Antimicrobial and cytotoxic effects of marine sponge extracts *Agelas clathrodes*, *Desmapsamma anchorata* and *Verongula rigida* from a Caribbean Island.

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Although marine sponges are known for their antimicrobial, antifungal and cytotoxic activity, very few studies have been carried out on endemic species of Martinique. Martinique is part of the Agoa Sanctuary, a marine protected area that includes the exclusive economic zones (EEZ) of the French Caribbean islands, making it an abundant source of marine species. To highlight the potential of this area for the discovery of marine biomolecules with antipathogenic and antitumor activities, we tested the aqueous and ethanolic extracts of sponge species *Agelas clathrodes*, *Desmapsamma anchorata* and *Verongula rigida*. Five bacterial strains: *Bacillus cereus* (CIP 78.3), *Escherichia coli* (CIP 54.127), *Pseudomonas aeruginosa* (CIP A22), *Staphylococcus aureus* (CIP 67.8) and *Staphylococcus saprophyticus* (CIP 76125) were evaluated, as well as four tumor cell lines: breast cancer (MDA-MB231), glioblastoma (RES259) and leukemia (MOLM14 and HL-60). Antimicrobial activity was evaluated using the disc diffusion technique by determining the minimum inhibitory and minimum bactericidal concentrations. Tumor cytotoxic activity was determined *in vitro* by defining the minimum concentration of extracts that would inhibit cell growth. Ethanolic extracts of *Agelas clathrodes* were bactericidal for *Staphylococcus aureus* and *Staphylococcus saprophyticus* strains, as well as strongly cytotoxic ($IC_{50} < 20$ μg/mL) on all cancer cell lines. *Verongula rigida* also showed strong cytotoxic activity on cell lines but no antimicrobial activity. These results are innovative for this species on these bacterial lines, highlighting the potential of sponge extracts from this area as bioactive compounds sources.

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Abstract

Although marine sponges are known for their antimicrobial, antifungal and cytotoxic activity, very few studies have been carried out on endemic species of Martinique. Martinique is part of the Agoa Sanctuary, a marine protected area that includes the exclusive economic zones (EEZ) of the French Caribbean islands, making it an abundant source of marine species. To highlight the potential of this area for the discovery of marine biomolecules with antipathogenic and antitumor activities, we tested the aqueous and ethanolic extracts of sponge species *Agelas clathrodes*, *Desmapsamma anchorata* and *Verongula rigida*. Five bacterial strains: *Bacillus cereus* (CIP 78.3), *Escherichia coli* (CIP 54.127), *Pseudomonas aeruginosa* (CIP A22), *Staphylococcus aureus* (CIP 67.8) and *Staphylococcus saprophyticus* (CIP 76125) were evaluated, as well as four tumor cell lines: breast cancer (MDA-MB231), glioblastoma (RES259) and leukemia (MOLM14 and HL-60). Antimicrobial activity was evaluated using the disc diffusion technique by determining the minimum inhibitory and minimum bactericidal concentrations. Tumor cytotoxic activity was determined *in vitro* by defining the minimum concentration of extracts that would inhibit cell growth. Ethanolic extracts of *Agelas clathrodes* were bactericidal for *Staphylococcus aureus* and *Staphylococcus saprophyticus* strains, as well as strongly cytotoxic ($IC_{50} < 20$ µg/mL) on all cancer cell lines. *Verongula rigida* also showed strong cytotoxic activity on cell lines but no antimicrobial activity. These results are innovative for this species on these bacterial lines, highlighting the potential of sponge extracts from this area as bioactive compounds sources.

Keywords: natural products, marine sponges, antimicrobial activity, cytotoxic activity, tumoral cell lines, Martinique, *Agelas clathrodes*, *Desmapsamma anchorata*, *Verongula rigida*.

58

Introduction

60   Martinique is part of the AGOA Sanctuary, a marine protected area recognized under the

61   SPAW (Specially Protected Areas and Wildlife) protocol that includes the entire exclusive

62   economic zones (EEZ) of the four French Caribbean islands (Saint-Martin, Saint-Barthelemy,

63   Guadeloupe and Martinique), making it one of the "hot spots" of worldwide marine diversity

64   (https://sanctuaire-agoa.fr/editorial/vast-territory) (Fig. 1). Indeed, new sponge species are

65   regularly discovered on the 47 000 Km^2 EEZ of the island by the active study of its biodiversity

66   (Grenier et al., 2020; Griffiths et al., 2021; Impact Mer, 2008; Impact Mer, 2012; Perez et al.,

67   2017; Perez & Ruiz, 2018). However, these marine organisms are poorly valued (Laville et al.,

68   2009). Therefore, the goal of this study is to improve knowledge of the island's sponge diversity

69   as a potential source of novel marine natural products with antibacterial activity on drug-resistant

70   pathogens or anticancer activity on common cancer cell lines.

71   Indeed, bioactive natural products are increasingly sought after, with a specific interest

72   in pharmacology, where they account for about 70% of approved drugs (Cortadellas et al., 2010).

73   In 2017, seven pharmaceuticals derived from marine substances were validated for clinical uses

74   by the FDA[1] (Dyshlovoy & Honecker, 2018). Marine sponges are of particular interest because

75   of the abundance of secondary metabolites they produce and their highly diversified chemical

76   nature (El-Amraoui et al., 2010; Mayer et al., 2013; Nweze et al., 2020; Sipkema et al., 2005).

77   The discovery of novel antibiotics from these organisms is one of the main goals of

78   current research. This is due to the increasing resistance of many strains to commercially

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[1] FDA : Food and Drug Administration

79   available antibiotics such as *Pseudomonas aeruginosa* or *Staphylococcus aureus* which are

80   ranked among the "priority pathogens" resistant to antibiotics. Marine organisms are a

81   historically rich source of compounds active against these drug-resistant pathogens such as those

82   found in bacteria, fungi, algae or invertebrates (Nweze et al., 2020).

83   Cancer treatment has also benefited from marine natural products with notable examples

84   like cytarabine, eribulin, and trabectedin. The anti-tumor and cytotoxic activity of sponges have

85   been known and exploited for years with the emergence of chemical derivatives used in cancer

86   treatments such as cytarabine (ARA-C) for the treatment of leukemia or eribulin mesylate as

87   treatment for breast cancer (Dyshlovoy & Honecker, 2018). Despite the existence of these and

88   other treatments, the National Estimates of Cancer Incidence and Mortality in Metropolitan

89   France states that women's breast cancer incidence increased by 0.6% per year since 2010.

90   During this same period, the incidence rate for acute leukemia increased by an average of

91   114.5% (Defossez et al., 2019). In light of this, there is an urgent need for new anti-tumor drugs

92   with novel targets and novel modes of action that could be filled by novel marine sources.

