs	Fluorescent	Orange	Positive								
RA3.ME	0.388	Yellow	Absorbs	Absorbs	Orange	Positive	0.188	Invisible	Invisible	Invisible	Violet	Absorbs	Fluorescent	Positive
	0.238	Orange	Absorbs	Absorbs	Orange	Positive								
	0.575	Invisible	Absorbs	Fluorescent	Orange	Positive	0.175							
RB1.ME	0.363	Yellow	Absorbs	Absorbs	Orange	Positive		Invisible	Invisible	Invisible	Violet	Absorbs	Fluorescent	Positive
	0.25	Orange	Absorbs	Absorbs	Orange	Positive								
Quinine	0.888	Invisible	Fluorescent	Fluorescent	Orange	Positive								
Thymol							0.713	Invisible	Fluorescent	Invisible	Red-Violet	Absorbs	Fluorescent	Positive

All extracts examined in this research demonstrated antibacterial activity against the tested gram-positive bacteria, with *B. subtilis* exhibiting higher susceptibility compared to *S. aureus* (Figureure 2). Conversely, among the gram-negative bacteria tested, the extracts only exhibited activity against *K. pneumoniae*, although some activity was observed against *P. aeruginosa* with the EM extracts. Notably, RA3.E displayed the highest antibacterial activity against *B. subtilis* (DOI 7.03 ± 0.33 mm), while the EM extract of RB1 exhibited the highest activity against *K. pneumoniae* (DOI 9.70 ± 1.1 mm).

Solvent selection plays a critical role in extracting bioactive compounds from marine sponges (Ebada *et al*., 2008). Ethanol is typically utilized for extracting polar secondary metabolites, whereas ethyl acetate is favored for semi-polar compounds. Conversely, as a polar solvent, methanol exhibits higher solubility for hydrophobic compounds than ethanol. Therefore, employing a mixture of ethyl acetate and methanol is anticipated to yield a diverse array of secondary metabolites, encompassing both polar and non-polar compounds (Ebada *et al*., 2018).

In this research, the ethanolic extracts exhibited better activity than the EM extracts when tested against *B. subtilis* and *S. aureus*. However, the opposite trend was observed with gram-negative bacteria. The EM extracts displayed better activity against *K. pneumoniae* and *P. aeruginosa* compared to the ethanolic extracts from both sponges (Figureure 2). These results suggest that the secondary metabolites produced by the sponges possess varying activities against different bacteria, potentially influenced by the choice of organic solvents for compound extraction, which in turn is influenced by compound polarity and extract solubility. It appears that ethanolic extracts contain compounds with enhanced activity against gram-positive bacteria, whereas those extracted by EM yield more active compounds against gram-negative bacteria. Particularly intriguing is the significantly higher activity of RB1.EM against *K. pneumoniae* compared to its ethanolic extract. Overall, the combination of ethyl acetate and methanol emerged as the more effective solvent, as crude extracts from both sponges RA3 and RB1 from Raja Ampat exhibited inhibition against both gram-positive and gram-negative bacteria. In a previous study, extracts of sponge *Aaptos sp*. from Maspari Island, Indonesia, demonstrated good antibacterial activity with a zone of inhibition (ZOI) of 15.9 ± 0.2 mm against *S. aureus* (Rozirwan *et al*., 2018). Further fractionation and analysis of fractions, as well as purification, are crucial for comprehensively identifying and characterizing the bioactive compounds extracted.

Chemical Constituents Profile of Extract Sponges

The TLC profile of active extracts from the marine sponge *Aaptos* has been extensively investigated. Alkaloid compounds were discovered in the sponge *A. suberitoides* from the Barang Lompo Islands, as evidenced by the presence of orange spots observed after the butanol extract was sprayed with dragendorff reagent (Rosmiati *et al*., 2015). In addition, alkaloid and terpenoid compounds were identified in *A. aaptos* in another study. These compounds were detected in both butanol and aqueous extracts, but not in the diethyl ether extract (Rosmiati *et al*., 2011). TLC was employed to identify all extracts and detect the presence of alkaloids and terpenoids.

Alkaloid

In the present research, RA3.E and RB1.E exhibited positive alkaloid presence, evidenced by the emergence of distinct orange spots upon application of the dragendorff reagent. This observation was consistent with the findings obtained from methanol:ethyl acetate extracts of RA3.EM and RB1.EM, which revealed three orange spots sharing similar characteristics. Notably, these findings closely mirrored the behavior of quinine, a well-established positive control, which also manifested an orange spot, thereby confirming its alkaloid classification (Table I) (Supplementary 1. 1.4. A; B; C; D; E). The consistent and parallel results obtained across different extraction methods further support the putative alkaloid content of RA3 and RB1.

