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Bioactivity Screening of Singapore Gorgonians: Antimicrobial Activity, Toxicity to *Artemia salina* and Efficacy against *Plasmodium berghei*

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ABSTRACT—Nine of ten gorgonian extracts from all four families tested showed antimicrobial activity. Inhibition of Gram positive bacteria was more widespread than that of Gram negative bacteria. Inhibition of the yeast *Saccharomyces cerevisiae* was confined to (and widespread within) the family Ellisellidae. No antifungal activity against *Aspergillus* sp. was found in all the extracts tested. Acute (6 hr) and chronic (24 hr) extract toxicity towards *Artemia salina* varied between extracts and showed no taxonomic correlations; compared to potassium dichromate, extract toxicity was low. Chronic exposure to the extracts was more effective in killing *A. salina*; chronic LD_{50} values ranged from 3.2 to 125.9 mg/ml. All ten extracts failed to extend survival, lower percentage parasitemia or affect parasite morphology in *Plasmodium berghei*-infected mice.

INTRODUCTION

The evolutionary success of Indo-Pacific octocorals, including gorgonians, has been attributed in part to the occurrence of toxic secondary metabolites which minimise predation, increase competitiveness and aid in reproduction (Sammarco and Coll, 1988; Coll, 1992). In particular, these metabolites help to prevent fouling (Bandurraga and Fenical, 1985), and deter feeding by various invertebrates (Lasker *et al.*, 1988; Harvell and Fenical, 1989; Vreeland and Lasker, 1989; Fenical and Pawlik, 1991; Van Alstyne and Paul, 1992) and fish (Pawlik *et al.*, 1987; Harvell and Fenical, 1989; Fenical and Pawlik, 1991). Green (1977) noted that the high diversity of fish on coral reefs would lead to higher competition for food resources. This would result in more specialised feeding habits, and in turn increase the grazing pressure by fish on coral reef marine invertebrates. One response of these sessile creatures would be to accumulate toxins or feeding deterrents. Gerhart and Coll (1993) reported that pukalide, a widely distributed gorgonian metabolite, caused emesis in fish at concentrations found in living gorgonian colonies. They also reported that pukalide contributed to ‘learned aversion’ in corallivorous fish. Anti-microbial metabolites may prevent potentially pathogenic bacterial or fungal build-up (Mitchell and Chet, 1975) on gorgonian colonies. Bacterial or fungal films may also be the precursor of secondary macro-fouling (Baier and Meyer, 1991). The ecological necessity of chemical defense in organisms that generally lack morphological defenses, and cannot physically escape predation increases the probability that extracts from gorgonians will contain useful bioactive compounds. In fact, out of five marine-derived compounds that have been, or are currently in clinical trials (McConnell *et al.*, 1994), two (pseudopterosins A and E) are from a gorgonian (*Pseudopterogorgia elisabethae*). Of nine compounds believed by the same authors to possess therapeutic potential (based on extensive biological and pharmacological tests), two were again from gorgonians (fuscosides A and B, from the Caribbean *Eunicea fusca*). The numerous novel bioactive compounds from gorgonians have been reviewed and discussed elsewhere (Goh and Chou, 1998).

The gorgonian fauna in Singapore is diverse and relatively abundant (Goh and Chou, 1994, 1996), with 31 species recognised. Compared with the 150 or more species of scleractinian or hard corals found on Singapore reefs, screening of gorgonians for bioactivity appears to be a poor choice. However, little or no predation by corallivorous fish has been observed in the field despite the fact that the Octocorallia, in comparison with the Scleractinia, are a potentially rich source of protein, fat, and carbohydrates (Coll, 1981). In addition, a review by Tursch *et al.* (1978) did not reveal significant levels of secondary compounds in the Scleractinia. These indicate the likelihood of metabolites within the tissue of gorgonians that deter feeding by fish.