93   This study focused on marine sponges collected on the Caribbean coast of Martinique.

94   We evaluated the activity of aqueous and ethanolic extracts of the three sponge species *Agelas*

95   *clathrodes*, *Desmapsamma anchorata* and *Verongula rigida* for their antimicrobial and antitumor

96   activity. We highlight the antibiotic and anticancer activity of several sponge extracts after

97   evaluation on five bacterial strains (*Bacillus cereus* (CIP 78.3), *Escherichia coli* (CIP 54.127),

98   *Pseudomonas aeruginosa* (CIP A22), *Staphylococcus aureus* (CIP 67.8) *and Staphylococcus*

99   *saprophyticus* (CIP 76125)), and on four tumor cell lines (breast cancer (MDA-MB231),

100   glioblastoma (RES259) and leukemia (MOLM14 and HL-60)), and are able to link some of our

101   findings to previously reported data.

102

Materials and Methods

Sampling and identification

105   The samples *Agelas clathrodes*, *Desmapsamma anchorata* and *Verongula rigida* were

106   collected on the site "Fond Boucher" (14°39'21.12"N-61°9'21.22"W) on the north Caribbean side

107   of Martinique (Fig 1. and Table 1). The samples were collected by dives between 15 and 20

108   meters deep during two campaigns, on October 7, 2017 by Dr. Romain Ferry (2019 Fishing

109   Order No. R02-2019-04-08-004, Martinique Sea Department). The samples were conditioned in

110   individual plastic bags and immediately stored in a cooler for transport to the laboratory of the

111   "Université des Antilles" (UA). They were then washed with fresh water and directly stored at -

112   20°C before extraction.

113   Sponges have been identified on the basis of their external morphology and skeletal

114   composition, in particular the type of spicule (Boury-Esnault & Rützler, 1997; Custódio et al.,

115   2007; Impact Mer, 2008; Impact Mer, 2012; Perez et al., 2017; Perez & Ruiz, 2018). The sponge

116   skeletons were studied at the laboratory on UA campus in Martinique, by extraction of the

117   spicules and by longitudinal and transverse sectioning of the tissue. Extractions are carried out

118   by three washes with bleach after freeze-drying 5 mg of sample. Observation was conducted

119   using an optical microscope equipped with a ZEISS camera. The pictures were analyzed with the

120   ZEN2012 software. The size of the spicules was estimated by calculation according to the lens

121   size. The determination of the species was realized with the external anatomic description and

122   observed spicules, as well as the bibliography analysis based on the inventories already

123   published in the area. No comparison was made with similar samples or holotypes. Internal

124   anatomy and cytology were not determined in this study as well.

125   Method for calculating the size of the spicules: size on the photo / magnification

126   (lens*objective).

127

128   **Preparation of sponge extracts**

129   The extracts were prepared by maceration in two solvents, distilled water and 100%

130   ethanol. The sponges were dried in a freeze-dryer, then 2 g were cut into small pieces, crushed

131   with a mortar, placed in a 15 mL falcon, then 10 mL of solvent were added. After repeated

132   manual turning and swirling, the tubes were placed under agitation for 24 hours at room

133   temperature. The extracts were then filtered on standard filter paper. The process was repeated 3

134   times. The extracts were then pooled and stored at -20°C before drying. The ethanolic extracts

135   (E) were dried in a rotary evaporator at 45°C. 1 mL of solvent was added to the flask twice and

136   passed through an ultrasonic bath to recover the residues and placed in glass vials. The solvent

137   residues were evaporated in a fume hood and the dry extract was stored at -20°C before testing.

138   The aqueous extracts (A) were freeze-dried and stored at -20°C.

139

140   **Antimicrobial activity**

141   ***Bacterial strains***

142   The five bacterial strains belong to group 1 and 2 of the classification of

143   microorganisms by risk groups (Article R4421-3 of the Decree n°2008-244 of March 7, 2008-art.

144   (V)). Strains came from the Institut Pasteur Collection (CIP): Paris: *Bacillus cereus* (CIP 78.3),

145   *Staphylococcus saprophyticus* (CIP 76125T), *Escherichia coli* (CIP 54.127) strains are listed in

146   group 1 (non-pathogenic) and *Pseudomonas aeruginosa* (CIP A22), *Staphylococcus aureus* (CIP

147   67.8) strains are listed in group 2 (pathogenic). Inoculums were prepared from strains cultivated

148   in agar nutrient at 37°C for all strains except for *Pseudomonas aeruginosa* (CIP A22) incubated

149   at 30°C.

150   ***Antimicrobial assay***

151   Antimicrobial activity was tested on Mueller Hinton agar using the disc diffusion

152   method according to Majali et al. (2015). Pure 18h culture inoculums on agar nutrient, were

153   seeded at a concentration of 10^7UCF/mL. Sterile 6mm diameter discs were soaked with 20 µL of

154   extract (at 500 µg/mL) and dried for 15min. The discs were then stored at 4°C before being

155   placed on the seeded agar and the petri dishes were incubated for 24h at 37°C for all strains

156   except for *Pseudomonas aeruginosa* (CIP A22) incubated at 30°C with agitation. The inhibition

157   diameter was then measured. Tests were performed in triplicates for each species. The standard

158   antibiotic ampicillin (10 µg, lot 7B5479) was used as a positive control for *S. saprophyticus*, *S.*

159   *aureus* and *E. coli*; chloramphenicol (30 µg, lot 5C5220) was used as a positive control for *B.*

160   *cereus* and fosfomycin (50 µg, lot 4L5252) was used as a positive control for *P. aeruginosa*.

161   Discs impregnated with 20 µL of solvent (H_2O and ethanol) were also used as negative controls.

162   An inhibition diameter greater than 9 mm around the disc indicated positive activity

163   according to the analysis of Cita et al. (2017).

164 ***Minimal inhibitory concentration (MIC)***

165   MIC was determined by the successive liquid microdilution method according to Majali

166 et al. (2015), modified for ethanolic extract of *Agelas clathrodes*. 2-in-2 dilutions of the crude

167 extract were performed in a 96-well plate. The plates were then incubated 24 hours at 37°C for

168 all strains except for *Pseudomonas aeruginosa* (CIP A22*)* incubated at 30°C. The optical density

169 was measured on a plate reader at 600 nm. The concentration range used was 0.488 µg/mL to

170 1000 µg/mL.

171   ***Minimal bactericidal concentration (MBC)***

172   MBC was also evaluated for the ethanolic extract of *Agelas clathrodes* by counting the

173 surviving bacteria in tubes with no visible growth using a method adapted from Majali et al.

