Evaluating the Antibacterial Properties of Deep-Sea Sponges *Dactylospongia elegants*, *Stelletta fibrosa*, and *Haliclona manglaris* from the Jordanian Gulf of Aqaba

Razan Ataallah Abuassaf [Equal first author, 1, 2, 3] , Fatima F. Al-Jamal [1, 3, 4] , Osama H. Abusara [5] , Malek Zihlif [6] , Ahmad A. Deeb [5] , Mamoon M.D. Al-Rshaidat [Corresp. Equal first author, 1, 3]

1 Department of Biological Sciences, School of Sciences, The University of Jordan, amman, Jordan

2 Department of Chemistry, Faculty of Science, Isra University, Amman, Jordan

3 Laboratory for Molecular and Microbial Ecology (LaMME), The University of Jordan, amman, Jordan

4 Department of Biological Sciences, Faculty of Science, Jerash private university, Jerash, Jordan

5 Department of Pharmacy, Faculty of Pharmacy, Al-Zaytoonah University of Jordan, Amman, Jordan

6 Department of Pharmacology, School of Medicine, The University of Jordan, Amman, Jordan

Corresponding Author: Mamoon M.D. Al-Rshaidat
Email address: m.rshaidat@ju.edu.jo

Marine sponges are known for their rich variety of secondary metabolites, many of which show potential for pharmaceutical applications. In this study, three deep-sea sponge species-- *Stelletta fibrosa*, *Dactylospongia elegans,* and *Haliclona manglaris* – were identified using DNA barcoding, and their ethanolic extracts were tested for antibacterial activity. The extracts were evaluated against Gram-positive (e.g., *Bacillus pumilus, Staphylococcus aureus, Staphylococcus epidermidis,* and methicillin-resistant *Staphylococcus aureus*, MRSA) and Gram-negative bacteria (e.g., *Escherichia coli* and *Klebsiella aerogenes)* using the agar well diffusion method. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also determined. Among the extracts, *D. elegans* exhibited the most potent antibacterial activity, with inhibition zones ranging from 6 to 21 mm against gram-positive bacteria and low MIC/MBC values from 0.25 to 3 mg/ml. Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis of *D. elegans* revealed the presence of bioactive compounds such as gallic acid, caffeic acid, bolinaquinone, dactyloquinone, and others, which are known for their antimicrobial properties. These findings suggest that *D. elegans* has promising antibacterial properties that could be valuable in combating antimicrobial resistance.

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[1] Department of Biological Sciences, School of Sciences, The University of Jordan, Amman, Jordan

[2] Department of Chemistry, Faculty of Science, Isra University, Amman , Jordan

[3] Laboratory for Molecular and Microbial Ecology (LaMME), The University of Jordan, Amman, Jordan

[4] Department of Pharmacy, Faculty of Pharmacy, Al-Zaytoonah University of Jordan, Amman, Jordan

[5] Department of Pharmacology, School of Medicine, The University of Jordan, Amman, Jordan

[6] Department of Biology, Faculty of Science, Jerash Private University, Jerash, Jordan

Corresponding Author:
Mamoon M.D. Al-Rshaidat[1,2]
Department of Biological Science, School of Science, Laboratory for Molecular and Microbial Ecology.
The University of Jordan, Amman, Jordan m.rshaidat@ju.edu.jo
* These authors contributed equally to this work

Abstract

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mm against gram-positive bacteria and low MIC/MBC values from 0.25 to 3 mg/ml. Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis of *D. elegans* **revealed the presence of bioactive compounds such as gallic acid, caffeic acid, bolinaquinone, dactyloquinone, and others, which are known for their antimicrobial properties. These findings suggest that** *D. elegans* **has promising antibacterial properties that could be valuable in combating antimicrobial resistance.**

Keywords: Deep-marine sponges, Gulf of Aqaba, Bioactive compounds, antimicrobial resistance (AMR), Marine natural products, DNA barcoding, Antibacterial activity.

1. Introduction

One of the major concerns for human health today is antimicrobial resistance (AMR) (Naser et al. 2024). Human activity is the main factor driving its accelerated appearance and spread, especially the improper and excessive use of antibiotics (Lakshmanan et al. 202) for treating, preventing, or managing human infections. The situation has escalated to a critical level as minor and severe infections become progressively more difficult to cure (Murray et al. 2022). Many of the currently available drugs have not been able to overcome microbial resistance and combat newly emerging diseases (Al-Zereini et al. 2023). AMR is projected to cause 10 million deaths each year by 2050. The declining discovery of new antibiotics and limited pharmaceutical investment exacerbate this worldwide threat (Tang et al. 2023). Without urgent innovation, resistant infections will surpass current treatments (Theuretzbacher et al. 2020).

Natural products, which offer a wide range of bioactive compounds obtained from various natural sources, have played a significant role in the field of drug discovery. These compounds have potential pharmacological characteristics that make them attractive candidates for further investigation (Thomford et al. 2018). Scientists have identified more than 30,000 natural products derived from marine organisms (Lindequist 2016). Several statistical analyses conducted on marine natural products indicate that sponges are highly prolific in terms of producing isolated biologically active compounds (Hu et al. 2015).

The Red Sea is recognized for its deep waters, which mark it as a hotspot for biodiversity and endemic species (Wooster et al. 2019) . The Gulf of Aqaba, which is located in the northeastern part of the Red Sea, is considered to be a deep, narrow, and somewhat isolated water body (Al-

67   Taani et al. 2020). A significant portion of the Gulf falls within the deep-sea category, which

68   describes marine habitats deeper than 200 meters (Moore & Squires 2016). which makes the Gulf

69   different from other similar deep-sea regions. The Gulf reveals an unusual thermal profile, which

70   keeps the water temperatures high even at its deepest points. The high amount of sunlight, along

71   with little circulation of water from the deeper regions, contributes to the warm temperatures. This

72   region also helps in supporting a wide variety of marine life to flourish (Sengupta et al. 2024).

