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Antibacterial and antibiotic potentiating activities of tropical marine sponge extracts

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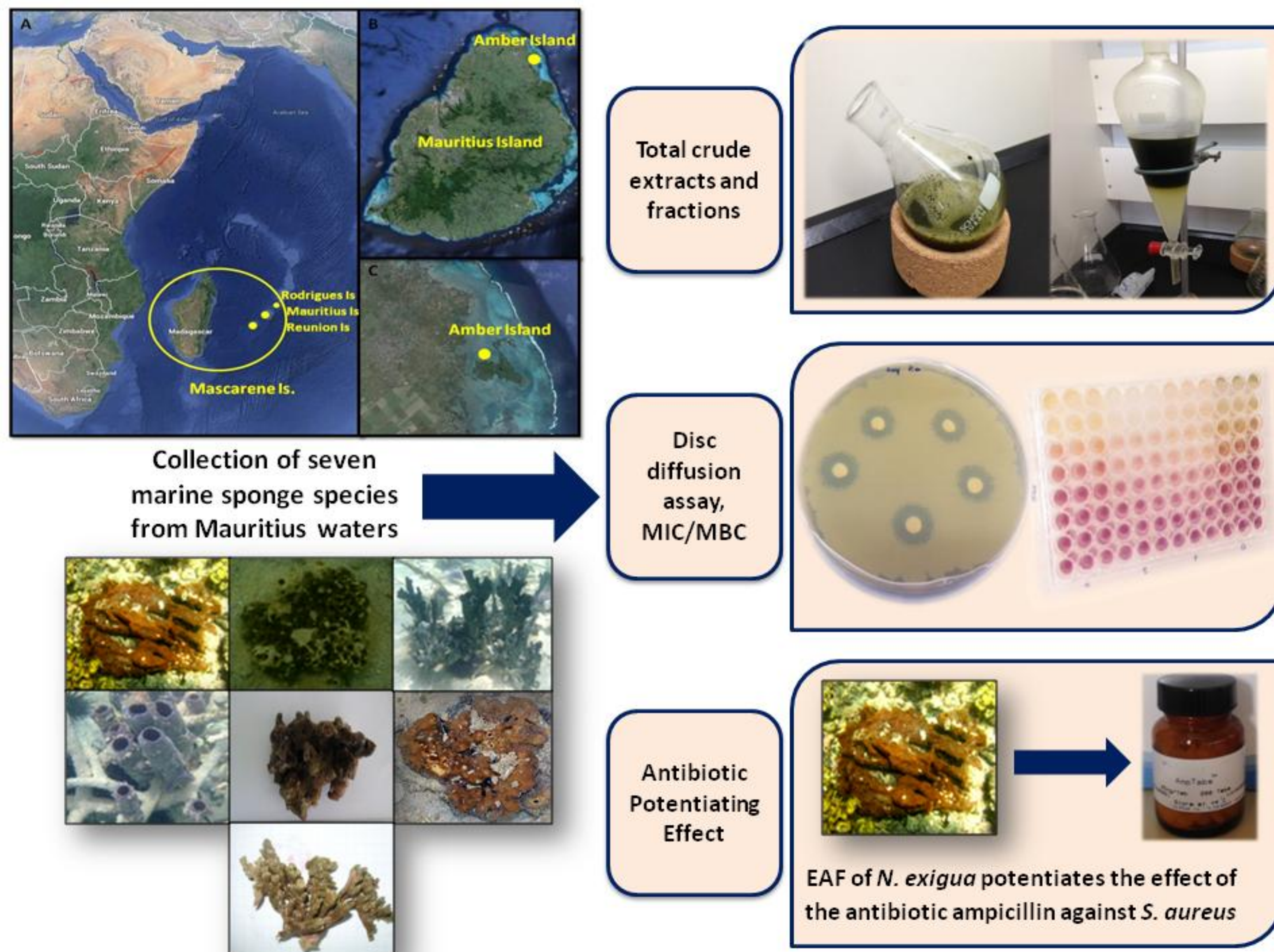
Graphical Abstract



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Abstract

Increasing prevalence of antibiotic resistance has led research to focus on discovering new antimicrobial agents derived from the marine biome. Although ample studies have investigated sponges for their bioactive metabolites with promising prospects in drug discovery, the potentiating effects of sponge extracts on antibiotics still remains to be expounded. The present study aimed to investigate the antibacterial capacity of seven tropical sponges collected from Mauritian waters and their modulatory effect in association with three conventional antibiotics namely chloramphenicol, ampicillin and tetracycline. Disc diffusion assay was used to determine the inhibition zone diameter (IZD) of the sponge total crude extracts (CE), hexane (HF), ethyl acetate (EAF) and aqueous (AF) fractions against nine standard bacterial isolates whereas broth microdilution method was used to determine their minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and antibiotic potentiating activity of the most active sponge extract. MIC values of the sponge extracts ranged from 0.039 to 1.25mg/mL. Extracts from *Neopetrosia exigua* rich in beta-sitosterol and cholesterol displayed the widest activity spectrum against the 9 tested bacterial isolates whilst the best antibacterial profile was observed by its EAF particularly against *Staphylococcus aureus* and *Bacillus cereus* with MIC and MBC values of 0.039 mg/mL and 0.078 mg/mL, respectively. The greatest antibiotic potentiating effect was obtained with the EAF of *N. exigua* (MIC/2) and ampicillin combination against *S. aureus*. These findings suggest that the antibacterial properties of the tested marine sponge extracts may provide an alternative and complementary strategy to manage bacterial infections.

Keywords Antibacterial activity, Antibiotic potentiating, Marine sponges, *Neopetrosia exigua*, Mauritius

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1. Introduction

The optimism of the 1950s and 1960s of a world without infections is gradually being replaced by an era of pessimism characterized by widespread emergence of antimicrobial resistance (Raghunath 2008). Even today, infectious diseases including HIV/AIDS, tuberculosis, and malaria continue to elude the prospect of morbidity and mortality worldwide killing around 700, 000 people annually (O'Neill 2016). Of particular concern is the high mortality rate associated with bacterial infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), extended spectrum β-lactamase (ESBL)-producing Enterobacteriaceae, vancomycin-resistant *Enterococcus*, penicillin-resistant *Streptococcus pneumonia* as well as multi drug resistant (MDR) *Salmonella* (Farooqui et al. 2015). According to the World Health Organisation "this is happening at a time when too few new drugs are being discovered *vis à vis* antimicrobial resistance" (WHO, 1996). Clearly, the reduced effectiveness of the current catalogue of clinically useful antibiotics motivates research for identifying novel molecules with antimicrobial properties to stem the tide against antimicrobial resistance.