174 (2015) and Marinho et al. (2010). A streak of a 10 µL aliquot was seeded onto PCA plates using

175 a calibrated plater, and incubated for 24h at 37°C for all strains except for *Pseudomonas*

176 *aeruginosa* (CIP A22*)* incubated at 30°C. After incubation, colonies were counted for each

177 streak. Only streaks between 30 and 300 colonies were considered. The extract was considered

178 having a bactericidal effect for MBC/MIC=1 and a bacteriostatic effect for MBC > MIC (Majali

179 et al., 2015).

180

181 **Cytotoxic evaluation**

182   ***Cell lines***

183   Four cell lines were selected for the cytotoxic evaluation. A human breast cancer cell

184 line MDA-MB-231, a glioblastoma cell line (RE259) and two leukemia cell lines: MOLM-14

185   acute myeloid leukemia (more precisely, a MOLM14 luc cell line, expressing the luciferase-GFP

186   gene) and HL-60 acute human promyelocytic cell line. Cell lines came from ATCC (CCL-240)

187   for HL-60; ECACC (cat n°= 92020424) for MDA-MB-231; MOLM-14 GFP/Luc = MOLM-14

188   were obtained from JE. Sarry and engineered to express luciferase (Stuani et al., 2021) and

189   Res259 (grade II, diffuse astrocytoma) were kindly provided by Chris Jones (The Institute of

190   Cancer Research, Sutton, UK. (Rakotomalala et al., 2021).

191   Cell lines were cultivated according to the following protocol established by the TrGET

192   facility in the Marseille Cancer Research Center (CRCM)[2]:

193   All cancer cell lines were tested negative for mycoplasma contamination. RES259 cells

194   were grown in MEM medium supplemented with 10% heat-inactivated foetal bovine serum and

195   1% non-essential amino acids. MDA-MB-231 cells were cultured in RPMI supplemented with

196   10% FCS, 1% L-Glutamine and 1% sodium pyruvate. MOLM-14 were maintained at a

197   concentration of 0.5 M/mL in MEM alpha medium supplemented with 10% FCS at 37°C 5%

198   CO_2. The HL-60 cell line was grown using the same procedure in IMDM (Iscove's Modified

199   Dulbecco's Medium, Gibco 12440053) supplemented with 20% FCS. Adherent cells were seeded

200   in Corning 3903 clear-bottom 96-well plates overnight prior to treatment at 1250 cells for

201   RES259 and 5000 cells for MDA MB231 in 90 µL of their respective medium. MOLM-14 and

202   HL-60 cells were seeded at 10,000 cells per well on the day of testing.

203   ***Dilution of the extracts***

204   During cytotoxic tests, only ethanolic extracts were analyzed because contaminations

205   have been noticed for aqueous extracts in the cell cultures. For each sponge sample, two

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[2] CRCM : Centre de Recherche en Cancérologie de Marseille

206 extractions were evaluated (X1 and X2) for a total of 3 performed experiments labelled as #1, #2

207 or #3. A 10X concentrated cascade dilution of the compounds was performed in medium at 10%

208 constant DMSO concentration and then 10 µL of these dilutions were added to the 90 µL of the

209 wells in triplicate, in order to get concentrations ranging from 500 µg/mL to 0.06 µg/mL (1%

210 final DMSO concentration).

211

212

213 ***Cytotoxic assay***

214   Experiments were performed as triplicates (except marked otherwise in the result table).

215 Doxorubicin was used as positive control for the MDA-MB231 assay, and Aracytin was used for

216 the three other cell lines. Plates were incubated for 72 h at 37°C with 5% CO_2, then after a 30

217 min room temperature reset, 50 µl of cell titer Glo (Promega) were added. Cell lysis was induced

218 for 2 min on an orbital shaker, then after 10 min of incubation at RT to allow the signal to

219 stabilize, the luminescence was measured on a Berthold centro LB960 luminometer. Curves

220 depending on the doses were plotted and the median inhibitory concentration corresponding to

221 the lowest concentration of active compound allowing to inhibit the growth of the cells by 50%

222 in vitro (IC_{50}) was calculated using the GraphPad PRISM software, as well as a confidence

223 interval range (CRI). For IC_{50} measurements, values were normalized and fitted with GraphPad

224 Prism (least squares regression) using the following equation $Y=100/(1+((X/IC_{50})^\wedge Hillslope))$.

225

226 **Results**

227 **Sponges identification**

228   Sponges were identified according to two criteria: their external morphology and the

229 composition of their skeleton.

230 The first sample was identified as ***Agelas clathrodes*** (Table 2). For this sample, the external

231 morphology was massive and the color was bright orange (Fig. 1A). Consistency was hard and

232 oscula were often fused in a comma shape. The skeleton was composed of spongin fibers,

233 achantostyles and achantoxes verticillates megascleres, mostly between 70 µm and 500 µm in

234 size (Fig. 1B). No microscleres were observed (Fig. 1C) (Hooper et Van Soest 2002; Hartman

235 2002; Van Soest 2002).

236 The second sample was identified as ***Desmapsamma anchorata*** (Table 2). External morphology

237 presented a branching of pink-lilac colour with oscules scattered at the surface (Fig. 1D).

238 Consistency was soft and the skeleton consisted of diactinal oxea megascleres (Fig. 1E) of

239 around 80 µm to 100 µm as well as miscrocleres: sigmas, isochelas, spherasters and tripods of

240 less than or equal to 20 µm (Fig. 1F) (Hooper & Van Soest, 2002; Van Soest, 2002).

241 Finally, the last sample was identified as ***Verongula rigida*** (Table 2). External morphology was

242 massive with a charcoal black exterior and a sulphur yellow interior (Fig. 1G). Consistency was

243 hard and oscula were located in crevices. They were round, wide and randomly distributed at the

244 surface. The skeleton was mainly composed of spongin fibers (Fig. 1H) and a few short and thick

245 oxeas of about 60-70 µm were observed but were not specific to the sample (Fig. 1I) (Van Soest,

246 1978; Bergquist & Cook, 1978; Bergquist & Cook, 2002).

247

248 **Antimicrobial activity**

249 ***Antimicrobial assay***

250 In order to evaluate the antibacterial activity of the three extract sponges in water or ethanol,

251 inhibition diameters were obtained by the disc diffusion method on three Gram+ bacterial

252 strains: *S. aureus, B. cereus* and *S. saprophyticus* (Fig. 2A); and on two Gram- bacterial strains:

253 *E. coli* and *P. aeruginosa* (Fig. 2B).

254 Different positive controls were used to match bacteria sensitivity (T+). Ampicillin has been

255 used for *S. saprophyticus*, *S. aureus* and *E. coli*; chloramphenicol for *B. cereus*; and fosfomycin

256 for *P. aeruginosa*. Water only (T1-) or ethanol only (T2-) were used as negative controls (Fig.

