Comparison of chemical compounds associated with sclerites from healthy and diseased sea fan corals (*Gorgonia ventalina*)

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Background: The roles of gorgonian sclerites as structural components and predator deterrents have been widely studied. Yet, their role as barriers against microbes has only recently been investigated, and even less is known about the diversity and roles of the chemical compounds associated with sclerites. **Methods:** Here, we examine the semi-volatile organic compound fraction (SVOCs) associated with sclerites from healthy and diseased *Gorgonia ventalina* sea fan corals to understand their possible role as a stress response or in defense of infection. We also measured the oxidative potential of compounds from diseased and healthy *G. ventalina* colonies. **Results:** The results showed that sclerites harbor a great diversity of SVOCs. Overall, 70 compounds were identified, the majority of which are novel with unknown biological roles. The majority of SVOCs identified exhibit multiple immune-related roles including antimicrobial and radical scavenging functions. The free radical activity assays further confirmed the anti-oxidative potential of some these compounds. The anti-oxidative activity was, nonetheless, similar across sclerites regardless of the health condition of the colony, although sclerites from diseased sea fans display slightly higher anti-oxidative activity than the healthy ones. **Discussion:** Sclerites harbor great SVOCs diversity, the majority of which are novel to sea fans or any other corals. Yet the scientific literature consulted showed that the roles of compounds found in sclerites vary from antioxidant to antimicrobial compounds. However, this study fell short in determine the origin of the SVOCs identified, undermining our capacity to determine the biological roles of the SVOCs on sclerites and sea fans.

1   **Comparison of chemical compounds associated with sclerites from healthy and diseased sea**

2   **fan corals (*Gorgonia Ventalina*).**

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13   **ABSTRACT**

14 **Background:** The roles of gorgonian sclerites as structural components and predator deterrents

15 have been widely studied. Yet, their role as barriers against microbes has only recently been

16 investigated, and even less is known about the diversity and roles of the chemical compounds

17 associated with sclerites.

18 **Methods:** Here, we examine the semi-volatile organic compound fraction (SVOCs) associated

19 with sclerites from healthy and diseased *Gorgonia ventalina* sea fan corals to understand their

20 possible role as a stress response or in defense of infection. We also measured the oxidative

21 potential of compounds from diseased and healthy *G. ventalina* colonies.

22 **Results:** The results showed that sclerites harbor a great diversity of SVOCs. Overall, 70

23 compounds were identified, the majority of which are novel with unknown biological roles. The

24 majority of SVOCs identified exhibit multiple immune-related roles including antimicrobial and

25 radical scavenging functions. The free radical activity assays further confirmed the anti-oxidative

26 potential of some these compounds. The anti-oxidative activity was, nonetheless, similar across

27 sclerites regardless of the health condition of the colony, although sclerites from diseased sea fans

28 display slightly higher anti-oxidative activity than the healthy ones.

29 **Discussion:** Sclerites harbor great SVOCs diversity, the majority of which are novel to sea fans

30 or any other corals. Yet the scientific literature consulted showed that the roles of compounds

31 found in sclerites vary from antioxidant to antimicrobial compounds. However, this study fell

32 short in determine the origin of the SVOCs identified, undermining our capacity to determine the

33 biological roles of the SVOCs on sclerites and sea fans.

34 **INTRODUTION**

35 Sea fans (*Gorgonia* sp.) are one of the top competitors for space within Caribbean coral reefs

36 ecosystems. Similar to their relatives, the scleractinian corals, sea fans have suffered several

37 environmental disturbances such as aspergillosis disease outbreaks (a fungal infection caused by

38 the saprophytic fungus *Aspergillus sydowy*) that seriously compromised the viability of many

39 populations across the Caribbean during the 1990s (Smith et al., 1996; Nagelkerken et al., 1997;

40 Geiser et al., 1998; Kim et al., 2004). Yet, in contrast to the scleractinian, sea fans have overcome

41 these disturbances and continue to thrive. In fact, gorgonians are the prevailing corals in many

42 reefs formerly dominated by scleractinian corals. Their relative success is provided in part, by

43 their strong immune defenses, which have allowed gorgonians to maintain their internal

44 homoeostasis and health (Ruiz-Diaz et al., 2013; Sabat & Toledo-Hernández, 2015).