Contributing to the global search for new antibiotics, natural products have historically been pharma's treasure trove for drug hunters (Newman and Cragg 2010). Drug discovery from natural sources has recently shifted to the marine biome which hosts a vast repertoire of biodiversity. Among marine organisms, sponges have been widely described as important producers of secondary metabolites with novel structural features and bioactivity (Sipkema et al. 2005). These organisms are sessile invertebrates which compensate for their lack of physical defence by producing chemicals indicating their ecological significance as anti-predation, competition for space and control of epibiont overgrowth (Majik et al. 2014). Sponge-associated microbiome, including bacteria, archaea, and fungi also play a role in the production of these compounds (Beesoo et al. 2014). These chemical adaptations involve chemical classes such as terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides,

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amino acid derivatives and halogenated metabolites (Sipkema et al. 2005). In the marine environment, antimicrobial and antifouling metabolites prevent sessile organisms such as sponges to host hazardous biofilms on their exposed surfaces (Majik et al. 2014). Hence, antimicrobial compounds isolated from sponges may represent an alternative source of novel antibiotics which can be used to supply the starved pharmaceutical market.

About 800 antimicrobial compounds have been isolated from sponges, a few of which has demonstrated potentials as promising therapeutic leads such as manzamine A and psammaplin A. Pharmaceutical interest in sponges was aroused in the early 1950s by the discovery of the nucleosides Ara-C, the first marine-derived anticancer agent, and the antiviral drug Ara-A from the marine sponge *Cryptotethia crypta* (Laport et al. 2009). These early promises have now been substantiated by a growing number of sponge derived compounds such as discodermolide (Martello et al. 2001), spongistatin 1 (Schyschka et al. 2008), laulimalide (Bajaj and Srayko 2013) that have also shown synergistic interactions with conventional anticancer drugs. Synergism of drug therapy and natural products may also give optimum results in the fight against existing and emerging infectious diseases. Regarding the application of natural products on bacterial infections, besides their direct antimicrobial activity, natural extracts have also been studied as antimicrobial drug resistance modifiers. Thus, various combinations of these extracts with synthetic antibiotics have been tested against bacterial proliferation, and many studies have demonstrated that compounds present in the extracts can modify the activity of antibiotics, increasing their efficacy (Coutinho et al. 2011; Morais Braga et al. 2012; Souza et al. 2012; Matias et al. 2013).

With most research in the area of marine natural product chemistry being conducted in Atlantic and Pacific Ocean regions, it is becoming increasingly important to survey the fauna of isolated regions of

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the globe for new chemotherapeutic agents (Leal et al. 2012). The Mascarene Islands (Mauritius, Rodrigues and Reunion) are the most isolated of the tropical marine flora and fauna of the South-west Indian Ocean and represent an extremely fertile reservoir of genetic and metabolic novelties (Beedessee et al. 2015). In our continuous exploration of antimicrobial agents from the marine ecosystem, the aims of this study were to evaluate the *in vitro* antibacterial activity of the total crude extracts, hexane, ethyl acetate and aqueous fractions from seven sponge species which are strongly represented in the Mascarene regions namely *Neopetrosia exigua*, *Aaptos chromis*, *Iotrochota birotulata*, *Haliclona tuberosa*, *Spheciospongia* sp., *Biemna fortis* and *Halichondria* sp. We also aimed to determine the antibiotic potentiating effect of the most active sponge extract in association with chloramphenicol, ampicillin and tetracycline. The present work is anticipated to establish important baseline information on the antibacterial properties of the studied sponges and preliminary data on the chemical constituents of the most active sponge extract.

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2. Material and methods

2.1 Sampling and Identification

Seven sponge samples namely *Neopetrosia exigua*, *Aaptos chromis*, *Iotrochota birotulata*, *Haliclona tuberosa*, *Spheciospongia* sp, *Biemna fortis* and *Halichondria* sp were collected at Amber Island (20°03'52.5"S and 57°41'19.7"E) on the northeast coast of Mauritius island in February 2013 (Figure 1). The sponges were collected at depth varying from 1-2 metres by snorkelling. They were transferred in sterile polyethene bags under sea water to the lab, cleaned of debris and frozen at -80°C prior to freeze drying and extraction. Taxonomic identification of the sponge species was made using the World Porifera Database (World Porifera Database, 2016).

2.2 Extraction and creation of fraction libraries

The sponges were cut into small pieces, weighed, freeze dried and ground into powder. The freeze dried sponge (250–1000g) was exhaustively macerated with methanol and dichloromethane (1:1) for 48 h. After maceration, the solution was filtered and evaporated to dryness on a rotatory vacuum evaporator set at a temperature of 37°C (LABORATA 4003, Heidolph, Germany). This constituted the crude extract, which was suspended in distilled water to be partitioned subsequently with *n*-hexane and ethyl acetate to afford non-polar, semi-polar and polar fractions respectively. The extracts were weighed and stored at −20°C until used.

2.3 Microorganisms

The microbial strains were obtained from American Type Culture Collection (ATCC): *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Pseudomonas fluorescens* (ATCC 13525), *Klebsiella oxytoca* (ATCC

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43086), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella enterica* (ATCC 29986) and *Serratia marcescens* (ATCC 14756). All isolates were maintained on nutrient agar slants at 4°C. The strains were activated by subculture at 37°C for 24 h with fresh Muller Hinton Agar (MHA) prior to any antimicrobial tests.

2.4 Preparation of inoculums

The inoculum size of the test isolates was standardized according to the Clinical Laboratory Standards Institute guidelines (CLSI, 2008). Briefly, the bacteria were inoculated in Muller Hinton Broth (MHB) and were incubated at 37°C for 24 h. The turbidity of the bacterial suspension was adjusted to match the tube of 0.5 McFarland turbidity standards at 600 nm using the spectrophotometer on a Helios-Alpha spectrophotometer (Unicam Ltd., UK) which equals to 1.5×10^8 colony-forming units (CFU)/mL. This was used for the standardisation of the antibacterial assay.

2.5 Antibacterial agar disc diffusion assay

The *in vitro* antibacterial activity of the marine extracts total crude extracts and fractions was evaluated via the disc diffusion method using Müeller–Hinton agar with determination of inhibition zones diameter measured in millimeter (mm) (CLSI, 2008). Sterile filter paper discs (6 mm) were impregnated with 10 µL of sponge extracts and then placed on inoculated Petri dishes containing 0.1 ml bacterial suspension adjusted to 1.5×10^8 CFU/mL. Chloramphenicol, ampicillin and tetracycline pre-dosed at 50 µg/mL per disc were used as positive control whereas discs without samples (5% DMSO (dimethyl sulfoxide) acted as negative control. The zones of inhibition including the diameter of the extract impregnated dics were compared with those of the controls after incubation at 37°C for

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24 h. The inhibition zone diameter (IZD) was used as criteria for the definition of active or inactive sponge extracts. The tests were carried out in triplicate for each extract.

