



# Antibiotic-resistant bacteria inhibited by extracts and fractions from Brazilian marine sponges

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**RESUMO: “Bactérias resistentes a antibióticos inibidas por extratos e frações de esponjas marinhas do Brasil”.** O número crescente de bactérias resistentes aos antibióticos tem se tornado um sério problema médico nos últimos anos. As esponjas marinhas são uma fonte rica em compostos bioativos e muitas espécies podem ser úteis para o desenvolvimento de novos antimicrobianos. Esse estudo descreve uma triagem *in vitro* de esponjas para a pesquisa de novas substâncias contra bactérias resistentes. Os extratos de esponjas foram testados sobre 44 estirpes bacterianas, incluindo quatorze resistentes a antibióticos. Dez entre doze espécies de esponjas apresentaram atividade em um ou mais bioensaios. Os extratos aquosos de *Cinachyrella* sp. e *Petromica citrina* apresentaram um amplo espectro de ação sobre estirpes bacterianas resistentes, tais como, *Staphylococcus aureus*, *Staphylococcus* coagulase-negativos e *Enterococcus faecalis*. O extrato aquoso de *P. citrina* foi fracionado e a fração aquosa apresentou atividade inibitória sobre estirpes de *Staphylococcus*. Esta fração, na concentração do CMI (16 µg/mL), demonstrou efeito bactericida sobre células de *S. aureus* na fase exponencial de crescimento. O mecanismo de ação da fração ainda não foi elucidado, mas nós observamos que esta afeta a síntese protéica de *Staphylococcus*. Nossos resultados demonstraram pela primeira vez que *Petromica citrina* é uma fonte potencial de novas drogas para o tratamento de infecções causadas por bactérias resistentes.

**Unitermos:** atividade antibacteriana, bactéria resistente a antibióticos, esponjas marinhas, *Petromica citrina*.

**ABSTRACT:** The growing number of bacterial strains resistant to conventional antibiotics has become a serious medical problem in recent years. Marine sponges are a rich source of bioactive compounds, and many species can be useful for the development of new antimicrobial drugs. This study reports the *in vitro* screening of marine sponges in the search for novel substances against antibiotic-resistant bacteria. Sponge extracts were tested against 44 bacterial strains, including fourteen antibiotic-resistant strains. Ten out of the twelve sponge species studied showed activity in one or more of the bioassays. Aqueous extracts of *Cinachyrella* sp. and *Petromica citrina* showed a large action spectrum over resistant-bacteria such as *Staphylococcus aureus*, coagulase-negative staphylococci and *Enterococcus faecalis*. Aqueous extract of *P. citrina* was fractionated and aqueous fraction showed a greatest inhibitory activity on *Staphylococcus* strains. In addition, this fraction demonstrated a bactericidal effect on exponentially growing *S. aureus* cells at the MIC (16 µg/mL). The mechanism of action of bioactive fraction is still unclear, but we showed that it affect protein biosynthesis of *Staphylococcus*. Our results demonstrated for the first time that *P. citrina* is a potential source of new drugs for the treatment of infections by antibiotic-resistant bacteria.

**Keywords:** antibacterial activity, antibiotic-resistant bacteria, marine sponges, *Petromica citrina*.

## INTRODUCTION

The increasing prevalence of multi-resistant bacteria made the search of new antibacterial agents an important strategy for the establishment of alternative therapies in difficult handling infections (Berlinck

et al., 2004). The incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) infections continues to rise in National Nosocomial Infections Surveillance System hospitals (Farr, 2006). Methicillin-resistant staphylococci infections mainly caused by *S. aureus* (MRSA strains)

and by coagulase-negative staphylococci (CNS) such as *Staphylococcus epidermidis* (MRSE) and *Staphylococcus haemolyticus* (MRSH) isolates have increased over the last two decades (Rice, 2006). These antibiotic-resistant strains of *Staphylococcus* spp. are the pathogens most frequently isolated from nosocomial bacteremias, with an attributable mortality rate ranging from 13% for CNS to 42% for MRSA. In these cases, the therapy is generally limited to the use of vancomycin and teicoplanin (Diekema et al., 2001; Weber et al., 2003). However, some *Staphylococcus* strains resistant to glycopeptides have been reported in Brazil and other countries. Community-acquired MRSA is also increasing (Zetola et al., 2005). The proportion of enterococci resistant to vancomycin continues to rise in hospital settings, with the overwhelming majority of infections due to *Enterococcus faecium* (NNIS, 2004). The search for new drugs against antibiotic-resistant bacteria is an area of great medical importance (Rice, 2006).