45 Gorgonians have a rather diverse repertoire of defensive mechanisms to cope and respond to

46 disturbances, both abiotic and biotic (Toledo-Hernández & Ruiz-Diaz 2014; Ruiz-Diaz et al.,

47 2016). For instance, to respond to pathogen infection, gorgonians are equipped with chemical

48 pathways such as the melanin cascade that prevent or reduce the pathogen dissemination

49 throughout the host tissue (Petes et al., 2003; Mydlarz et al., 2008). Concomitant to this response,

50 gorgonians have the ability to activate the production of peroxidase enzymes, which induce the

51 production of reactive oxygen species with cytotoxic roles and cellular signaling (Mydlarz &

52 Harvell, 2007). Furthermore, gorgonians are also characterized for their diverse production of

53 secondary metabolites, many of which are thought to be immune-related compounds due to their

54 anti-predation and microbial properties (Harvell, Fenical & Green, 1988; Kim, 1994; Jensen et

55 al., 1996; Smith et al., 1996; Geiser et al., 1998; Kim et al., 2000a, 2000b, Alker et al., 2004).

56 Gorgonians also have sclerites, which are minute skeletal elements mainly composed of calcium

57 carbonate surrounding an organic matrix made up primarily of glycoproteins, spread throughout

58 the gorgonians' epidermis, mesoglea, and axial skeleton (Kingsely & Watabe, 1982, 1984;

59 Harvell & Suchanek, 1987, Van Alstyne & Paul, 1992). Sclerites are thought to have multiple

60 defensive roles. For instance, sclerites provide structural support to gorgonians by reducing the

61 elasticity and stiffness of the axial skeleton against water motion (Koehl, 1982; Lewis & Willis,

62 1991). In addition, sclerites play a role in deterring predation from gastropods and reef fishes

63 (Harvell, Fenical & Greene, 1988; Van Alstyne & Paul, 1992; Koh et al., 2000). Furthermore, the

64 purpling of sclerites (i.e., darkening of sclerites through the accumulation of pigments; Leverette

65 et al., 2008) has also been linked to defensive mechanisms against several biological threats

66 (Petes et al., 2003; Mydlarz & Harvell, 2007; Mydlarz et al., 2008).

67 Yet, the role of sclerites as chemical barriers against microbes has only recently been

68 investigated. For instance, Toledo-Hernández et al., (2016) showed that sea fan sclerites treated

69 with acetone and placed on top of the surfaces of culture media as to block the entrance of

70 microbes, were less effective at delaying the crossing and further colonization of fungi in the agar

71 than sclerites not treated with any solvent. However, no screening of chemical compounds in

72 sclerites was conducted in that study. In fact, studies devoted to surveying the chemical

73 compounds found in gorgonian sclerites are rather scarce, with only a few of them describing the

74 chemical composition of the core of sclerites (Kingsley & Watabe, 1982, 1984; Kingsley et al.,

75 1990). Here we perform for the first time, a screening of the semi volatile organic compounds

76 (SVOCs) fraction found in sclerites from healthy and diseased *G. ventalina* colonies.

77 Furthermore, we performed a scientific literature survey of the SVOCs found in sclerites to

78 determine their potential biological roles. Finally, we conducted radical scavenging activity

79 assays to examine the oxidative potential of chemical compounds associated with sclerites from

80 healthy and diseased fans.

81 **METHODS**

82 **Tissue collection and sclerite isolation**

83 Sclerites used for this study were isolated from ten healthy and ten diseased *Gorgonia ventalina*

84 colonies located at a depth of 1.5-2.0 m from El Escambrón beach, San Juan, Puerto Rico

85 (18º28'00''N, 66º05'12''W). The tissue samples were collected under permit 2012-IC-086 issued

86 to Claudia P. Ruiz Diaz, University of Puerto Rico (UPR) Rio Piedras campus, given by the

87 Puerto Rico Department of Natural Resources, Commonwealth of Puerto Rico. Healthy colonies

88 showed neither lesions nor tissue purpling, whereas diseased colonies showed at least one lesion

89 usually overgrown by algae and surrounded by a ring of purple tissue. Previous studies have used

90 the aforementioned pathology to diagnose algal tumor affliction, sea fan aspergillosis, as well as

91 competitive interactions between inter and conspecific individuals (Morse et al., 1977; Smith et

92 al., 1996; Alker et al., 2004). However, since we performed no microbiological or histological

93 analyses to determine the precise etiology of the injured tissue collected for this study, we

94 avoided the use of any term that could be associated with the afflictions previously described for

95 sea fans. One tissue fragment approximately 4 cm^2 was cut from the edge of each healthy fan

96 (hereafter HH). In diseased fans, 2 cm^2 samples were cut from both healthy tissue at the edge of

97 the fan (healthy-diseased or HD) as well as from the diseased area (diseased-diseased or DD).

