Ethanolic extracts from deep marine sponges: A new frontier in antibacterial discovery from the Jordanian Gulf of Aqaba

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Abstract. The urgent need for new antibiotics to counter bacterial resistance has led to renewed interest in marine natural products. The present study evaluated the antibacterial potential of ethanolic extracts from three deep-sea sponges: *Stelletta sp*., *Dactylospongia cf. elegans* (*D. cf. elegans*) and *Axinella sp*., which were collected from the Gulf of Aqaba off the coast of Jordan. Antibacterial activity was assessed against Gram-negative and Gram-positive bacteria using the well diffusion method, followed by determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Only *D. cf. elegans* exhibited potent activity, which was limited to Gram-positive bacteria and showed inhibition zones of 7 to 21 mm and MIC and MBC values of 1 and 2 mg/ml, respectively. *Stelletta sp.* showed no detectable activity, and *Axinella sp.* displayed minimal effects. DNA barcoding (28S rRNA) confirmed that all three species belong to the class Demospongiae. LC-MS/MS analysis of the extract from *D. cf. elegans* identified bioactive constituents, including bolinaquinone, dactyloquinone, gallic acid and caffeic acid, which are compounds known for antibacterial properties and likely contributed to the observed activity. Thus, *D. cf. elegans* could be a promising source of antibacterial agents against Gram-positive pathogens and warrants further evaluation of the mechanisms involved, its toxicity, and its effects *in vivo*.

Introduction

Antibiotics are central to the management of severe infections and have transformed human health (1,2). However, the rise and rapid spread of antimicrobial resistance (AMR) have made numerous infections increasingly difficult to treat. This crisis stems from the emergence and proliferation of resistant microorganisms and is exacerbated by inappropriate use of antibacterial agents in both clinical and community settings (3-6). Numerous currently available antibiotics have failed to overcome established resistance mechanisms, which underscores the need for prudent antimicrobial stewardship and the discovery of new agents (7,8). There is an urgent need for prudent antibiotic use and the development of novel antibacterial agents to safeguard human, animal and agricultural health (9-11).

Natural products have long been recognized as an important source of bioactive compounds for modern medicine (12), and their unique chemical and structural diversity provides opportunities to develop agents with novel mechanisms of action (10,13). Modifications of existing natural compounds can temporarily overcome resistance (2,14), but the discovery of entirely new natural molecules remains essential to address the global threat of AMR (7,15,16). Marine organisms, and particularly sponges, have attracted increasing attention for their ability to produce antibacterial metabolites (17-19). The marine ecosystem represents a promising reservoir of novel antibiotics, and there is a need to explore less-studied habitats to meet the demand for new therapeutics (10,20,21).

Compared with terrestrial environments, marine ecosystems offer greater potential for discovering unique bioactive molecules (12,22,23). The Gulf of Aqaba is a relatively isolated deep basin in the Red Sea and exhibits unique thermal and ecological features that distinguish it from other deep-sea regions (24,25). Its unusual conditions support high biodiversity and make it an exceptional setting for exploring novel bioactive metabolites (26,27).

As sessile organisms, marine sponges are constantly exposed to diverse microbial communities in their aquatic habitats, including predators, biofouling microorganisms and pathogens (28-30). Because they lack an innate immune system, their primary defence strategy is the production of secondary metabolites, which act as chemical defences and enable them to adapt to environmental pressures (9,31-33). Symbiotic microorganisms associated with sponges also contribute to

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their defence, nutrition and metabolism (34). The compounds derived from the hosts and symbionts include terpenoids, peptides, alkaloids, macrolides and steroids that have shown potential as drug leads for the treatment of diseases such as malaria, cancer and infections caused by antibiotic-resistant pathogens (8,35,36). Numerous sponge species have demonstrated antibacterial, anticancer, antifungal, anti-inflammatory and antimalarial properties, supporting their pharmaceutical relevance (37-41). Given the escalating problem of bacterial resistance, marine biotechnology ('blue biotechnology') is increasingly a focus to discover bioactive molecules from marine organisms as sources of novel antibacterial agents (11,13).

Several studies have demonstrated the antibacterial activity of sponge extracts against both Gram-positive and Gram-negative bacteria (42-44). For instance, extracts from the marine sponge *Acanthella cavernosa* exhibit inhibitory effects against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (45). Similarly, extracts from the sponge *Callyspongia plicifera* have been reported to have antibacterial activity against *S. aureus*, *Bacillus subtilis* and *Klebsiella pneumoniae* (46,47). According to studies conducted in the Gulf of Aqaba, several shallow-water sponge species have demonstrated notable antibacterial properties. For example, the ethanolic crude extract of *Grayella cyathophora* exhibits strong activity, particularly against *Pseudomonas aeruginosa* (48). By contrast, deep-sea environments, which are characterized by high pressure, low temperature, and the absence of light, favour the production of unique secondary metabolites. This further supports the rationale for investigating deep-sea sponges as promising sources of biologically active compounds (49,50).