73   Sponges are aquatic invertebrates, belong to the phylum Porifera. Sponges in the ocean are soft-

74   bodied, mostly sedentary animals with a complex mesh of canals and chambers. This structure

75   enables them to pump and filter immense amounts of water, often rich and diverse in bacterial life.

76   These bacteria serve different purposes, including organic matter decomposition, essential nutrient

77   formation, and providing the sponge with protection against harmful bacteria and diseases

78   (Karleskint 2013). However, sponges are a source of novel secondary metabolites with intriguing

79   chemical structures, with different biological activities, such as anticancer and antibacterial

80   activities (Hong et al. 2022).

81   According to previous studies done in the Gulf of Aqaba, several shallow-water sponge species

82   have significant antibacterial properties. For instance, *Grayella cyathophora's* ethanolic crude

83   extract showed strong antibacterial activity, especially against *Pseudomonas aeruginosa* (El-

84   Damhougy et al. 2017). Furthermore, the distinct conditions of the deep sea, such as its high

85   pressure, low temperature, and lack of light, drive the production of even more unique bioactive

86   compounds, which would support the scientific interest in studying deep-sea sponges for possible

87   biological uses (Back et al. 2021; Skropeta 2008; Steffen et al. 2022).

88   This study focuses on identifying three marine sponges collected from the deepest depths of the

89   Jordanian Gulf of Aqaba, a relatively unexplored environment. By investigating the ethanolic

90   extracts of these sponges, the research aims to evaluate their antibacterial potential and uncover

91   the chemical composition of the most active one. The novelty of this work lies in exploring deep-

92   sea sponges from this distinctive habitat, which may have unique bioactive compounds with

93   significant antibacterial potential.

94   **2. Materials & Methods**
95   **2.1 Sponge collection.**

96   Deep-sea Sponge specimens were collected in July 2022 from the Jordanian Gulf of Aqaba using
97   manned submersibles equipped with robotic arms during the OceanXplorer expedition under
98   permit number (9795), issued by Aqaba special economic zone authority. Ecological parameters
99   were recorded at each collection site. The samples included Sponge 1, collected at a depth of 260
100   m (temperature: 21.17°C, salinity: 40.7 PSU, dissolved oxygen: 5.96 mg/L); Sponge 2, collected
101   at 290 m (temperature: 21.1 °C, salinity: 40.7 PSU, dissolved oxygen: 5.92 mg/L); and Sponge 3,
102   collected at 140 m (temperature: 22.5 °C, salinity: 40.66 PSU, dissolved oxygen: 6.21 mg/L). All
103   samples were preserved in 70% ethanol at -4 °C and placed in the Laboratory for Molecular and
104   Microbial Ecology (LaMME), The University of Jordan, Amman. **Figure 1** illustrates the location
105   of the sample collection, with an image of the sample.

106
107   **2.2 Identification of sponge samples**

108   To determine the species of sponge, 25 mg of sponge tissue was divided into tiny fragments. The
109   DNeasy Blood and Tissue Kit from Qiagen (Manchester, UK) was then used to extract the DNA
110   from sponge tissue (Harper et al., 2023). The 28S ribosomal RNA gene served as the molecular
111   marker (Yang et al., 2017), with the primers listed in **Table 1.** This marker is commonly employed
112   in sponge barcoding for species identification (Timmers et al. 2022).

113   Gene amplification was performed using the polymerase chain reaction (PCR), veriti™ 96-Well
114   Thermal Cycler (Applied Biosystems, USA) with the Enti-link PCR master mix from ELK
115   Biotechnology Co. (Wuhan, China). The PCR was conducted under the following conditions: 3
116   minutes of initial denaturation at 94°C, 35 cycles of denaturation at 94°C for 30 seconds, annealing
117   at 56°C (depending on the gene) for 30 seconds, 83 seconds of extension at 72°C, and a final 10
118   minutes of extension at 72°C. A 1% agarose gel from Cleaver Scientific (Warwickshire, UK) was
119   used to verify the PCR results, which were then examined using a gel documentation system. After
120   that, the PCR products were shipped to Macrogen, South Korea, for sequencing. Finally, we
121   aligned the acquired sequences with sponge species using Molecular Evolutionary Genetics
122   Analysis (MEGA) software by the Center for Evolutionary Medicine and Informatics, Arizona
123   State University, Tempe, AZ, USA. and the Basic Local Alignment Search Tool (BLAST)
124   database from the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).
125   A phylogenetic tree was constructed using the neighbor-joining method and the aligned 28S rRNA

126 sequences of closely related sponges obtained from the GenBank database collections
127 (Cristianawati et al. 2019).

2.3 Ethanolic Extraction

129 After collecting the sponges, the specimens were immersed in ethanol/water solution (70% v/v)
130 and kept at -20°C for storage. Ethanol was used to extract both hydrophilic and hydrophobic
131 components in optimum yield (Bashari et al. 2019). The samples were then blended and heated at
132 55-60°C for 2 hours while being soaked in ethanol, followed by dark resting overnight. The
133 samples were filtered afterward, and the concentrated rinse was rotary evaporated; the paste extract
134 was lyophilized to yield the powder.

2.4 Preparation of extract

136 To make the stock solution, 25 mg of extract powder was dissolved in 1 ml of dimethyl sulfoxide
137 (DMSO) from Scharlau (Barcelona, Spain) to reach a concentration of 25 mg/ml. This liquid was
138 stored in a Falcon tube after being vortexed and filtered with a 0.45μm nylon syringe filter. After
139 that, sterile distilled water was used to dilute the stock solution to different concentrations (5, 10,
140 15, and 20 mg/mL).