Under UV illumination at 365 nm, RA3.E, RA3.EM, RB1.E, RB1.EM, and quinine exhibited distinct blue fluorescence (Supplementary 1.3. A; B; C; D; E) (Table I). Conversely, under UV at 254 nm, the samples appeared dark, but the TLC plate F254nm showed fluorescence (Supplementary 1.2. A; B; C; D; E) (Table I). This dual-wavelength response sheds light on the specific properties of the tested extracts and highlights their potential for differentiation and identification in analytical applications. Moreover, under UV illumination at 254 nm, RA3.E, RA3.EM, RB1.E, RB1.EM, and quinine appeared dark, but the TLC plate F254nm exhibited fluorescence (Supplementary 1.2. A; B; C; D; E) (Table I). Interestingly, while one alkaloid

spot in the samples displayed blue fluorescence, two alkaloid spots showed absorbance under UV at 365 nm (Supplementary 1.3. A; B; C; D; E) (Table I). These different results may indicate differences in chemical composition (Wagner & Bladt, 1996), suggesting that these samples may contain more than one type of alkaloid. This dual-wavelength response underscores the specific characteristics of the tested extracts and their potential for differentiation and identification in analytical applications.

A previous study on bioactive substances has revealed the isolation of alkaloids belonging to the aaptamine class from marine sponges *Aaptos* found in Kupang and South Sulawesi. Seven known aaptamines, along with a new class of aaptamine alkaloid, were isolated from methanolic extracts. These seven aaptamines were demonstrated to possess antibacterial properties against shrimp pathogenic bacteria, *Vibrio harveyi* and *Vibrio* sp. (Arai *et al*., 2014). The mechanism underlying the antibacterial activity of alkaloids involves disrupting cell division, inhibiting cellular respiration, suppressing enzyme activity, damaging bacterial membranes, and even influencing bacterial virulence genes (Othman *et al*., 2019).

Terpenoid

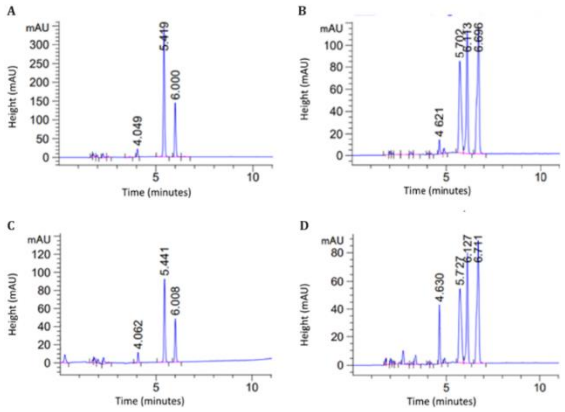
After treating the samples with anisaldehyde spray and subjecting them to heating at 105 °C, a violet spot was observed in RA3.ME and RB1.ME (Supplementary. 2.4. A; B; 2.5. A; B; 2.6. A; B) (Table I). However, no such compound was detected in RA3.E and RB1.E (Supplementary. 2.4. C; D; 2.5. C; D; 2.6. C; D) (Table I). When heated to 105 °C to speed up the reaction, terpenoids produce stains of different colors when treated with an anisaldehyde reagent (Ebada *et al*., 2008). The aromatic group of anisaldehyde acts as a catalyst in this reaction, facilitating the condensation with the targeted organic molecules. Consequently, this reaction produces compounds like triphenylmethane, characterized by conjugated double bonds. The color intensity of these compounds becomes more pronounced as the number of double bonds increases, resulting in violet, blue, brown, or green spots on the TLC plate under visible light observation (Harborne, 1998).

According to Wagner & Bladt (1996), thymol, the positive control, appeared as a reddish-violet spot due to its classification as a monoterpenoid compound. Anisaldehyde spraying is conducted after the thymol spot becomes visible under UV light at 254 nm. This is necessary because UV light at 254 nm can detect the double bonds present in thymol. However, since thymol lacks color, its spots are not visible under visible light or UV light at 365 nm. Therefore, applying an anisaldehyde reagent is essential to render thymol spots visible and detectable under UV light at 365 nm (Harborne, 1998). Similar results were observed with RA3.ME and RB1.ME, where the spot became visible under UV light at 365 nm following treatment with the anisaldehyde reagent.