A recent study selected Red Sea sponge species at shallower depths (140-290 m) and focused primarily on antibacterial screening (51). The present investigation provides the first comprehensive chemical; molecular and biological characterization of deep-sea sponges collected at previously unexplored depths (345-362 m) from the Jordanian Gulf of Aqaba during the OceanXplorer Jordan Expedition 2022. DNA-barcoding (28S rRNA, GenBank accession numbers PX278188.1-PX278188.3) confirmed that the analyzed specimens represent genetically independent lineages relative to earlier samples. LC-MS/MS metabolite profiling revealed a distinct secondary-metabolite fingerprint, including Hyatellaquinone, Manoalide, Motualevic acid, Manzamine A and Popolohuanone, which were not reported in the previous study. Furthermore, the present study broadens the biological scope by assessing antibacterial activity against multidrug-resistant *S. aureus* (including MRSA), thereby delivering a more complete pharmacological assessment. Collectively, the integration of deeper-water sampling, molecular verification, metabolomic differentiation, and expanded bioassays establishes the present study as an independent and novel contribution to marine-derived drug-lead discovery in the Gulf of Aqaba.

The aim of the present study was to investigate the antibacterial potential of three deep-sea sponges collected from the Gulf of Aqaba, Jordan, one of the deepest and relatively unexplored marine environments in the region. By evaluating their bioactivity and chemical composition, it was aimed to identify safe and biocompatible agents with potential therapeutic applications. Special emphasis was placed on the ethanolic extract of the most active sponge, *Dactylospongia cf. elegans* (*D. cf. elegans*), which was chemically characterized using LC-MS/MS.

Materials and methods

Sampling of sponge specimens. In July 2022, as part of the OceanXplorer Jordan Expedition, three deep-sea sponge samples were collected from Stations 1 and 2, located in the deepest bottom of the Gulf of Aqaba, Jordan, aboard the research vessel OceanXplorer (Fig. S1). Sampling was conducted at depths ranging from 345 to 362 m using the robotic arm on the manned submersible. Specifically, Sponge 1 was collected at 345 m, Sponge 2 at 362 m, and Sponge 3 at 362 m, as detailed in Table I, all retrieved on 14 July 2022. Samples were immediately placed in sterile labeled containers, frozen at -20˚C onboard and transferred on ice to the Laboratory for Molecular and Microbial Ecology, The University of Jordan, for subsequent analyses. Sampling was authorized under the OceanXplorer permit no. 9795, issued by the Aqaba Special Economic Zone Authority. The morphological characteristics of these samples are summarized in Table SI, and representative morphological images are provided in Figs. S2-4 to enhance the visualization and clarity of Table SI.

Molecular identification of sponge samples by DNA barcoding. To identify the sponge species, ~25 mg of tissue was fragmented before DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Inc.) following established protocols (22). The 28S ribosomal RNA gene was selected as the molecular marker (52) with primers listed in Table II. This marker was chosen because of its reliability in resolving taxonomic relationships within the class Demospongiae, and its successful application in previous sponge barcoding studies (53,54). It is acknowledged, however, that species-level identification of Porifera should not rely solely on a single locus. Ideally, such assignments are corroborated by morphological traits (Table SI) and, where possible, multi-locus data (for example, COI + 28S ± ITS). Accordingly, the authors' species assignments were made conservatively and supported by both molecular and morphological evidence.

PCR was used to amplify partial fragments of the 28S rRNA gene, which has proven effective in classifying sponge taxa from underexplored habitats (12,55,56). The PCR amplification was performed using EntiLink™ PCR Master Mix (ELK Biotechnology, Co., Ltd.) with the following cycling condition: An initial denaturation at 94˚C for 3 min; 35 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, and extension at 72˚C for 83 sec; followed by a final extension at 72˚C for 10 min. Amplification products were visualized by 1% agarose gel electrophoresis and documented using a Gel Documentation system.

PCR products were subsequently purified and sent to Macrogen (South Korea) for Sanger sequencing. Sequence analysis was performed using MEGA software and BLAST searches against the GenBank database. A phylogenetic tree was constructed by the neighbor-joining method based on 28S

Table I. Sponge sample ID and their collection depths.

Sample ID	Sponge 1	Sponge 2	Sponge 3
Depth of the collection area	345 m	362 m	345 m
Station No.	Station 1	Station 2	Station 1

rRNA sequences from closely related sponge taxa available in GenBank (57).

Ethanolic extraction and preparation of the sponges' crude extracts. Freshly chopped sponges were soaked in a 70% ethanol/water solution for storage at -20˚C. An adapted extraction procedure was performed (14,20,58). Initially, the sponges blended while immersed in the ethanol solution. Subsequently, the mixture was heated at 55-60˚C for 2 h to optimally extract hydrophilic and hydrophobic compounds with ethanol solution. The resulting solution was then filtered, and the filtrate was concentrated using a rotary evaporator.

The concentrated extract was further processed by lyophilization (freeze drying), resulting in a powder (59). A concentration of 25 mg/ml was achieved by dissolving 25 mg of extracted powder in 1 ml of Dimethyl Sulfoxide (DMSO) to create the stock solution. This liquid was vortexed and filtered through a 0.45-μm nylon syringe filter before being placed in a Falcon tube for storage. The stock solution was then diluted into various quantities (5, 10, 15 and 20 mg/ml) using sterile distilled water.