Most gorgonian species are found within easily accessible SCUBA diving depths in Singapore, in contrast to other reefs in the region (Goh and Chou, 1994). This facilitates their collection and hence the study of their bioactivities. They also have a relatively high rate of growth (Goh and Chou, 1995) and regeneration (unpublished data), allowing the possibility of sustainable harvesting of these organisms if useful metabolites are found.

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MATERIALS AND METHODS

Collection and species tested

Ten commonly found species were tested for bioactivity. These were: from the Subergorgiidae, *Subergorgia suberosa* and *S. mollis*; from the Melithaeidae, *Acabaria robusta*; from the Ellisellidae, *Junceella* (*Dichotella*) cf. *gemmacea*, *Ctenocella* (*Umbracella*) cf. *umbraculum* and *Ctenocella* (*Umbracella*) sp. A; from the Plexauridae, *Euplexaura* cf. *pinnata*, *Echinogorgia* sp. A, *Echinogorgia* sp. C and *Echinogorgia* sp. E. Voucher specimens of all species are lodged in the Zoological Reference Collection of the Department of Biological Sciences, National University of Singapore. Gorgonian colonies were hand-collected from reefs south of the main island of Singapore using SCUBA. Each species was collected from only one reef site to prevent inter-locality differences that would complicate results. Any debris attached to the colonies was removed immediately at collection. When brought to the surface, metabolic activity was arrested by pouring liquid nitrogen over the collections of each species (hereinafter known as samples). These samples were then stored in ice until they were transferred to a freezer at –30°C in the laboratory.

Extract preparation

Frozen samples were ground, homogenised, and the weight of the ground sample noted. Samples were extracted overnight in acetone. The mixture of sample and solvent (acetone) was then filtered under vacuum. The residue was re-extracted in fresh acetone overnight while the filtrate was stored. This process was repeated so that a total of three extractions were performed. The three resulting filtrates were combined, and the acetone removed by evaporation under vacuum using a rotary evaporator, giving the crude extracts.

Extract concentrations

Test concentrations of extracts were prepared with respect to wet weights of crude samples. This enabled a standardised measurement that could be applied to compare extract activity in any bioassay. It also allowed direct comparisons between different organisms, and facilitated ecological inferences from bioassay results. Using extract dry wieghts would have allowed greater precision and reproducibility in assays, but it would not yield ecologically-relevant information.

To allow for comparisons of results with work using freeze-dried material, the wet and freeze-dried weights of the ten species tested were compared. Wet/dry weight ratios for each species are: *S. suberosa*: 1.6; *S. mollis*: 2.0; *A. robusta*: 1.4; *J.* (*Dichotella*) cf. *gemmacea*: 2.1; *C.* (*Umbracella*) cf. *umbraculum*: 1.7; *C.* (*Umbracella*) sp. A: 2.1; *Euplexaura* cf. *pinnata*: 2.8; *Echinogorgia* sp. A: 2.3; *Echinogorgia* sp. C: 2.2; *Echinogorgia* sp. E: 2.6.

Anti-microbial activity

The ten extracts were tested for toxicity against representative Gram-positive (*Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria, a yeast (*Saccharomyces cerevisiae*) and a fungus (*Aspergillus* sp.) using the disc assay method. An aliquot (equivalent to 0.5 g wet weight of tissue) of each test extract was spotted onto a filter paper disc (Difco concentration discs, 1/4", 1599-33) and allowed to air-dry. Three replicate discs were used for each test extract. Discs were placed on a lawn of the growing microbe and a positive assay was measured in terms of the diameter of a clear zone of microbial growth inhibition around the disc after overnight incubation at the appropriate temperature (bacteria: 37°C; yeast, fungus: 28°C). Controls using seawater and acetone were also included.