The potential of marine organisms as a source of new substances is huge and has been barely investigated. Marine species comprise approximately half of the total global biodiversity, and thus the sea offers many potential sources for discovery of novel compounds (Aneiros & Garateix, 2004). The world ocean is considered the largest reservoir of natural molecules remaining to be evaluated for drug activity (Gerwick, 1987).

Marine sponges (Porifera) are sessile, filter-feeding invertebrates with limited physical defenses and highly developed chemical defenses against predators and larval settlement of other sessile organisms (e.g., Pawlik et al., 1995; Becerro et al., 2003; Wulff, 2006; Thoms & Schupp, 2007). Marine sponges are among the richest sources of pharmacologically-active chemicals from marine organisms; they are known to produce many different compounds with antitumoral, antiviral, antifungal, and antibacterial activities (e.g., Kobayashi, 2000; Berlinck et al. 2004; Blunt et al. 2007; Selegim et al. 2007).

Screenings of marine sponges for antibacterial activity led to the isolation and characterization of a wide range of active compounds, including some promising therapeutic leads (Munro et al. 1999; Berlinck et al. 2004; Mayer & Hammann, 2004; Sipkema et al., 2005; Moura et al., 2006). Up to 800 antibiotic compounds have been isolated from marine sponges (Torres et al., 2002). In the continuing effort by the marine natural products community, many antibacterial agents have been identified from sponges. Despite the high number of marine natural products discovered, none of them has yet led to an antibacterial product, but many are currently under investigation. Recently, some examples of substances with antibacterial activity, as manzamine A and psammaphin A, isolated from marine sponges were reviewed for our group (Laport et al., 2009).

In contrast with the great number of compounds with general antibacterial activity found in sponges or

associated microorganisms, relatively few substances active against antibiotic-resistant bacteria have been isolated from sponges. The Arenosclerins A-C and haliclonyclamine E isolated from the Brazilian sponge *Arenosclera brasiliensis* inhibited growth of twelve antibiotic-resistant bacteria isolated from a hospital (Torres et al., 2002). Crude extracts of less than 3% of 215 Brazilian sponge species tested showed activity against antibiotic-resistant bacteria (Selegim et al., 2007).

So far, pure compounds isolated from one specie (Torres et al., 2002) and crude extracts of 215 species of Brazilian sponges have been investigated through bioassays against antibiotic-resistant bacteria (Selegim et al., 2007). Although a growing number of studies investigated the cytotoxic, neurotoxic and antimicrobial activities of sponges from the Brazilian coast (Muricy et al., 1993; Rangel et al., 2001; Monks et al., 2002; Berlinck et al., 2004; Selegim et al., 2007), most Brazilian sponges remain unstudied. The city of Rio de Janeiro harbours a sponge fauna distinct from that of other areas in Southeastern Brazil such as Arraial do Cabo and São Sebastião, where other screenings have been conducted (Monteiro & Muricy, 2004). Many species from Rio de Janeiro therefore have not been tested so far for antibacterial activities, particularly against antibiotic-resistant bacteria. In this study we performed a screening for antibacterial activity of crude extracts or active fraction of marine sponges from Brazil against bacterial strains of medical importance.

## MATERIAL AND METHODS

### Sponge sampling

Samples of twelve sponge species (Table 1) were collected through SCUBA diving from 4-20 m depth at Praia Vermelha (22° 57' S - 43° 09' W) and Cagarras Archipelago (23° 01' S - 43° 11' W), both located in Rio de Janeiro, Southeastern Brazil.