2.6 Determination of minimal inhibitory concentration (MIC)

Sponge extracts which showed the ability to inhibit the growth of microorganisms used in the present study (IZD > 8 mm) were tested for their minimum inhibitory concentration (MIC) by the microdilution technique (Eloff 1998). The extracts were diluted with 5% dimethyl sulfoxide to obtain concentrations from 0.0195 to 2.5 mg/mL. 100 µL of the extracts dilutions were placed into a 96-well microplate and inoculated with 100 µL of each bacterial strain at a density adjusted to 0.5 of the McFarland scale (1.5×10^8 CFU/mL). The microplate was incubated at 37°C for 24 h. The p-Iodonitrotetrazolium chloride (0.2 mg/mL) was used as an indicator of microbial growth. Viable bacteria reduced the yellow dye to a purple colour. The MIC was defined as the lowest concentration of extract that inhibits bacterial growth. This test was performed in triplicates. Chloramphenicol, ampicillin and tetracycline (stock concentration: 2.048 mg/mL) were used as positive control.

2.7 Minimum Bactericidal Concentration (MBC)

Sponge extracts which showed antibacterial activity below100 µg/mL were further assessed for their minimum bactericidal concentration (MBC). MBC is the smallest concentration of the drug necessary for elimination of 99.9% of the microorganisms tested. To establish MBC of the extracts, 20 µl of each culture medium with no visible growth was removed from each well and inoculated in MHA agar plates. After 16–20h of incubation at 37 °C, the number of surviving organisms was determined. If MIC = MBC or if MBC = 1, 2 or 3 dilutions above MIC, the activity is considered bactericidal (Isenberg 1992).

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2.8 Antibiotic potentiating assay

Sponge extract/fraction showing significant antibacterial activities as compared to the positive controls in the previous assays was associated with conventional antibiotics in view of evaluating any possible synergistic effect as previously described (Tankeo et al. 2015). In this experiment, a preliminary study (using the microbroth dilution assay) of the bacteria tested (*E. coli*, *P. fluorescens*, *S. aureus*, and *B. cereus*) with chloramphenicol, ampicllin and tetracycline were investigated to select the sub inhibitory MIC at which the experiment will be performed. Briefly, the sponge extract was tested at sub inhibitory concentrations of MIC/2 and MIC/4 in association with antibiotics against each bacterial isolate. Fractional inhibitory concentration (FIC) was calculated as the ratio of $MIC_{\text{Antibiotic in combination}}/MIC_{\text{Antibiotic alone}}$ and the results were discussed as follows: synergy (≤0.5), indifferent (>0.5 to 4), or antagonism (>4). All assays were performed in triplicate.

2.9 Gas chromatography–mass spectrometry (GC–MS) analyses of the ethyl acetate (EAF) fraction of N. exigua

The EAF fraction of *N. exigua* was analyzed by GC–MS coupled to an Agilent MS model 6975 C MSD with triple axis detector (Agilent Technologies, USA). The GC column is a 30 m HP5-MS column made from (5%-phenyl)-methylpolysiloxane (i.d.= 0.25mm, df=0.25µm). 1 mg of the *N. exigua* EAF fraction was weighed and added to 20µL pyridine and 50µL of BSTFA (with 1% TMCS). The prepared sample was then incubated at 60 °C for 1 hour in order to yield trimethylsilyl derivatives (TMSi) of the compounds in the sample (Abulaimi 2017). Sample derivatization was carried out at GC initial temperature of 240°C for 3 minutes and incremented to 280°C for 25 minutes at a rate of 20°C/min. The mass spectrometer operated using an electron ionization source at 70eV. The ion source temperature was 230°C. Analysis preceded using MSD ChemStation and by matching the MS spectra of the peaks with those stored in the NIST 2011 Mass Spectral Library (Agilent Technologies, USA).

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2.9 Data Analysis

All experiments were conducted in triplicate and statistical analyses were conducted using analysis of variance (ANOVA). The results were expressed as means of triplicate determinations ± standard deviation (SD). The confidence limits used in this study were based on 95% ($p < 0.05$). The software SPSS v.19 (SPSS-IBM, Orchard Road-Armonk, New York, NY, USA) was used for statistical analysis.

3. Results

3.1 Antibacterial agar disc diffusion assay

All of the 28 sponge total crude extracts and fractions screened for antibacterial activity showed significant activity against at least one bacterial strain ranging from weak to strong activities based on the following arbitrary criterion: Very-weak-6-8 mm, weak-8-12 mm, good-12-16 mm, and strong > 16 mm inhibition activities respectively (Table 3.1). 17 and 22 of the total crude extracts and fractions exhibited very weak and weak antibacterial activity respectively. On the other hand, 11 extracts showed good antibacterial activities while 2 displayed strong propensity. EAF of *N. exigua* gave the most potent action spectra yielding the largest IZD particularly against the Gram-positive *S. aureus* (19.57 ± 0.25 mm). The antibacterial potential of this sponge fraction was comparable to that of ampicillin (20.47 ± 1.75 mm) but significantly better than chloramphenicol (17.73 ± 0.64 mm) and tetracycline (17.43 ± 0.40 mm) ($p < 0.05$). CE and EAF of *N. exigua* inhibited *B. cereus* significantly with IZD of 17.74 ± 0.60 mm and 18.36 ± 0.66 mm respectively compared to the positive control tetracycline (16.20 ± 1.18 mm). Inhibitory efficacy of EAF *N. exigua* was similarly more pronounced on *E. coli* (IZD: 15.80 ± 1.91 mm) than tetracycline (IZD: 12.77 ± 1.15 mm) and statistically similar (IZD: 15.47 ± 0.42 mm) to chloramphenicol (IZD: 16.07 ± 0.31 mm) against *P. fluorescens*. Besides extracts of *N.*

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exigua, CE of *I. birotulata* also showed significantly comparable antibacterial activity (IZD*:* 14.60 ± 0.53 mm) to tetracycline (IZD*:* 14.27 ± 0.31 mm) against *P. fluorescens* ($p < 0.05$).

Significant differences in antimicrobial activity were found among the crude extracts and solvent fractions ($P < 0.05$). While the sponge total crude extracts showed minimal antibacterial, selected sub fractions of extracts showed promising results against the pathogens. In most cases, EAFs revealed stronger antibacterial activities while AFs exhibited weaker activities. It was also generally observed that Gram-positive *B. cereus* and *S. aureus* were the most susceptible bacteria to the sponge extracts while *P. pneumonia* and *E. coli* were the most resistant Gram-negative strains.

3.2 Minimum Inhibitory Concentration

The antibacterial activity of each sponge extract with IZD greater than 8 mm was further probed to determine its MIC using the micro-dilution assay. The sponge extracts exhibited antibacterial activity within a range of concentrations of 0.039 to 1.25 mg/mL (Table 3.2). *N. exigua* had the most potent antibacterial activity with its EAF displaying MIC values below 100 µg/mL particularly against *S. aureus* (0.039 mg/mL), *B. cereus* (0.039 mg/mL), *E. coli* (0.78 mg/mL), *P. fluorescens* (0.78 mg/mL). These MIC values were in some cases better or comparable to the reference antibiotics used in the present study. Furthermore, CE of *I. birotulata* also inhibited the growth of *P. fluorescens* with MIC value (0.078 mg/mL) lower than tetracycline (0.128 mg/mL). The overall MIC results of the sponge extracts confirmed the screening done by the disc diffusion method.