Invertebrate (*Artemia salina*) toxicity

A. salina (brine shrimp) cysts (Argentemia, Argent Chemical Laboratories, Redmond, WA, USA) were hydrated in a petri dish with filtered artificial seawater and placed under a lamp. Hatching occurred in about 12 hr. Sets of at least ten freshly hatched nauplii were collected using a Pasteur pipette and placed in separate wells of a multiwell container. Test solutions were made up to 2 ml and added to each of three wells giving a minimum total sample size of 30 brine shrimp for each test solution. Percentage mortalilty in each of the three replicate wells were consistent with each other; using the three replicates prevented unforseeable differences in individual wells from affecting results. Results from the three replicate wells were pooled for data analysis. Both positive (potassium dichromate solution, various concentrations) and negative (artificial seawater) controls were also set up. Mortality was scored in each well after 6 hr (acute exposure) and 24 hr (chronic exposure). Nauplii were counted under a microscope. A nauplius was considered dead if it lay immobile at the bottom of the well or if the appendages, e.g., the antennae, antennulae and mandibles were inactive. Results were analysed using the Reed-Muench method described in Teng (1993).

Anti-malarial activity

A preliminary test of all ten extracts was conducted on malaria-infected mice. Because of the limited amount of extract available, only one mouse was used for each extract. Mice were infected with *Plasmodium berghei* by direct blood passage and extract-treated two days post-infection (once *P. berghei* was detected in the blood). Extracts were administered as Tween 60/saline solutions (5%/0.9%) intraperitonially on three consecutive days. The survival time of the extract-treated mice was compared with control mice which were treated with PBS.

Subsequent re-collection and extraction using freeze-dried material of *S. mollis* and *Echinogorgia* sp. E (both of which showed T/C > 160 in the primary assay) was carried out to validate these preliminary results. The extraction protocol was modified from the original as follows: Six grams of ground, freeze-dried material from each of the two species were extracted overnight (with continuous shaking) in 20 ml acetone, filtered, and the filtrate collected. This was repeated twice to obtain three filtrates, which were combined. Acetone was removed from the combined filtrate by vacuum concentration under low heat until the extract was dry. An equivalent of 100 mg of each freeze-dried extract, resuspended in 0.5 ml of Tween 60/saline solution (5%/0.9%) was used to make up each daily dose. Five animals were used for each treatment, and five doses were planned for each animal, but because the mice appeared very weak on the fifth day, the last dose was withheld. Dosing began one day post-infection (once *P. berghei* was detected in the blood). A control with five mice given daily doses of the same volume of PBS was also set up. In addition to comparing survival time in treated versus control animals, blood smears allowed estimation of percentage parasitemia and parasite morphology monitoring in red blood cells of individual animals over the treatment period.

RESULTS

Anti-microbial activity

Anti-bacterial activity was recorded in representatives from all four families studied (Table 1). Of the ten species tested, only *Euplexaura* cf. *pinnata* was inactive against all microorganisms tested; in particular, it was inactive against the Gram-positive *B. subtilis* whereas all other extracts inhibited this bacterium. Both subergorgiid species, as well as *J.* (*Dichotella*) cf. *gemmacea* and *Echinogorgia* sp. E also inhibited the Gram-negative bacterium *E. coli*. Zones of inhibition around the extract-loaded discs ranged (where inhibitory activity was present) from 7.7 mm to 12 mm in diameter. Activity against the yeast *S. cerevisiae* was confined to members of the family Ellisellidae; moreover, all three species of this family tested showed activity against the yeast, with zones of inhibition rang-

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Table 1. Antimicrobial activity of crude gorgonian extracts using the standard disc diffusion assay