98 Fragments were placed individually in 50 mL centrifuge tubes and brought to the laboratory on

99   ice. Once in the laboratory, each fragment was further cut into smaller fragments of 2-3 cm^2 in

100 size and placed in 20.0 mL-sterilized leveled vials filled with 16.0 mL of distilled water. Then, to

101 disassociate sclerites from the soft tissue matrix, vials were vortexed at room temperature,

102 sonicated for 5 min, and then allowed to settle for approximately 4 hrs. This procedure was

103 repeated over a period of 5-7 days to minimize impacts on the structure of the sclerites. After

104 disassociated, sclerites were rinsed several times with distilled water, individually transferred to

105 clean 55 mm Pyrex petri dishes, and dried at 35 ºC for 72 hrs.

106 **Gas chromatography-mass spectrometry analysis**

107 From each dried sclerite sample, 0.10 g were grounded in a clean porcelain mortar and the

108 homogenized powder was transferred to a 1.5 mL micro-test tube with 1 mL of acetone. Each

109 tube was vortexed and centrifuged at 6,000 rpm for 5 min. Then, 1.5 mL of the resulting extract

110 from each sample was filtered and transferred without introducing air bubbles to clean amber

111 vials (32 x12 mm). Afterward, samples were purged with nitrogen to remove dissolve oxygen and

112 then septum caps were fitted to each vial and these were inverted to ensure no headspace was

113 visible and finally, vials were hermetically sealed and not opened until analyzed to preserve the

114 solution. Prior to injecting the solvent used for the extraction, reagents, glassware and other

115 samples processing hardware were blank tested to ensure these materials will not add artifacts to

116 the sclerite extracts analyses. Then, vials with sclerite extracts were placed in an Agilent 7890A

117 gas chromatograph (GC) coupled to an Agilent 5975 C mass-selective detector for splitless

118 injection. Because removing a sample aliquot from the vials may compromise the integrity of the

119 sample, multiple aliquots were injected to allow for the screening and re-analysis of the samples.

120 The injection temperature was 250 °C. Separation of samples was performed using a fused silica

121 capillary column (SLB®-5 ms 30 m × 0.32 mm × 0.25 µm film thickness; Supelco, Bellefonte,

122 PA). The initial column temperature was held at 70 °C for 1 min, and then increased by 8 °C min$^-$

123 ^{1} until reaching 270 °C, where it was held for 3 min, for a total run time of 29 min per sample.

124 Ultrapure helium at constant-flow of 1.2 mL min^1 was used as the carrier gas. Analysis of the

125 generated chromatograms was performed using the AMDIS version 2.7/NIST version 2.0

126 computer program and the criteria for selection in the analysis for the screening compounds were:

127 (1) R- match >800; (2) S/N > 15; and (3) peak areas >1000. Compound identification was

128 confirmed through the use of NIST library appropriated standards and retention time index

129 (http://www.nist.gov/srd/nist1a.cfm). To determine the potential biological roles of each of the

130   detected compounds, a scientific literature review using the IUPAC names of each compound was

131   conducted.

Free radical scavenging assay

133   Free radical scavenging activity of sclerite extracts was estimated using 1,1,-diphenyl-2-

134   picrylhydrazyl (DPPH) (MacDonald-Wisk, Wood & Garg, 2006). Briefly, 0.10 g of homogenized

135   powder not previously used from each of the sclerite samples was placed into labeled and clean

136   50 mL centrifuge tubes filled with 5.0 mL methanol. Then, each tube was vortexed for 10 sec,

137   placed in a water bath at 40 °C for 40 min, and sonicated afterward for 30 min. The samples were

138   then centrifuged for 10 min (6,000 rpm) at 25 °C. Afterwards, 1.0 mL of the supernatant ("sclerite

139   extract") was transferred to a clean 50 mL centrifugal tube containing 5.0 mL methanol. Then, 1

140   µL of the sclerite extract and 39 µL of DPPH (0.09 mM) were added to a 96-well microplate and

141   the absorbance of the each sample was recorded at 0, 1, 4 and 5 minutes and then every 5 minutes

142   for a one-hour period at 560 nm in a Thermo Scientific Multiscan FC spectrophotometer. Only

143   those samples with absorbances with absorbance < 2 were used in the analysis. Scavenging

144   activity in this study was expressed as IC_{50}, which represents the concentration of extracts

145   (mg/mL) needed to inhibit 50% of the DPPH radicals. To calculate IC_{50} for each sclerite sample,

146   the percent inhibition of radical production (%I) was estimated using the following equation

147   (Mishra et al., 2012):

$$\%I = (A_i - A_f) \cdot 100/A_i \, ,$$

149   where A_i and A_f represent the initial and final absorbance, respectively. A linear regression

150   analysis was performed with extract concentration as the independent variable and %I as the

151   dependent variable. Finally, the resulting linear regression equations with a correlation coefficient

152    $R^2 > 80\%$ were used to estimate the IC_{50}.