**Table 1.** Species of marine sponges screened for antibacterial activity in this study.

Species	Family
<i>Cinachyrella</i> sp.	Tetillidae
<i>Cliona celata</i> complex	Clionidae
<i>Dragmacidon reliculatus</i>	Axinellidae
<i>Geodia corticostylifera</i>	Geodiidae
<i>Guitarra sepia</i>	Guitarridae
<i>Haliclona</i> sp.	Chalinidae
<i>Hymeniacidon heliophila</i>	Halichondriidae
<i>Mycale microsigmatosa</i>	Mycalidae
<i>Oceanapia nodosa</i>	Phloeodictyidae
<i>Petromica citrina</i>	Desmanthidae
<i>Polymastia janeirensis</i>	Polymastiidae
<i>Tedania ignis</i>	Tedaniidae

### Crude sponge extract preparation

After collection, the specimens were processed under aseptic conditions as follows: macro-organisms were removed from the fresh material and the specimens were weighted; 5-10 g of sponge was macerated in sterile distilled water or ethanol to obtain a final concentration of approximately 1 g/mL of each crude extract. Extracts were centrifuged at 5.000 xg for 5 min and the supernatant was filtered (Millipore 0.22 µm) and stored at 4 °C.

### Bacterial strains and culture conditions

Eighteen reference (Table 2) and 26 clinical (Table 3) strains were used as test organisms in the antibacterial activity assays. Bacteria were grown in BHI (Brain Heart Infusion - Difco) medium at 37 °C for 18 h.

### Assay for antibacterial activity

Antibacterial activity of crude extracts from sponges (200 mg/mL) or fractions from bioassay-guided fractionation was determined against the growth of bacteria by the agar-diffusion method as described previously by Giambiagi-deMarval et al. (1990). Briefly, 20 µL of the crude extracts or fractions were spotted on BHI-agar. After growth of each strain at 37 °C, 105 cells of the indicator strains in BHI soft agar were poured over the plates. Plates were incubated at 37 °C for 18 h and the inhibition zones around the spotted strain were observed. Inhibition zones  $\geq 8$  mm were considered indicative of inhibitory activity. Since the aqueous extract of *Petromica citrina* presented the largest action spectra and very little data about this species were published, it was selected for subsequent analysis using the indicator strains of genus *Staphylococcus*.

### Extraction and bioassay-guided fractionation

The frozen sponge *P. citrina* (4.86 g) was lyophilized and extracted with 200 mL of methanol/water (9:1) at room temperature. The crude extract was partitioned between n-hexane (9x 50 mL), chloroform (5x 50 mL) and ethyl acetate (5x 50 mL). The solutions were completely evaporated (in a rotatory evaporator under reduced pressure) to give the respective fractions, n-hexane (897.8 mg), chloroform (271.7 mg), ethyl acetate (255.1 mg) and aqueous phase (1.09 g). All organic solvents used were of analytical grade. Antibacterial activity of all filtered fractions (125 mg/ml) was assayed as described previously by Giambiagi-deMarval et al., (1990).

### Determination of Minimum Inhibitory Concentration (MIC)

Antimicrobial activity is measured by determined the smallest amount of agent needed to inhibit the growth

of a test microorganism, a value called MIC. The MIC was evaluated by the dilution method in Mueller-Hinton broth (Difco) medium, according to CLSI (2006), for the sponge selected and with different concentrations of active fraction. Bacteria (104 CFU/mL) were inoculated in the broth with active fraction, and incubated at 37 °C for 18 h.

### Determination of Minimum Bactericidal Concentration (MBC)

MBC is the smallest concentration of the drug necessary for elimination of 99.9% of the microorganisms tested. The MBC was determined after the MIC assays. In tubes where MIC results showed no bacterial growth, an aliquot of 0.1 mL was seeded in Mueller-Hinton agar without addition of drugs and the bacterial growth was evaluated for the MBC determination. After 18 h at 37 °C, if MIC = MBC or if MBC = 1, 2 or 3 dilutions above MIC, the activity is considered bactericidal (Isenberg, 1992).

### Effects of antibacterial active fraction from *P. citrina*

The active fraction (final concentration MIC value) from *P. citrina* was sterile filtered and it was added to early log growth phase cells of *S. aureus* 42AE in 20 mL BHI medium. A culture in early log growth phase cells of *S. aureus* 42AE in 20 mL BHI medium without antibacterial fraction or sterile BHI medium were added to control. The cultures were then either maintained at 37 °C and at 1 h intervals, the O.D. of 600 nm were determined.