Antibacterial effect of sponges' ethanolic extracts. The antibacterial efficacy of ethanolic extract was assessed using six bacterial strains, including *S. aureus* (ATCC 29213), *Staphylococcus epidermidis* (*S. epidermidis*; ATCC 51625) and *Bacillus pumilus* (isolate) as Gram-positive bacteria, and *Klebsiella aerogenes* (isolate) and *E. coli* (ATCC 25922) as Gram-negative bacteria. In addition, methicillin-resistant *S. aureus* (MRSA; ATCC 1026) was included as a clinically relevant resistant strain.

The agar well diffusion method was used to evaluate the antibacterial activity of the extracts (60). Briefly, Muller-Hinton Broth (MHB) was inoculated with the bacterial strains and incubated at 37˚C overnight. The bacterial culture density was adjusted to the 0.5 McFarland turbidity standard (≈1.5x10^8 CFU/ml) (61), and the inoculum was spread onto Muller-Hinton agar plates. Wells (8 mm) were created in the agar and filled with different extract concentrations (5, 10, 15 and 20 mg/ml). Plates were left at room temperature for 1-2 h to allow pre-diffusion before incubation at 37˚C for 24 h.

After incubation, antibacterial activity was determined by measuring the inhibition zones around the wells (52,62). Gentamycin (10 μg) served as the positive control for all bacteria (63,64), and vancomycin (30 μg) was used as the MRSA-specific positive control (65). Furthermore, 80% DMSO was used as the negative control, as it was required to ensure complete dissolution of the crude sponge extracts for accurate antimicrobial testing. To exclude any solvent-related effects, pure 80% DMSO was tested against all bacterial strains and showed no antibacterial activity. In addition, Sponge 1 extract, despite being dissolved in 80% DMSO, exhibited no detectable antibacterial effect, further supporting the lack of interference from the solvent itself (66). All experiments were performed in triplicate.

Determination of MBC and MIC. The MIC of the sponge extracts was determined using the standard 96-well microdilution method (67). Each well contained 100 μl of bacterial culture (~6.0 log10 CFU/ml) obtained from an overnight culture. Following the protocol of Balouiri *et al* (68), serial dilutions of the extracts were prepared in MHB. The concentration ranges were selected based on the results of the agar well diffusion assay. For Sponge 2, concentrations of 5, 4, 3, 2, 1, 0.5, 0.25 and 0.125 mg/ml were tested against *S. aureus* and MRSA, while 10-1 mg/ml dilutions were tested against *S. epidermidis*. Negative controls (80% DMSO and un-inoculated MHB) and positive control (bacterial suspension only) were included to ensure accuracy (65,69). The plates were sealed and incubated at 37˚C for 24 h. Similarly, MBC was determined as described previously (67). The MBC assay was designed to distinguish between bactericidal and bacteriostatic effects of the sponge extracts. Samples from wells with concentrations at or above the MIC were subcultured and evenly spread onto fresh agar plates (70). The lack of bacterial colonies on the agar after 24 h at 37˚C indicated the MBC, defined as the lowest extract concentration capable of completely eliminating the tested microorganism (71).

LC-MS-MS analysis of sponge extracts. The most bioactive sponge extract's chemical makeup was assessed utilizing LC-MS-MS. Smart Labs Group conducted this analysis using a Shimadzu LC system that comprised the following parts: SIL-30AC (autosampler), CBM-20A (control bus module), LCMS-8030 (Triple Quadrupole Mass Spectrometer), LC-30AD (liquid chromatograph), and CTO-30A (column oven). A total of ~30 mg of the sponge extract was fully extracted using methanol (MeOH) to prepare the sample. To purify the resulting crude extract, a solid-phase extraction column was used. In this stage, the methanolic fraction (100% MeOH) was preserved, whereas the aqueous fraction (100% H_2O) was discarded. A final working concentration of 2.5 mg/ml of the crude extract was used for the LC-MS-MS analysis. Using a gradient elution protocol that lasted 15 min, the LC-MS-MS technique moved from 100% water (containing 0.1% formic acid) to 95% acetonitrile (also containing 0.1% formic acid). Main details, such as particular molecular ion masses (with an accuracy of <5 ppm), compound retention periods (in minutes), and MS-MS daughter ion patterns for structural clarification, were all supplied by the ensuing LC-MS spectra.

Statistical analysis. All data were processed and visualized using GraphPad Prism™ software (version 10.3.0; GraphPad Software Inc.; Dotmatics). Inhibition zone diameters obtained from the agar well diffusion assay were expressed as the mean ± standard error of the mean (SEM) based on at least three independent replicates. Since the study was exploratory in nature and aimed at characterizing biological trends rather than testing predefined hypotheses, no formal statistical comparisons (for example, ANOVA or t-tests) were performed.

Table II. Primer sequences for 28S ribosomal RNA gene.