3.3 Minimum Bactericidal Concentration (MBC)

Result from the MBC assay (Table 3.2) showed that EAF of *N. exigua* possessed bactericidal activities against *E. coli* (0.625 mg/mL), *P. fluorescens* (0.313 mg/mL), *B. cereus* (0.078 mg/mL) and *S. aureus*

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(0.078 mg/mL). Bactericidal activity was also registered by CE of *N. exigua* towards *S. aureus* and *B. cereus* with MBC value of 0.312 mg/mL. CE of *I. birotulata* was however bacteriostatic against *P. fluorescens* (MBC> 2.5 mg/mL).

3.4 Antibiotic potentiating effect

Based on its low MIC values, EAF of *N. exigua* was selected to further determine its antibiotic potentiating activity at sub inhibitory concentrations MIC/2 and MIC/4 against selected bacterial strains including *E. coli*, *P. fluorescens*, *B. cereus* and *S. aureus*. A synergistic effect was observed in most of the different combinations when associated with chloramphenicol, ampicillin and tetracycline as indicated by the FIC values (Table 3.3).

However, when the concentrations of EAF of *N. exigua* were varied, the synergistic effect observed was not same for all the bacteria. In some cases the synergistic activity was enhanced in a dose dependent manner but in others there was a decrease or no change in activity as compared to the MIC values of the antibiotics alone. For instance, when chloramphenicol was associated with EAF of *N. exigua* at MIC/4 and assessed against *P. fluorescens* the MIC value decreased from 0.064 to 0.016 mg/mL but when the concentration of the sponge extract was doubled (MIC/2) there was still a decrease in MIC compared to the MIC of chloramphenicol alone.

On the other hand, when chloramphenicol and ampicillin were associated with the sponge extract at both MIC/4 and MIC/2 against *B. cereus* and *S. aureus* respectively, a decrease in the MIC value of the antibiotic was observed but within the two doses, the MIC values remained unchanged (Table 3.3). Furthermore, EAF of *N. exigua* when combined with ampicillin at both concentrations did not show any improvement in activity against *E. coli* (0.064 mg/mL) and *P. fluorescens* (0.016 mg/mL) as compared to the MIC values of the antibiotic alone. Similarly the combination of the sponge extract

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and tetracycline resulted in indifferent effects against *E. coli* (0.256 mg/mL). Overall, the most significant increase in antibiotic activity in the presence of the sponge extract was noted with the EAF *N. exigua* (MIC/2) and ampicillin combination on *S. aureus*, (MIC: 0.004 mg/mL; FIC: 0.125) with a four-fold decrease of the MIC value of the antibiotic alone (0.032 mg/mL).

3.5 Chemical constituents of the EAF of N. exigua

Preliminary chemical screening of EAF of the sponge *N. exigua* was performed using GC–MS. The number and nature of chemical constituents in the sponge EAF as depicted by the peaks were characterized and identified by comparing the mass spectra of the constituents with NIST library (Figure 2). Table 4 lists the compound names, retention time and percentage compositions. Overall, 13 TMSi derivatives of compounds (1-13) were detected including fatty acids (n-hexadecanoic acid (**1**), trans-9-octadecenoic acid trimethylsilyl ester (**2**)), and sterols (cholesterol trimethylsilyl ether (**7**), silane, [(3.beta.)-cholesta-5,24-dien-3-yloxy]trimethyl (**9**), silane, [[(3.beta., 24R)-ergost-5-en-3-yl]oxy]trimethyl- (**10**), beta-sitosterol trimethylsilyl ether (**13**)). Of all these compounds, beta-sitosterol trimethylsilyl ether (**13**) was identified as the most significant component of the EAF representing 23.9%.

4. Discussion

The emergence of multiple drug resistance in human pathogenic microorganisms has added momentum to search for new antimicrobial substances from natural sources. There have been several mechanisms proposed for the antibacterial activity of potent drugs including natural extracts (Kumar et al. 2014). In many cases, natural extracts can be more effective than chemically synthesized pure compounds because they are a complex mixture of components. Their complexity enables them to interact with

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multiple molecular targets thus conveying accrued difficulty for target microorganisms to develop resistance due to multiple response sites (Lagunin et al. 2016). Intensive efforts during the past decades have led to the discovery of promising antimicrobial extracts and compounds from marine organisms. In particular marine sponges have been recently the subject of several antimicrobial screening studies worldwide (Tangman et al. 2015; Putra et al. 2016). Here, we present data indicating the antibacterial efficacy and antibiotic potentiating activity of crude extracts and fractions obtained from seven tropical sponges from Mauritius waters.

The diversity in antimicrobial activity shown by natural extracts is usually subjected to differences in their chemical concentration and composition (Smolskaite et al. 2015). In general, the total CEs demonstrated smaller IZDs and higher MIC values as compared to the HFs and EAFs. The MIC of crude extracts is rarely found to be < 0.1 mg/ml but with more refined fractions or individual compounds, MICs of < 0.1 mg/ml may be reflective of good antimicrobial activity and thus can be considered worthy for further investigations (Webster et al. 2007). However, it is striking that the total CE of *I. birotulata* demonstrated noteworthy antibacterial activity (MIC: 0.078 mg/mL) as compared to its fractions with MIC values below 0.1 mg/mL. This may potentially suggest a synergistic effect arising from several compounds present in the total CEs could account for the high antibacterial activities. It is noteworthy that a number of antimicrobial compounds have been isolated and characterised from the genus *Iotrochota*, such as the alkaloids purpurones (Shen et al. 2012). Other sulphated alkaloids are baculiferins isolated from the Chinese marine sponge *I. baculifera*. Baculiferins were found to be potent inhibitors against HIV IIB in both MT4 and MAGI cells (Fan et al. 2010).

Of all the marine crude extracts and fractions, EAF of *N. exigua* displayed the greatest antibacterial effect with the largest IZD and lowest MIC/MBC values. The antibacterial effect of EAF of *N. exigua*

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was more pronounced against the Gram positive bacteria *S. aureus* and *B. cereus* with IZD and MIC values comparable to the reference antibiotics used in the present study (Table 3.1 and 3.2). This is quite remarkable considering that antibiotics are in the purified and concentrated form whereas the extracts are crude and may harbour both pharmacologically and non-pharmacologically active compounds with the chance of some compounds having a masking effect over others (Njume et al. 2011). This is an indication that the EAF of *N. exigua* may contain therapeutically useful compounds against *S. aureus* and *B. cereus* associated infections (Tangman et al. 2015).