### Analysis of protein synthesis by SDS-PAGE

Overnight cultures of *S. aureus* ATCC 29213, *S. aureus* 42AE, *S. epidermidis* ATCC 1228 or *S. epidermidis* 70S were diluted in BHI to 107 CFU/mL and incubated for 1 h at 37 °C. For labeling, cells were concentrated to 108 CFU/mL in a methionine-free medium (MEM, Gibco) containing 200 µCi/mL of [35S] methionine (Amersham) and subject to active fraction addition (16 µg/mL) at 37 °C. Samples controls were maintained at 37 °C without inhibitory fraction addition. In all the tests, the cells were labeled for 1 h and collected by centrifugation at 12,000 g for 5 min. The cells were lysed with the addition of 40 ng/mL of lysostaphin (Sigma) during 2 h at 37 °C. After incubation period, equal volumes of 0.5 M Tris-HCl (pH 7.2) buffer containing 4% SDS, 10% β-mercaptoethanol, 20% glycerol and 0.1 % bromophenol blue were added, and the samples were boiled for 5 min (Laport et al., 2001). Aliquots from each set of samples were subjected to SDS-10% polyacrylamide gel electrophoresis (PAGE) as described by Ausubel et al., (1997), loading an amount corresponding to an equal number of cells onto each lane. Gels were stained with Coomassie blue, destained, dried, and exposed to X-ray film.

## RESULTS

### Antibacterial activity

Ten of the twelve sponge species tested demonstrated inhibitory activity against at least one bacterial strain (Tables 2-3). Only *Hymeniacidon heliophila* and *Oceanapia nodosa* were inactive against all strains tested. *Cliona celata* complex was active against *Corynebacterium fimi* NCTC 7547 only (Table 2). Six sponge species showed significant antibacterial activity against antibiotic-resistant bacteria, including, VRE and MRSA strains (Table 3).

Aqueous extracts of *Petromica citrina* and *Cinachyrella* sp. presented the largest action spectra: they inhibited 64% and 50% of the 44 bacterial strains tested, respectively (Tables 2-3). Most strains inhibited by these sponges were Gram-positive cocci, including VRE, MRSA, MRSE and various others CNS strains.

The aqueous extract of *P. citrina* inhibited growth of nineteen clinical strains, being ten antibiotic-resistant strains, including *S. aureus*, CNS, *Enterococcus faecalis* and *Escherichia coli*. As this extract showed the best spectra on antibiotic-resistant strains and very little data about this specie were published, it was selected for subsequent analysis.

### Extraction and bioassay-guided fractionation

Antibacterial activity of all fractions from aqueous extract of *P. citrina* was assayed on reference and clinical strains, being the active aqueous fraction. The results showed a similar spectrum of inhibition those observed with crude extract, being inhibited mainly *S. aureus* and CNS strains. Moreover, inhibitory activity of active fraction was higher at about 0.5-fold when compared to inhibitory activity of crude extract. The Figure 1 shows the antibacterial effect of aqueous fraction on *S. aureus* 42AE.

### MIC and MBC determination

The MIC of active fraction of *P. citrina* over *S. aureus* 42AE was 16 µg/mL and the MBC was 3 64 µg/mL. This value indicates bactericidal activity of aqueous fraction of *P. citrina*, because MBC was two dilutions above MIC.

### Effect of the inhibitory active fraction on *S. aureus*

Studies were conducted to examine the effect of active fraction from *P. citrina* upon the growth of *S. aureus* 42AE. Addition of active fraction at the MIC (16 µg/mL) to log-phase cultures resulted in a 90% decrease of absorbance in comparative analysis with the control sample (without active fraction addition) (Figure 2).

## Analysis of protein synthesis

To investigate a possible mechanism of action of antibacterial fraction from *P. citrina*, radiolabelling studies were performed to determine if active fraction, at 16 µg/mL, induced an inhibition protein biosynthesis. The autoradiography showed that aqueous fraction inhibited a total protein synthesis of all strains tested, including reference and antibiotic-resistant strains (Figure 3).

## DISCUSSION

The discovery of new antibiotics is important due to the increasing incidence of multiple-resistance of pathogenic microorganisms to drugs that are currently in clinical use (Burgess et al., 1999). Marine sponges are among the most promising sources of new antimicrobial substances. Although Brazil has the world's second most extensive coastline after Australia, the development of the chemistry and pharmacology of Brazilian marine organisms has been hampered for many years because the main focus of Brazilian natural product chemists has been directed to the study of medicinal plants and microorganisms. Only recently, a few Brazilian research groups have focused on the chemistry of marine organisms (Torres et al., 2002; Berlinck et al., 2004; Selegim et al., 2007). Our results add new information to the growing database of known biological activities of Brazilian sponges.

