Journal of Marine Science and Technology

Volume 26 | Issue 1 Article 13

ANTIMICROBIAL, ANTIVIRAL AND CYTOTOXIC ACTIVITIES OF SELECTED MARINE ORGANISMS COLLECTED FROM THE COASTAL AREAS OF MALAYSIA

Yik Sin Chan
Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Bandar Barat, Kampar, Perak, Malaysia.

Cheong Wei Ong
Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Bandar Barat, Kampar, Perak, Malaysia.

Bee Ling Chuah
Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Bandar Barat, Kampar, Perak, Malaysia

Kong Soo Khoo
Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Bandar Barat, Kampar, Perak, Malaysia

Fook Yee Chye
Faculty of Food Science & Nutrition, Universiti Malaysia Sabah, UMS Road, Kota Kinabalu, Sabah, Malaysia.

See next page for additional authors

ANTIMICROBIAL, ANTIVIRAL AND CYTOTOXIC ACTIVITIES OF SELECTED MARINE ORGANISMS COLLECTED FROM THE COASTAL AREAS OF MALAYSIA

Acknowledgements

This study was supported partly by UTAR Research Fund(IPSR/RMC/UTARRF/2012-C2/K03) and by a research grant from the Kuok Foundation (Vote: 4393/000). The authors thank Ministry of Higher Education, Malaysia for the MyMaster scholarship granted to Ms. Yik Sin Chan for her Master of Sciencecandidature. The technical assistance provided by Ms. Chee Kei Kong is much appreciated

Authors

Yik Sin Chan, Cheong Wei Ong, Bee Ling Chuah, Kong Soo Khoo, Fook Yee Chye, and Nam Weng Sit

Journal of Marine Science and Technology, Vol. 26, No. 1, pp. 128-136 (2018)
DOI: 10.6119/JMST.2018.02_(1).0012

ANTIMICROBIAL, ANTIVIRAL AND CYTOTOXIC ACTIVITIES OF SELECTED MARINE ORGANISMS COLLECTED FROM THE COASTAL AREAS OF MALAYSIA

Yik Sin Chan[1], Cheong Wei Ong[1], Bee Ling Chuah[1], Kong Soo Khoo[2], Fook Yee Chye[3], and Nam Weng Sit[1]

Key words: antibacterial, antifungal, seaweed, sponge, soft coral.

ABSTRACT

Many marine organisms have developed the capability of producing unique metabolites and thus are highly likely to contain anti-infective agents. This study was conducted to investigate extracts of three seaweeds (*Caulerpa racemosa*, *Caulerpa sertularioides*, *Kappaphycus alvarezii*), two soft corals (*Lobophytum microlobulatum*, *Sarcophyton auritum*) and a marine sponge (*Spheciospongia vagabunda*) collected from Malaysian coast for antibacterial, antifungal, antiviral and cytotoxic activities. The samples were subjected to sequential solvent extraction in order to obtain hexane, chloroform, ethyl acetate, ethanol, methanol and water extracts. The antibacterial and antifungal activities were studied using a colorimetric broth microdilution method. The hexane extract of *L. microlobulatum* had the strongest antibacterial activity and exhibited the lowest minimum inhibitory concentration (0.04 mg/mL) and minimum bactericidal concentration (0.08 mg/mL) against *Staphylococcus aureus* and *Bacillus cereus*, respectively. For antifungal activity, the lowest MIC and minimum fungicidal concentration values were produced by the hexane extract of *S. auritum* against the dimorphic yeast *Cryptococcus neoformans*, both with 0.04 mg/mL. None of the extracts were active against the filamentous fungus *Aspergillus fumigatus*. Only the hexane and ethanol extracts of *L. microlobulatum* and the ethyl acetate extract of *S. auritum* exhibited strong inhibition on the cytopathic effect induced by the Chikungunya virus (a re-emerging mosquito-borne virus) with 50% effective concentrations of 14.3 ± 0.2, 124.3 ± 1.9 and 176.6 ± 9.7 µg/mL, respectively. Extracts from the two soft corals, *L. microlobulatum* and *S. auritum* possessed stronger antibacterial, antifungal and antiviral activities compared to the seaweeds and the sponge.

I. INTRODUCTION

Despite advances in medical technology and scientific knowledge, infectious diseases remain as a leading cause of morbidity and mortality (Tekwu et al., 2012). Bacteria have been recognized as one of the major etiological agents for lower respiratory tract infections and diarrheal diseases, which are among the top 10 killers according to the World Health Organization (WHO) estimates (WHO, 2015a). The incidence of fungal infections, such as candidiasis, cryptococcosis and aspergillosis has significantly increased over the past few decades (Debourgogne et al., 2016; Vallabhaneni et al., 2016). Candidiasis is a major health threat for human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) patients.

Antibiotics or antimicrobial drugs used against many bacterial or fungal diseases are rapidly losing their effectiveness as microorganisms develop resistance towards them. Due to the advent of new resistance mechanisms, this has resulted failure of treatment, leading to prolonged illness and increased risk of death (Tanwar et al., 2014). In the European Union alone, an estimated 25,000 deaths were caused by drug-resistant microorganisms and costing more than US 1.5 billion dollars every year in healthcare expenses and productivity losses (WHO, 2015b). One of the measures to minimize the increasing rate of microbial resistance is to have a continuous investigation for new, safe and effective alternative agents.

The Chikungunya virus is a re-emerging mosquito-borne virus that causes Chikungunya fever, typically with acute and chronic musculoskeletal pain in humans. Since 2004, the virus

Paper submitted 01/06/17; revised 07/18/17; accepted 09/29/17. Author for correspondence: Nam Weng Sit (e-mail: sitnw@utar.edu.my).
[1] *Department of Biomedical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Bandar Barat, Kampar, Perak, Malaysia.*
[2] *Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman, Bandar Barat, Kampar, Perak, Malaysia.*
[3] *Faculty of Food Science & Nutrition, Universiti Malaysia Sabah, UMS Road, Kota Kinabalu, Sabah, Malaysia.*

has caused millions of cases of disease in the Indian Ocean region and emerged to Europe, Middle East and Pacific regions (Morrison, 2014). The latest outbreaks of Chikungunya fever occurred in Mandera East sub-county of Kenya (WHO, 2016) and in Karachi, Pakistan (Aamir et al., 2017). Since many patients are not reporting to healthcare facilities, it is likely that the incidence of cases is underestimated (WHO, 2016). Patients with Chikungunya fever are only treated symptomatically with anti-inflammatory and analgesic drugs as there are no specific drugs against the virus (Caglioti et al., 2013).