2.5 Antimicrobial activity

142 To cover both Gram-positive and Gram-negative bacteria, six bacterial strains were chosen.
143 Among the Gram-positive bacteria were *Bacillus pumilus* (isolate), *Staphylococcus aureus*
144 American Type Culture Collection (ATCC 29213), and *Staphylococcus epidermidis* (ATCC
145 51625). Among the Gram-negative bacteria were *Escherichia coli* (ATCC 25922) and *Klebsiella*
146 *aerogenes* (isolate). Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 1026) is a
147 multidrug-resistant bacterium (MDR) showing resistance to β-lactams, macrolides,
148 fluoroquinolones, and often clindamycin (Chambers & DeLeo 2009). It was subjected to additional
149 testing after the extract showed antibacterial efficacy against *Staphylococcus aureus*.

150 The antibacterial activity of the three sponge extracts was assessed using the agar well diffusion
151 method. The Muller-Hinton broth (MHB) from Oxoid (Basingstoke, UK) was first mixed with the
152 bacterial strain, and it was then incubated overnight at 37°C (Magaldi et al. 2004). Following
153 incubation, the density of the culture was modified to satisfy the McFarland turbidity criteria of
154 0.5 (Salameh et al. 2024).

155   Using sterile cotton swabs, the test organisms were cultured on Muller-Hinton agar plates,

156   followed by a 10-minute drying period. The agar plates were then prepared with 8 mm-diameter

157   wells made with a sterile cork borer. Following that, various concentrations of the sponge extracts

158   (5, 10, 15, and 20 mg/ml) were added to these wells using micropipettes. To allow for pre-

159   diffusion, the culture plates were set on the bench for an hour before being incubated for 24 hours

160   at 37°C (Ibrahim et al. 2022)

161   Following an 18-hour incubation period, the diameter of the inhibition zones (measured in

162   millimeters) around the wells was used to determine the antibacterial activity (Geetha & Roy

163   2013). For all microorganisms, 10µg gentamycin (Yassine et al. 2013) was utilized as the positive

164   control, and MRSA-specific 30µg vancomycin (Hannan et al. 2008) was used. Additionally, 80%

165   DMSO was used as the negative control, which was the highest solvent concentration utilized in

166   the extract dilutions. This ensured that any antimicrobial activity observed was due to the extract

167   itself and not the solvent. To reduce the possibility of experimental errors, each experiment was

168   run three times.

2.6 The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC)

171   The minimal inhibitory concentration (MIC) procedure, done as in a (Balouiri et al. 2016) protocol

172   with some adjustments, involves the preparation of various extract concentrations using sterile

173   MHB. These concentrations were then added to a 96-well plate in a specific order: for Sponge 2

174   concentrations: 5,4,3,2,1.0.5,0.25, 0.12, ng/ml for all bacterial strains, while Sponge 3

175   concentrations:20, 19,18,17,16,15,14,13,12 mg/ml for *S. aureus* and 15,14,13,12,11,10,9,8,7

176   mg/ml for *S. epidermidis*. The selection of these concentrations depended on the results obtained

177   from the well diffusion agar method. Negative controls included 80% DMSO and sterile MHB,

178   while positive control comprised a bacterial suspension.

179

180   The minimal bactericidal concentration (MBC) was established by transferring and evenly

181   spreading the treated culture broth from the wells with concentrations equal to or greater than the

182   MIC onto agar plates. The MBC was defined as the lowest concentration of the fraction needed

183   to entirely eradicate the test microorganism, indicated by no growth observed on the agar plate

184   following incubation at 37°C for 24 hours (Majali et al. 2019).

185

2.7 Sponge extract chemical composition using LC-MS-MS

187 Liquid chromatography with tandem mass spectrometry (LC-MS-MS) was performed by Smart
188 Labs Group (Amman, Jordan) using Shimadzu LC (Kyoto, Japan) for the most active sponge
189 extract. It consisted of CBM-20A (control bus module), CTO-30A (column oven), LC-30AD
190 (liquid chromatograph), SIL-30AC (autosampler), and LCMS-8030 (liquid chromatograph mass
191 spectrometer - Triple Quad MS). 50 mg of the sponge extract was extracted with methanol
192 (MeOH). Then, the crude extract was cleaned using a solid phase extraction column, in which the
193 100% MeOH fraction was retained. A working concentration of 2.5 mg/mL of the crude extract
194 was used. LC-MS spectra consisted of retention time (minutes), accurate molecular ions (> 5 ppm
195 accuracy), and MS-MS daughter ions. The LC method used for the analysis spanned 15 minutes
196 with a gradient of 100% H_2O (with 0.1% formic acid) to 95% acetonitrile (ACN).

2.8 Statistical Analysis

198 All statistical analyses were conducted using GraphPad Prism™ version 10.3.0 (GraphPad
199 Software Inc., San Diego, CA, USA). Two-way ANOVA was used to analyze differences between
200 multiple groups, followed by Tukey's test for post-hoc comparisons. Results were expressed as the
201 mean ± standard error of the mean (SEM), with statistical significance defined as $p \leq 0.05$.

202

3. Results

3.1 Sponge DNA barcoding

205 The 28S rRNA gene was successfully amplified from three sponge samples *via* PCR, on agarose
206 gel electrophoresis, yielding bands ranging from 1000 to 1300 base pairs. **(Supplementary 1)**.
207 Our results confirm high-quality DNA amplification for sequencing.
208 The 28S rRNA sequences from the three specimens showed similarities with other Spongillidae
209 species when analyzed using BLAST. The sequences were matched to Spongillidae GenBank
210 sequences that already existed. The following are the findings of the BLAST analysis for the three
211 sponge samples' 28S rRNA sequence alignment with GenBank. Sponge 1 was determined to be
212 *Stelletta fibrosa*, which is a member of the genus *Stelletta,* class Demospongiae, order
213 Tetractinellida, and family Ancorinidae. The sponge 2 was found to be *Dactylospongia elegans*,
214 which is also a member of the class Demospongiae but belongs to the family Thorectidae, order

215   Dictyoceratida, and genus *Dactylospongia*. Sponge 3 was found to be *Haliclona manglaris*, which

216   is a member of the family Chalinidae, genus *Haliclona*, and order Haplosclerida under the class

217   Demospongiae .The phylogenetic tree constructed using the Neighbor-joining method with 1000

218   bootstrap support indicates high 28s rRNA sequence similarities with previously mentioned

219   species (**Figure 2**).