EXTRACT	Inhibition diameter (mm) (mean of 3 replicates each)			
	B. subtilis	*E. coli*	*S. cerevisiae*	*Aspergillus* sp.
Subergorgiidae				
S. suberosa	10.3	11	0	0
S. mollis	12	9.5	0	0
Melithaeidae				
A. robusta	9	0	0	0
Ellisellidae				
J. (*Dichotella*) cf. *gemmacea*	10.3	9	16.7	0
C. (*Umbracella*) cf. *umbraculum*	11	0	12.3	0
C. (*Umbracella*) sp. A	9.7	0	14.3	0
Plexauridae				
Echinogorgia sp. A	8	0	0	0
Echinogorgia sp. C	7.7	0	0	0
Echinogorgia sp. E	10.3	12	0	0
Euplexaura cf. *pinnata*	0	0	0	0
Controls				
Seawater	0	0	0	0
Acetone	0	0	0	0

Table 2. Percentage mortality of *A. salina* larvae to acute (6 hr) and chronic (24 hr) exposure to various concentrations of crude gorgonian extracts

EXTRACT	Extract Conc. (mg/ml)					
	6 hr Exposure			24 hr Exposure		
	10	50	100	10	50	100
Subergorgiidae						
S. suberosa	1.5	12.5	31.5	1.5	60.3	100
S. mollis	0	0	6.8	11	34	79
Melithaeidae						
A. robusta	0	0	2.9	1.1	58.6	89.3
Ellisellidae						
J. (*Dichotella*) cf. *gemmacea*	0	2.1	6.8	2	17.7	58.9
C. (*Umbracella*) cf. *umbraculum*	0	0	2.7	2.1	31.5	90.7
C. (*Umbracella*) sp. A	0	16.3	40.9	32.7	87.2	99.2
Plexauridae						
Echinogorgia sp. A	0	0	0	0	1	15.1
Echinogorgia sp. C	1.3	1.8	4.1	3.7	47.1	91.3
Echinogorgia sp. E	0	13	53.1	36.4	91.9	100
Euplexaura cf. *pinnata*	0	0	7.3	1.2	34.4	74.2

Note: 1. A negative control using artificial seawater showed 0% mortality for both 6 hr and 24 hr exposures.
2. A positive control using various dilutions of potassium dichromate solution is presented in Table 3.

ing from 12.3 mm to 16.7 mm. None of the ten species tested was active against the fungus *Aspergillus*. Both controls did not inhibit growth of any of the micro-organisms tested.

Invertebrate (*Artemia salina*) toxicity

Percentage mortality of *A. salina* larvae varied from extract to extract (Table 2). In the 6 hr exposure, three extracts (from three different families) were markedly more toxic than the other seven extracts at 50 mg/ml and 100 mg/ml: *S. suberosa* (Subergorgiidae), *C.* (*Umbracella*) sp. A (Ellisellidae), and *Echinogorgia* sp. E (Plexauridae). These three extracts were again among the most toxic in the 24 hr exposure at the same concentrations; toxicity of the extracts of *A. robusta*, *C.* (*Umbracella*) cf. *umbraculum* and *Echinogorgia* sp. C were comparable to the three after this longer exposure. Percentage mortality of extracts at corresponding concentrations showed a general increase with exposure time, although the magnitude of increase varied with species. In general, for both acute and chronic exposures, increasing the concentration from 10 mg/ml to 50 mg/ml caused a disproportionally greater response than an increase from 50 mg/ml to 100 mg/ml. In particular, after 24 hr exposure, *A. robusta*, *Euplexaura* cf. *pinnata* and *S. suberosa* showed very significantly higher rates of increase from 10-50 mg/ml than from 50-100 mg/ml (53.8, 29.2, and 22.7-fold increases versus 1.5, 2.2, and 1.6-fold increases, respectively).

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Extract toxicity towards *A. salina* was generally low when compared to potassium dichromate (Tables 2, 3), especially in the shorter (6 hr) exposure period: the highest mortality (53.1%; seen in *Echinogorgia* sp. E at 100 mg/ml) never exceeded mortality levels seen in 0.6 mg/ml potassium dichromate solution. Where mortality was recorded in extracts for the acute exposure assay, mortality rates were comparable to that of 0.2-0.6 mg/ml potassium dichromate solution. Chronic (24 hr) exposure to all concentrations of potassium dichromate solution tested elicited a 100% mortality rate except at 0.025 mg/ml, where mortality was 79%; in the gorgonian extracts, mortality ranged from 0% to 36.4% at a concentration of 10 mg/ml.