The marine environment is a remarkable pool of a wide variety of organisms which are producers of novel and bioactive secondary metabolites with diverse chemical structures (Devi et al., 2011). However, when compared with terrestrial plants, the use of marine organisms in folk medicine is very restricted because of their relative inaccessibility (Blunden, 2001). Many marine organisms produce secondary metabolites with great pharmaceutical potential. There are four marine natural product-derived drugs approved for clinical use: cytarabine which has anticancer properties, vidarabine which acts against herpes simplex and varicella zoster viruses, ziconotide which has analgesic activity and trabectedin which is used for the treatment of ovarian carcinoma and soft tissue sarcoma (Cavalcante-Silva et al., 2013).

Seaweeds are classified into three main divisions: green (Chlorophyta), brown (Phaeophyta) and red (Rhodophyta) (Wang et al., 2014). *Caulerpa racemosa* and *Caulerpa sertularioides*, belonging to Chlorophyta are commonly known as sea grapes and green feather alga, respectively. *Kappaphycus alvarezii* belongs to the Rhodophyta and is commonly known as Elkhorn sea moss. It is a main source of *kappa* carrageenan (Hayashi et al., 2010). Recent reviews highlight that marine algae possess anticoagulant, antihyperlipidemic, antihyperglycemic, anti-inflammatory, antioxidative, antitumor, antiviral, immunomodulating and hepatoprotective activities (Wang et al., 2014; Wells et al., 2017).

Spheciospongia vagabunda is a marine sponge belonging to the family Clionaidae. Marine sponges are sessile organisms that lack of circulatory, nervous and digestive systems. Marine soft corals such as *Sarcophyton auritum* are also sessile organisms that have less rigid calcium carbonate structure and with a soft and leathery appearance (Cooper et al., 2014). Extracts of *S. vagabunda* and *S. auritum* have shown strong activity against liver and breast cancer cell lines (Eltamany et al., 2014). Soft corals of the genus *Lobophytum* produce many cembranoid diterpenes, such as crassumolide E, an inhibitor of acetylcholinesterase, and lobohedleolide, (7Z)-lobohedleolide and 17-dimethylaminolobohedleolide which have anti-HIV activity (Rashid et al., 2000; Bonnard et al., 2010).

Malaysia has one of the largest continental shelf areas in the tropical world, which is a region rich in biodiversity (Mazlan et al., 2005). As such, the potential of marine organisms in the coastal areas of Malaysia should be explored, in order to have a better understanding on their antimicrobial and antiviral properties. In this study, three species of seaweeds, i.e., *Caulerpa racemosa*, *Caulerpa sertularioides* and *Kappaphycus alvarezii*, two species of soft corals, *Lobophytum microlobulatum* and *Sarcophyton auritum*, and a marine sponge *Spheciospongia vagabunda* were evaluated for antibacterial activity against Gram-positive and Gram-negative bacteria, antifungal activity against yeasts and filamentous fungi, antiviral activity against the Chikungunya virus and cytotoxicity on the African monkey kidney epithelial (Vero) cells.

II. MATERIALS AND METHODS

1. Collection and Identification of Marine Organisms

Caulerpa racemosa, *Caulerpa sertularioides*, *Lobophytum microlobulatum*, *Sarcophyton auritum* and *Spheciospongia vagabunda* were collected from the coastal area of Port Dickson, Negeri Sembilan, West Malaysia. *Kappaphycus alvarezii* was collected from the coastal area of Semporna, Sabah, East Malaysia. The fresh materials were transported in sea water to the laboratory located at Kampar, Perak, West Malaysia for processing except for *K. alvarezii* which was oven-dried and delivered to the laboratory via courier service. Species identification was performed by Dr. Kong Soo Khoo (one of the researchers) for *C. racemosa* (Forsskål) J.Agardh and *C. sertularioides* (S.G.Gmelin) M.Howe; Dr. Suhaimi Md Yasir (Seaweed Research Unit, Universiti Malaysia Sabah) for *K. alvarezii* (Doty) Doty ex P.C.Silva; Dr. van Ofwegen Leen (Naturalis Biodiversity Center, RA Leiden, The Netherland) for *L. microlobulatum* (Tixier-Durivault, 1970); Dr. Ping-Jyun Sung (National Museum of Marine Biology and Aquarium, Pingtung, Taiwan) for *S. auritum* (Verseveldt & Benayahu, 1978); and Dr. de Voogd Nicole (Naturalis Biodiversity Center, RA Leiden, The Netherland) for *S. vagabunda* (Ridley, 1884).

2. Preparation of Marine Organism Extracts

The marine organisms were cleaned thoroughly before freeze drying, except for *K. alvarezii* which was oven-dried at 40°C. The dry samples were blended into powder form and subjected to sequential solvent extraction using hexane, chloroform, ethyl acetate, ethanol, methanol and distilled water in order to extract bioactive compounds. The maceration was carried out at room temperature for three cycles (one day per cycle). The filtrate for each solvent was evaporated using a rotary evaporator while the water extract was lyophilized. All the dried extracts were kept at -20℃ prior to bioassay.

3. Bioassays

1) Antibacterial Assay

Two species of Gram-positive bacteria (*Bacillus cereus* ATCC® 11778™ and *Staphylococcus aureus* ATCC® 6538™) and four species of Gram-negative bacteria (*Acinetobacter baumannii* ATCC® 19606™, *Escherichia coli* ATCC® 35218™, *Klebsiella pneumoniae* ATCC® 13883™ and *Pseudomonas aeruginosa* ATCC® 27853™) were evaluated in the study. Prior to inoculum preparation, all the bacteria were sub-cultured on Mueller-Hinton agar (MHA) at 37℃ for 24 h. The bacterial inocula were

prepared according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2013).