257 2A and 2B).

258 Large inhibition diameters were observed for the positive controls and no inhibition diameters

259 were obtained for negative controls as expected (Fig. 2A and 2B).

260 For the sponge extracts, only the ethanolic extract of *Agelas clathrodes* showed inhibition

261 diameter for *S. aureus* and *S. saprophyticus* (Fig. 2A).

262 The inhibition diameter was then measured (Table 3) with an inhibition diameter greater than 9

263 mm around the disc indicating positive activity (Cita et al., 2017).

264 Table 3 shows the antimicrobial activity results obtained on the five bacterial strains. Only the

265 ethanolic extract of *Agelas clathrodes* shows high specific activity on the two Gram + strains: *S.*

266 *aureus* (inhibition diameter: 10.7 mm) and *S. saprophyticus* (inhibition diameter: 9.5 mm).

267 ***MIC and MBC***

268   Only *Agelas clathrodes* showed inhibition discs, it was therefore possible to measure

269   MIC and MBC values. The MIC determined for *S. aureus* was 15.62 µg/mL and was higher than

270   the 7.81 µg/mL obtained for *S. saprophyticus* (Table 4). This result was consistent with the 15.62

271   µg/mL MBC on *S. saprophyticus* and the 31.25 µg/mL on *S. aureus*. We could also note that the

272   MBC/MIC ratios were equal to 2, indicating a bacteriostatic effect for the ethanolic extract of

273   *Agelas clathrodes*.

274

275

276   **Cytotoxic activity**

277   ***Cytotoxic assay***

278   A complete cytotoxicity evaluation of each sponge ethanolic extract was performed on

279   four cancer cell lines: MDA-MB-231 (breast cancer), RE259 (glioblastoma), MOLM-14 (acute

280   myeloid leukemia) and HL-60 acute (human promyelocytic leukemia). Figures 3 and 4 display

281   these cytotoxic dose response evaluations. Results for synthetic anticancer drugs used as

282   references (doxorubicin and Ara-C) are also indicated. These control experiments were in

283   agreement with previous internal evaluations, thus strengthening the sponge extracts results and

284   conclusions. Several extracts could be linked to strong to moderate cytotoxic activity with cell

285   viability percentages reduced in a dose response manner. All the IC_{50} extracted from the dose

286   response analysis were kept independent to illustrate the good homogeneity observed across

287   different evaluation experiments and for several extraction campaigns. Table 5 lists all these

288   measured IC_{50} (per experiment and per extract) as well as the 95% confidence interval range

289   (CIR) generated during the fitting. Overall, the *Agelas clathrodes* extracts exhibited a strong

290   cytotoxicity on all evaluated cell lines. The *Verongula rigida* extracts also exhibited strong to

291   moderate cytotoxicity on three cell lines, while the *Desmapsamma anchorata* extracts were

292   mostly inactive. A detailed individual analysis is presented below for each cell line.

293   ***Antiproliferative activity on the human breast cancer line (MDA-MB-231)***

294   The results reported in Table 5, show that the ethanolic extracts of the two species: *A.*

295   *clathrodes* and *V. rigida* were active on cell viability for this cell line. On the other hand, the *D.*

296   *anchorata* extracts were inactive. Indeed, the MDA-MB-231 cells were more sensible to *A.*

297   *clathrodes* with IC_{50} values in the µg/mL range (3.42 to 7.91 µg/mL). The *V. rigida* extracts

298   were also active but with 1 log less potency, exhibiting IC_{50} values ranging from 18.62 to 64.41

299   µg/mL.

300   ***Antiproliferative activity on the glioblastoma cell line (RES259)***

301   The activity of the extracts on the RES259 glioblastoma cell line was similar to the

302   results observed for the breast cancer cell line. Strong cytotoxicity properties were observed for

303   *A. clathrodes* and *V. rigida* with IC_{50} values lower than 10 µg/mL as shown in Table 5. *A.*

304   *clathrodes* is once again the most active extract with IC_{50} values in the µg/mL range (1.45 to

305   6.16 µg/mL) followed by *V. rigida* with again 1 log weaker potency (16.33 to 25.89 µg/mL). In

306   contrast, *D. anchorata* did not exhibit significant antiproliferative properties.

307   ***Antiproliferative activity on the leukemia cell lines (MOLM-14 and HL-60)***

308   The MOLM-14 leukemia cell line was the most sensitive to all sponge extracts, closely

309   followed by HL-60. Indeed, the species *A. clathrodes* exhibited similar antiproliferative

310   properties on both cell lines in the µg/mL range (0.42 to 1.57 µg/mL on MOLM-14 and 1.82 to

311   2.5 µg/mL on HL-60 as seen in Table 5). While *V. rigida* was active on both cell lines with the

312   same activity range, a slight preference was measured for MOLM-14 over HL-60 (3.18 to 11.94

313   µg/mL for MOLM-14 versus 11.03 to 36.19 µg/mL for HL-60). While one experiment (#3, X2)

314   could generate a dose response curve for *D. anchorata* with an IC_{50} below 100 µg/mL (47.72

315   µg/mL), this result could not be confirmed for the two other evaluations confirming the overall

316   lack of activity of this species extracts during all the cytotoxicity evaluation and on all cell lines.

317

318   **Discussion**

319   **Antimicrobial activity**

320   The results obtained highlight the effects of *Agelas clathrodes* whose ethanoic extract is

321   the only sample presenting a specific activity on three strains of staphylococcus.

322   In similar tests carried out with the *Agelas sventres* species, such strain-specific activity

323   on staphylococcus had not been observed. In fact, an activity had been observed on *E. coli* (CIP

324   54.127) for methanolic extracts and in n-hexane. Similar results were obtained on *S. aureus* (CIP

325   67.8) with chloroform and hexane extracts, and finally on *C. albicans* (ATCC 10231) with

326   chloroform extracts (Galeano & Martínez, 2007). The specificity observed with *A. clathrodes*

327   probably indicates a difference in active biomolecule composition due to either the species or the

328   nature of the solvent. Indeed, with the aqueous extract having no effect, we can conclude that the

329   nature of the solvent plays a crucial part in the extraction of active molecules. A test performed

330   using a crude methanolic extract of *Agelas sp.* showed a significant effect on the same *S. aureus*

331   strain (CIP 67.8), but again these were not specific to staphylococcus (Balansa et al., 2020).