Table 3. Percentage mortality of *A. salina* to acute (6 hr) and chronic (24 hr) exposure to various concentrations of potassium dichromate solution (positive control)

Dilution (mg/ml)	Exposure Time	
	6 hr	24 hr
4.0	100	100
2.0	100	100
1.0	99	100
0.8	97	100
0.6	54	100
0.4	4	100
0.2	0	100
0.1	0	100
0.05	0	100
0.025	0	79

The chronic (24 hr) LD_{50} values for eight of the extracts [except *Junceella* (*Dichotella*) cf. *gemmacea* and *Echinogorgia* sp. A, where data collected did not permit estimation of the LD_{50}] were estimated graphically using the Reed-Muench method (Teng, 1993). LD_{50} values ranged from 3.2 to 125.9 mg/ml; standard deviations were negligible (Table 4).

Anti-malarial activity

The preliminary anti-malarial assay indicated a T/C of 167 for both the *S. mollis* and *Echinogorgia* sp. E extracts (Table 5). Subsequent treatment and analysis of mice using new preparations of these extracts failed to reproduce these T/C percentages (Table 6a). Mice treated with either extract did not survive significantly longer than control mice treated only with PBS. The percentage parasitemia of red blood cells was also compared between treatments and control, and found to be not significantly different (Table 6b). Parasite morphology also did not vary significantly between control and treatments.

Table 4. Chronic (24 hr) LD_{50} for *A. salina* larvae exposed to crude gorgonian extracts

EXTRACT	$LD_{50} \pm$ SD (mg/ml)	n (mean of 3 replicates)
Subergorgiidae		
S. suberosa	56.2 ± 0.002	49.3
S. mollis	125.9 ± 0.002	40.7
Melithaeidae		
A. robusta	56.2 ± 0.002	61
Ellisellidae		
C. (*Umbracella*) cf. *umbraculum*	125.9 ± 0.001	47
C. (*Umbracella*) sp. A	5.0 ± 0.006	51.3
Plexauridae		
Echinogorgia sp. C	112.2 ± 0.002	76
Echinogorgia sp. E	3.2 ± 0.003	41.7
Euplexaura cf. *pinnata*	125.9 ± 0.002	43.3

Note: 1. LD_{50} was estimated graphically using the Reed-Muench method described in Teng (1993).
2. The LD_{50} of two species, *J.* (*D.*) cf. *gemmacea* and *Echinogorgia* sp. A could not be estimated using this method.

Table 5. Effect of crude gorgonian extracts on survival of *P. berghei* - infected mice

EXTRACT	Survival time after infection (d)	Treatment / Control (T/C) percentage
S. suberosa	6	100
S. mollis	10	167
A. robusta	7	117
J. (*Dichotella*) cf. *gemmacea*	6	100
C. (*Umbracella*) cf. *umbraculum*	6	100
C. (*Umbracella*) sp. A	6	100
Echinogorgia sp. A	6	100
Echinogorgia sp. C	6	100
Echinogorgia sp. E	10	167
Euplexaura cf. *pinnata*	6	100
Control (PBS)	6	–

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Table 6. Effect of *S. mollis* and *Echinogorgia* sp. E crude extracts on *P. berghei* - infected mice

a. Survival

EXTRACT	Mean survival time after infection (d)	Treatment / Control (T/C) percentage
S. mollis	5.8	100
Echinogorgia sp. E	6.0	103
Control (PBS)	5.8	100

b. Red blood cell infection rate

EXTRACT	Mean percentage infected red blood cells				
	Day 1	Day 2	Day 3	Day 4	Day 5
S. mollis	2.6	12.2	32.8	70.8	75.0
Echinogorgia sp. E	2.2	13.0	29.4	74.4	87.0
Control (PBS)	2.4	12.0	37.0	63.0	91.7