The colorimetric broth microdilution method, with some modifications, of Eloff (1998) was used. A stock solution of each extract at 10 mg/mL was prepared in a methanol-water mixture (2:1, v/v). The stock solution was serially diluted two-fold with Mueller-Hinton broth (MHB) in 96-well microplates to obtain eight concentration values ranging from 0.02 to 2.50 mg/mL. Chloramphenicol/tetracycline with a final concentration range of 1 to 128 μg/mL was used as the positive control. Prepared bacterial inoculum (50 μL) was then added to the wells, making the total volume of each well 100 μL. Sterility control (medium only), growth control (bacterium only) and negative control (extract only) were also included in each 96-well microplate. The microplates were incubated at 37°C for 24 h. Twenty μL of iodonitrotetrazolium chloride (INT) at 0.4 mg/mL was added into each well after incubation. The color change of the indicator was observed and minimum inhibitory concentration (MIC) was recorded. Twenty μL from the wells which showed inhibitory effect (no color change) was inoculated onto MHA and incubated for 24 h. Formation of bacterial colony on the agar surface was observed and minimum bactericidal concentration (MBC) was determined. The assay was conducted in three independent experiments.

2) Antifungal Assay

Four species of yeasts (*Candida albicans* ATCC® 90028™, *Candida parapsilosis* ATCC® 22019™, *Candida krusei* ATCC® 6258™ (teleomorph) and *Cryptococcus neoformans* ATCC® 90112™) and two species of filamentous fungi (*Aspergillus fumigatus* ATCC® 204305™ and *Trichophyton interdigitale* ATCC® 9533™) were used in the study. Prior to inoculum preparation, the yeasts and filamentous fungi were sub-cultured on Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA), respectively. The fungal inocula were prepared according to the guidelines published by Clinical Laboratory Standards Institute (CLSI, 2002a; CLSI, 2002b).

The colorimetric broth microdilution method of Eloff (1998) was adopted with modifications. A stock solution of each extract at 10 mg/mL was prepared in a methanol-water mixture (2:1, v/v). The stock solution was then serially diluted two-fold with RPMI-1640 medium in 96-well microplates to achieve eight concentration values ranging from 0.02 to 2.50 mg/mL. Amphotericin B with a final concentration range of 0.06 to 8 μg/mL was used as the positive control. Prepared fungal inoculum (50 μL) was added to the wells, making the total volume of each well 100 μL. Sterility control (medium only), growth control (fungus only) and negative control (extract only) were included in each microplate. The microplates were incubated at 35°C and 48 h for *Candida* spp.; 35°C and 72 h for *C. neoformans* and *A. fumigatus*, and 28°C and 7 d for *T. interdigitale*. Twenty μL of INT at 0.4 mg/mL was added into each well after incubation. The color change of the indicator was observed and minimum inhibitory concentration (MIC) was recorded. Twenty μL from the wells which showed inhibitory effect (no color change) was then inoculated onto SDA/PDA and incubated accordingly. Formation of fungal colony on the agar surface was observed and minimum fungicidal concentration (MFC) was determined. The assay was performed in three independent experiments.

3) Cytotoxicity Assay

African monkey kidney epithelial (Vero) cell line (ATCC®CCL-81™) was used for cytotoxicity testing of the extracts (Chan et al., 2015). The cell line was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% of fetal bovine serum (FBS), 1% of penicillin-streptomycin solution and 3.7 g/L of sodium bicarbonate, and grown at 37°C in a humidified atmosphere with 5% of carbon dioxide.

The cytotoxicity assay was carried out to determine non-cytotoxic concentrations of the extracts to be used in the antiviral assay (Section 3(4)). Vero cells were seeded in each well of 96-well microplates (4×10^4 cells/well) and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. Extracts (100 μL each) of eight different concentrations ranging from 5 to 640 μg/mL, obtained by two-fold serial dilution in DMEM supplemented with 1% of FBS, were added after 24 h of cell seeding. The control wells contained Vero cells without any extract treatment. The microplates were incubated at 37°C in a humidified incubator with 5% CO_2 for 72 h. The cell viability was determined by the Neutral Red uptake assay (Repetto et al., 2008) after incubation. The assay was conducted in three independent experiments with duplicates for each experiment.

4) Antiviral Assay

The antiviral assay was carried out in the Virology & Immunology Laboratory with restricted access and equipped with Biosafety Level 2 facilities. The Chikungunya virus used belonged to the Bagan Panchor strain (accession number EU703761; Asian genotype) and provided by Professor Shamala Devi of Faculty of Medicine, University of Malaya, Malaysia. The virus was propagated in the Vero cells and harvested after cytopathic effect had developed and stored at -80°C prior to bioassay.

Non-cytotoxic concentrations of the extracts with cell viability ≥ 90%, as determined from the cytotoxicity assay, were deployed in the cytopathic inhibition effect assay (Chan et al., 2016). Vero cells (4×10^4 cells/well) were seeded in 96-well microplates and incubated at 37°C and 5% CO_2 for 24 h. Extracts of six different concentrations, obtained by two-fold serial dilution in DMEM supplemented with 1% of FBS, were then added together with Chikungunya virus at a multiplicity of infection of one. The virus titre was determined according to the method of Reed and Muench (1938). Medium (DMEM only), virus (cells with virus only) and cell (cells with medium only) controls were incorporated in each microplate. Chloroquine with a final concentration range of 0.39 to 12.4 μM was used as the positive control. The microplates were then incubated at 37°C and 5% CO_2 for 72 h. Determination of cell viability was carried out by the Neutral Red uptake assay (Repetto

et al., 2008). The assay was performed in three independent experiments with duplicates for each experiment. Upon comple-

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of marine organism extracts against medically-important bacteria.