A medley of studies performed on marine sponges of the genus *Neopetrosia* showed that several compounds conferring antimicrobial potencies were isolated from its extracts. Leone et al (2008) isolated exiguaquinol from the sponge *N. exigua* which inhibited the bacterial enzymes *Helicobacter pylori* (Glutamate racemase that inter converts L- and D- glucose) needed for the construction of bacterial cell walls. Renieramycin J, tetrahydroisoquinoline alkaloid and araguspongine M, both of which showed strong antifungal activities were also isolated from the same species from Japanese waters. A *Synechococcus*-like cyanobacterium has been detected in the tissue of *N. exigua* by visible and fluorescent microscopic observations (Oku et al. 2003). Hence these compounds or their precursors may be produced by these symbiotic microorganisms.

From the foregoing findings, it was observed that the extracts had more pronounced effect on the Gram-positive bacteria than the Gram-negative bacteria. This is in congruence with most of the available reports on antibacterial property of sponges that revealed that Gram-positive bacteria are more susceptible to sponge extracts as compared to Gram negative ones (Liu et al. 2004). Extracts derived from 28 marine sponge species collected along the French coast had high antibacterial activity against Gram positive bacteria (77%) than Gram negative bacteria (53%) (Lippert et al. 2003). Moreover, McCaffrey and Endean (Amade et al. 1987) also showed that Gram-positive bacteria were

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more sensitive to sponge extracts of subtropical and tropical species than Gram-negative. Similarly in this study, the Gram positive *S. aureus* and *B. cereus* exhibited higher sensitivity as compared to the Gram-negative bacteria. Gram-negative bacteria *K. pneumoniae* and *E. coli* were most resistant to the tested extracts possibly because of the outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to antimicrobial compounds (McCaffrey et al. 1987).

Bacterial resistance can occur against natural extracts/compounds as well thereby increasing the need to develop newer and more powerful therapies to combat resistant microorganisms. Therefore, a novel concept which has recently been explored is the synergism between known antimicrobial drugs and bioactive natural products. This therapy can be employed to expand the antimicrobial spectrum, to prevent the surfacing of bacterial mutants and to minimize toxicity, thereby exhibiting antimicrobial action greater than what would be normally expected from each individual antibiotic (Chopra and Greenwood, 2001). Synergy between marine natural products and antimicrobial drugs has been frequently reported. Song et al. (2010) showed that 7-O-methylkoninginin D and trichodermaketones A-D isolated from the marine-derived fungus *Trichoderma koningii*, combined with ketoconazole showed synergistic antifungal activity against *Candida albicans.* Another study led by Choi et al. (2015) demonstrated that fucoidans isolated from brown seaweeds drastically enhanced the antibacterial activity of oxacillin and ampicillin to combat MRSA. Additionally the methanolic extract of the seaweed *Eisenia bicyclis* also influenced the activity of erythromycin and lincomycin and thus may be used as adjuvant in antibiotic therapy against acne-related bacteria (Choi et al. 2015).

In the present study, based on the evidence of EAF of *N. exigua* as a strong antimicrobial, its potentiating effect was further assessed against *E. coli*, *P. fluorescens, S. aureus* and *B. cereus* using

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chloramphenicol, ampicillin and tetracycline. Overall, the highest synergistic activity was observed by EAF of *N. exigua* (MIC/2) and ampicillin against *S. aureus* suggesting that EAF of *N. exigua* may have potential for use as an adjunct in the treatment of *S. aureus* related bacterial infections. This finding is particularly pertinent as multi-drug resistant strains of this microorganism are on the rise in both hospital and community environments against orthodox antibiotics and its control is very difficult by therapeutic means (Tangman et al. 2015).

The continuous increasing susceptibility of several antibiotics to bacterial resistance including ampicillin, tetracycline and chloramphenicol has been recently confirmed by official reports of the Center for Disease Dynamics, Economics and Policy (CDDEP, 2015). The resistance mechanisms against these drugs include: reduction of the cell membrane permeability, enzyme degradation, and activation of efflux pumps. Therefore, the synergistic effects that have been achieved by the association of EAF of *N. exigua* with these antibiotics could be related to an increase in drug influx, because a set of constituents present in the extracts, might exert a destabilizing effect on the bacterial cell wraps, causing increased permeability to tested antibiotics and thus, potentiating their effect (Lee et al. 2014). Of note, at present no other study regarding the antibiotic modulatory effect of marine sponges has been reported, as well as, additional studies aimed at identifying the molecular mechanism underlying the antimicrobial property of this sponge extract remain to be carried out.

So far, the GC-MS analysis of the EAF of *N. exigua* has revealed two previously identified antibacterial compounds namely hexadecanoic acid (and its derivatives) and β-sitosterol. Hexadecanoic acid is one of the most common saturated fatty acids found in animals and plants and has shown antibacterial activity against a panel of pathogenic bacteria including *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* sp (Zong hui et al. 2010). Similarly Abou-Elela and his colleagues

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reported the antibacterial activity of hexadecanoic acid derivatives isolated from the sponge *Spongia officinalis* and the brown algae *Cytosoria compressa* against *S. aureus, S. faecalis, P. aeruginosa* and *E.coli* (Abou-Elela et al. 2009). β-sitosterol (23.9%) which is known for its pluri-pharmacological properties (Saeidnia et al. 2014) was predominant. Subramaniam et al. (2014) reported promising antibacterial activity of β-sitosterol derivatives particularly against *E. faecalis, S. dysenteriae*, and *P. aeruginosa* (MIC: 10-15 µg/ml) and its synergistic activity with commonly used antibiotics such as ciprofloxacin, polymyxin B and chloramphenicol. This molecule has been an interesting lead for chemical and biological investigations and over the years, several modified β-sitosterol have been isolated (Ododo et al. 2016, Subramaniam et al 2014, Sanches et al. 2005). Sponges of the genus *Neopetrosia* are still yielding novel bioactive molecules; neopetrocyclamines A and B (Liang et al., 2015), 9'-epi-3β, 3'β-dimethylxestospongin C (Li et al., 2011) and exiguamine A (Brastianos et al., 2006) and this was the impetus for the investigation of a member of this genus. Liquid chromatography-MS analyses of the active EAF of *N. exigua* will be carried out with the aim to identify other constituents (undetected by GC-MS) contributing to its antibacterial property. Further bioassay guided fractionation will also be needed to isolate and identify the active constituents present in this extract and elucidate their mode of action.