Primer	Sequence
28F63mod (Forward)	5'-ACCCGCTGAAYTTAAGCATATHANTMA-3'
28R1072 (Reverse)	5'-GCTATCCTGAGGGAAACTTCGG-3'

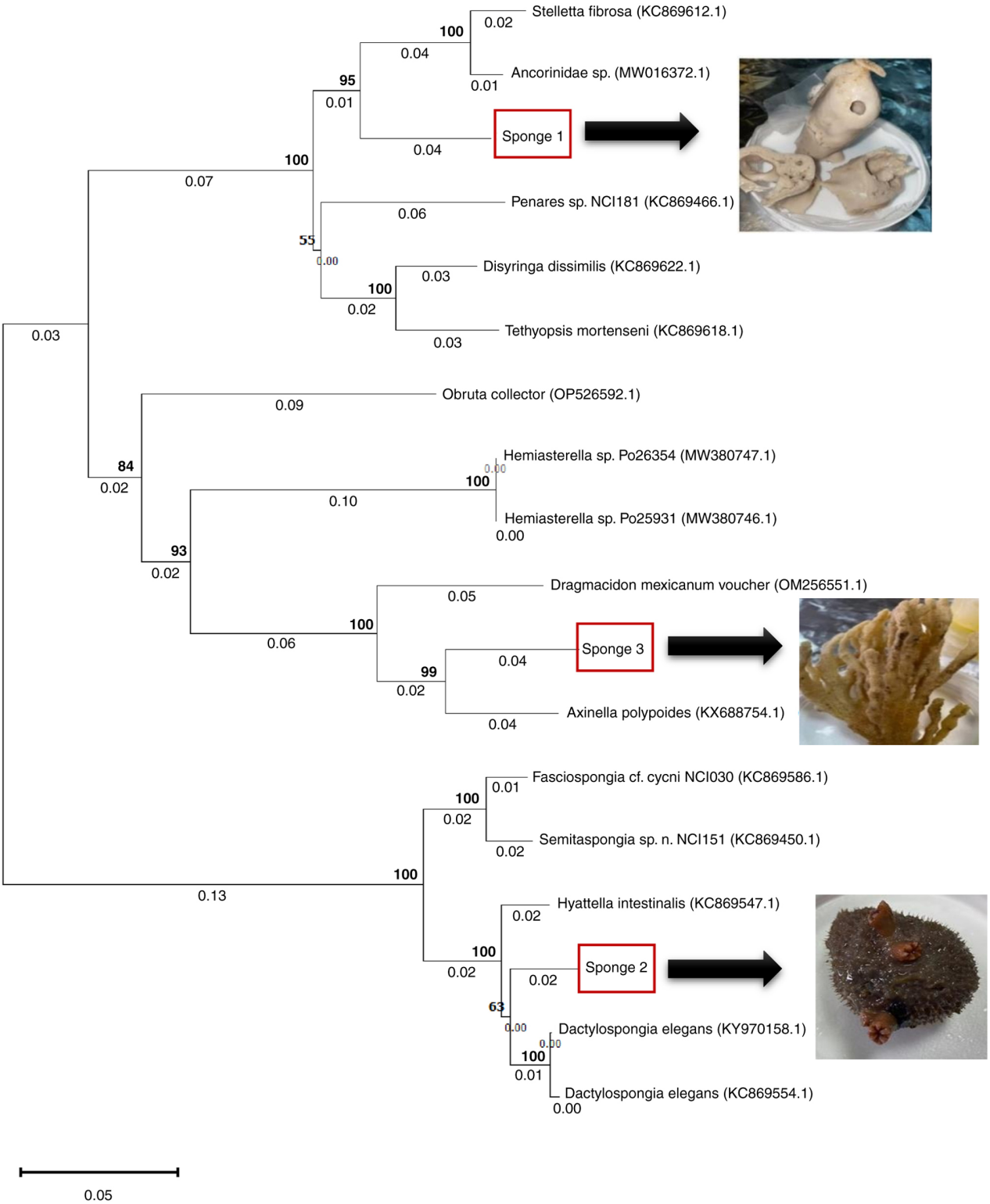

Figure 1. Phylogenetic Analysis of 28S rRNA gene sequences from three marine sponge samples: A phylogenetic tree was constructed using MEGA11 software with the UPGMA method, and evolutionary distances were computed via the Neighbor-Joining algorithm. Bootstrap values (based on 1,000 replicates) are displayed in bold. Red boxes and corresponding morphological images characterize the three sponge samples.