220   **3.2 Antimicrobial Activity**

221   The extracts' antibacterial activity at various concentrations was evaluated using the agar well

222   diffusion technique, and the diameter of the inhibition zone was measured in millimeters (mm).

223   The results are depicted in **Table 2** and **Supplementary 2.**

224   *S. fibrosa* (Sponge 1) demonstrated no positive results against the selected bacteria. In contrast, *D.*

225   *elegans* (Sponge 2) exhibited significant activity against Gram-positive bacteria (S. *aureus, B.*

226   *pumilus, S. epidermidis*, and MRSA). However, *H. manglaris* (Sponge 3) showed only modest

227   activity against two bacterial strains (*S. aureus and S. epidermidis*).

228   Positive controls, gentamycin (10 μg) and vancomycin (30 μg), showed clear inhibitory zones

229   against all tested microorganisms, validating the technique. In contrast, the negative control (80%

230   DMSO) exhibited no inhibition, **Table 3**, showing that the observed antibacterial activity was

231   primarily due to sponge extracts.

232
233   **Figure 3** presents the (MIC) and (MBC) values for the extracts from *D. elegans* (Sponge 2) and

234   *H. manglaris* (Sponge 3), both of which exhibited antibacterial activity against the tested bacteria.

235   Statistical analysis revealed significant differences ($p < 0.05$) between the two sponge extracts for

236   each bacterial strain. At lower concentrations, *D. elegans* (Sponge 2) yielded promising results,

237   especially against *B. pumilus*, with a MIC of 0.125 mg/mL and an MBC of 0.5 mg/mL. In contrast,

238   *H. manglaris* (Sponge 3) showed notable effectiveness against *S. aureus*, with both its MIC and

239   MBC values reaching 8 mg/mL.

240
241   **3.3 LC-MS-MS**

242   Through LC-MS-MS analysis (**Supplementary 3**), the ethanol extract of *D. elegans* (sponge 2)

243   was characterized as in **Table 3.** The prominent compounds identified were a high proportion (over

244   7%) of gallic acid, caffeic acid, dactyloquinone, and bolinaquinone. Other significant metabolites,

245   including indole-3-carbaldehyde, hyatellaquinone, linoleic acid, chromazonarol, ilimaquinone,

246   isospongiaquinone, mamanuthaquinone, cyclospongiaquinone, pelorol, ergosterol, manoalide, and

247   lectin, are also present at more than 2% in the extract.

248

4. Discussion

250   The marine environment is incredibly rich in a diverse array of species, offering a vast reservoir

251   of biometabolites. Many of these biometabolites possess unique chemical structures that are not

252   found in terrestrial sources. This study demonstrated that *D. elegans* ethanolic extract exhibited

253   promising selective antibacterial activity against gram-positive bacteria *S. aureus, S. epidermidi*,

254   *B. pumilus,* and MRSA, and *H. manglaris* ethanolic extract exhibited weak antibacterial activity

255   against *S. aureus* and *S. epidermidis*.

256   The marine sponge *Haliclona manglaris* has antibacterial properties, especially against Gram-

257   positive bacteria (Nazemi et al. 2014). This action stems from bioactive substances produced by

258   the sponge itself, such as lectins (Carneiro et al. 2015), which shows promising activity against

259   biofilm formation (Andrade et al. 2023), as well as from compounds produced by its symbiotic

260   microbes (Gupta 2019).

261   *Haliclona sp*. from the Persian Gulf was tested against gram-positive and gram-negative bacteria

262   in aqueous, diethyl ether, and methanol extracts. With MIC = 5 mg/ml, the diethyl ether extract

263   exhibited the highest activity level, especially against *B. subtilis* and *S. aureus*. (Nazemi et al.

264   2014).

265   Dactylospongia sponges, notably *D. elegans*, are well-known for producing a vast spectrum of

266   metabolites with potent bioactivities. The majority of these metabolites are made up of

267   sesquiterpenes, which include hydroquinones, quinones, and tetronic acids, as well as a few

268   sesterterpenes, sterols, and pregnanes. These compounds have shown substantial bioactivities,

269   including anticancer, cytotoxic, antibacterial, and anti-inflammatory properties. (Ibrahim et al.,

270   2022).

271   The LC-MS-MS analysis of the *D. elegans* ethanolic extract shows it contains high concentration

272   of novel bioactive metabolites, particularly since it was collected from a unique marine

273   environment with stable warm temperatures (above 20°C) and high oxygen levels (~6 mg/L), so
274   the conditions that may have impacted the production of metabolites. The antibacterial activity of
275   these metabolites is deeply linked to their unique ecological conditions, acting as a defense against
276   biofouling and microbial hazards, with their particular chemical arsenal shaped by environmental
277   stressors like nutrient levels and temperature (Faulkner 1984; Tähtinen et al. 2018).

278   Since phenolic chemicals like gallic acid, caffeic acid, chromazonarol, and pelorol are often found
279   in terrestrial plants, their discovery in a marine-derived extract is especially remarkable. This
280   compound shows strong antibacterial activity, especially against Gram-positive bacteria like
281   *Staphylococcus aureus* (Pinho et al. 2014). This result is in line with earlier research that has
282   documented the potency of these molecules against related bacterial strains.