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5. Conclusion

This study demonstrated the antibacterial effects of seven sponge crude extracts and fractions, in addition to the synergistic action against numerous bacterial strains when combined with conventional antibiotics. Among the sponge species investigated *N. exigua* was the most promising species tested, advocating the possibility of finding potent antibacterial agents particularly from its ethyl acetate fraction. However, it must be emphasized that interactions between natural products and synthetic drugs depend on several factors including pharmacokinetics and employed doses and therefore,

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combinations established *in vitro* may not have the same effect *in vivo* or in clinical trials. Further research should be undertaken to ensure the safety and efficacy of the sponge extracts by delineating the active constituents in the extract. Overall, the implication of this study opens a new and fertile area of future research to pursue in search of novel antimicrobial lead compounds. In this perspective, further in depth studies are warranted to elucidate the molecular mechanisms underlying the antibacterial effects of the marine extracts which may yield more insights into their chemotherapeutic as potential antimicrobial agents.

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Conflict of interests

None declared.

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Table 1 Inhibition Zone Diameter (mm) of the sponge total crude extracts and fractions using the agar disc diffusion assay

Sponge Species/ Standard Antibiotics	Extracts (10µL/Disc)	Inhibition Zone Diameter (mm)								
Test		12.67±1.15 [c]	14.27±0.31[c]	17.17±0.78 [a]	20.40±1.14[a]	17.60±1.14[b]	17.30±0.35[b]	16.00±0.71 [c]	16.20±1.18[d]	17.43±0.40[b]
		Gram negative bacteria						Gram positive bacteria		
		E.c	***P.f***	***P.a***	***K.o***	***K.p***	***S.m***	***S.e***	***B.c***	***S.a***
Neopetrosia exigua	**CE**	11.40±0.53[c]	12.47±0.42[de]	9.67±1.16[b]	13.16±0.23[d]	7.86±0.39[d]	12.20±1.01[c]	10.64±0.56[h]	17.74±0.60[c]	15.33±0.32[b]
	HF	11.70±0.36[c]	11.97±0.35[e]	10.57±0.57[b]	14.36±0.46[c]	8.60±0.36[c]	10.60±0.60[d,e]	11.34±0.27[g]	14.50±0.35[e]	14.53±0.25[b]
	EAF	15.80±1.91[b]	15.47±0.42[b]	11.43±0.45[b]	14.74±0.15[c]	9.64±0.34[c]	14.10±0.36[c]	12.02±0.19[f]	18.36±0.66[c]	19.57±0.25[a]
	AF	10.67±2.08[c]	12.97±0.25[d]	7.20±0.62[c,d]	9.82±0.29[h]	6.48±0.22[d]	7.97±0.85[e,f]	7.08±0.58[m]	14.22±0.64[e,f]	11.40±0.46[e]
Iotrochota birotulata	**CE**	7.33±0.58[d]	14.60±0.53[c]	7.57±1.04[c,d]	11.54±0.29[f]	ND	10.30±1.37[d,e]	8.58±0.27[j,k]	12.10±0.46[g]	13.50±0.44[d]
	HF	7.60±1.28[d]	11.97±0.65[e]	7.00±0.30[c,d]	10.56±0.48[g]	ND	12.30±0.30[c,d]	8.00±0.25[k]	13.08±0.34[f,g]	14.03±2.61[b]
	EAF	8.37±1.00[d]	11.33±0.42[f]	9.70±0.20[b]	9.56±0.22[h,i]	ND	14.47±0.42[c]	8.40±0.30[j,k]	14.44±0.50[e]	14.83±0.55[c]
	AF	6.70±0.89[d]	6.13±0.12[j]	6.60±0.30[d]	7.22±0.36[k,l]	ND	6.53±0.61[f]	6.64±0.19[n]	7.46±0.18[k]	7.00±0.61[g]
Aaptos chromis	**CE**	ND	9.13±0.15[g]	ND	10.62±0.23[g]	ND	8.40±0.35[e]	9.38±0.19[i]	12.72±0.18[f,g]	14.33±0.45[c]
	HF	ND	10.07±0.31[g]	ND	8.44±0.42[j]	ND	9.50±0.30[e]	10.36±0.13[h]	10.88±0.75[h]	10.30±0.44[e]
	EAF	ND	11.23±0.32[f]	ND	8.16±0.25[j,k]	ND	7.23±0.32[e,f]	13.50±0.39[e]	14.44±0.38[e]	14.50±0.46[c]
	AF	ND	7.37±0.35[i]	ND	6.80±1.16[l,m]	ND	6.20±0.36[f]	6.64±0.17[n]	8.62±0.36[j]	8.17±0.21[f]
Biemna fortis	**CE**	ND	8.20±0.0.20[h]	ND	10.30±0.29[g,h]	ND	ND	6.80±0.34[m,n]	11.69±0.44[h]	10.20±0.17[e]
	HF	ND	10.10±0.56[g]	ND	11.46±0.19[f]	ND	ND	7.56±0.19[l,m]	12.32±0.18[g,h]	14.77±0.93[c]
	EAF	ND	10.93±0.40[f]	ND	11.16±0.23[f,j]	ND	ND	9.50±0.28[i]	14.34±0.25[e,f]	13.80±0.80[c,d]
	AF	ND	6.20±0.20[j]	ND	6.98±0.27[l]	ND	ND	ND	7.42±0.19[k]	6.10±0.10[h]
***Spheciospongia* sp**	**CE**	ND	8.30±0.30[h]	ND	8.46±0.21[j]	ND	ND	8.36±0.18[j,k]	12.62±0.66[g]	13.23±0.21[d]
	HF	ND	9.10±0.56[h]	ND	8.76±0.19[i,j]	ND	ND	8.72±0.29[j]	12.38±0.83[g]	8.43±0.38[f]
	EAF	ND	12.00±0.50[e]	ND	9.04±0.21[j]	ND	ND	9.18±0.26[i]	12.88±0.36[f,g]	11.40±0.53[e]
	AF	ND	ND	ND	ND	ND	ND	ND	6.72±0.25[h]	6.37±0.35[g]
Haliclona tuberosa	**CE**	ND	6.20±0.20[j]	ND	7.46±0.21[k,l]	ND	ND	8.18±0.33[k]	11.90±0.66[g,h]	8.17±0.45[f]
	HF	ND	7.30±0.44[i]	ND	7.76±0.19[k]	ND	ND	7.16±0.34[m]	6.70±0.31[k]	9.23±0.21[f]
	EAF	ND	10.20±0.20[g]	ND	8.24±0.11[j,k]	ND	ND	7.80±0.16[k,l]	12.08±0.40[g,h]	11.27±1.03[e]
	AF	ND	6.30±0.36[j]	ND	ND	ND	ND	ND	6.92±0.31[k]	6.37±0.35[g]
***Halichondria* sp**	**CE**	ND	ND	ND	ND	ND	ND	ND	8.82±0.36[j]	7.37±0.12[g]
	HF	ND	ND	ND	ND	ND	ND	ND	7.52±0.24[k]	8.42±0.33[f]
	EAF	ND	ND	ND	ND	ND	ND	ND	9.76±0.40[i]	9.50±0.36[f]
	AF	ND	ND	ND	ND	ND	ND	ND	6.82±0.15[k]	6.27±0.29[g]
Chl		14.50±0.87[b]	16.07±0.31[b]	16.33±1.63[a]	20.80±1.30[a]	16.80±0.84[b]	16.40±0.40[b]	18.00±0.71[b]	20.40±1.34[b]	17.73±0.64[b]
Amp		18.27±1.12[a]	21.10±0.26[a]	18.40±0.56[a]	17.40±1.52[b]	20.20±0.84[a]	20.17±1.76[a]	21.20±0.84[a]	22.20±1.84[a]	20.47±1.75[a]