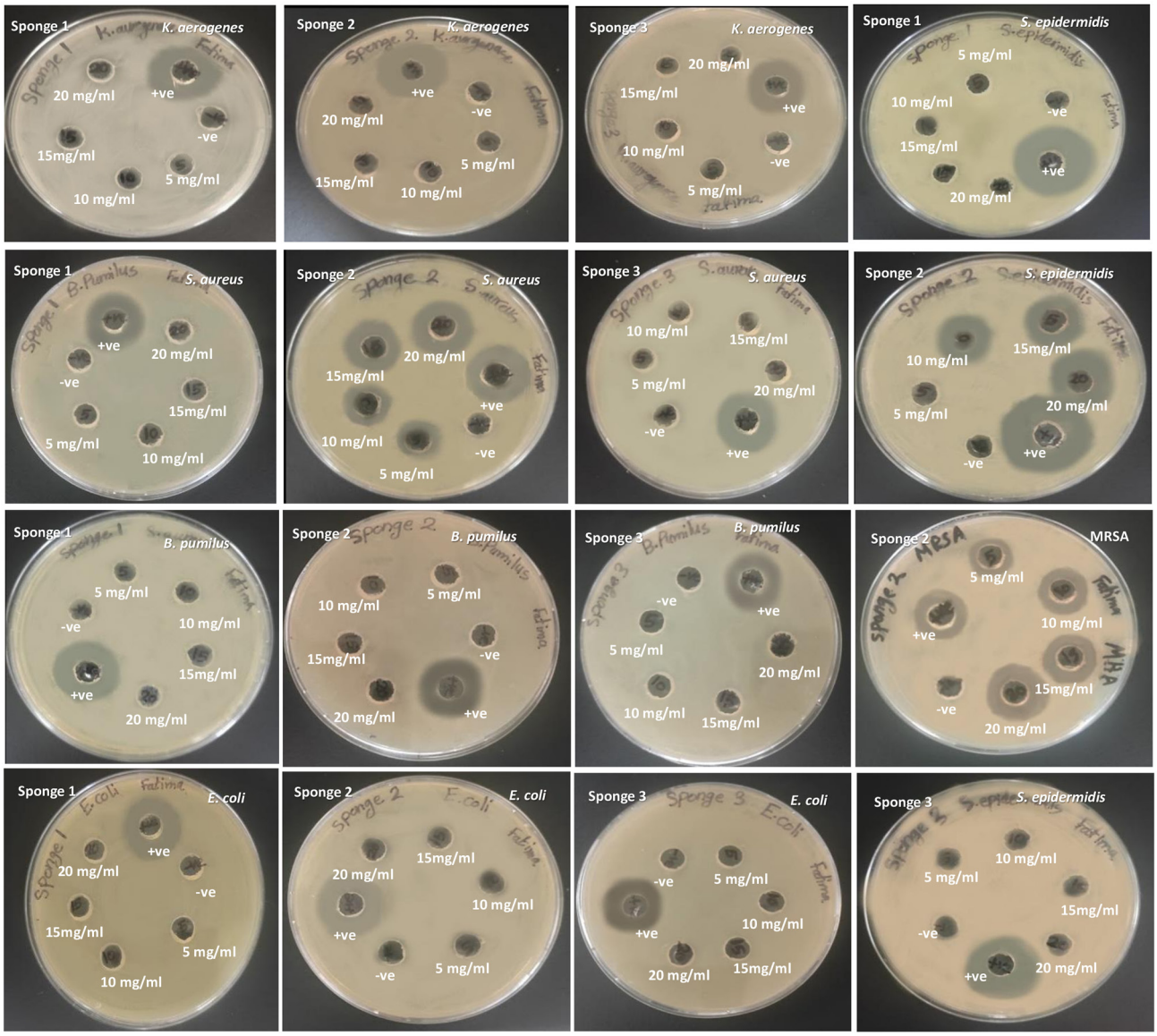

Figure 2. Results of the agar well diffusion assay for antibacterial activity against: *Escherichia coli*, *Staphylococcus epidermidis*, *Klebsiella aerogenes*, *Staphylococcus aureus*, *Bacillus pumilus* and methicillin-resistant *Staphylococcus aureus*. Where *Stelletta sp*. (Sponge 1), *D. cf. elegans* (Sponge 2), *Axinella sp*. (Sponge 3) with concentrations (5, 10, 15, 20 mg/ml). Gentamycin (10 μg): Positive control for all bacteria, Vancomycin (30 μg): MRSA-specific positive control, and 80% DMSO: Negative control. MRSA, methicillin-resistant *Staphylococcus aureus*.

The results are therefore presented descriptively, and MIC and MBC values are shown as representative data from replicate experiments to illustrate reproducibility and relative potency. Although triplicate measurements were obtained, they represent technical replicates of the same sample and thus were not subjected to inferential statistical testing. The study was exploratory and descriptive in design.

Results and Discussion

Sponge identification (DNA barcoding and phylogenetic analysis). The 28S rRNA gene was successfully amplified from all three sponge specimens, which produced single amplicons of ~1,000-1,300 bp and were confirmed by agarose gel electrophoresis (Fig. S5). The purified PCR products were sequenced, and the resulting sequences were analysed using BLAST against the NCBI GenBank database. The BLAST results demonstrated high similarity to known representatives of the class Demospongiae with query coverage values of 99%. Specifically, Sponge 1 displayed the highest similarity to *Stelletta fibrosa* (90.04% identity), Sponge 2 was most similar to *Dactylospongia elegans* (95.74% identity) and Sponge 3 was most similar to *Axinella polypoides* (91.32% identity).

To refine taxonomic placement, a phylogenetic tree was constructed using the neighbour-joining method with 1,000 bootstrap replicates (Fig. 1). The tree topology supported the BLAST results and clustered Sponge 1 within the genus *Stelletta* (family Ancorinidae, order Tetractinellida), with Sponge 2 grouped closely with D. elegans (family Thorectidae, order Dictyoceratida), and Sponge 3 within the genus Axinella (family Axinellidae, order Axinellida). Given the limitations of single locus barcoding for Porifera, we conservatively report the three specimens at the genus level: *Stelletta sp*., *Dactylospongia cf. elegans* and *Axinella sp*. This conservative

Table III. Inhibition zone diameters (mm) for different bacterial strains treated with sponge 2 ethanolic extracts in different concentrations.