Extracts	Gram-positive bacteria				Gram-negative bacteria							
	Bacillus cereus		*Staphylococcus aureus*		*Acinetobacter baumannii*		*Escherichia coli*		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*	
	MIC[a]	MBC[a]	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
						Caulerpa racemosa						
Hex	0.31	0.31	0.63	NA	NA	-	NA	-	0.31	0.31	NA	-
Chl	0.63	0.63	0.63	NA	NA	-	NA	-	0.63	NA	NA	-
EA	0.31	0.31	0.63	NA	NA	-	NA	-	0.63	2.50	NA	-
EtOH	2.50	2.50	NA	-	NA	-	NA	-	NA	-	NA	-
MeOH	1.25	1.25	NA	-	NA	-	NA	-	2.50	2.50	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.004	-	0.004	-	0.002	-	0.002	-	0.004	-	0.002	-
						Caulerpa sertularioides						
Hex	0.16	0.63	0.16	NA	NA	-	NA	-	0.31	NA	NA	-
Chl	0.08	0.16	0.16	NA	NA	-	NA	-	0.63	0.63	NA	-
EA	0.16	0.16	0.31	NA	NA	-	NA	-	0.31	NA	NA	-
EtOH	0.31	0.63	0.63	NA	NA	-	NA	-	1.25	1.25	NA	-
MeOH	1.25	1.25	NA	-	NA	-	NA	-	2.50	NA	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.004	-	0.004	-	0.002	-	0.002	-	0.004	-	0.002	-
						Kappaphycus alvarezii						
Hex	1.25	1.25	NA	-	NA	-	NA	-	2.50	2.50	NA	-
Chl	1.25	1.25	NA	-	NA	-	NA	-	2.50	2.50	NA	-
EA	2.50	2.50	NA	-	NA	-	NA	-	NA	-	NA	-
EtOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
MeOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.004	-	0.004	-	0.002	-	0.002	-	0.004	-	0.002	-
						Lobophytum microlobulatum						
Hex	0.08	0.08	0.04	NA	NA	-	NA	-	0.08	NA	2.50	NA
Chl	0.31	0.31	0.16	2.50	1.25	NA	NA	-	0.31	NA	1.25	NA
EA	1.25	1.25	0.63	NA	1.25	NA	NA	-	1.25	2.50	1.25	NA
EtOH	NA	-	NA	-	2.50	NA	NA	-	NA	-	1.25	NA
MeOH	NA	-	NA	-	NA	-	NA	-	NA	-	1.25	NA
Water	NA	-	NA	-	NA	-	NA	-	NA	-	1.25	NA
Antibio	0.008	-	0.008	-	0.008	-	0.004	-	0.016	-	0.032	-
						Sarcophyton auritum						
Hex	1.25	1.25	0.16	NA	2.50	NA	NA	-	1.25	NA	2.50	2.50
Chl	0.63	0.63	0.31	NA	NA	-	0.63	NA	0.63	2.50	1.25	2.50
EA	0.63	NA	0.63	NA	2.50	NA	0.63	NA	NA	-	0.63	2.50
EtOH	NA	-	0.63	NA	NA	-	0.63	NA	NA	-	1.25	NA
MeOH	NA	-	NA	-	NA	-	0.63	NA	NA	-	2.50	NA
Water	NA	-	NA	-	NA	-	1.25	NA	NA	-	2.50	NA
Antibio	0.002	-	0.016	-	0.001	-	0.001	-	0.004	-	0.016	-
						Spheciospongia vagabunda						
Hex	1.25	1.25	2.50	NA	NA	-	NA	-	1.25	1.25	NA	-
Chl	1.25	2.50	2.50	NA	NA	-	NA	-	1.25	1.25	NA	-
EA	0.63	0.63	2.50	NA	NA	-	NA	-	0.63	1.25	NA	-
EtOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
MeOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.004	-	0.004	-	0.002	-	0.002	-	0.004	-	0.002	-

[a] The MIC and MBC values are expressed as mean of three consistent independent replicates in mg/mL. Abbreviations: Hex, hexane extract; Chl, chloroform extract; EA, ethyl acetate extract; EtOH, ethanol extract; MeOH, methanol extract; Antibio, chloramphenicol (positive control) was used for *B. cereus, S. aureus* and *K. pneumoniae* whereas tetracycline (positive control) was used for *A. baumannii*, *E. coli* and *P. aeruginosa*. NA denotes no activity while "-" denotes not performed.

tion of the experiments, all the microplates were autoclaved prior to disposal.

4. Data Analysis

The percentages of cell viability at different extract concentrations were analyzed by one-way analysis of variance (ANOVA) using IBM® SPSS® Statistics (Version 20) software. The two-tailed significance level was set at $P < 0.05$. Post hoc test, either with Tukey's (equal variance assumed) or Dunnett's (equal variance not assumed) test was further conducted to determine which concentration of extract was effective.

III. RESULTS

1. Antibacterial Activity

The bacteriostatic and bactericidal activities of an extract are denoted by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), respectively. As shown in Table 1, not all the extracts possess both activities. By considering one extract against one bacterial species as one bioassay, 37.0% (80/216) of the bioassays showed bacteriostatic activity while only 17.1% (37/216) showed bactericidal activity. At the concentrations used for this study, none of the extracts were able to kill *A. baumannii* and *E. coli*.

Table 2. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of marine organism extracts against medically-important fungi.