The consistency observed in the TLC patterns, both in the alkaloid and terpenoid profiles, supports the classification of RA3 and RB1 within the same genus. Their morphological characteristics further corroborate this finding. Previous research on marine sponges from Terengganu Island successfully utilized a combination of morphological examination and chemical profiling to identify specimens as *Aaptos sp*. (Mohd *et al*., 2005). The similarity in chemical composition between these sponges suggests that, in addition to the morphological examination, chemical profiling is a valuable tool for dereplication, helping to avoid confusion arising from species variation (Bobzin *et al*., 2000).

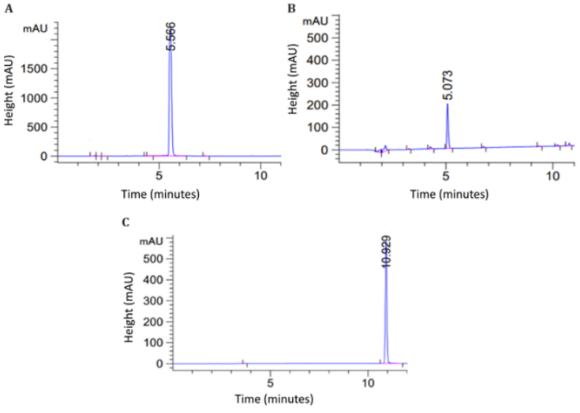
HPLC-DAD

Based on the HPLC-DAD results, distinct peak heights were observed for each extract, although the chromatograms exhibited striking similarities. Specifically, RA3.E demonstrated retention times of 4.049, 5.419, and 6.000 min, while RB1.E exhibited retention times of 4.062, 5.441, and 6.008 min. Both RA3.E and RB1.E displayed three prominent peaks with closely matching retention durations, suggesting the presence of analogous compounds in both samples (Figureure 3a and 3b). Similarly, RA3.ME and RB1.ME displayed four dominant peaks with retention periods of 4.621, 5.702, 6.113, and 6.696 min for RA3.ME, and 4.630, 5.727, 6.127, and 6.711 min for RB1.ME, respectively (Figure 3c and 3d). These findings indicate comparable retention durations among the compounds in RA3.E, RB1.E, RA3.ME, and RB1.ME, suggesting shared chemical properties based on similar retention times. Moreover, variations in peak and retention times were evident between ethanol and methanol:ethyl acetate extracts, suggesting potential impacts of solvent extraction techniques on sample chemical profiles, and subsequently, on biological activities.



Figureure 3. Chromatogram results of extracts: (a)RA3.E; (b) RA3.ME; (c) RB1.E; (d) RB1.ME using diode-array detection (DAD)

In the HPLC-DAD analysis, standard antibiotics such as ampicillin (AMP), ciprofloxacin (CIP), and chloramphenicol (CMP) were included for comparison with the extracts. The retention times for these antibiotics were noted as follows: CIP at 5.566 min, AMP at 5.073 min, and CMP at 10.929 min (Figure 4a, 4b, 4c). Interestingly, discrepancies were observed between the retention times of the antibiotic standards and the chromatograms of the RA3 and RB1 extracts. Specifically, the retention time of CIP closely resembled that of RB1.E (5.441 min) and RA3.E (5.419 min).



Figureure 4. Chromatogram results of antibiotics: a. Ciprofloxacin (CIP); b. Ampicillin (AMP); c. Chloramphenicol (CMP) using diode-array detection (DAD)

The similarity in retention time indeed suggests potential similarities in physicochemical properties, particularly in terms of compound polarity and functional groups. Compounds with similar retention times may belong to the same class, indicating similarities in their chemical structure and potentially in their bioactivity, especially if the central functional group plays a crucial role in determining bioactivity. However, it is important to note that retention time alone cannot establish the identity of a compound. Therefore, it would be premature to dismiss the potential importance or bioactivity of compounds solely based on the absence of a correlation between their retention times and those of antibiotics.