332   Thus, the alcohol extracts appear to have a greater effect on staphylococci than on other strains.

333 Alcoholic solvents would probably allow a better extraction of alkaloid type biomolecules.

334 Indeed, the alkaloids present in marine sponges are known for their antibacterial properties,

335 particularly on *S. aureus*. For example, bromo-pyrrole alkaloids extracted from the species

336 *Agelas dispar*, are known for their moderate antimicrobial activity on Gram +: *B. subtilis* and *S.*

337 *aureus* (Chairman et al., 2012). This suggests the potential presence of similar biomolecules in

338 the *A. clathrodes* extract or a biomolecule with similar effect. It could also be a combination of

339 biomolecules. The lack of effect on these same strains with the aqueous extract confirms the

340 necessity of an alcoholic solvent for this type of biomolecules. Similarly, in *Agelas dilatata*,

341 pyrrole-imidazoles were tested on two pathogenic strains of *P. aeruginosa* (CIP A22 and PAO1)

342 and showed a moderate to strong activity. More specifically, oroidin 1 (pryrrole-imidazole which

343 was first isolated in *Agelas oroides* in 1971) has also showed a moderate activity against

344 laboratory strains of *P. aeruginosa* (PA01 and PA14) (Melander et al., 2016). Bromoageliferin is

345 an isolated molecule that showed significant activity, specifically on the *P. aeruginosa* strain CIP

346 A22 (Pech-Puch, Pérez-Povedano, et al., 2020). This could indicate the absence of this molecule

347 in *A. clathrodes*.

348   Concerning the species *V. rigida*, this is the first study to analyze the antibacterial activity

349 of this extract on non-marine bacterial strains (Newbold et al., 1999). Indeed, this species is

350 known for its antibacterial effect on some sponge pathogenic strains, in particular *Bacillus sp*.

351 and *Vibrio alginolyticus*, but no work has been done on the evaluation of their possible activity

352 on non-marine strains. In addition, antiparasitic (Putra et al., 2016a; Bianco et al., 2015) or

353 antidepressant (Zhang, 2020) effects have also been described. Also, based on our results, we

354 find that this species does not possess activity on non-marine bacterial strains.

355   Regarding *D. anchorata* species, we confirmed its lack of activity against Gram- bacteria,

356   including *E. coli* and *P. aeruginosa* (Bianco et al., 2015). Indeed, it is becoming increasingly

357   difficult to find antibacterial molecules effective against Gram- bacteria, as confirmed by our

358   results. Moreover, antimicrobial activity tests performed with *D. anchorata* extracts revealed no

359   effect on *S. aureus*, a Gram+. This study confirmed the inactivity of the extract on the strains

360   selected for this study but it may be active on other bacterial strains not used here.

361   The determination of MIC and MBC could only be performed on *A. clathrodes* showing

362   bioactivity on the selected bacterial strains. The extracts were more potent for *S. saprophyticus*

363   than *S. aureus*. Given the quality of the extractions performed and the quantity of extracts

364   obtained after drying, a higher MIC for *S. aureus* does not mean an absence of active

365   biomolecules but maybe a lower concentration. Indeed, a higher MIC has already been obtained

366   for an alcoholic extract on *E. coli* for the *S. massa* sponge (Putra et al., 2016a). Similarly for the

367   aqueous extract, the number of biomolecules may be too low to observe a significant effect on

368   the bacteria.

369   The bacteriostatic effect of this extract may, again, indicate the presence of biomolecules

370   different from those known to exist in the other studied *Agelas* genus species.

371   Moreover, *Agelas* genus seems to have a specific type of alkaloid biomolecules or a

372   specific combination of them, hence the absence of effect for the species *D. anchorata* and *V.*

373   *rigida*.

374

375   **Cytotoxic activity**

376   The cytotoxicity evaluation highlighted that the *Agelas clathrodes* species has a strong

377   cytotoxic activity in addition to antimicrobial properties as previously demonstrated. *Agelas*

378   *clathrodes* species is also the one with the highest activity on all cell lines followed by

379   *Verongula rigida*. Their order of activity for both these sponges is the same, namely, a stronger

380   activity on the leukemia cell lines (MOLM-14 and HL-60) followed by the activity on the

381   RES259 glioblastoma cells and finally on the MDA-MB231 breast cancer lineage.

382   The sponges of the *Agelas genus* are known for their cytotoxic effect on many cancerous

383   cells associated with several extracted molecules such as agelasins, agelasidins and agelines. The

384   Agelasphins extracted from the *A. mauritianus* species exhibit, notably, antitumor and

385   immunostimulatory effects (Natori et al., 1994). Agelasine B extracted from *A. clathrodes*

386   species has demonstrated effects on the MCF-7 line of human breast cancer cells (Dipolo et al.,

387   2012). Moderate effects of *A. clathrodes* extracts were also observed on the MDA-MB-435 cell

388   line (Custódio et al., 2007).

389   In our study, we observed similar effects on a different breast cancer cell line.

390   Nevertheless, working with a crude extract, we could not establish here if it was an effect of

391   agelasine B or if it was due to a different molecule or set of molecules. Indeed, other tests

392   conducted on several species of the Agelas genus (*A. citrina, A. clathrodes, A. dilatata and A.*

393   *sceptrum*) on the same MCF-7 cell line showed more negligible effects, except for *A. citrina*

394   which stood out with a percentage of inhibition of 100% at 30 and 15 µg/mL (Pech-Puch,

395   Berastegui-Cabrera, et al., 2020). Similar results for extracts of the *Agelas clathrodes* species

396 were observed in the MDA-MB-435 cancer cell line, as well as for *Agelas sp.* in the same study

397 (Custódio et al., 2007). Furthermore, a moderate activity had been found for *A. clathrodes* in the

398 HL-60 cell line (IC$_{50}$: 48.51 µg/mL) as well as in a glioblastoma cell line SF-295 (IC$_{50}$: 62.36

399 µg/mL) (Custódio et al., 2007). The stronger activity in our evaluation campaign could be

400 explained by a synergy of active biomolecules.

401   The ethanolic crude extract of *Verongula rigida* showed a high cytotoxic response in

402 this study. This activity is specific to leukemic (MOLM-14 and HL-60), glioblastoma (RES 259)

403 and breast cancer (MDA-MB231) cell lines. Although no specific studies have been performed

404 on these cells, the published results of Galeano et al. 2011 on the cytotoxicity of *V. rigida* on

405 U937 cells of the human myeloid linage, could sustain our observations. Indeed, Galeano

406 showed that this property would be due to aeroplysinin-1, a protein tyrosine kinase inhibitor

407 found in *V. rigida* extracts. These active biomolecules will have to be purified from the extracts

408 and evaluated to confirm our preliminary results.

409   As for the antimicrobial and cytotoxic activities of crude ethanolic extract of

410 *Desmapsamma anchorata*, we validated a lack of activity on our cell lines for the first time, but

411 this was also shown on other cell lines in previous studies (Lhullier et al., 2020; Marques et al.,

412 2016). This was presumably due to the presence of relatively low amount of active biomolecules.