Statistical analyses

154   Nonmetric Multi Dimensional Scaling (nMDS) along with an Analysis of Similarity (ANOSIM)

155   with Bray-Curtis distances measurement were utilized to compare the composition of chemical

156   compounds among the sclerites from different tissues. We used nMDS ordination, achieved by

157   the *metaMDS* wrapper function from the package vegan in R version 3.2. The ordination was

158   applied such that the data was scaled down to two dimensions.

159   For the analysis of free radical scavenging, a Kruskal-Wallis was performed to detect differences

160   between IC_{50} index from sclerites from different tissues. In this analysis, IC_{50} index was assigned

161   as the dependent variable and the origin of the sclerite was assigned as the independent variable.

162   Finally, a Chi^2 test was conducted to determine statistical differences in the relative abundances

163   of alcohols, alkanes and alkenes and sclerite origin. In all analyses, P-value was fixed at 0.05.

164   Statistical analyses were performed using R version 3.2 (R Core Team, 2014). For the statistical

165   analyses, HD and DD samples from the same colony where considered independent and analyzed

166   accordingly. In modular organisms such as gorgonian corals, modules are semi-autonomous units.

167   Consequently, any particular stressors (abiotic or biotic) may trigger or have a differential

168   consequence in tissue subjected to different health states (Van Valen, 1978; Tuomi & Vuorisalo,

169   1989).

170   **RESULTS**

171   **Semi-volatile organic compounds**

172   A total of 70 SVOCs were found in the sampled sclerites; 37% of these were identified from DD

173   sclerites, while 35% and 29% were identified from DH and HH sclerites, respectively. Yet, the

174   nMDS and ANOMIS analyses failed to group the chemical compounds according to their origin

175   (e.g., HH, HD and DD sclerites). This holds true even when compounds found only once or twice

176   across the samples were eliminated from the analysis.

177   In general, three major groups were identified: alcohols, alkanes and alkenes. When data was

178   organized based on sclerite origin, alcohols and secondarily alkanes were the most frequently

179   identified SVOCs in all samples, although the number of identified alkenes was higher in HD

180   sclerites (Figure 1). However, the Chi^2 test analysis revealed no statistical differences between

181   alcohols, alkanes and alkenes and the sclerites origin. Furthermore, DD sclerites exhibited the

182   highest number of exclusive compounds with a total of 17 unique SVOCs, followed by HH and

183   HD sclerites with 15 and 8 unique SVOCs, respectively (Table S1; Figure 2). The remaining

184   SVOCs were either shared across all sclerites or between two distinct combinations of sclerites

185   (Figure 2).

186   Evidence from the scientific literature concerning the possible biological roles of the SVOCs

187   identified in sclerites was obtained for 44% the compounds. Most were associated with immune

188   related roles such as antimicrobials and antioxidants. Less represented roles were related to

189   cytotoxic and apoptotic compounds, cell membrane components such as steroids and oxidized

190   steroids derivatives, and intermediate signaling molecules.

191   **Free radical scavenging assay**

192   Thirteen out of the 30 sclerites sampled tested had R^2 >80% and thus were used for the free

193   radical scavenging assays. Of these, six samples were HH sclerites, whereas four and three were

194   DD and HD sclerite samples, respectively. The free radical scavenging IC_{50} ranged from 0.21 mg

195   dry extract/mL to 0.01 mg dry extract/mL DPPH. Observed differences in antioxidant activity

196   among sclerites were not statistically different, however on average there was a trend for higher

197   values in HH and lowest in DD sclerites (Figure 3).

198   **DISCUSSION**

199   Overall, 70 SVOCs associated with sclerites were identified. The majority of compounds have

200   not been reported previously in corals and consequently little is known of their biological roles

201   (Table S1). Several of these SVOCs were common to all sclerites samples, whereas others were

202   found in two or exclusively in one of sclerite types (Table S1; Figure 2). Yet, no clusters between

203   the compounds and health states were formed, suggesting that the chemical composition of

204   sclerites is similar across the samples independent of the health state of the colony. Nonetheless,

205   the concentration of these compounds may vary among colonies with contrasting health state, but

206   our data do not provide sufficient details to validate or reject this argument.