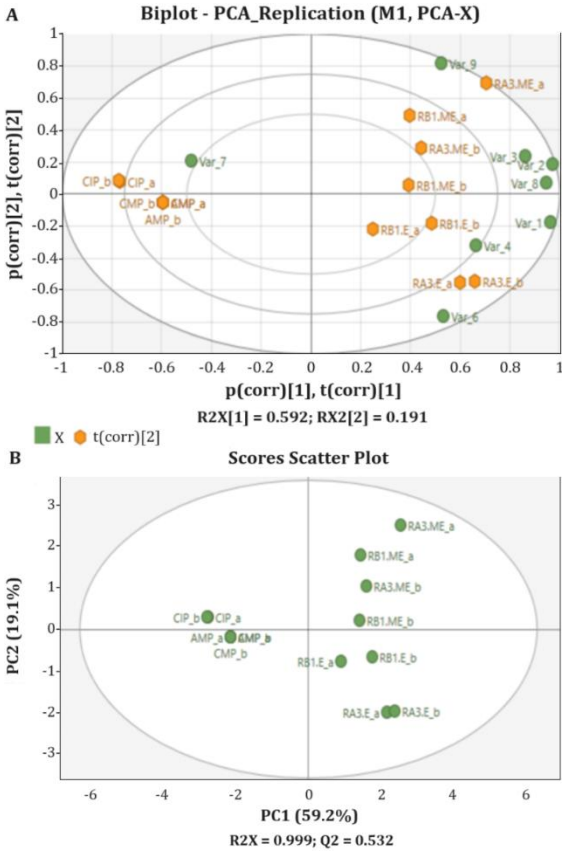
As Perera *et al*. (2019) highlighted, HPLC profiling can guide the selection of appropriate isolation protocols for crude extracts when the types of compounds present are unknown. Nevertheless, further analyses such as mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectroscopy are necessary to comprehensively characterize the compounds detected in the chromatograms. These techniques would provide crucial information for identifying the compounds and elucidating their potential biological activities (Ebada *et al*., 2008).

Correlation of Antibacterial Activity and Chemical Profile of Extract Sponges

PCA was employed in this research to explore the correlation between the distribution of compounds present in extracts RA3 and RB1 and their respective antibacterial activities. The data used for PCA were derived from the HPLC-DAD chromatograms, providing a comprehensive overview of the compounds present in the extracts. By extracting the principal components from the chromatographic data, PCA condensed the complex information into a smaller set of variables that captured the majority of the variance in the dataset. These principal components were arranged in order of significance, enabling their graphical representation in a score plot.

In the PCA analysis, the similarity of compounds present in sponge extracts from two different locations, RA3 and RB1, is condensed into a set of new descriptive variables known as principal components (PCs). The first principal component (PC1) captures the most significant variation in the data, while the second principal component (PC2) captures the second most important variation. Together, these principal components summarize the majority of the variability in the dataset. By representing this variation graphically in a score plot, PCA enables

the visualization of distinct groups or patterns in the data (Fick *et al*., 2004).). The score plot shows that the PCA result was obtained from several groups (Figureure 5).



Figureure 5. Principal Component Analysis (PCA) of extracts: RA3 in ethyl acetate: methanol (RA3.ME); RA3 in ethanol (RA3.E); RB1 in ethyl acetate: methanol (RB1.ME); RB1 in ethanol (RB1.E) and antibiotics Ciprofloxacin (CIP); Chloramphenicol (CMP); Ampicillin (AMP). (a). The results of a scatter plot of sponge extracts and antibiotics. (b). The PCA biplot of sponge extracts and antibiotics.

In this research, the PCA model successfully described over 85% of the variance in the dataset, with PC1 accounting for 59.2% of the variance and PC2 accounting for 19.1%. The correlation between the experimental data and the PCA results was assessed using R2 and Q2 values. Ideally, high R2 and Q2 values (>0.5 = good, >0.9 = excellent) are desirable, with the difference between them ideally not exceeding 0.2-0.3, too much noise has been incorporated into the model (Fick *et al*., 2004). In the current research, the R2X value was exceptionally high at 0.999, indicating that the model explains nearly all the data variance. However, the Q2 value of 0.532 suggests good predictability but also indicates some degree of noise in the model, as there is a significant difference between R2X and Q2. This discrepancy may arise from imprecise model fitting or the presence of outliers in the dataset (Figure 5).

The loading plot assesses the correlation between components and variables to gauge the extent of shared information. Variables are depicted as points in component space, with their loadings serving as coordinates (Abdi & Williams, 2010). This graphical representation offers insight into the importance of each variable for every extract and antibiotic. The proximity of samples in the plot reflects their similarity, while clustering variables with similar properties facilitates the evaluation of overarching trends and chemical correlations and the identification of outliers.

Variables 1, 4, and 6 exhibit a notable presence in positive PC1 and negative PC2, indicating their significant discriminatory effect on RA3.E and RB1.E extracts. In RA3.E, these variables correspond to retention times of 1.703, 4.049, and 5.419 min, respectively, while in RB1.E, they align with retention times of 1.700, 4.062, and 5.441 min, respectively. Hence, variables 1, 4, and 6 are likely to have discriminatory effects similar to those observed in RA3.E and RB1.E. Conversely, variables 2, 3, 8, and 9 demonstrate a strong discriminatory effect on RA3.ME and RB1.ME extracts, as they are situated in positive PC1 and positive PC2. In RA3.ME, these variables correspond to retention times of 1.831, 2.316, 6.113, and 6.696 min, respectively, while in RB1.ME, they align with retention times of 1.832, 2.270, 6.127, and 6.711 min, respectively. Thus, variables 2, 3, 8, and 9 are likely to exert a discriminatory effect similar to that observed in RA3.ME and RB1.ME (Figure 5a).