283   Gallic acid, in particular, is highly effective even at low dosages, primarily by rupturing the
284   structural integrity of the bacterial cell membrane, which eventually results in bacterial cell
285   death.(Luís et al. 2014). Furthermore, other research has shown its ability to inhibit bacterial
286   growth by changing bacterial metabolism and lowering the formation of biofilms
287   (Keyvani-Ghamsari et al., 2023). Caffeic acid exhibits strong antibacterial properties individually
288   and with other drugs (Aijaz et al., 2022). As a comprehensive review conducted by (Khan et al.
289   2021), caffeic acid has antibacterial activity through several mechanisms, such as bacterial cell
290   membrane disruption, inhibiting essential enzymes, and interfering with microbial pathogenicity.
291   Strong activity of caffeic acid against *S. aureus* with MIC and MBC (0.625 and 1.25mg/mL) was
292   recorded, most likely as a result of hyper-acidification that caused bacterial cell disruption.
293   Additionally, it was effective against *S. epidermidis* (MIC and MBC: 0.625 mg/mL), presumably
294   by donating protons that increase the internal acidity of the cell (Pinho et al. 2014). Gallic acid and
295   caffeic acid have not been previously isolated from this species, as reported by Ibrahim et al., 2022,
296   but have been isolated previously from sponge-associated bacteria from the sponge *Hyrtios erectus*
297   (Ma et al. 2019b). Pelorol also showed moderate to strong antimicrobial activity, specifically
298   against S. *aureus* and *E. faecalis,* with MICs of 3.125 and 12.5 µM, respectively (Ebada et al.
299   2017).

300   Bolinaquinone,   dactyloquinone,   hyatellaquinone,   ilimaquinone,   isospongiaquinone,
301   mamanuthaquinone, and cyclospongiaquinone are marine sesquiterpene quinones. Among them,
302   hyatellaquinone showed comparatively high action against MRSA and *S. aureus* (Ma et al. 2019a).

303   Likewise, ilimaquinone shows antibacterial properties against *Streptococcus pyogenes* and *S.*

304   *aureus* (Chen et al. 2022), but isospongiaquinone demonstrated specific activity against *S. aureus*

305   only (Ebada et al. 2017). Additionally, quinone derivatives have been proven to target essential

306   bacterial proteins, such as LptA and Top IV, suggesting a dual-target mechanism of antibacterial

307   action (Yang et al. 2019). The variety in structure among these compounds, especially in their

308   quinone moieties and terpenoid skeletons, may contribute to their effectiveness and lower the

309   threat of resistance development (Emami et al. 2005).

310   Linoleic acid has activity against *S. aureus* by rupturing bacterial membranes with MIC values as

311   low as 0.01 mg/mL (Dilika et al. 2000). Indole-3-carbaldehyde has been recorded to inhibit

312   bacterial growth and biofilm formation, with MIC values of 100 and 150 µg/mL against *S. aureus*

313   and *B. subtilis*, respectively, suggesting it interferes with cell signaling and virulence factors

314   (Rattanaphan et al. 2020). Lectins are sugar-binding proteins that effectively inhibit bacterial

315   adhesion and biofilm formation (Hasan et al. 2023). Ergosterol has shown antibacterial effects,

316   likely through disrupting the membrane integrity and interfering with bacterial metabolism

317   (Andrade et al. 2019). However, to the best of our knowledge, no studies have reported the

318   antibacterial activity of bolinaquinone, dactyloquinone, mamanuthaquinone,

319   cyclospongiaquinone, chromazonarol, and manoalide, so further research is needed to assess their

320   potential as antimicrobial agents.

321   Moreover, apart from individual compounds' reported activity in literature, the antibacterial

322   activity of the extract may have risen due to synergistic activity between two or more compounds

323   present in the extract. These could be of high or low abundance within the extract. Further studies

324   in terms of purifying the extract and isolating each compound to analyze it alone, MS analysis,

325   and antibacterial activity, would be worth investigating. Also, combining isolated compounds

326   study any possible synergistic antibacterial activity would be worth studying

327   In conclusion, this study opens the door for further research into novel antimicrobial drugs by

328   demonstrating the antibacterial activity of the deep *D. elegans* ethanolic extract and highlighting

329   its bioactive components discovered through LC-MS/MS analysis. Although bolinaquinone,

330   dactyloquinones, and other molecules have mostly been examined for their anti-inflammatory

331   activities and cytotoxicity, their potential antibacterial action remains unexplored. More research

332   is necessary to determine their antibacterial properties and to investigate if they have synergistic

333   interactions with gallic acid, caffeic acid, and others. Such investigations could provide important
334   information about their combined therapeutic potential as novel antibacterial agents.

335   5. **Acknowledgments**

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337   support in providing access to their research vessel for sample collection. We also wish to
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339   biotechnology research lab/ Isra University, for their expertise in conducting the experiments.