413 Conclusion

414   Taken together, the results of this study suggest that crude extracts of marine sponges

415 from Martinique have a potential antimicrobial and cytotoxic effect. Two sponge species stood

416 out: *Agelas clathrodes* for both antimicrobial and antitumor properties, and *Verongula rigida* for

417 its antitumor properties. The existence of such active compounds in this species extracts would

418   respond to a need for natural antibacterial and antitumor molecules, with limited side effects or

419   drug resistances. Hypotheses have also emerged concerning the biomolecular composition of

420   *Agelas clathrodes* and *Verongula rigida* such as the presence of alkaloids and aeroplysin-1, or

421   biomolecules with similar effects. Moreover, having identified these species in Martinique

422   constitutes a key element for the valorization of the marine biodiversity of the island within the

423   Caribbean area. As this study is a preliminary work never done before in the French West Indies,

424   especially in Martinique, it would be interesting to widen the spectrum of study to other species

425   of the region and to carry out complementary chemical analyses in order to isolate and confirm

426   the bioactive candidate or to identify other biomolecules of interest.

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List of species collected and GPS localization data.

Species	Site	GPS data
Agelas clathrodes	Fond Boucher	61° 9'21.22"W 14°39'21.12"N
Desmapsamma anchorata	Fond Boucher	61° 9'21.22"W 14°39'21.12"N
Verongula rigida	Fond Boucher	61° 9'26.55"W 14°39'23.67"N

1   **Table 1: List of species collected and GPS localization data.**

Identification of sponge species collected.

Sample 1	Class: *Demospongiae* Sollas, 1885 Sub-class: *Heteroscleromorpha* Cárdenas, Pérez & Boury-Esnault, 2012 Order: *Agelasida* Hartman, 1980 Familly: *Agelasidae* Verrill, 1907 Genus: *Agelas* Duchassaing & Michelotti, 1864 ***Agelas clathrodes*** Schmidt, 1870
Sample 2	Class: *Demospongiae* Sollas, 1885 Sub-class: *Heterocleromorpha* Cárdenas, Pérez & Boury-Esnault, 2012 Order: *Poecilosclerida* Topsent, 1928 Familly: *Desmacididae* Schmidt, 1870 Genus: *Desmaspamma* Burton, 1934 ***Desmapsamma anchorata*** Carter, 1882
Sample 3	Class: *Demospongiae* Sollas, 1885 Sub-class: *Verongimorpha* Erpenbeck, Sutcliffe, De Cook, Dietzel, Maldonado, Van Soest, Hooper & Wörheide, 2012 Ordre: *Verongiida* Bergquist, 1978 ***Verongula rigida*** Esper, 1794

1 **Table 2: Identification of sponge species collected.**

Inhibition diameter of aqueous and ethanolic extracts of the different sponge species on the five strains (mm).

A: aqueous extract. E: ethanolic extract. R: resistant. -: not used for this strain.

Extracts sponges and controls	Extract solvent	Strains				
		Gram +			Gram -	
		S. aureus	*B. cereus*	*S. saprophyticus*	*E. coli*	*P. aeruginosa*
Ampicillin (T+)		46 ± 1	-	40 ± 1	26 ± 0	-
Chloramphenicol (T+)		-	25 ± 0	-	-	-
Fosfomycin (T+)		-	-	-	-	17,5 ± 0,71
(H2O) T1-	A	R	R	R	R	R
(EtOH) T2-	E	R	R	R	R	R
A. clathrodes	A	R	R	R	R	R
	E	10,66 ± 0,58	R	9,5 ± 0,5	R	R
D. anchorata	A	R	R	R	R	R
	E	R	R	R	R	R
V. rigida	A	R	R	R	R	R
	E	R	R	R	R	R

2 **Table 3: Inhibition diameter of aqueous and ethanolic extracts of the different sponge species on the**
3 **five strains (mm).**

4 A: aqueous extract. E: ethanolic extract. R: resistant. -: not used for this strain.

MIC and MBC for the ethanolic extract (E) of Agelas clathrodes.

Strain	MIC (µg/mL)	MBC (µg/mL)	MBC/MIC ratio
S. saprophyticus	7.81	15.62	2
S. aureus	15.62	31.25	2

5   **Table 4: MIC and MBC for the ethanolic extract (E) of Agelas clathrodes.**

Cytotoxic evaluation results.

Half maximal inhibitory concentration (IC$_{50}$) and confidence interval range (CRI) measured for the ethanolic extracts (E) on different cancer cell lines (MDA-MB231 (breast cancer), RES259 (glioblastoma), MOLM-14 (leukemia) and HL-60 (leukemia)). For each sponge sample, two extractions were evaluated (X1 and X2) for a total of 3 experiments labelled as #1, #2 or #3. All values are listed as µg/ml concentrations and 'ND' is specified for non-determined values and 'NC' for not converged values during the fitting process. Doxorubicin and Ara-C are used as positive control anticancer agents.

		MDA-MB-231		RES259		MOLM-14		HL-60	
		IC$_{50}$**	95% CIR**	IC$_{50}$**	95% CIR***	IC$_{50}$**	95% CIR***	IC$_{50}$**	95% CIR***
Doxorubicin	**#1**	**0.20**	*0.14 to 0.29*	**ND**	-	**ND**	-	**ND**	-
	#2	**0.18**	*0.13 to 0.23*	**ND**	-	**ND**	-	**ND**	-
Ara-C	**#1**	**ND**	-	**0.07***	*0.05 to 0.08*	**0.085***	*0.06 to 0.11*	**ND**	-
	#2	**ND**	-	**0.08**	*0.07 to 0.10*	**0.222**	*0.15 to 0.25*	**0.12**	*0.10 to 0.15*
Agelas clathrodes	**#1 (X1)**	**3.42**	*2.38 to 4.91*	**1.45**	*0.91 to 2.07*	**0.41**	*0.34 to 0.51*	**ND**	-
	#2 (X1)	**7.91**	*2.02 to 30.99*	**3.52**	*2.64 to 4.66*	**1.57**	*1.30 to 1.85*	**1.82**	*1.58 to 2.18*
	#3 (X2)	**7.71**	*NC*	**6.16**	*NC*	**1.21**	*1.06 to 1.37*	**2.5**	*2.20 to 2.85*
Desmapsamma anchorata	**#1 (X1)**	**>100**	-	**>100***	-	**>100***	-	**ND**	-
	#2 (X1)	**>100**	-	**>100**	-	**>100**	-	**>100**	-
	#3 (X2)	**>100**	-	**97.15**	*NC*	**47.72**	*40.04 to 56.95*	**>100**	-
Verongula rigida	**#1 (X1)**	**18.62**	*16.77 to 20.67*	**16.33**	*NC*	**3.83**	*2.74 to 5.20*	**ND**	-
	#2 (X1)	**30.68**	*26.06 to 36.11*	**11.83**	*NC*	**3.18**	*2.18 to 4.56*	**11.03**	*8.25 to 14.50*
	#3 (X2)	**64.41**	*54.41 to 76.25*	**25.89**	*NC*	**11.94**	*6.40 to 20.65*	**36.19**	*NC*