The biplot analysis effectively combined the loading plot and score plot results. Within the same quadrant of the principal component (PC), RA3.ME and RB1.ME were positioned adjacent to RA3.E and RB1.E, suggesting similar characteristics among their compounds and indicating no discernible differences in compound properties between different regions of the sponge. Notably, ethanol and methanol:ethyl acetate extracts were distributed across separate quadrants. The positive PC1 and negative PC2 quadrants were occupied by ethanol extracts (RA3.E and RB1.E), while the positive PC1 and positive PC2 quadrants were characterized by methanol:ethyl acetate extracts

(RA3.ME and RB1.ME). In addition, antibiotic compounds, including CIP, were located in the positive PC1 and negative PC2 quadrants, while CMP and AMP were situated in the negative PC1 and negative PC2 quadrants, respectively. Interestingly, no extracts were found in the same quadrant as the antibiotics. This pattern was consistent with the observations in the profile chromatograms (Figure. 5b).

The PCA conducted in this research offered valuable insights into the physicochemical properties of natural products sourced from diverse origins, thereby facilitating a more holistic comprehension of their interrelations. The discernible clustering patterns unveiled by PCA not only revealed similarities in compound attributes across varying geographical locales but also facilitated the systematic classification of ethanol and methanol:ethyl acetate extracts. The positioning of antibiotic compounds within specific quadrants underscores the discriminative capability of PCA in distinguishing chemical profiles. Further investigation employing a wide array of antimicrobial agents as controls could prove beneficial in analyzing specific compounds with analogous characteristics.

The metabolomic analysis aimed at enhancing TLC and HPLC analysis yielded a compound similar to alkaloids in both RA3 and RB1 sponge extracts. Identified as N1-[4-(1,3-Oxazol-5-yl)phenyl]cyclopropane-1-carboxamide, this compound exhibited retention times of 4,703 min for RA3 and 4,911 min for RB1, with a consistent m/z value of 229. These findings strongly suggest the presence of alkaloids in the RA3 and RB1 sponge extracts. Of particular significance are the implications drawn from previous research on aaptamine, an alkaloid sourced from *Aaptos* (Rajivgandhi *et al*., 2019), which underscored its robust antibacterial efficacy and bactericidal properties, rendering it a promising natural agent for combating drug-resistant pathogens. The synergy between PCA results and the distinctive attributes of aaptamine underscores the effectiveness of the analytical methodology in compound prediction. Furthermore, this research not only enriches our understanding of natural product diversity but also underscores the instrumental role of PCA in identifying and prognosticating valuable compounds, with aaptamine emerging as a frontrunner for prospective therapeutic interventions against bacterial infections.

CONCLUSION

Marine sponge *Aaptos spp*. represents a significant source of natural compounds with potential applications in drug discovery. This research reported the antibacterial properties of *Aaptos* sponge extracts sourced from Raja Ampat island, unveiling the presence of alkaloid compounds similar to aaptamine. To further characterize the bioactive compounds present, molecular docking and dereplication processes utilizing preparative HPLC can be employed, facilitating the isolation and purification of target/lead compounds through fractionation. In addition, HRMS profiling offers a valuable tool for characterizing and distinguishing compounds within the active extracts. The methodologies outlined in this research provide a comprehensive framework for evaluating the therapeutic potential of natural products and offer an innovative approach for advancing drug discovery research in the future.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

AUTHOR CONTRIBUTIONS

All authors mentioned in this manuscript are contributed equally as **main contributors**. AA, KA, STU, EWP, and NS contributed to conceive and to design the experiment, to conduct the compound extraction and to perform the antibacterial activity, to prepare Figureure and data for presentation, to analyze the data, to write the first draft of the

manuscript, to edit and to review the manuscript. AA, KA, NS, MBP, and FNM contribute to performing compound extraction and antibacterial screening. AA, AN, KW, SAH, and IH contribute to the field work, to prepare and to provide the sponge raw materials. AA, EWP, NG, DS, STU, and IH contribute to provide chemicals and consumables. AA, EWP, CNA, NG, DS, and STU contribute to supervision. AA, KA, NG, FNM, and STU contributed to edit the manuscript. All authors read, review, and agree with the content of the manuscript.

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