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Figure 1

Figure 1 Gulf of Aqaba map and sponge image

Map of the Gulf of Aqaba showing the locations of sample collection and an image of the collected sample. The map was supplied by the OceanX vessel. Numbers 1–8 indicate the ship's stations during the expedition.
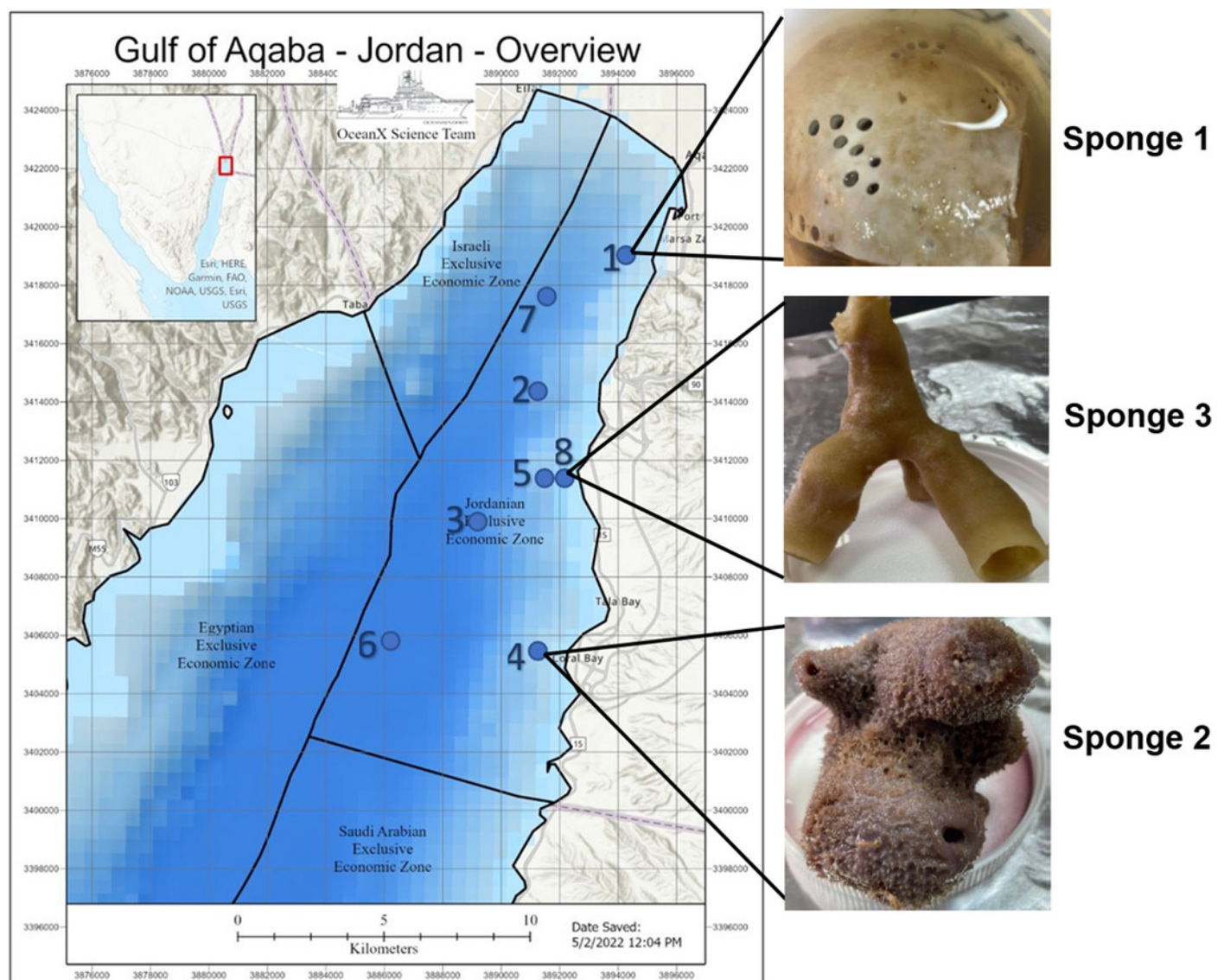
Figure 2

Figure 2: Phylogenetic tree of 28S rRNA sequences from three sponge samples

The phylogenetic tree was constructed using MEGA11 software, with the three sponge samples highlighted in red circles. The tree was generated using the UPGMA method and computed with the Neighbor-Joining algorithm, with 1000 bootstrap replications (indicated by bold numbers).
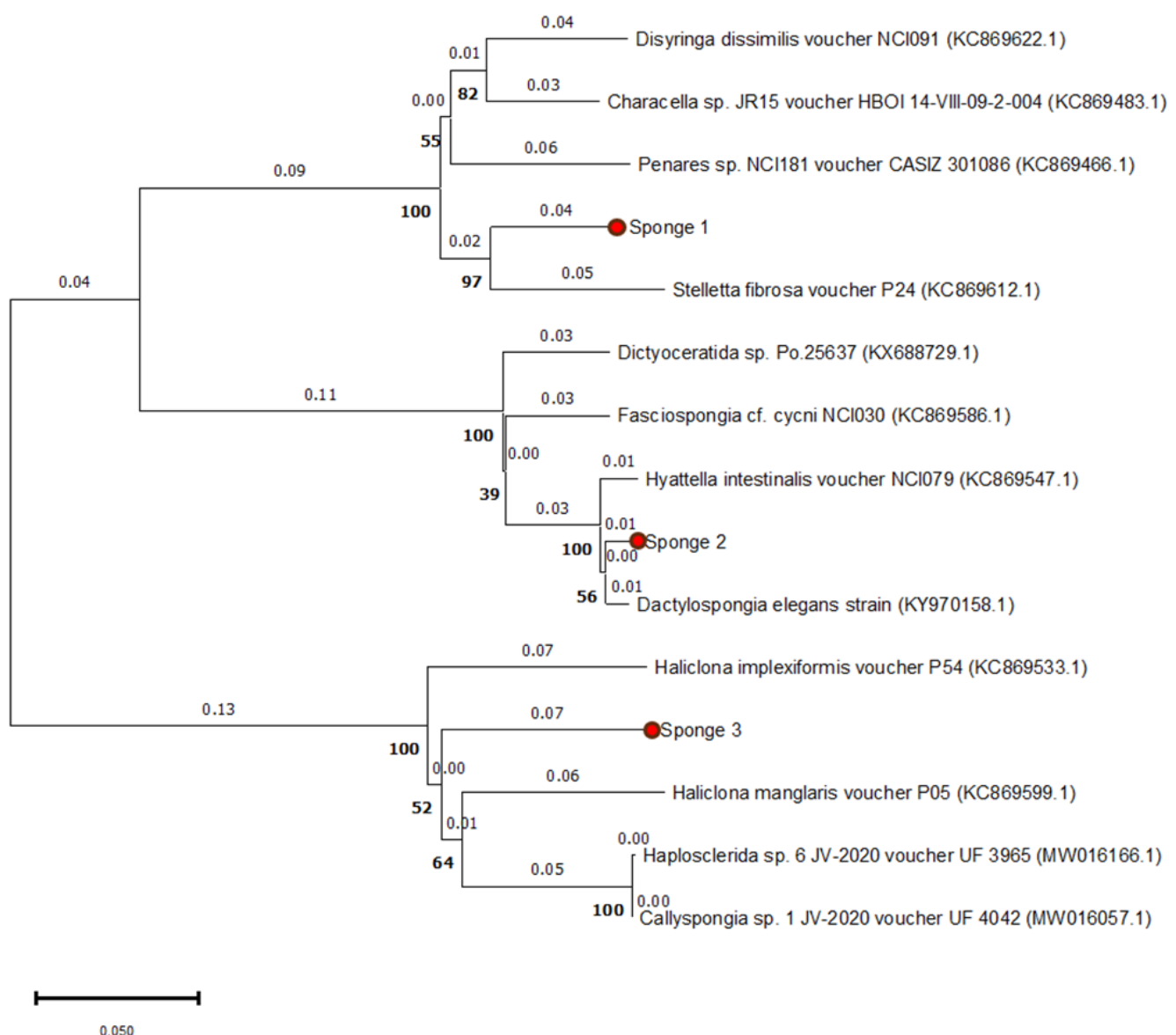
Figure 3