In this study, ten out of twelve sponge species screened exhibited antibacterial activity. This high percentage, 83%, of active species was also found in other screenings for antimicrobial-active sponge species, independently of the geographical region (Bergquist & Bedford, 1978; Amade et al., 1982, 1987; Thompson et al., 1985; Uriz et al., 1991; Muricy et al., 1993; Monks et al., 2002; Berlinck et al., 2004; Selegim et al., 2007). Few studies have shown a lower percentage of active species in antimicrobial assays (e.g., 20% in Monks et al., 2002). Activity against antibiotic-resistant bacteria is much less common, in the order of 3-15% of the species tested (Selegim et al., 2007). Seven out of twelve sponges from Rio de Janeiro (58.3%) were active against such resistant bacteria, a high percentage when compared with other studies. Probably, the methodologies used by other authors (e.g., Monks et al., 2002; Torres et al., 2002 and Selegim et al., 2007) may be not as sufficiently sensitive as the methodology described by Giambiagi-deMarval et al., (1990), where each crude extract of sponge was spotted on the surface of agar, instead of using filter paper discs impregnated with the crude extracts. The general problems inherent to antimicrobial screening of plant extracts have been reviewed by Cos et al., (2006).

Six sponge species, *Cinachyrella* sp., *Guitarra sepia*, *Haliclona* sp., *M. microsigmatosa*, *P. citrina* and *T. ignis*, showed significant antibacterial activity against antibiotic-resistant bacteria, including *E. coli*, VRE,

MRSA, MRSE and various others CNS strains. These bacteria species are the most isolated in hospital infections in the world (Goldrick, 2004).

In the present work, the sponge species with potential for research of antibacterial substances were *P. citrina* and *Cinachyrella* sp. These species were active against bacteria of medical importance, including fourteen reference strains and 21 strains isolated from hospital patients. Aqueous extract of *P. citrina* inhibited growth of ten antibiotic-resistant strains, including strains of *E. coli*, *S. aureus*, CNS, and *E. faecalis*. Moreover, *P. citrina* extract

inhibited ten reference strains, including *S. aureus*, CNS, *E. faecalis* and *M. tuberculosis*. Surprisingly, specimens of *P. citrina* from Santa Catarina state (South Brazil) showed antichemotatic and cytotoxic properties, but no activity against the five bacterial strains tested (*E. coli*, *S. aureus*, *S. epidermis*, *B. subtilis*, and *M. luteus*) (Monks et al., 2002). This discrepancy may be due either to differences in extraction or bioassay protocols, or to a natural variability in the concentration of the active substances or fractions. Natural variations in chemical profiles are commonly found in sponges (reviewed in Thoms & Schupp, 2007).