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Values are the mean of three independent determinations N.B: *E.c (Escherichia coli*); *P.f* (*Pseudomonas fluorescens*); *P.a* (*Pseudomonas aeruginosa*); *K.o* (*Klebsiella oxytoca*); *K.p* (*Klebsiella pneumoniae*); *S.m* (*Serratia marcescens); S.e* (*Salmonella enterica*); *B.a* (*Bacillus cereus*); *S.a* (*Staphylococcus aureus*) ; Chl: Chloramphenicol, Amp: Ampicillin, Tet: Tetracycline; CE: Crude extract; HF: Hexane fraction; EAF: Ethyl acetate fraction; AF: Aqueous fraction; (ND): Not Detected. Note: Diameter of inhibition zones include diameter of discs (6mm). For each bacterium, different letters in the same column indicate significant differences ($p < 0.05$) between the mean values.

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Table 2 Minimum Inhibitory/Bactericidal Concentration of the most efficacious sponge total crude extracts and fractions.

Sponge Species/ Standard antibiotics	Sponge extracts	Minimum Inhibitory/Bactericidal Concentrations (mg/mL)								
		Gram-negative bacteria							Gram-positive bacteria	
		E.c	*P.f*	*P.a*	*K.o*	*K.p*	*S.m*	*S.e*	*B.c*	*S.a*
Neopetrosia exigua	**CE**	0.313	0.156	NT	0.156	1.25	0.313	0.625	**(0.078)** (0.312) BC	**(0.078)** (0.312) BC
	HF	0.313	0.313	>2.5	0.313	0.625	0.156	0.313	0.156	0.156
	EAF	**(0.078)** (0.625) BC	**(0.078)** (0.312) BC	1.25	0.156	0.313	0.156	0.156	**(0.039)** (0.078) BC	**(0.039)** (0.078) BC
	AF	0.625	0.313	NT	0.313	NT	1.25	NT	0.156	0.313
Iotrochota birotulata	**CE**	NT	**0.078** (>2.5) BS	-	0.313	NT	0.625	1.25	0.313	0.313
	HF	NT	0.313	-	0.313	NT	0.313	NT	0.313	0.156
	EAF	>2.5	0.313	-	0.625	0.625	0.156	0.625	0.156	0.156
	AF	NT	0.625	-	NT	-	NT	NT	NT	NT
Aaptos chromis	**CE**	-	0.312	-	0.313	-	>2.5	1.25	0.313	0.156
	HF	-	1.25	-	1.25	-	NT	0.625	0.625	0.313
	EAF	-	0.313	-	0.625	-	0.625	0.156	0.156	0.156
	AF	-	NT	-	NT	-	NT	NT	0.625	1.25
Biemna fortis	**CE**	-	>2.5	-	1.25	-	-	NT	0.625	0.625
	HF	-	1.25	-	0.625	-	-	NT	0.313	0.156
	EAF	-	0.625	-	0.313	-	-	0.313	0.156	0.156
	AF	-	NT	-	NT	-	-	-	NT	NT
***Spheciospongia* sp**	**CE**	-	>2.5	-	>2.5	-	-	>2.5	0.313	0.625
	HF	-	1.25	-	>2.5	-	-	>2.5	NT	0.625
	EAF	-	0.313	-	0.625	-	-	1.25	0.313	0.313

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	AF	-	-	-	-	-	-	-	NT	NT
Haliclona tuberosa	**CE**	-	NT	-	NT	-	-	>2.5	0.625	1.25
	HF	-	NT	-	NT	-	-	NT	NT	0.625
	EAF	-	0.625	-		-	-	NT	0.625	0.313
	AF	-	NT	-	-	-	-	-	-	-
Chl		0.128 (0.256) BC	0.064 (0.512) BS	0.128 (0.512) BC	0.016 (0.128) BC	0.128 (0.256) BC	0.032 (0.256) BC	0.032 (0.128) BC	0.016 (0.016) BC	0.064 (0.064) BC
Amp		0.064 (0.256) BC	0.016 (0.128) BC	0.064 (0.256 BC	0.032 (0.064) BC	0.064 (0.128) BC	0.016 (0.064) BC	0.016 (0.032) BC	0.016 (0.128) BC	0.032 (0.128) BC
Tet		0.256 (0.512) BS	0.128 (>0.512) BS	0.128 (>0.512) BS	0.032 (>0.512) BS	0.064 (0.128) BC	0.064 (0.128) BC	0.064 (0.256) BC	0.064 (0.512) BC	0.064 (0.256) BC

Values are the mean of three independent determinations N.B: *E.c (Escherichia coli*); *P.f* (*Pseudomonas fluorescens*); *P.a* (*Pseudomonas aeruginosa*); *K.o* (*Klebsiella oxytoca*); *K.p* (*Klebsiella pneumoniae*); *S.m* (*Serratia marcescens); S.e* (*Salmonella enterica*); *B.a* (*Bacillus cereus*); *S.a* (*Staphylococcus aureus*). CE: Crude Extract; HF: Hexane Fraction; EAF: Ethyl acetate Fraction; AF: Aqueous Fraction; Chl: Chloramphenicol, Amp: Ampicillin, Tet: Tetracycline; (-): Not evaluated because marine extract did not show any inhibitory effect (MIC) upto 2.5 mg/ml. NT: Not tested because marine extract showed an inhibitory effect with inhibition zone <8 mm by disc diffusion method. (): MBC value (mg/mL). BS: Bacteriostatic; BC: Bacteriocidal. Values in bold represent significant antibacterial activity of the sponge crude extracts or fractions.