DISCUSSION

The present study utilises the strategy of directed screens to test for toxicity of crude extracts towards micro-organisms (representative gram-positive and gram-negative bacteria, a fungus, and a yeast), and an invertebrate (*Artemia salina*). Whole organism assays, rather than more selective enzyme or receptor-based bioassays were used since the non-specificity of these assays would enable detection of a wider range of active substances (Garcia-Alonso *et al*., 1993). Anti-microbial activity has long been used as an indication of bioactivity (Burkholder and Burkholder, 1958; Thomson *et al*., 1985; Kim, 1994; Jensen *et al*., 1996), as has the *A. salina* screen (McLaughlin *et al*., 1993; Teng, 1993 and references therein). Both these assays have the advantages of being fast, cheap, and simple. In addition, both the anti-microbial assay and the *A. salina* toxicity assay lend themselves to statistical analyses. These assays were conducted to map bioactivity in Singapore gorgonians as a preliminary analysis of the potential applications of these extracts. A more specialised screen involving in vivo action against malarial parasites in mice was also carried out. Angerhofer *et al*. (1992a) were of the opinion that marine natural products were under-explored as a resource for novel anti-malarial drugs - most screening for anti-malarials have been carried out on plant extracts. The only known compounds of marine origin showing anti-malarial activity are sesquiterpenes from the sponge *Acanthella klethra* (Angerhofer *et al*., 1992b). Sesquiterpenoid compounds are commonly found in gorgonians (Scheuer, 1973), providing a rationale for this assay.

Anti-microbial activity

Like Bergquist and Bedford (1978) and Reichelt and Borowitzka (1984) in their studies of sponges and marine algae, respectively, anti-bacterial activity was found in a wide taxonomic spectrum of gorgonians in Singapore. Extracts from the Ellisellidae were different from the subergorgiids, melithaeids and plexaurids in that, without exception, all three ellisellid extracts inhibited eukaryotic yeast (*S. cerevisiae*) cells, with relatively large inhibition diameters (12.3-16.7 mm; Table 1). This could indicate a distinct class of bioactive compounds present only in this family. Confirmation of this chemotaxonomic distinction, the identification of the specific compound(s) responsible, and their ecological roles would make interesting follow-up studies. In extracts of *A. robusta*, *C.* (*Umbracella*) cf. *umbraculum*, *C.* (*Umbracella*) sp. A, and *Echinogorgia* spp. A and C, all of which show selective inhibition of Gram positive but not Gram negative bacteria, it is possible that the active compounds act on the bacterial peptidoglycan (Wilkinson, 1986) in a manner similar to that of penicillin, which only inhibits Gram positive but not Gram negative bacterial strains. The eukaryotic *S. cerevisiae* and *Aspergillus* also do not possess this layer and would be insensitive to compounds that affect this layer.

Four previous studies (Burkholder and Burkholder, 1958; Burkholder, 1973; Kim, 1994; Jensen *et al*., 1996) reporting anti-microbial activity in gorgonian extracts have been published. The average inhibition diameters (only for "positive" extracts) in disc diffusion assays against the bacteria *B. subtilis*, *E. coli*, and the yeast, *S. cerevisiae* in the present study were 9.8 mm, 10.4 mm and 14.4 mm, respectively. This compares to averages worked out from Burkholder's (1973) results: 6.1 mm, 8.2 mm, and 2.6 mm, against *B. subtilis*, *E. coli*, and *Candida* sp. (a different species of yeast), respectively. It should be noted that although activity against *S. cerevisiae* far exceeded that against *Candida*, a larger proportion of the extracts tested by Burkholder (1973) showed at least some activity against the yeast compared to its restriction to the three ellisellid species here. Kim (1994) found that average inhibition diameters of the Gram positive bacteria *B. megaterium* and *S. aureus* was < 1 mm for both. Non-marine Gram negative bacteria were not tested in Kim's non-polar extracts. Jensen *et al*. (1996) employed mostly naturally-occurring marine strains, making comparisons with purely clinical strains inappropriate.