Extracts	Yeasts								Filamentous Fungi			
	Candida albicans		*Candida parapsilosis*		*Candida krusei*		*Cryptococcus neoformans*		*Aspergillus fumigatus*		*Trichophyton interdigitale*	
	MIC[a]	MFC[a]	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
						Caulerpa racemosa						
Hex	0.31	NA	0.63	0.63	0.31	0.31	0.16	0.31	NA	-	0.31	NA
Chl	0.63	NA	1.25	NA	0.31	0.31	0.16	0.63	NA	-	0.63	NA
EA	0.63	NA	2.50	NA	0.31	0.31	0.31	0.63	NA	-	0.63	NA
EtOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
MeOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.0005	-	0.0005	-	0.001	-	0.0003	-	0.001	-	0.002	-
						Caulerpa sertularioides						
Hex	NA	-	NA	-	NA	-	0.31	0.63	NA	-	0.31	NA
Chl	1.25	NA	2.50	NA	0.63	0.63	0.63	1.25	NA	-	0.63	NA
EA	2.50	NA	2.50	NA	0.31	0.31	0.31	0.63	NA	-	0.31	NA
EtOH	2.50	NA	2.50	NA	1.25	NA	1.25	1.25	NA	-	1.25	NA
MeOH	NA	-	NA	-	1.25	NA	2.50	NA	NA	-	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.0005	-	0.0005	-	0.001	-	0.0003	-	0.001	-	0.002	-
						Kappaphycus alvarezii						
Hex	NA	-	NA	-	NA	-	2.50	2.50	NA	-	2.50	NA
Chl	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
EA	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
EtOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
MeOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.0005	-	0.0005	-	0.001	-	0.0003	-	0.001	-	0.002	-
						Lobophytum microlobulatum						
Hex	NA	-	NA	-	NA	-	0.16	2.50	NA	-	NA	-
Chl	NA	-	NA	-	NA	-	0.08	0.31	NA	-	0.63	2.50
EA	0.08	0.31	0.08	0.16	1.25	2.50	0.16	0.31	NA	-	0.63	1.25
EtOH	0.08	0.31	0.16	0.31	0.04	0.16	0.04	0.08	NA	-	0.08	0.31
MeOH	1.25	2.50	0.63	2.50	0.31	1.25	0.16	0.31	NA	-	1.25	NA
Water	2.50	NA	0.63	1.25	2.50	NA	2.50	NA	NA	-	NA	-
Antibio	0.001	-	0.001	-	0.001	-	0.0003	-	0.002	-	0.004	-
						Sarcophyton auritum						
Hex	0.63	1.25	0.63	0.63	0.63	1.25	0.04	0.04	NA	-	1.25	1.25
Chl	0.16	1.25	1.25	1.25	0.16	1.25	0.08	0.08	NA	-	1.25	1.25
EA	0.16	0.63	0.08	0.63	1.25	1.25	0.08	0.08	NA	-	0.63	1.25
EtOH	0.16	NA	0.08	1.25	0.16	2.50	0.63	1.25	NA	-	NA	-
MeOH	1.25	2.50	0.31	0.63	1.25	NA	2.50	2.50	NA	-	NA	-
Water	1.25	2.50	0.63	NA	2.50	NA	2.50	NA	NA	-	NA	-
Antibio	0.0005	-	0.0005	-	0.004	-	0.0005	-	0.001	-	0.004	-
						Spheciospongia vagabunda						
Hex	NA	-	NA	-	2.50	NA	2.50	NA	NA	-	2.50	NA
Chl	NA	-	NA	-	2.50	NA	2.50	NA	NA	-	2.50	NA
EA	NA	-	NA	-	2.50	NA	1.25	NA	NA	-	2.50	NA
EtOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
MeOH	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Water	NA	-	NA	-	NA	-	NA	-	NA	-	NA	-
Antibio	0.0005	-	0.0005	-	0.001	-	0.0003	-	0.001	-	0.002	-

[a] The MIC and MFC values are expressed as mean of three consistent independent replicates in mg/mL. Abbreviations: Hex, hexane extract; Chl, chloroform extract; EA, ethyl acetate extract; EtOH, ethanol extract; MeOH, methanol extract; Antibio, Amphotericin B (positive control); NA denotes no activity while "-" denotes not performed.

Extracts from the soft corals were found to be more active towards the bacteria compared to the seaweeds and marine sponge. The chloroform and ethyl acetate extracts of *L. microlobulatum*, and the hexane, chloroform and ethyl acetate extracts of *S. auritum* exhibited broad spectrum antibacterial activity as they inhibited the growth of five out of six bacteria tested with their MIC ranges of 0.16-1.25, 0.63-1.25, 0.16-2.50, 0.31-1.25 and 0.63-2.50 mg/mL, respectively (Table 1). The chloro-

form extract of *L. microlobulatum* was the only extract that showed bactericidal effect against *S. aureus*. The three extracts

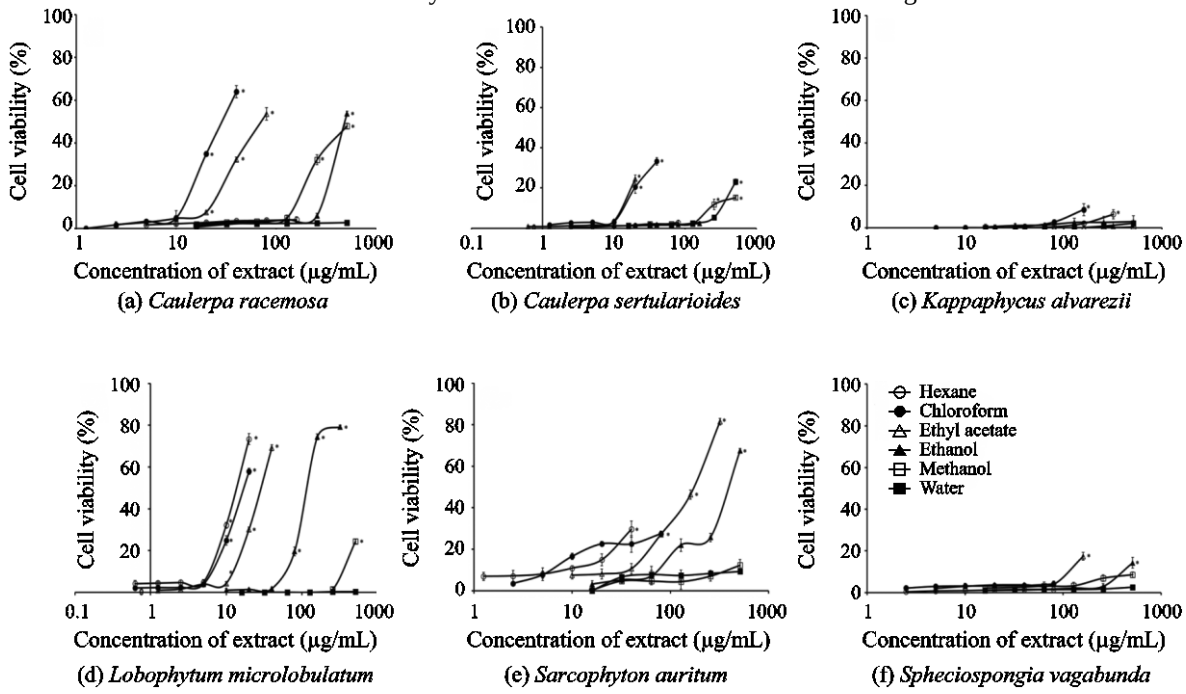

Fig. 1. Viability of African monkey kidney epithelial (Vero) cells co-incubated with the Chikungunya virus and marine organism extracts. The cells were incubated at 37℃ and 5% CO_2 for 72 h and the cell viability was assessed by the Neutral Red uptake assay. The asterisk mark indicates significant difference ($P < 0.05$) when analyzed with one-way ANOVA test. The x-axis is displayed in log scale.

derived from *S. auritum* were the only extracts that killed *P. aeruginosa*. In addition, the strongest bacteriostatic and bactericidal activities, as evident in the lowest MIC and MBC values, were observed in the hexane extract of *L. microlobulatum* against *S. aureus* (0.04 mg/mL) and *B. cereus* (0.08 mg/mL), respectively.