* Duplicate instead of triplicate   ** Half maximal inhibitory concentration (μg/mL)   *** CIR: confidence interval range (μg/mL)

1   **Table 5: Cytotoxic evaluation results.**

2   Half maximal inhibitory concentration (IC$_{50}$) and confidence interval range (CRI) measured for the
3   ethanolic extracts (E) on different cancer cell lines (MDA-MB231 (breast cancer), RES259
4   (glioblastoma), MOLM-14 (leukemia) and HL-60 (leukemia)). For each sponge sample, two extractions
5   were evaluated (X1 and X2) for a total of 3 experiments labelled as #1, #2 or #3. All values are listed as
6   μg/ml concentrations and 'ND' is specified for non-determined values and 'NC' for not converged values
7   during the fitting process. Doxorubicin and Ara-C are used as positive control anticancer agents.

Figure 1

Identification of the sponges.

Map of the sampling location (Top) and morphological analysis of each sponge (Bottom) showing: (A, D, G) photos after freezing; (B, E, H) micrographs of the skeleton x100; (C, F, I) micrographs of the skeleton x4000. The analysis shows: (C) whorled achantostyles and whorled achantoxes in formation; (F) oxes, isocheles, tripods and spheriaster; (I) thick and short oxes.

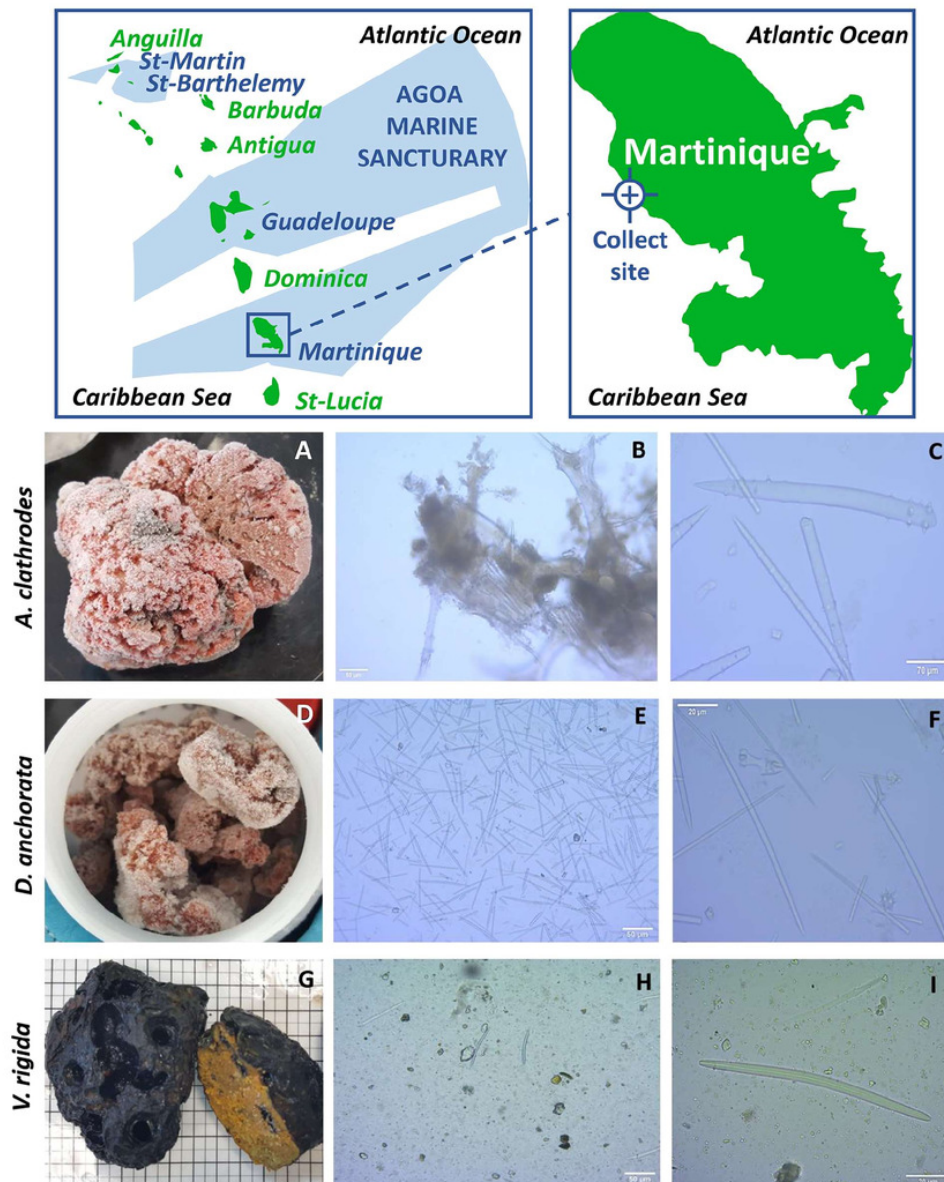



Figure 2

Antimicrobial screening and antibacterial activity of sponge species by the disc diffusion method on five bacterial strains.

(A) Three Gram+ bacterial strains: S. aureus, B. cereus and S. saprophyticus. T+ (Positive control): Ampicillin for S. aureus and S. saprophyticus; Chloramphenicol for B. cereus. **(B)** Two Gram- bacterial strains: E. coli and P. aeruginosa. T+ (Positive control): Ampicillin = for E. coli; Fosfomycin for P. aeruginosa. **(A)** and **(B)** T1- (Negative control 1): H_2O only. T2- (Negative control 2): EtOH only. S1 H_2O: A.clatrodes aqueous extract / S1 EtOH: A.clatrodes ethanolic extract. S2 H_2O: D.anchorata aqueous extract / S2 EtOH: D.anchorata ethanolic extract. S3 H_2O: V.rigida aqueous extract / S3 EtOH: V.rigida ethanolic extract. For each panel, visible inhibition discs are marked with yellow arrows.