Figure 3. MIC and MBC of *D. elegans* (sponge 2) and *H. manglaris* (sponge 3) ethanolic extracts: (A) MIC and (B) MBC for the ethanolic extracts of *D. elegans* (Sponge 2) against *S. aureus, B. pumilus, S. epidermidis,* and MRSA, as we

: (A) MIC and (B) MBC for the ethanolic extracts of *D. elegans* (Sponge 2) against *S. aureus, B. pumilus, S. epidermidis,* and MRSA, as well as *H. manglaris* (Sponge 3) against *S. aureus* and *S. epidermidis*. Statistical analysis revealed significant differences between the two sponge extracts, with p values indicated as follows: p = 0.033 (*), p = 0.002 (**), and p <0.001 (***). SEM values were zero in all cases; therefore, error bars are not displayed
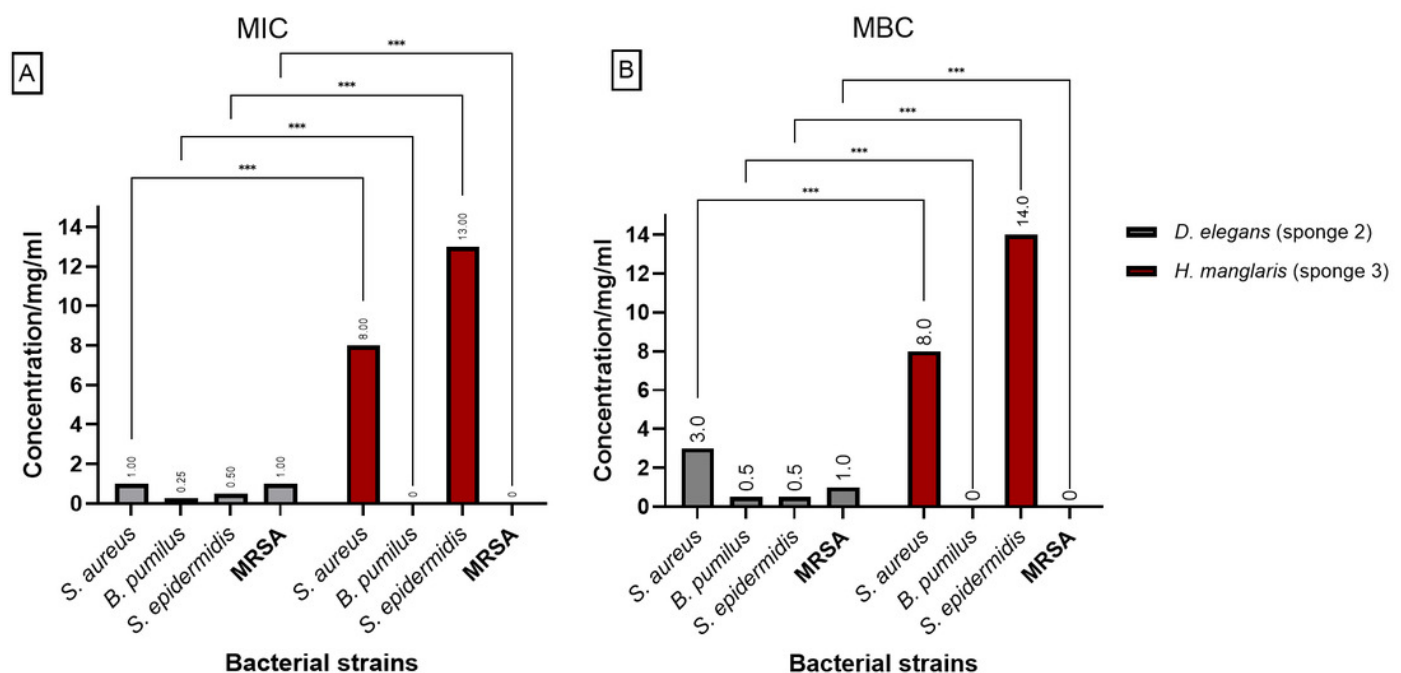
Table 1. Forward and reverse primer sequences for 28S ribosomal RNA amplification.

1   **Table 1. Forward and reverse primer sequences for 28S ribosomal RNA amplification.**

Primer	Sequence	Reference
28F63mod (Forward)	5'-ACCCGCTGAAYTTAAGCATATHANTMA-3'	(Timmers et al., 2022)
28R1072 (Reverse)	5'-GCTATCCTGAGGGAAACTTCGG-3'	

Table 2:Inhibition zone diameters (mm) for different bacterial strains treated with sponge ethanolic extracts in different concentrations: Data are expressed as mean ± standard error of the mean (SEM) based on three independent replicates (n=3), Posit

Data are expressed as mean ± standard error of the mean (SEM) based on three

independent replicates (n=3), Positive control for all bacteria: Gentamycin (10μg), MRSA-

specific positive control: Vancomycin (30μg) , and Negative control: 80%DMSO

1 **Table 2:Inhibition zone diameters (mm) for different bacterial strains treated with sponge**
2 **ethanolic extracts in different concentrations:** Data are expressed as mean ± standard error of
3 the mean (SEM) based on three independent replicates (n=3), Positive control for all bacteria:
4 Gentamycin (10μg), MRSA-specific positive control: Vancomycin (30μg), and Negative control:
5 80%DMSO.