Mean of the inhibition zone diameter (mm) for sponge 2						
Bacterial strains	5 mg/ml	10 mg/ml	15 mg/ml	20 mg/ml	Positive control	Negative control
E. coli	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	19.67±1.20	0.00±0.00
S. aureus	11.00±0.58	15.33±0.88	17.67±1.85	21.00±1.53	20.00±3.00	0.00±0.00
K. aerogenes	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	19.67±0.88	0.00±0.00
B. pumilus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	18.33±0.88	0.00±0.00
S. epidermidis	12.66±2.33	7.00±3.51	14.00±0.58	17.33±0.33	27.00±0.58	0.00±0.00
MRSA	12.33±0.33	15.33 ± 0.33	17.00±0.58	18.33±0.33	15.67±0.33	0.00±0.00

Data are expressed as the mean ± standard error of the mean based on three independent replicates (n=3). Positive control for all bacteria: Gentamycin (10 μg), MRSA-specific positive control: Vancomycin (30 μg), and Negative control: 80% DMSO.

Table IV. Bioactive compounds were detected in the ethanolic extract of sponge 2 by LC-MS-MS.

Compounds	Molecular formula	Molecular weight (g/mol)	%	RT
Gallic acid	$C_7H_6O_5$	170.12	7	1.3
Dactyloquinone	$C_{22}H_{28}O_4$	356.5	6.2	3
Chromazonarol	$C_{21}H_{30}O_2$	314.5	5.1	2.35
Manoalide	$C_{25}H_{36}O_5$	416.5	5.1	5.4
Caffeic acid	$C_9H_8O_4$	180.16	5	1.4
Bolinaquinone	$C_{22}H_{30}O_4$	358.5	5	3.6
Mamanuthaquinone	$C_{22}H_{30}O_4$	358.5	4.2	3.7
δ-humulene	$C_{15}H_{24}$	204.35	4.1	1.6
Ergosterol	$C_{28}H_{44}O$	396.6	4.1	5.1
Linoleic acid	$C_{18}H_{32}O_2$	280.4	3.6	1.9
Ilimaquinone	$C_{22}H_{30}O_4$	358.5	3.3	3.22
Pelorol	$C_{23}H_{32}O_4$	372.5	3.3	4.5
Hyatellaquinone	$C_{12}H_{14}O_4$	222.24	3.2	1.7
Ellagic Acid	$C_{14}H_6O_8$	302.19	3.2	2.1
Cyclospongiaquinone	$C_{22}H_{30}O_4$	358.5	3.1	4
indole-3-carbaldehyde	C_9H_7NO	145.16	3	1.2
Petasitolone	$C_{15}H_{24}O_2$	236.35	2.8	1.75
Ocimene	$C_{10}H_{16}$	136.23	2.2	1.1
Scopoletin	$C_{10}H_8O_4$	192.17	2.2	1.45
Isospongiaquinone	$C_{22}H_{30}O_4$	358.5	2.1	3.5
Lectin	$C_{26}H_{28}O_6$	436.5	2.1	5.7
Dysideamine	$C_{21}H_{29}NO_3$	343.5	2	2.8
Stelletin	$C_{30}H_{38}O_4$	462.6	1.7	6.3
Nakijiquinone D	$C_{25}H_{35}NO_6$	445.5	1.6	6
Ferulic acid	$C_{10}H_{10}O_4$	194.18	1.5	1.5
Clathric acid	$C_{20}H_{30}O_2$	302.5	1.5	2.2
Motualevic acid	$C_{16}H_{23}Br_2NO_2$	421.2	1.3	5.5
Smenospongimine	$C_{22}H_{31}NO_3$	357.5	1.2	3.1
Limonene	$C_{10}H_{16}$	136.23	1.1	1
p-coumaric acid	$C_9H_8O_3$	164.16	1.1	1.25
Catechin	$C_{15}H_{14}O_6$	290.27	1	2
Smenospongine	$C_{21}H_{29}NO_3$	343.5	1	2.6
Dactyltronic acid	$C_{21}H_{30}O_5$	362.5	1	4.15
Popolohuanone	$C_{42}H_{57}NO_3$	623.9	1	6.7
Chlorogenic acid	$C_{16}H_{18}O_9$	354.31	0.9	2.9
Luffariellolide	$C_{25}H_{38}O_3$	386.6	0.8	4.8
Rutin	$C_{27}H_{30}O_{16}$	610.5	0.5	6.5
Dictyoceratin A	$C_{23}H_{32}O_4$	372.5	0.3	4.35
Squalene	$C_{30}H_{50}$	410.7	0.3	5.3
Manzamine A	$C_{36}H_{44}N_4O$	548.8	0.3	6.4

identification is further supported by morphological traits (Table SI). The final sequences generated in the present study were deposited in GenBank under accession numbers PX278187 (*Stelletta sp.*), PX278188 (*D. cf. elegans*) and PX278189 (*Axinella sp.*).

Antibacterial activity. The antibacterial activity of the three ethanolic sponge extracts was evaluated against six clinically relevant bacterial strains, including Gram-positive strains (*S. aureus, B. pumilus*, *S. epidermidis* and MRSA and Gram-negative strains (*E. coli* and *K. aerogenes*). As shown in Fig. 2, only the extract of *D. cf. elegans* exhibited marked antibacterial activity, which was limited to Gram-positive bacteria. The inhibition zones ranged from 7 to 21 mm in a concentration-dependent manner against *S. aureus*, MRSA and *S. epidermidis* (Table III). By contrast, no inhibitory effect was observed against *B. pumilus* or the Gram-negative strains (*E. coli* and *K. aerogenes*).