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Table 3 Antibiotic potentiating activity of EAF *N. exigua*

Antibiotics	Concentration of EAF *N. exigua* (mg/mL)	Bacterial strains, MIC (mg/mL) of antibiotics in the absence and presence of EAF *N. exigua* extract and FIC in parenthesis			
		E.c	***P.f***	***S.a***	***B.c***
Chl	0	0.128	0.064	0.064	0.016
	MIC/4	0.064 $(0.5)^S$	0.016 $(0.25)^S$	0.032 $(0.5)^S$	0.008 $(0.5)^S$
	MIC/2	0.032 $(0.25)^S$	0.032 $(0.5)^S$	0.016 $(0.25)^S$	0.008 $(0.5)^S$
Amp	0	0.064	0.016	0.032	0.016
	MIC/4	0.064 $(1)^I$	0.016 $(1)^I$	0.008 $(0.25)^S$	0.004 $(0.25)^S$
	MIC/2	0.064 $(1)^I$	0.016 $(1)^I$	0.004 $(0.125)^S$	0.004 $(0.25)^S$
Tet	0	0.256	0.128	0.064	0.064
	MIC/4	0.256 $(1)^I$	0.064 $(0.5)^S$	0.032 $(0.5)^S$	0.032 $(0.5)^S$
	MIC/2	0.256 $(1)^I$	0.064 $(0.5)^S$	0.016 $(0.25)^S$	0.016 $(0.25)^S$

Values are the mean of three independent determinations. N.B: Chl: Chloramphenicol, Amp: Ampicillin, Tet: Tetracycline; Bacterial strains: *E.c (Escherichia coli*); *P.f* (*Pseudomonas fluorescens*); *B.a* (*Bacillus cereus*); *S.a* (*Staphylococcus aureus*); Potentiating effect: S; Indifferent: I (-); FIC: Fractional Inhibitory Concentration.

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Table 4 Chemical constituents of EAF of *N. exigua* detected by GC-MS.

Peak	Retention time (min)	Compound name	Relative percentage ± SD
1	3.571	Hexadecanoic acid, trimethylsilyl ester	1.8±0.5
2	4.515	trans-9-Octadecenoic acid, trimethylsilyl ester	2.0±0.1
3	4.627	Octadecanoic acid, trimethylsilyl ester	0.5±0.0
4	5.605	Hexanedioic acid, bis(2-ethylhexyl) ester	6.8±1.0
5	6.767	Bis(2-ethylhexyl) phthalate	22.6±0.2
6	8.598	1-Monooleoylglycerol trimethylsilyl ether	0.5±0.3
7	15.71	Cholesterol trimethylsilyl ether	16.2±1.9
8	16.361	Silane, [(3.beta.)-cholesta-5,24-dien-3-yloxy]trimethyl-	4.9±0.4
9	16.696	Silane, trimethyl[[(3.beta.,4.alpha.,5.alpha.)-4-methylcholesta-8,24-dien-3-yl]oxy]-	2.3±0.3
10	18.354	Silane, [[(3.beta.,24R)-ergost-5-en-3-yl]oxy]trimethyl-	2.5±0.3
11	19.292	Stigmasterol trimethylsilyl ether	0.6±0.0
12	20.626	beta-Sitosterol	1.5±0.6
13	21.532	beta.-Sitosterol trimethylsilyl ether	23.9±3.8

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Figure Captions

Fig.1. Satellite images showing the Mascarene region (A), Mauritius is located at 20.2000° S, 57.5000° E in the Indian Ocean (B), Research site map area of Amber Island located at 20°03'52.5"S and 57°41'19.7"E (C) (Source: Google Earth 2016).

Fig.2. GC-MS chromatogram of EAF of *N. exigua*. The inset shows the chemical structure of compounds 1, 5, 7 and 13.

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Figure 1

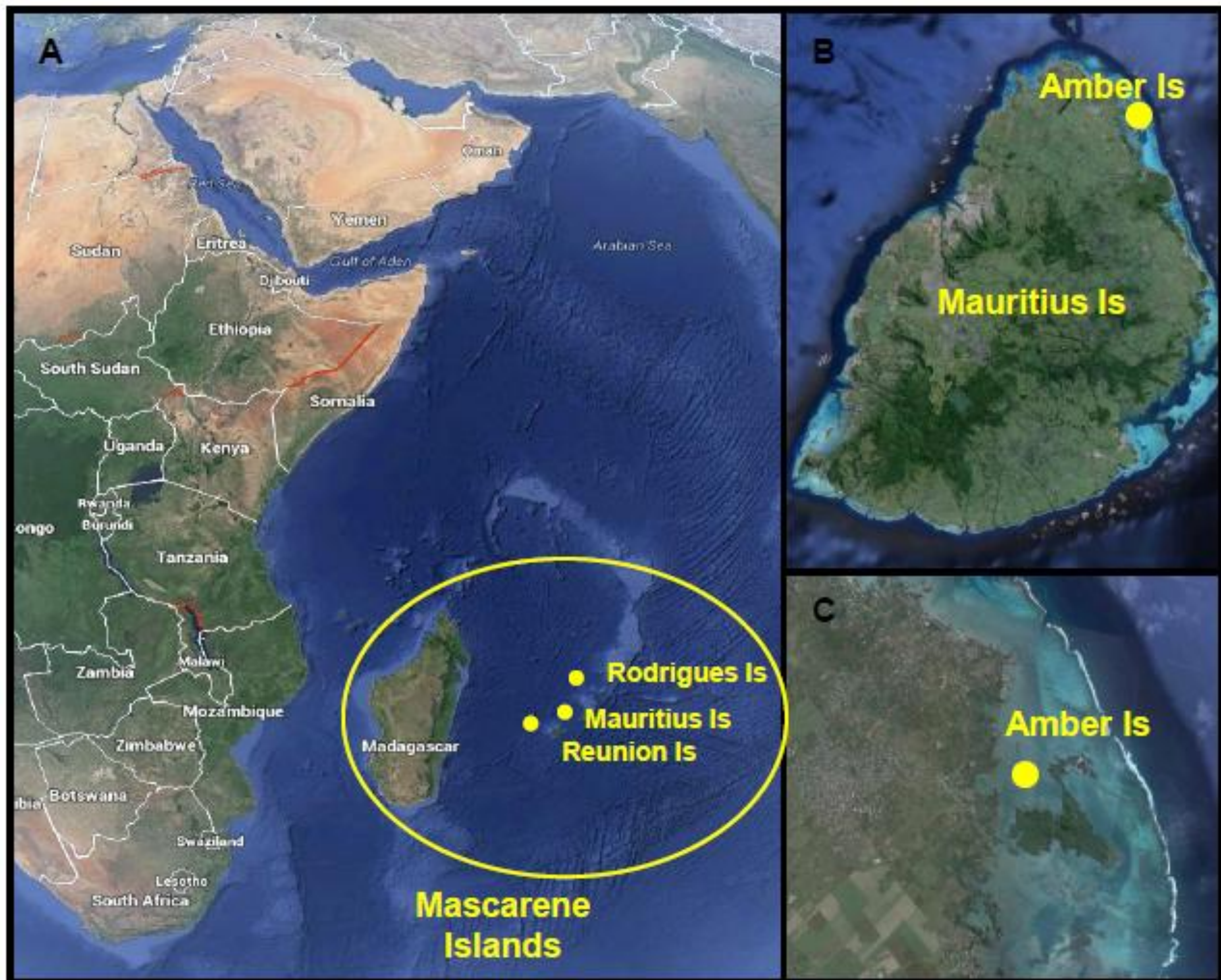

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Figure 2