2. Antifungal Activity

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) are used to illustrate the potency of an extract with regard to its fungistatic and fungicidal activities, respectively. Fungistatic activity was observed in 43.5% (94/216) of the bioassays whereas only 25.0% (54/216) of the bioassays showed fungicidal activity (Table 2). *Aspergillus fumigatus* was the most resistant fungus tested, as none of the extracts were effective against it.

Extracts from the two soft corals also appeared to be more active towards the fungi than the seaweeds and sponge. The lowest MIC and MFC values (0.04 mg/mL) were shown by the hexane extract of *S. auritum* against the dimorphic yeast, *C. neoformans*. All extracts of *S. auritum* showed antifungal activity against all the yeasts with MIC ranges of 0.16-1.25 mg/mL against *C. albicans*, 0.08-1.25 mg/mL against *C. parapsilosis*, 0.16-2.50 mg/mL against *C. krusei* and 0.04-2.50 mg/mL against *C. neoformans*. All extracts of *L. microlobulatum* were active against *C. neoformans* with a MIC range of 0.04-2.50 mg/mL. In contrast, neither fungistatic nor fungicidal activity was exhibited by the water extract of *C. sertularioides*, ethanol, methanol and water extracts of *C. racemosa* and *S. vagabunda*, and all the extracts (except the hexane extract) of *K. alvarezii*. The hexane extract of *K. alvarezii* showed antifungal activity against *C. neoformans* and *T. interdigitale* with a MIC value of 2.50 mg/mL.

3. Cytotoxicity and Antiviral Activity

The antiviral activity of the extracts was evaluated based on the inhibition of cytopathic effect caused by the Chikungunya virus on the Vero cells. As the Neutral Red uptake assay measures the viability of cells and does not differentiate the virus-induced cytopathic effect and the cytotoxicity caused by extracts, it is essential to use non-toxic concentrations of the extracts for the antiviral assay. The cytopathic inhibition effect can be classified on an arbitrary basis as strong when cell viability is 70% or higher, moderate when cell viability is between 31% to 69%, and weak when cell viability is 30% or lower. Three of the 36 extracts (8.3%) showed significant strong inhibition effect, while 22.2% of the extracts demonstrated moderate inhibition effect, i.e., the chloroform, ethyl acetate, ethanol and methanol extracts of *C. racemosa*, the chloroform extract of *C. sertularioides*, the chloroform and ethyl acetate extracts of *L. microlobulatum* and the ethanol extract of *S. auritum* (Fig. 1). Other extracts exhibited only weak inhibition effect.

Three extracts showed strong antiviral activity. These were derived from the two soft corals, hexane and ethanol extracts of *L. microlobulatum* with cell viabilities (mean ± s.d.) of 73.3% ± 2.6% and 79.2% ± 0.9%, respectively, and ethyl acetate extract

of *S. auritum* with cell viability of 81.4% ± 1.6% (Fig. 1). The corresponding 50% effective concentrations (EC_{50}), as determined from the plot of percentage of cell viability against extract concentration, were 14.3 ± 0.2, 124.3 ± 1.9 and 176.6 ± 9.7 μg/mL, respectively. Results from the cytotoxicity assay indicated that the 50% cytotoxic concentrations (CC_{50}) for the hexane extract of *L. microlobulatum* was 34.5 ± 0.2 μg/mL while the CC_{50} for the other two extracts exceeded the highest concentration used in the study, 640.0 μg/mL. All extracts from the seaweed *K. alvarezii* and the marine sponge *S. vagabunda* showed weak cytopathic inhibition effect with the mean cell viability being less than 20%.

IV. DISCUSSION

This study reports for the first time the antiviral activity of seaweeds (*C. racemosa* and *C. sertularioides*) and soft corals (*L. microlobulatum* and *S. auritum*) against the Chikungunya virus, and the antibacterial and antifungal activities of *L. microlobulatum* against human pathogens.

Saraiva et al. (2011) proposed a classification for plant extracts based on the potency of antimicrobial activity according to MIC values, whereby an extract can be regarded as highly active (MIC ≤ 0.1 mg/mL), active (0.1 mg/mL < MIC ≤ 0.5 mg/mL), moderately active (0.5 mg/mL < MIC ≤ 1 mg/mL), weakly active (1 mg/mL < MIC ≤ 2 mg/mL) and inactive (MIC > 2 mg/mL). By using the proposed classification, only the chloroform extract of *C. sertularioides* and hexane extract of *L. microlobulatum* were classified as extracts having highly active antibacterial activity. The extracts that were classified as having highly effective against fungi were the chloroform, ethyl acetate, and ethanol extracts of *L. microlobulatum*, and hexane, chloroform, ethyl acetate and ethanol extracts of *S. auritum*. It is evident that soft coral extracts show promising antibacterial and antifungal properties compared to the seaweeds and sponge.

Despite the paucity of reports on the biological activities of extracts from *L. microlobulatum* and *S. auritum* (Eltamany et al., 2014), several chemical constituents have been isolated from these soft corals. Among these compounds, lobane diterpenoids, norsesquiterpene and steroid glycosides have been reported from *L. microlobulatum* (Anjaneyulu and Kameswara Rao, 1996; Anjaneyulu and Raju, 1996). Four diterpene cembranoids and a ceramide have been isolated from the methanol/dichloromethane (1:1) extract of *S. auritum* (Eltahawy et al., 2014; Eltahawy et al., 2015). Lobane diterpenoids and cembranoids are unique secondary metabolites found abundantly in many genera of soft corals (Blunt et al., 2013). They exhibit a wide spectrum of biological activities, including antimicrobial activities (Bonnard et al., 2010; Hussien and Mohamed, 2015), and thus may account for the effects observed in this study. The results also suggest that the activity may be attributed to chemical constituents other than lobanes and cembranoids, due to the diversity of solvent polarity used in the extraction. The type of chemicals likely extracted from the marine organisms is determined by the nature of the solvent used (Grosso et al., 2015).