The extracts of *Stelletta sp.* and *Axinella sp.* also failed to show any detectable antibacterial effect. This absence of activity may be attributed to differences in the secondary metabolite composition of these sponges, ecological variation, or the presence of compounds with specificity toward microbial taxa that were not included in the present panel. The

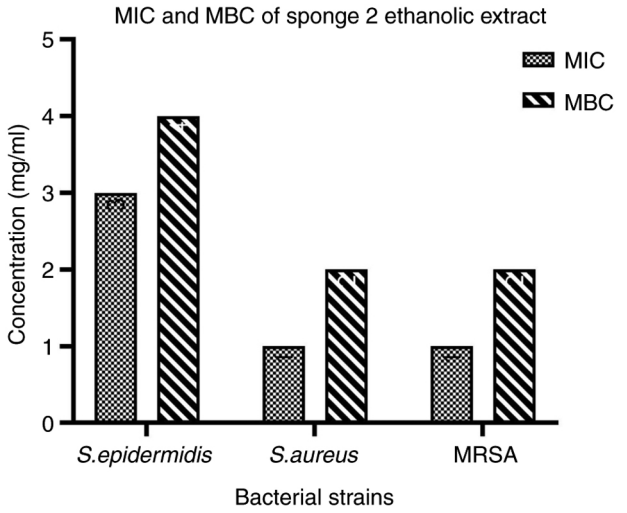

Figure 3. Comparative MIC and MBC value of sponge 2 ethanolic extract against gram-positive bacteria: MIC and MBC for the ethanolic extracts of sponge 2 against *S. aureus*, *S. epidermidis* and methicillin-resistant *Staphylococcus. S. aureus*, *Staphylococcus aureus; S. epidermidis, Staphylococcus epidermidis*; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

MIC and MBC assays further supported the diffusion results. The ethanolic extract of *D. cf. elegans* exhibited MIC and MBC values of 1 and 2 mg/ml against Gram-positive bacteria, respectively (Fig. 3), which confirmed its potent bactericidal potential. All inhibition-zone data are presented as the mean ± SEM with 95% confidence intervals from three independent replicates, while MIC and MBC values are reported descriptively according to standard practice in antimicrobial susceptibility testing.

These findings align with previous investigations on sponge-derived metabolites. For instance, sesquiterpene quinones from *Acanthella cavernosa* and diterpenoids from *Haliclona sp.* displayed selective activity against Gram-positive bacteria, but often at higher MIC ranges (2-8 mg/ml) (72,73). Similarly, extracts from *D. elegans* have been reported to yield structurally diverse compounds with broad-spectrum activity, including against resistant strains of *S. aureus* (74). Mechanistic studies have shown that drimane meroterpenoids, such as pelorol, inhibit bacterial dihydrofolate reductase, a key enzyme in folate metabolism and DNA synthesis in pathogens (75,76). This proposed mechanism may explain the pronounced activity of *Dactylospongia*-derived metabolites observed in the present study.

The strong bioactivity of *Dactylospongia cf. elegans* is also consistent with its metabolite composition, which was confirmed by LC-MS/MS analysis (Table IV). Compounds such as bolinaquinone, dactyloquinone, gallic acid and caffeic acid were detected in the extract, which are metabolites that have been extensively reported for their antibacterial properties (77-79). Their co-occurrence in the ethanolic extract suggests a possible synergistic contribution to the selective activity against Gram-positive pathogens.

LC-MS/MS-based chemical profiling of D. cf. elegans extract. LC-MS/MS analysis of the ethanolic extract of *D. cf. elegans* (Sponge 2) revealed a chemically diverse profile comprising ~40 metabolites (Table IV, Fig. S6). These included terpenoids (for example, limonene, squalene, δ-humulene), alkaloids (for example, manzamine A, smenospongine), quinones (for example, hyatellaquinone, bolinaquinone, ilimaquinone and dactyloquinone), and phenolic acids (for example, gallic, caffeic, ferulic, p-coumaric and chlorogenic). Compounds such as gallic acid (7%), dactyloquinone (6.2%), bolinaquinone (5.0%), chromazonarol (5.1%), manoalide (5.1%) and δ-humulene (4.1%) were detected in relatively high abundance. Interestingly, phenolic compounds such as gallic and caffeic acid, which are commonly associated with terrestrial plants, were also present. The occurrence of gallic acid and chromazonarol in marine sponges has been attributed to sponge-associated symbionts or the uptake of dissolved organic matter (78,80,81).

Several of the metabolites detected in this extract are well recognized for their antibacterial properties. Gallic acid and caffeic acid disrupt bacterial membranes, interfere with metabolism, inhibit biofilm formation, and have potent effects against *S. aureus* and *S. epidermidis* (82-85). Manzamine A impairs protein synthesis in Gram-positive bacteria, while quinones such as ilimaquinone and hyatellaquinone generate reactive oxygen species that compromise membrane integrity (86,87). Therefore, the selectivity of the extract for Gram-positive bacteria in our assays may be explained by these mechanisms, which also explain the absence of activity against Gram-negative bacteria due to their impermeable outer membrane (73).