207   On the other hand, the scientific literature surveyed revealed potential roles for some of the

208   compounds. For instance, some of the compounds identified in this study have been characterized

209   as free radical scavengers (Table S1). The oxidative analysis assays performed in this study,

210   which revealed that extracts from the three sclerite origins exhibit antioxidant activity, further

211   confirms this fact.

212   Antioxidant production in gorgonians is thought to have mitigating roles during several immune

213   responses including phagocytosis, peroxidase enzymatic activity, and melanization (Olano &

214   Bigger, 2000; Midlarz & Harvell, 2007). These immune responses produce reactive oxygen

215   species (ROS) and other free radical molecules, inducing oxidative stress in the coral.

216   Consequently, to counteract this oxidative stress, sea fans have developed regulatory systems

217   such as increased production of non-enzymatic antioxidant scavengers, which neutralize the

218   potential harmful impact of ROS and other free radical molecules (Yost, Jones & Mitchelmore,

219   2010; Shahbudin et al., 2011).

220   Antioxidant capacity in corals has also been linked to thermal stress. Several studies have

221   reported that corals under thermal stress exhibit higher antioxidant potential than those under

222   normal temperature conditions (Downs et al., 2002; Griffin & Bhagooli, 2004). In fact, over-

223   expression of genes involved in the oxidative-stress response has been reported to increase in

224   acroporids after they have undergone thermal stress (Császá, Senaca & Oppen, 2009).

225   Alternatively, microbes associated with sea fans, both prokaryotes and eukaryotes, may be

226   producing the antioxidant compounds found in sclerites. In the case of eukaryotes such as

227   zooxanthellae for instance, antioxidants may be synthesized as a mitigating mechanism against

228   ROS produced during photosynthesis (Foyer and Shigeoka, 2011).

229   Compounds known to have antimicrobial properties such as alcohols and esters were also

230   identified in sclerites (Table S1). Many of these compounds have been isolated from terrestrial

231   plants. Nonetheless, the fact that gorgonians produce chemical compounds with antimicrobial

232   activities is not surprising. Multiple culture-based studies using extracts from several gorgonians

233   species have revealed the antimicrobial potential of these extracts (Kim1994, 2000; Jensen et al.,

234   1996; Alker, Smith & Kim, 2001; Alker et al., 2004; Ward et al., 2007). Nonetheless, in this

235   study, no relationship could be established between the origin of compounds (i.e., diseased or

236   healthy corals) and their microbial roles, as the majority of the potential roles of the compounds

237   could not be obtained from the scientific literature. Many of these compounds could be

238   precursors or intermediaries of potentially biologically relevant molecules that were trapped

239   within the sclerites during their formation. If indeed some of the compounds isolated from

240   sclerites are precursors of biologically active molecules, as well as environmental cues or internal

241   stressors, sclerites could be used as archives of metabolic pathways or past climatic and biotic

242   stress events.

243   **CONCLUSIONS**

244   Sclerites harbor a great SVOC diversity, the majority of which are novel to sea fans or any other

245   corals. However, a major drawback of the study is our inability to determine the origins of these

246   SVOCs. For instance, it is uncertain which SVOCs, if any, were synthetized by microbes

247   associated with sea fans. On the other hand, certain compounds may be derivatives from other

248   compounds. For instance, 1-undercene, 9 methyl, could be a derivative of other compounds such

249   as alcohols which have undergone esterification reactions to form esters that are import fractions

250   of lipids and steroids of living cells. In either case, not knowing the origin of the SVOCs hinder

251   our capacity to understand the underlying mechanism of their biological function. However, as

252   research progresses and our understanding of sclerite formation is improved, coupled with the

253   identification of other chemical compounds using different extraction solvents (polar and non-

254   polar),

255   our understanding of the roles the chemical compounds from sclerites and the corals in general

256   will improve. Yet, the present study represents a necessary first step in that process.