In this study, three extracts which exhibited strong antiviral activity were obtained from two soft corals, i.e., the hexane and ethanol extracts of *L. microlobulatum* and the ethyl acetate extract of *S. auritum*. Although the potency (based on EC_{50} values) for the latter two extracts was lower than the hexane extract, they also possessed lower cytotoxicity (based on CC_{50} values) compared to the hexane extract. Secondary metabolites of soft corals have attracted much attention from researchers due to their remarkable pharmacological activities. Two norcembranoids, 5-epi-sinuleptolide and sinuleptolide, isolated from a soft coral of the genus *Sinularia* exert moderate inhibition on the Chikungunya virus replicon (Lillsunde et al., 2014). Further studies are needed to isolate and characterize the active compounds from the two soft corals, and to investigate the mechanisms of their action.

The antifungal results of *S. vagabunda* in this study are corroborated qualitatively by the study of Eltamany et al. (2014) which used percentage of growth inhibition instead of MIC as the end-point measurement. The extract which was obtained using methanol-dichloromethane (1:1) showed 19% and 6% of inhibition against *C. krusei* and *C. neoformans*, respectively, which can be considered as non-effective as an antifungal agent. The extract also did not show any inhibitory activity against *C. albicans* and *A. fumigatus* (Eltamany et al., 2014). None of the 36 marine organism extracts used (Table 2) exhibited inhibitory activity against *A. fumigatus*, the causative agent of invasive aspergillosis with a mortality rate of 50%-95% (Kwon-Chung and Sugui, 2013). Such resistance has also been noted in studies using plant extracts (Muschietti et al., 2005; Webster et al., 2008). The resistance might be due to the defence mechanisms of filamentous fungi, such as production of melanin pigment. Melanin is an essential component that maintains the integrity of the conidial walls and mycelia. It protects fungi against damage from ultraviolet irradiation, high temperature, chemical and biochemical stresses (Allam and Abd El-Zaher, 2012). The capacity of the fungus to grow and adapt to stress conditions, its mechanisms for circumventing the effects of immune systems and its ability to cause damage to host cell make *A. fumigatus* a ubiquitous pathogen (Abad et al., 2010).

V. CONCLUSION

Extracts from the two soft corals, *Lobophytum microlobulatum* and *Sarcophyton auritum* possessed stronger antibacterial, antifungal and anti-Chikungunya virus activities compared to the seaweeds and the sponge used in the study. They could serve as potential sources of new anti-infective compounds.

ACKNOWLEDGEMENTS

This study was supported partly by UTAR Research Fund (IPSR/RMC/UTARRF/2012-C2/K03) and by a research grant

from the Kuok Foundation (Vote: 4393/000). The authors thank Ministry of Higher Education, Malaysia for the MyMaster scholarship granted to Ms. Yik Sin Chan for her Master of Science candidature. The technical assistance provided by Ms. Chee Kei Kong is much appreciated.

REFERENCES

Aamir, U. B., N. Badar, M. Salman, M. Ahmed and M. M. Alam (2017). Outbreaks of Chikungunya in Pakistan. The Lancet Infectious Diseases 17(5), 483.

Abad, A., J. V. Fernández-Molina, J. Bikandi, A. Ramírez, J. Margareto, J. Sendino, F. L. Hernando, J. Pontón, J. Garaizar and A. Rementeria (2010). What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. Revista Iberoamericana de Micología 27(4), 155-182.

Allam, N. G. and E. H. F. Abd El-Zaher (2012). Protective role of *Aspergillus fumigatus* melanin against ultraviolet (UV) irradiation and *Bjerkandera adusta* melanin as a candidate vaccine against systemic candidiasis. African Journal of Biotechnology 11(24), 6566-6577.

Anjaneyulu, A. S. R. and K. V. S. Raju (1996). A new lobane diterpene acid from the soft coral *Lobophytum microlobulatum* of the Andaman and Nicobar Islands. Indian Journal of Chemistry 35(1), 45-48.

Anjaneyulu, A. S. R. and N. S. Kameswara Rao (1996). Four new lobane diterpenoids from the soft coral *Lobophytum microlobulatum* of the Havellock Island of the Andaman and Nicobar group of Islands. Indian Journal of Chemistry 35(12), 1294-1303.

Blunden, G. (2001). Biologically active compounds from marine organisms. Phytotherapy Research 15(2), 89-94.

Blunt, J. W., B. R. Copp, R. A. Keyzers, M. H. Munro and M. R. Prinsep (2013). Marine natural products. Natural Product Reports 30(2), 237-323.

Bonnard, I., S. B. Jhaumeer-Laulloo, N. Bontemps, B. Banaigs and M. Aknin (2010). New lobane and cembrane diterpenes from two Comorian soft corals. Marine Drugs 8(2), 359-372.

Caglioti, C., E. Lalle, C. Castilletti, F. Carletti, M. R. Capobianchi and L. Bordi (2013). Chikungunya virus infection: an overview. The New Microbiologica 36(3), 211-227.

Cavalcante-Silva, L. H., A. C. de Carvalho Correia, J. M. Barbosa-Filho, B. A. da Silva, B. V. de Oliveira Santos, D. P. de Lira, J. C. Sousa, G. E. de Miranda, F. de Andrade Cavalcante and M. S. Alexandre-Moreira (2013). Spasmolytic effect of caulerpine involves blockade of Ca^{2+} influx on guinea pig ileum. Marine Drugs 11(5), 1553-1564.

Chan, S. M., K. S. Khoo and N. W. Sit (2015). Interactions between plant extracts and cell viability indicators during cytotoxicity testing: Implications for ethnopharmacological studies. Tropical Journal of Pharmaceutical Research 14, 1991-1998.

Chan, Y. S., K. S. Khoo and N. W. Sit (2016). Investigation of twenty selected medicinal plants from Malaysia for anti-Chikungunya virus activity. International Microbiology 19(3), 175-182.

Clinical and Laboratory Standards Institute (2002a). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-Second Edition. CLSI Document M27-A2. 2nd Ed. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (2002b). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi; Approved Standard. CLSI Document M38-A. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Clinical and Laboratory Standards Institute (2013). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI Document M100-S23. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania.

Cooper, E. L., K. Hirabayashi, K. B. Strychar and P. W. Sammarco (2014). Corals and their potential applications to integrative medicine. Evidence-Based Complementary and Alternative Medicine 2014, 184959.