In addition to phenolics and quinones, other metabolites identified in the extract have reported antibacterial activities. Linoleic acid disrupts bacterial membranes with MIC values as low as 0.01 mg/ml (87). Indole-3-carbaldehyde inhibits bacterial growth and biofilm formation by interfering with signalling pathways (88), while lectins prevent adhesion and biofilm development (89). Ergosterol also exerts antibacterial effects by compromising membrane integrity metabolism (90). Notably, no studies to date have examined the antibacterial potential of several compounds detected in our extract, including bolinaquinone, dactyloquinone, manoalide and chromazonarol, which underscores the novelty of these findings.

The ecological setting of the sponge may also have shaped its secondary metabolite profile. *Dactylospongia* specimens were collected from a coral-rich reef with high biodiversity and low anthropogenic impact, where they are exposed to intense microbial competition, UV radiation, and other stressors that are known to upregulate biosynthetic gene clusters (80,91). Such conditions may explain the abundance of terpenoids (for example, δ-humulene) and quinones, which are compounds that are typically linked to chemical defence strategies. Some identified metabolites, such as pelorol and smenospongimine, are rarely reported in Dactylospongia species (92,93), and their detection here underscores the chemical novelty of this extract. Together with known classes (sterols, pregnanes, sesterterpenes), these compounds extend the pharmacological repertoire of *Dactylospongia*, which has been associated with antibacterial, anticancer, cytotoxic and anti-inflammatory properties (94).

It is noteworthy that *D. cf. elegans* (Sponge 2) exhibited unusually high bioactivity compared with the other sponges analysed in this study and even compared with previous

studies on conspecifics from non-reef habitats. The specimens were physically associated with coral structures in a reef-rich site of the Gulf of Aqaba. Coral-associated sponges have been shown to host distinct microbiomes and metabolomes compared with free-living conspecifics (95,96). The ecological interactions, including microbial symbiosis, nutrient exchange, and exposure to coral-derived dissolved organic matter, can significantly alter the sponge's chemical output (97-99).

The unusually high abundance of compounds such as dactyloquinone, bolinaquinone, gallic acid and chromazonarol in this extract were underrepresented in previous accounts of *Dactylospongia sp*. (98,99) and supports the hypothesis that reef proximity and environmental complexity are crucial drivers of metabolomic diversity. Similar findings have been reported for sponges from the Pacific and Caribbean, where coral-associated sponges biosynthesize higher levels of cytotoxic sesterterpenes and brominated alkaloids than reef-margin species (100). These ecological insights provide a plausible explanation for the enhanced antibacterial profile observed in the present study and reinforce the concept of sponges as holobionts with pharmacological potential that is shaped by both their taxonomy and their biotic environment.

Although numerous of the compounds detected are individually known for antibacterial effects, the overall bioactivity of the extract may also result from synergistic interactions. The combination of phenolic acids and quinones, for example, could potentiate the antibacterial potency against Gram-positive pathogens beyond the activity of each metabolite alone. Therefore, future studies should focus on purification, isolation and testing of individual compounds, as well as combinatorial assays to evaluate their synergy. In summary, LC-MS/MS profiling revealed a rich array of secondary metabolites in the ethanolic extract of *Dactylospongia sp*., including phenolic acids, quinones, terpenoids and alkaloids. These findings provide a strong chemical rationale for the selective antibacterial activity observed in this sponge, particularly against Gram-positive bacteria, and highlight its promise as a source of novel bioactive agents.

In conclusion, the present study highlighted the ethanolic extract of *Dactylospongia sp.* as a promising source of antibacterial compounds with selective activity against Gram-positive bacteria, including *S. aureus*, *S. epidermidis* and MRSA. LC-MS/MS profiling revealed a chemically diverse metabolite composition, including phenolic acids, quinones, terpenoids and alkaloids, several of which are well known for their antimicrobial activity. Importantly, some compounds identified in this extract, such as bolinaquinone, dactyloquinone, manoalide and chromazonarol, have not been previously reported for antibacterial effects, which underscores the novelty of these findings.

Future studies should focus on the purification and isolation of individual compounds, mechanistic investigations, and evaluation of synergistic interactions among metabolites such as gallic and caffeic acids with sponge-derived quinones. Toxicity profiling and validation *in vivo* are also necessary to fully assess their therapeutic potential. Collectively, these findings emphasize the value of *Dactylospongia sp*. in the search for novel antimicrobial agents and support the broader concept that marine sponges, particularly those associated with coral reef environments, represent important reservoirs for bioactive metabolites that could help to address the urgent global challenge of antibiotic resistance.

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Availability of data and materials

The data generated in the present study may be found in the NCBI GenBank database under accession numbers PX278187.1, PX278188.1 and PX278189.1 or at the following URL: https://www.ncbi.nlm.nih.gov/nuccore/PX278187.1, https://www.ncbi.nlm.nih.gov/nuccore/PX278188.1 and https://www.ncbi.nlm.nih.gov/nuccore/PX278189.1. The data generated in the present study may be requested from the corresponding author.

Authors' contributions

FFA, RAA, MMDA, MZ and OHA conceptualized the study and developed the methodology. FFA conducted the formal analysis. AAD conducted the LC-MS/MS analysis. MMDA and MZ were responsible for project administration. MMDA, OHA, AAD and MZ supervised the study. FFA and RAA wrote the original draft. MMDA, OHA and MZ wrote, reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. FFA and RAA confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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