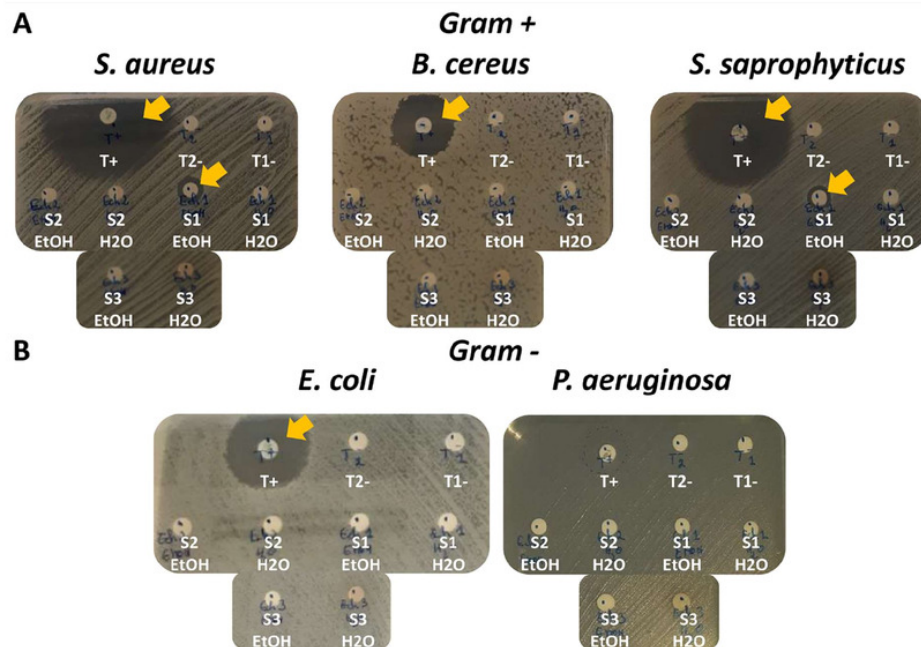



Figure 3

Cytotoxic evaluation of the sponge extracts on the for MDA-MB-231 and RES259 cell lines.

For each sample, three dose response experiments (1st blue; 2nd red; 3rd green) were performed as triplicates. Doxorubicin and Ara-C and were evaluated as positive controls for MDA-MB-231 and RES259 respectively, in two independent experiments.

MDA-MB-231

Agelas clathrodes

Verongula rigida

Desmapsamma anchorata

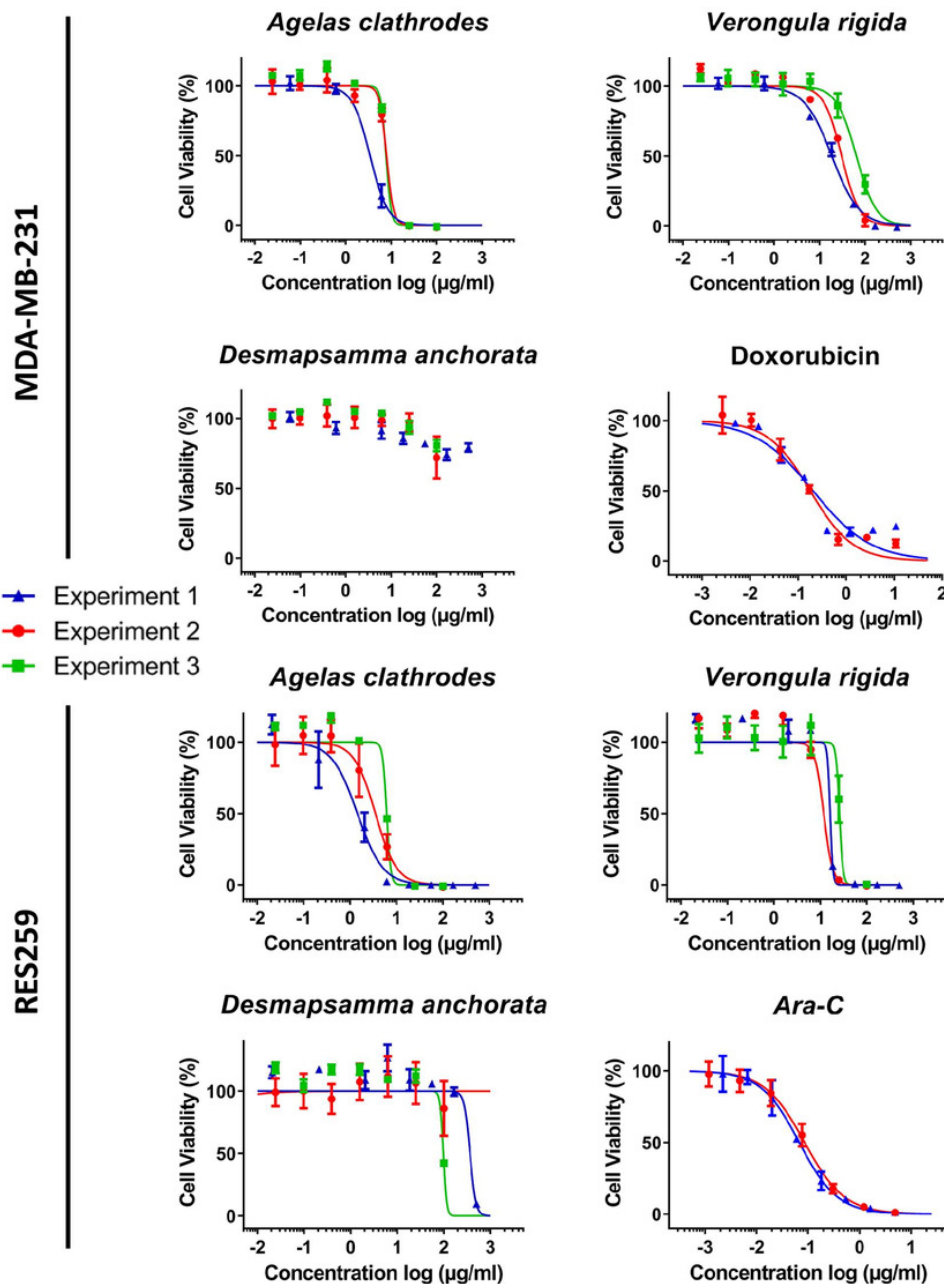
Doxorubicin

Experiment 1
Experiment 2
Experiment 3

RES259

Agelas clathrodes

Verongula rigida

Desmapsamma anchorata

Ara-C

Cytotoxic evaluation of the sponge extracts on the MOLM-14 and HL60 cell lines.

For each sample, two or three dose response experiments (1st blue; 2nd red; 3rd green) were performed as triplicates. Ara-C was evaluated as positive control in two independent experiments for MOLM14 and one experiment for HL60.

MOLM-14

Agelas clathrodes

Verongula rigida

Desmapsamma anchorata

Ara-C

▲ Experiment 1
● Experiment 2
■ Experiment 3

HL-60

Agelas clathrodes

Verongula rigida

Desmapsamma anchorata

Ara-C