Debourgogne, A., J. Dorin and M. Machouart (2016). Emerging infections due to filamentous fungi in humans and animals: only the tip of the iceberg? Environmental Microbiology Reports 8(3), 332-342.

Devi, P., S. Wahidulla, T. Kamat and L. D'Souza (2011). Screening marine organisms for antimicrobial activity against clinical pathogens. Indian Journal of Geo-Marine Sciences 40, 338-346.

Eloff, J. N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64(8), 711-713.

Eltahawy, N. A., A. K. Ibrahim, M. M. Radwan, M. A. Elsohly, H. A. Hassanean and S. A. Ahmed (2014). Cytotoxic cembranoids from the Red Sea soft coral, *Sarcophyton auritum*. Tetrahedron Letters 55(29), 3984-3988.

Eltahawy, N. A., A. K. Ibrahim, M. M. Radwan, S. A. Zaitone, M. Gomaa, M. A. ElSohly, H. A. Hassanean and S. A. Ahmed (2015). Mechanism of action of antiepileptic ceramide from Red Sea soft coral *Sarcophyton auritum*. Bioorganic & Medicinal Chemistry Letters 25(24), 5819-5824.

Eltamany, E. E., N. A. Eltahawy, A. K. Ibrahim, T. Temraz, H. A. Hassanean, M. M. Radwan and S. A. Ahmed (2014). Anticancer activities of some organisms from Red Sea, Egypt. Catrina 9(1), 1-6.

Grosso, C., P. Valentão, F. Ferreres and P. B. Andrade (2015). Alternative and efficient extraction methods for marine-derived compounds. Marine Drugs 13(5), 3182-3230.

Hayashi, L., A. Q. Hurtado, F. E. Msuya, G. Bleicher-Lhonneur and A. T. Critchley (2010). A review of *Kappaphycus* farming: Prospects and constraints. In: Seaweeds and their Role in Globally Changing Environments, edited by Israel, A., R. Einav and J. Seckbach, Springer, Dordrecht, 255-283.

Hussien, T. A. and T. Mohamed (2015). The bioactive natural products and biological activities of the Red Sea soft coral Sarcophyton: A review. Research Journal of Pharmaceutical, Biological and Chemical Sciences 6(4), 1301-1319.

Kwon-Chung, K. J. and J. A. Sugui (2013). *Aspergillus fumigatus* - What makes the species a ubiquitous human fungal pathogen? PLoS Pathogens 9(12), e1003743.

Lillsunde, K. E., C. Festa, H. Adel, S. de Marino, V. Lombardi, S. Tilvi, D. A. Nawrot, A. Zampella, L. D'Souza, M. V. D'Auria and P. Tammela (2014). Bioactive cembrane derivatives from the Indian Ocean soft coral, *Sinularia kavarattiensis*. Marine Drugs 12(7), 4045-4068.

Mazlan, A.G., C. C. Zaidi, W. M. Wan-Lofti and B. H. Othman (2005). On the current status of coastal marine biodiversity in Malaysia. Indian Journal of Marine Sciences 34, 76-87.

Morrison, T. E. (2014). Reemergence of Chikungunya virus. Journal of Virology 88(20), 11644-11647.

Muschietti, L., M. Derita, V. Sülsen, J. de Dios Muñoz, G. Ferraro, S. Zacchino and V. Martino (2005). *In vitro* antifungal assay of traditional Argentine medicinal plants. Journal of Ethnopharmacology 102(2), 233-238.

Rashid, M. A., K. R. Gustafson and M. R. Boyd (2000). HIV-inhibitory cembrane derivatives from a Philippines collection of the soft coral *Lobophytum* species. Journal of Natural Products 63(4), 531-533.

Reed, L. J. and H. Muench (1938). A simple method of estimating fifty percent endpoints. American Journal of Hygiene 27, 493-497.

Repetto, G., A. D. Peso and J. Zurita (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols 3(7), 1125-1131.

Saraiva, A. M., R. H. A. Castro, R. P. Cordeiro, T. J. S. P. Sobrinho, V. T. N. A. Castro and E. L. C. Amorim (2011). *In vitro* evaluation of antioxidant, antimicrobial and toxicity properties of extracts of *Schinopsis brasiliensis* Engl. (Anacardiaceae). African Journal of Pharmacy and Pharmacology 5, 1724-1731.

Tanwar, J., D. Das Shrayanee, Z. Fatima and S. Hammed (2014). Multidrug resistance: An emerging crisis. Interdisciplinary Perspectives on Infectious Diseases 2014, 541340.

Tekwu, M. T., A. C. Pieme and V. P. Beng (2012). Investigations of antimicrobial activity of some Cameroonian medicinal plants extracts against bacteria and yeast with gastrointestinal relevance. Journal of Ethnopharmacology 142(1), 265-273.

Vallabhaneni, S., R. K. Mody, T. Walker and T. Chiller (2016). The global burden of fungal diseases. Infectious Disease Clinics of North America 30(1), 1-11.

Wang, L., X. Wang, H. Wu and R. Liu (2014). Overview on biological activities and molecular characteristics of sulfated polysaccharides from marine green algae in recent years. Marine Drugs 12(9), 4984-5020.

Webster, D., P. Taschereau, R. J. Belland, C. Sand and R. P. Rennie (2008). Antifungal activity of medicinal plant extracts; preliminary screening studies. Journal of Ethnopharmacology 115(1), 140-146.

Wells, M. L., P. Potin, J. S. Craigie, J. A. Raven, S. S. Merchant, K. E. Helliwell, A. G. Smith, M. E. Camire and S. H. Brawley (2017). Algae as nutritional and functional food sources: revisiting our understanding. Journal of Applied Phycology 29(2), 949-982.

World Health Organization (2015a). Global Health Observatory Data Repository. Available from http://www.who.int/gho/en/. Accessed October 1, 2016.

World Health Organization (2015b). Antibiotic Resistance. Available from http://www.who.int/mediacentre/factsheets/antibiotic-resistance/en/. Accessed July 31, 2016.

World Health Organization (2016). Chikungunya - Kenya. Available from http://www.who.int/csr/don/09-august-2016-chikungunya-kenya/en/. Accessed October 1, 2016.