**Table 2.** Antibacterial activity of crude extracts from marine sponges against reference strains.

Bacteria	Sponges											
	Cin.sp	C.cel	D.ret	G.cor	G.sep	Hal.sp	H.hel	M.mic	O.nod	P.cit	P.jan	T.ign
Reference strains												
<i>Acinetobacter calcoaceticus</i> ATCC 14293	-	-	-	-	-	-	-	-	-	-	-	-
<i>Corynebacterium fimi</i> NCTC 7547	+A,E	+A	+A	+A,E	+A,E	+A,E	-	+A	-	+A,E	+A,E	+A,E
<i>Enterobacter cloacae</i> ATCC 25355	-	-	-	-	-	-	-	-	-	-	-	-
<i>Enterococcus faecium</i> ATCC 19434	-	-	-	-	-	-	-	-	-	-	-	-
<i>Escherichia coli</i> ATCC 25922	+E	-	-	-	-	-	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i> ATCC 700603	+E	-	-	-	-	-	-	-	-	-	-	-
<i>Micrococcus luteus</i> ATCC 4698	+A,E	-	+A	+A,E	+A,E	+A,E	-	+A	-	+A,E	-	+A,E
<i>Mycobacterium tuberculosis</i> H37Rv	-	nt	nt	nt	nt	-	nt	-	nt	+A	nt	nt
<i>Pseudomonas aeruginosa</i> ATCC 27853	-	-	-	-	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 29213	+A,E	-	-	-	+E	+E	-	+A,E	-	+A,E	+A	+E
<i>Staphylococcus aureus</i> ATCC 25923	-	-	-	-	-	-	-	+A	-	+A,E	-	-
<i>Staphylococcus cohnii</i> ATCC 29974	+A,E	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus epidermidis</i> ATCC 12228	+E	-	-	-	+E	+E	-	-	-	+A	-	+E
<i>Staphylococcus haemolyticus</i> CCM 2737	+A,E	-	-	-	+E	-	-	+A	-	-	-	-
<i>Staphylococcus hominis</i> ATCC23844	+A,E	-	-	-	-	-	-	+A	-	-	-	-
<i>Staphylococcus lugdunensis</i> DSM 4804	-	-	-	-	-	-	-	+A	-	+A	+A	-
<i>Staphylococcus schleiferi</i> DSM4807	+A,E	-	-	-	-	-	-	+A	-	+A	-	-
<i>Staphylococcus simulans</i> ATCC 27851	+A,E	-	-	-	-	-	-	+A	-	+A	+A	-
<b>Controls</b>												
Distilled water	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-	-	-	-

+, Inhibition; -, no inhibition; nt, not tested. A, aqueous extract; E, ethanolic extract. Sponge species: Cin.sp, *Cinachyrella* sp.; C.cel, *Cliona celata* complex; D.ret, *Drumacidon reliculatus*; G.cor, *Geodia corticostylifera*; G.sep, *Guitarra sepia*; Hal.sp, *Haliclona* sp.; H.hel, *Hymeniacidon heliophila*; M.mic, *Mycale microsigmatosa*; O.nod, *Oceanapia nodosa*; P.cit, *Petromica citrina*; P.jan, *Polymastia janeirensis*; T.ign, *Tedania ignis*.