		Mean of the inhibition zone diameter (mm) (Mean ± SEM)					
	Bacterial strains	*E.coli*	*S.aureus*	*K.aerogenes*	*B.pumilus*	*S.epidermidis*	MRSA
D. elegans (sponge 2)	**5 mg/ml**	**0.0** ± 0.0	**8.6** ± 4.4	**0.0** ± 0.0	**6** ± 2.5	**12.6** ± 2.3	**11.6** ± 1.6
	10 mg/ml	**0.0** ± 0.0	**16.3** ± 2.1	**0.0** ± 0.0	**11.3** ± 0.6	**15.6** ± 2.4	**13.6** ± 1.2
	15 mg/ml	**0.0** ± 0.0	**18** ± 2.0	**0.0** ± 0.0	**14.6** ± 1.7	**19.3** ± 1.2	**14.6** ± 0.6
	20 mg/ml	**0.0** ± 0.0	**19.6** ± 1.3	**0.0** ± 0.0	**18** ± 1.1	**21** ± 0.5	**15.3** ± 0.8
	Positive control	**14** ± 0.57	**17.6** ± 2.9	**12.3** ± 2.1	**21.6** ± 0.6	**21.3** ± 1.6	**21.6** ± 0.3
	Negative control	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0
H. manglaris (sponge 3)	**5 mg/ml**	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0
	10 mg/ml	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0
	15 mg/ml	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**12.0** ± 0.0	**0.0** ± 0.0
	20 mg/ml	**0.0** ± 0.0	**10.6** ± 0.3	**0.0** ± 0.0	**0.0** ± 0.0	**13.0** ± 0.0	**0.0** ± 0.0
	Positive control	**14.3** ± 0.8	**17.1** ± 1.0	**17.6** ± 1.7	**18.6** ± 1.8	**20.6** ± 0.8	**21.6** ± 0.3
	Negative control	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0	**0.0** ± 0.0

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Table 3. Compounds detected in the ethanol extract of *D. elegans* (Sponge 2)

1 **Table 3. Compounds detected in the ethanol extract of *D. elegans* (Sponge 2)**

	Compounds	Molecular formula	Molecular Wright	%	RT
1	Limonene	$C_{10}H_{16}$	136.23g/mol	1.1	1
2	Ocimene	$C_{10}H_{16}$	136.23g/mol	0.2	1.1
3	Indole-3- carbaldehyde	C_9H_7NO	145.16g/mol	2.2	1.2
4	Gallic acid	$C_7H_6O_5$	170.12g/mol	10.1	1.3
5	Caffeic acid	$C_9H_8O_4$	180.16g/mol	8	1.4
6	Hyatellaquinone	$C_{12}H_{14}O_4$	222.24g/mol	3.2	1.5
7	Linoleic acid	$C_{18}H_{32}O_2$	280.4g/mol	6.6	1.6
8	Catechin	$C_{15}H_{14}O_6$	290.27g/mol	1	1.68
9	Clathric acid	$C_{20}H_{30}O_2$	302.5g/mol	0.5	1.7
10	Chromazonarol	$C_{21}H_{30}O_2$	314.5g/mol	5.1	1.8
11	Smenospongine	$C_{21}H_{29}NO_3$	343.5g/mol	1.5	1.9
12	Dysideamine	$C_{21}H_{29}NO_3$	343.5g/mol	2	2
13	Chlorogenic acid	$C_{16}H_{18}O_9$	354.31g/mol	0.9	2.1
14	Dactyloquinone	$C_{22}H_{28}O_4$	356.5g/mol	7.1	2.25
15	Smenospongimine	$C_{22}H_{31}NO_3$	357.5g/mol	1.2	2.3
16	Ilimaquinone	$C_{22}H_{30}O_4$	358.5g/mol	4.3	2.4
17	Isospongiaquinone	$C_{22}H_{30}O_4$	358.5g/mol	2.1	2.6
18	Bolinaquinone	$C_{22}H_{30}O_4$	358.5g/mol	7.4	2.8
19	Mamanuthaquinone	$C_{22}H_{30}O_4$	358.5g/mol	6.1	2.9
20	Cyclospongiaquinone	$C_{22}H_{30}O_4$	358.5g/mol	3.1	3.1
21	Dactyltronic acid	$C_{21}H_{30}O_5$	362.5g/mol	0.6	3.15
22	Dictyoceratin A	$C_{23}H_{32}O_4$	372.5g/mol	0.3	3.3
23	Pelorol	$C_{23}H_{32}O_4$	372.5g/mol	5.1	3.5
24	Luffariellolide	$C_{25}H_{38}O_3$	386.6g/mol	0.8	3.65
25	Ergosterol	$C_{28}H_{44}O$	396.6g/mol	4.1	3.8
26	Manoalide	$C_{25}H_{36}O_5$	416.5g/mol	6	4
27	Motualevic acid	$C_{16}H_{23}Br_2NO_2$	421.2g/mol	1.3	4.2
28	Lectin	$C_{26}H_{28}O_6$	436.5g/mol	3	4.4

29	Nakijiquinone D	$C_{25}H_{35}NO_6$	445.5g/mol	1.6	4.6
30	Stelletin	$C_{30}H_{38}O_4$	462.6g/mol	1.7	4.9
31	Manzamine A	$C_{36}H_{44}N_4O$	548.8g/mol	0.3	5.2
32	Rutin	$C_{27}H_{30}O_{16}$	610.5g/mol	0.5	5.4
33	Popolohuanone	$C_{42}H_{57}NO_3$	623.9g/mol	1	5.6