The presence of compounds inhibiting the Gram positive

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bacterium *B. subtilis* appears to be widespread in that all four gorgonian families assayed showed this activity; only one extract (*Euplexaura* cf. *pinnata*) did not inhibit this bacterium (Table 1). Inhibition of the Gram negative *E. coli* was less widespread, with fewer extracts causing growth inhibition. The pattern of more widespread activity in marine extracts against Gram positive bacteria compared to Gram negative strains is consistent with the observations of Burkholder and Ruetzler (1969), McCaffrey and Endean (1985), Thomson *et al.* (1985), Kim (1994) and Jensen *et al.* (1996), but opposite to that of Bergquist and Bedford (1978); Amade *et al.* (1982) found an equal number of both groups of bacteria being inhibited by the sponge extracts they tested.

If we consider this activity to have a direct ecological function of preventing bacterial growth, these results are unexpected since the majority of bacteria occurring naturally in the marine environment are not Gram positive but Gram negative (Burkholder, 1973). The question then arises as to why compounds active against bacterial types that gorgonians would normally encounter may (it is conceded that *E. coli* is a terrestrial bacterium that gorgonians would not normally encounter, but for the purpose of discussion, and since no marine bacterial strains were available for assay, some generalisation is necessary) be absent. Jensen *et al.* (1996) proposed that the significantly greater efficacy of gorgonian extracts against Gram positive strains may simply be due to the lack of a protective outer membrane in the Gram positive cell. The benefits of bacterial-gorgonian symbiosis may also account for this apparent lack of activity against Gram negative (compared with Gram positive) bacteria. Preliminary transmission electron micrographs have indicated the presence of bacteria within the tissue of gorgonians from Singapore that appear to be symbiotic to their gorgonian hosts (no macro- or microscopic evidence of pathogenicity; C.R. Wilkinson and J. Vacelet, pers. comm.). If the relationship between bacteria and host is analogous to that of zooxanthellae and hermatypic corals, with bacteria providing nutritional benefit to the host, then it makes ecological sense for gorgonians not to produce metabolites that would inhibit these potential bacterial symbionts. On the other hand, microbial colonisation has been implicated as the precursor of secondary macrofouling (Baier and Meyer, 1991), and possession of selective anti-microbial metabolites that deter colonisation by microfouling organisms would also prevent subsequent macrofouling. Low percent cover of fouling organisms on sponges in San Diego was positively correlated with the possession of anti-bacterial metabolites (Thomson *et al.*, 1985). No similar trend between anti-bacterial activity and fouling was observed in the ten gorgonian species tested here. It has, however, been observed that in general, secondary macrofouling on gorgonians in Singapore only occurs where living gorgonian tissue has been scraped off or abraded. This provides circumstantial evidence that metabolites produced within the living tissue of gorgonians prevent fouling growth. Work on gorgonians elsewhere (Bandurraga and Fenical, 1985; Rittschof *et al.*, 1985) also appear to support this hypothesis.