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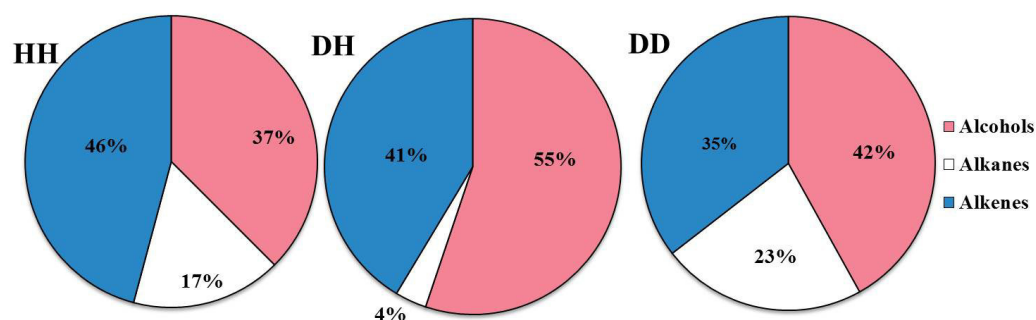
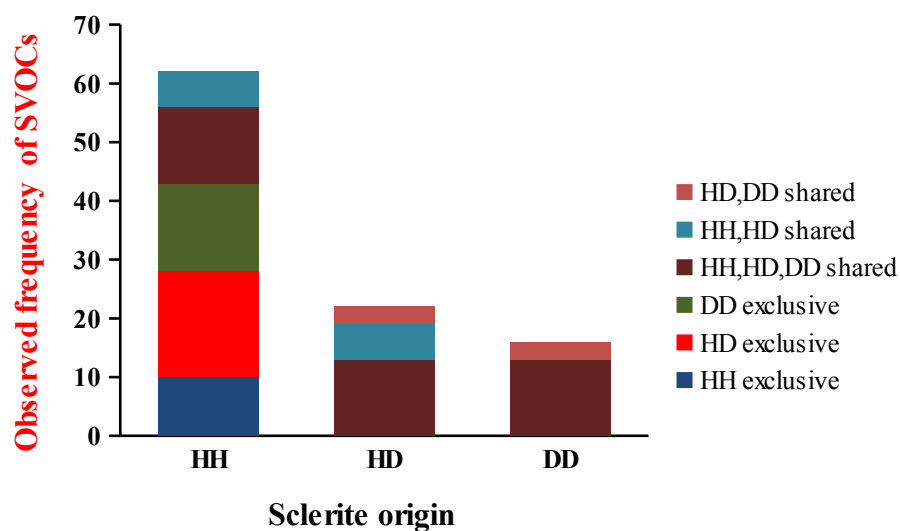
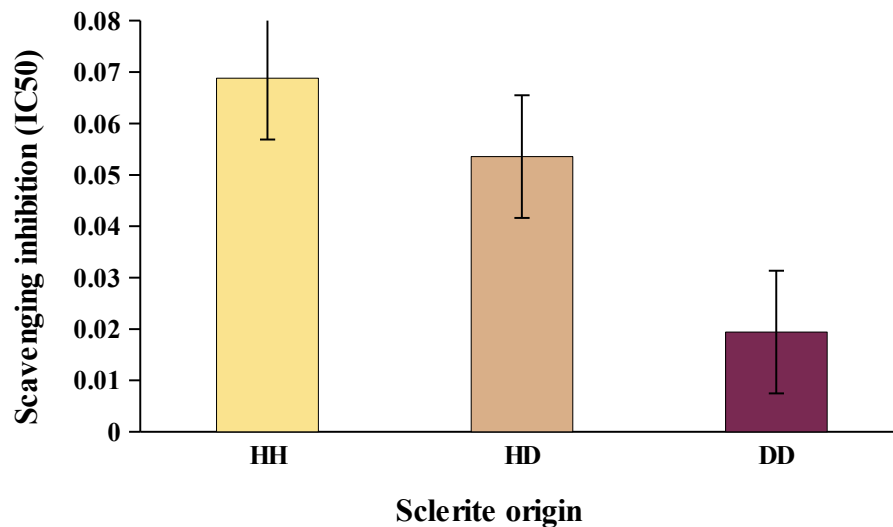

359 **Figure 1.** Percentages of the main functional chemicals in sclerites from healthy sea fans (HH),

360 from healthy tissue from diseased sea fans (HD), and diseased tissue from diseased sea fans

361 (DD).

362 **Figure 2.** Number of volatile organic compounds (VOCs) in sclerites from healthy sea fans (HH),
363 healthy tissue from diseased sea fans (HD), and diseased tissue from diseased sea fans (DD).

364   **Figure 3.** Average of free radical scavenging analysis expressed as IC_{50} of sclerites from healthy
365   sea fans (HH), healthy tissue from diseased fans (HD) and diseased tissue from diseased fans
366   (DD). Bars denote standard errors.