**Table 3.** Antibacterial activity of crude extracts from marine sponges against clinical strains.

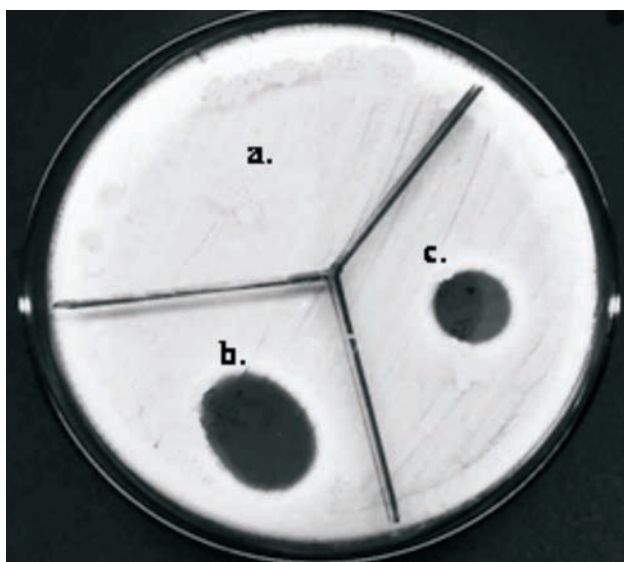
<i>Bacteria</i>	<i>Sponges</i>											
	Cin.sp	C.cel	D.ret	G.cor	G.sep	Hal.sp	H.hel	M.mic	O.nod	P.cit	P.jan	T.ign
Clinical strains (R*)	Cin.sp	C.cel	D.ret	G.cor	G.sep	Hal.sp	H.hel	M.mic	O.nod	P.cit	P.jan	T.ign
<i>Acinetobacter calcoaceticus</i>	+E	-	-	-	-	-	-	-	-	+A	-	-
<i>Citrobacter freundii</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Enterobacter cloacae</i> 43AE (AP, CFO, CT, TT)	-	-	-	-	-	-	-	-	-	-	-	-
<i>Enterobacter hafnia</i>	-	-	-	-	-	-	-	-	-	+A	-	-
<i>Enterococcus faecalis</i> OG1X (STR)	-	-	-	-	-	-	-	-	-	-	-	-
<i>Enterococcus spp.</i> A256 (VC, TEI)	+A	-	-	-	+E	+E	-	+A	-	-	-	+E
<i>E. faecalis</i> V583 (VC, ET, CLO, KN)	-	-	-	-	-	+E	-	+A	-	+A	-	+A
<i>Escherichia coli</i> 54AE (AP, CLO, SXT, TT)	+E	-	-	-	-	-	-	-	-	+A	-	-
<i>Klebsiella pneumoniae</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Pseudomonas aeruginosa</i> 2AE (AZ, P/T)	-	-	-	-	-	-	-	-	-	-	-	-
<i>Staphylococcus aureus</i> 42AE (PN, AP, OX)	-	-	-	-	+E	+A,E	-	+A,E	-	+A	+A	+E
<i>Staphylococcus aureus</i> 1Hp (PN)	+A,E	-	-	-	-	-	-	-	-	+A,E	-	-
<i>Staphylococcus aureus</i> 3Hp	+A	-	-	-	-	-	-	-	-	+A,E	-	-
<i>Staphylococcus aureus</i> 47Hp (PN)	+A,E	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus cohnii</i> 9S	+A,E	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus cohnii</i> 242S	+A,E	-	-	-	-	-	-	-	-	+E	-	-
<i>Staphylococcus cohnii</i> 338S	+A	-	-	-	-	-	-	-	-	+A,E	-	-
<i>Staphylococcus epidermidis</i> 57S (PN, CIP, AP, TT)	+A	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus epidermidis</i> 70S (PN, AP, OX, CFO, CR, KN, GN, IMP, CIP)	+A	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus epidermidis</i> 207S	+A	-	-	-	-	-	-	+A	-	+A	-	-
<i>Staphylococcus haemolyticus</i> 89S	+A	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus haemolyticus</i> 179S	+A	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus hominis</i> 60S (TT)	+A,E	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus hominis</i> 79S (PN, AP)	+A,E	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus hominis</i> 178S	+A	-	-	-	-	-	-	-	-	+A	-	-
<i>Staphylococcus simulans</i> 188S (CR, ET)	-	-	-	-	-	-	-	+A	-	+A	-	-
<b>Controls</b>												
Distilled water	-	-	-	-	-	-	-	-	-	-	-	-
Ethanol	-	-	-	-	-	-	-	-	-	-	-	-

+ Inhibition; - no inhibition; A aqueous extract; E ethanolic extract; CNS coagulase-negative staphylococci. Species of sponges as in Table 2. Clinical strains (R\*) resistant to: AP, ampicillin; AZ, aztreonam; CFO, cefoxitin; CIP: ciprofloxacin; CLO, chloramphenicol; CR, ceftriaxone; CT, cephalothin; ET, erythromycin; GN, gentamicin; IMP, imipenem; KN, kanamycin; OX, oxacillin; PN, penicillin; P/T, piperacillin/tazobactam; STR, streptomycin; SXT, trimethoprim/sulfamethoxazol; TEI, teicoplanin; TT, tetracycline; VC, vancomycin.

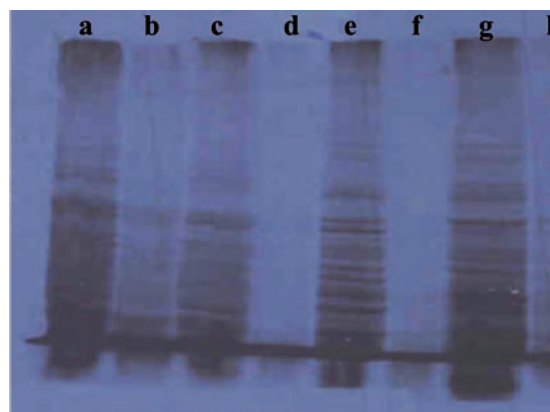
The other sponge species screened that demonstrated antibacterial activity against resistant-bacteria were *G. sepia*, *Haliclona* sp., *M. microsigmatos* and *T. ignis*. All these species inhibited VRE and MRSA strains. Three of these sponge species have already been found antibacterial activity in crude extracts (Muricy et al., 1993; Selegim et al., 2007). However, this is the first time that antibacterial activity from *G. sepia* is documented.