Invertebrate (*Artemia salina*) toxicity

No distinction in toxicity between gorgonian families towards *A. salina* was observed (Table 2), suggesting that compounds toxic to *A. salina* are not phylogenetically linked. This contrasts with results from the antimicrobial assays where activity against *S. cerevisiae* was confined to extracts from the Ellisellidae. In general, mortality increased between acute and chronic exposure for extracts at the three concentrations tested (except for *S. suberosa* and *Echinogorgia* sp. A at 10 mg/ml). The significantly greater efficacy of *S. suberosa*, *C.* (*Umbracella*) sp. A and *Echinogorgia* sp. E in the acute exposure assay may indicate the presence of compounds with fast-acting modes of action. This contrasts with the low mortality seen in *A. robusta*, *C.* (*Umbracella*) cf. *umbraculum* and *Echinogorgia* sp. C which exhibited significant toxicity levels only after chronic (24 hr) exposure. In particular, at 100 mg/ml, increases from 6 hr to 24 hr exposures were more pronounced for *A. robusta* (approx. 31-fold increase), *C.* (*Umbracella*) cf. *umbraculum* (approx. 34-fold increase) and *Echinogorgia* sp. C (approx. 22-fold increase) in comparison with the relatively gradual increases in *S. suberosa*, *C.* (*Umbracella*) sp. A and *Echinogorgia* sp. E (approx. 3.2, 2.4, and 1.9-fold increase, respectively). This sudden, pronounced increase in mortality in the former three extracts may indicate a slow acting toxin or one where sufficient quantities need to be absorbed before it is effective and contrasts with action of *S. suberosa*, *C.* (*Umbracella*) sp. A and *Echinogorgia* sp. E. Increase in mortality with increased extract concentration generally followed a typical sigmoidal pattern. This concentration-dependent activity suggests a receptor-based mode of action of the toxic compound(s) in the extracts. Lee *et al.* (1981) also recorded similar patterns of increase in toxicity of octocoral cembranolides with both concentration and exposure time against a rotifer and an amphipod species.

These toxicity assays were carried out at apparently very high concentrations (10-100 mg/ml) in order to obtain reasonable dilution profiles of the bioactivity. It must be remembered that these concentrations are with respect to crude, wet samples. Furthermore, any bioactive metabolite present would represent only a percentage (often small) of the crude extract. That the potassium dichromate solution (positive control; Table 3) elicited a much higher rate of mortality in the *A. salina* assay than any of the extracts tested (Table 2) is expected since the comparison is between a pure compound and a crude, wet extract. Thomson *et al.* (1985) reported positive assay results against *A. salina* for purified metabolites and extracts from sponges collected off the coast of California. Test concentrations in their study ranged from 10-100 μg/ml. McCaffrey and Endean (1985) found that Compound A from a *Callyspongia* sp. was toxic to *A. salina* larvae at 20 g/ml (extremely high for a pure compound; probably a typographical error which should read 20 μg/ml). No percentage values were provided for the mortalities reported in both studies; a positive assay was described simply as causing ‘death’ in *A. salina*. No direct quantitative comparisons can therefore be made with the present study.

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Anti-malarial activity

The initial encouraging results (Table 5) for extracts of *S. mollis* and *Echinogorgia* sp. E could not be repeated in the subsequent experiment. No significant therapeutic effects were detected in extracts of the two gorgonians, both in terms of survival time (Table 6a) and red blood cell infection rate (Table 6b). It is possible that the preliminary results were false positives; only one mouse was used for each preliminary assay, and the survival of the two mice injected with extracts of *S. mollis* and *Echinogorgia* sp. E could have been due to individual mice variability rather than extract efficacy. The subsequent test with six replicate mice for each treatment is therefore a more accurate reflection of extract efficacy.

To date, except for a preliminary study (Goh *et al*., 1995), no work on gorgonian bioactivity in Singapore has been carried out; nor have any of their chemical structures been elucidated. It must be emphasized that the bioassays that have been carried out are by no means exhaustive or comprehensive. It is possible that useful species may not be detected if screening is dependant on such limited bioassays. However, it must be remembered that the objective of the present study is to map the occurrence of bioactivity in crude extracts of gorgonians in Singapore using simple primary bioassays. Results will enable future workers to narrow down the search for novel bioactive compounds to species that have shown some activity in these assays, and to follow up on the leads generated in the areas of pharmaceutical or agrichemical screening, natural products chemistry, pharmacology, or chemical ecology.

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