In this study, extracts of sponges with antibacterial activity showed a large action spectrum against Gram-positive cocci. It has been well-established that Gram-positive bacteria are much more sensitive to drug action

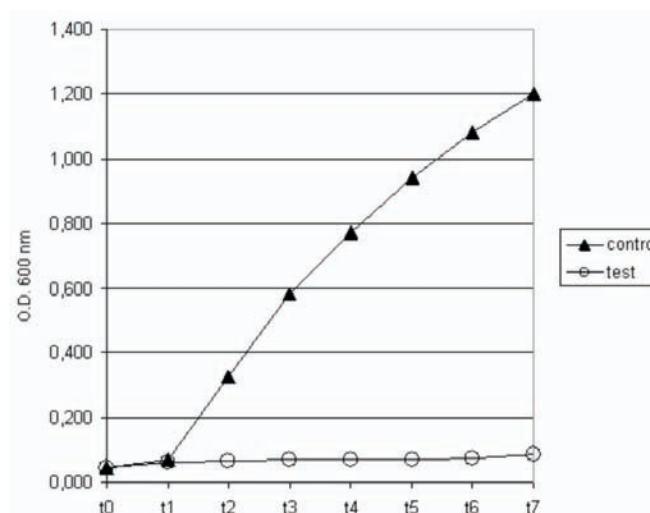
than Gram-negative bacteria (Cos, 2006). Gram-positive cocci such as *S. aureus*, CNS, and *Enterococcus* spp. are extremely important pathogens in hospital environments. The number of strains of these species that are resistant to standard antibiotics has increased, leading to increased morbidity and mortality in nosocomial infections. In some cases, such as hospital-acquired MRSA, infection control measures appear to be the most important mechanisms for limiting spread. In others, such as VRE, both infection control and antimicrobial exposures are important (Rice, 2006). New antibacterial agents effective for treating serious Gram-positive infections are also highly needed.



**Figure 1.** Antibacterial effect of aqueous fraction from extract *P. citrina* on *S. aureus* 42AE. (a) water, negative control; (b) test: aqueous fraction, inhibition zone around spot at 25 mm; (c) positive control: aqueous extract, inhibition zone around spot at 16 mm.



**Figure 3.** Influence of activity fraction from *P. citrina* on protein synthesis. Autoradiogram of a SDS-PAGE protein profile of the *S. aureus* ATCC 29213 (a,b), *S. aureus* 42AE (c,d), *S. epidermidis* ATCC 1228 (e,f) or *S. epidermidis* 70S (g,h) strains labeled in the presence of [<sup>35</sup>S] Met (200 µCi/mL) for 1 h at 37 °C. Control cells (lanes a, c, e, g) or cells treated with inhibitory fraction (16 µg/mL) (lanes b, d, f, h).



**Figure 2.** Effects of antibacterial activity of aqueous fraction from *P. citrina* on *S. aureus* 42AE. The cultures treated with inhibitory fraction (circle) or control (triangle) were then either maintained at 37 °C and at 1 h intervals, the O.D. of 600 nm were determined. Each point represents the mean of three independent experiments.

Our results indicate at least one promising new candidate for research of antibacterial substances. Fractionation of the crude extract from *P. citrina* showed activity of aqueous fraction. This fraction presented inhibitory effects on *Staphylococcus* sp., including MRSA strain isolated from a hospital. Moreover, bacterial protein synthesis was inhibited by substances present in the aqueous phase. This effect could be a direct mode of action or the active fraction could interfere with the integrity of the cell wall or the cytoplasmic membrane. In the latter case, the effect could cause the efflux of small molecules (for example, potassium and amino acids) and dissipation of the membrane potential, resulting in an arrest of all cellular biosyntheses. Therefore, inhibited protein biosynthesis could be a secondary effect. Few studies showed the mode of action of antibacterial substances isolated from sponges. A study with the sceptrin, an antimicrobial agent isolated from the *Agelas mauritiana*, showed a mechanism of action on the cell wall with subsequent damage to the membrane (Bernan et al., 1993). Future studies investigating the possible role of antibacterial substance from *P. citrina* on growth bacteria would provide greater insight into the precise mechanism of action of this antibiotic. In addition, the aqueous fraction is currently being studied chemically for identification of the active substance with the aim to use it to treat multiresistant-bacteria infections in the future.

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