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DETERMINATION OF *IN VITRO* ANTIMICROBIAL ACTIVITY OF SELECTED MARINE SPONGES FOUND IN SRI LANKA

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Abstract - Indiscriminate use of antibiotics leads to drug resistance in microorganisms. Therefore, discovery of new molecules to challenge the drug resistance is critically important. Sponges have developed efficient defense mechanisms which largely depend on the production of chemical compounds against foreign attackers. The aim of this work is to study the antimicrobial activities of Clathria rugosa, Clathria foliacea, Acanthella acuta and Antho dichotoma, collected from Dehiwala, Sri Lanka, against ten human pathogens and to separate and study the active ingredients using Bioautographic TLC assay. Mixture of methanolic and dichloromethane extracts of all four sponges were prepared using cold maceration technique. Using the standard disc diffusion method, extracts were tested against five Gram negative bacteria which includes Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella sp. and Escherichia coli, three Gram positive bacteria which includes Staphylococcus aureus, Methicillin Resistant Staphylococcus aureus (MRSA) and Staphylococcus saprophyticus and two fungal species namely Candida albicans and Aspergillus niger. Out of the sponges tested, only C. foliacea was active against S. aureus (Inhibition Zone Diameter = 11.7 mm) and MRSA (IZD= 9.0 mm). A. dichotoma was active against S. aureus (IZD = 9.0 mm), MRSA (IZD = 10.0 mm) and C. albicans (IZD = 13.0 mm). All Gram-negative bacteria, S. saprophyticus and A. nigerhave shown resistance to extracts of all four sponges. The best separation in TLC was observed with a mixture of Hexane, Toluene, Dichloromethane, Diethyl Ether, Methanol and Water mixed in a ratio of 2: 0.05: 0.6: 0.3: 0.1: 0.002. Among the 12 spots of A. dichotoma, visualized under UV light, six spots inhibited the growth of MRSA and all the spotshave shown the antimicrobial activity against C. albicans in TLC bioautography. Three out of seven visualized spots of C. foliacea were active against MRSA. Among the four sponges studied, A. dichotoma showed higher antimicrobial activity in terms of inhibition of bacterial growth, then number of microbial species inhibited as well as higher number of antimicrobial compounds. None of sponges tested were active against Gram negative bacteria in the microbial panel used. S. aureus was the most susceptible pathogen among the tested microorganisms. Even though, S. aureus and MRSA were susceptible for two sponge extracts, S. saprophyticus has shown resistance for all the sponge extracts tested.

Keywords - Sponge, Antimicrobial activity, Cold maceration, TLC bioautography

I. INTRODUCTION

Oceans cover more than 70% of the earths' surface and by volume it represents more than 95% of the biosphere. Still it comprises unexplored mysteries as it is a hard attempt to discover them. Sponges are such mysterious organisms where many of their defense mechanisms are still unknown.

A. What are Sponges?

Sponges are multicellular organisms belonging to Kingdom Animalia, Phylum Porifera. The Phylum comprises several extant classes, i.e. Calcarea, Hexactinellida, Demospongiae and one fossil class, Archaeocyatha, 7 subclasses, 25 orders, 127 families and 682 genera and approximately 8300 species are currently recognized (Hooper et al., 2004).
Sponges are aquatic species. Majority of them are found in marine environments and are important members of reef

ecosystems, while minority found in fresh water habitats. Their cells are embedded in a gelatinous matrix and are arranged around a system of water canals. Therefore the body is porous in nature.

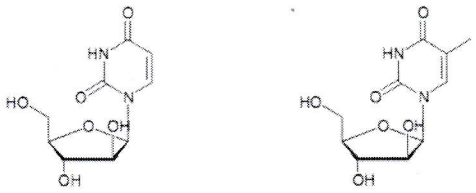
They do not have organs or true tissues. These are sessile filter feeders, which have developed a number of efficient defense mechanisms largely depend on the production of chemical compounds against foreign attackers such as viruses, bacteria, or eukaryotic predators (Bacuset al., 1974). Except for that, secondary metabolites can be produced by sponge associates, like bacteria and fungi (Thakur et al., 2003). These secondary metabolites are also involved in competition for space, providing protection from ultraviolet light etc. Most of the bioactive components of marine sponges are still to be identified.

B. Theoretical and Historical Background of Sponges in the Field of Medicine

Usage of sponges for medicinal purposes has a long history. Approximately 400 BC, Hippocrates recommended to dress soldiers' wounds with certain sponges since they had antibiotic effects. (Riddle 1987).

Marine sponges are known to be a rich source of pharmacologically active metabolites. So far many such compounds with potential biomedical application have been isolated. These include proteins with hemolytic activity, hemagglutinin, compounds that express antibacterial, antifungal, antiviral, anticancer activities etc.

The first influential finding of bioactive compounds from sponges was the isolation and, identification of arabinose containing bioactive nucleosides namely, spongouridine and spongothymidine from *Tethya crypta* (Caribbean sponge) in early 1950s byBergmann (Bhakuni et al., 2005)



Spongouridine Spongothymidine

Figure 01: Arabinose containing bioactive nucleosides (Image source: http://rushim.ru/books/biochemie/bioactive-marine-natural-products.pdf)

In many studies, secondary metabolites of sponges with such activities have been identified. Examples for secondary metabolites are calyculins from *Discodermia calyx* (Kato et al., 1986), discodermolide from *Discodermia dissolute* (Gunasekara et al., 1990), latrunculins from *Latrunculia magnifica* (Spector et al., 1983), spongistatins from *Spongia sp.* and *Spirastrella sp.* (Petit et al., 1994). These are cytotoxic and inhibit cell proliferation and therefore have the potential to be used as chemotherapeutics.

In the study of biological activities of aqueous and organic extracts from tropical marine sponges, Kristina Sepčić and colleagues, (2010) found that almost all the samples were able to prevent the growth of at least one bacterial strain. Many inhibited the growth of Gram positive bacteria, but a few were active against Gram negative bacteria. The extracts that showed considerable activity towards Gram negative strain were those from *Topsentia ophiraphidites* and *Aplysina archeri.*

The strongest antimicrobial activity was found in the organic extracts of Ircinia and Verongula. Brominated compounds isolated from marine organisms have shown a broad spectrum of antimicrobial activity (Bhakuni et al., 2005). But most of them are toxic. Aerothionin, aerophobin-2, aplysinamisin-1, isofistularin-3 from *Aplysiaaerophoba and A. cavernicola* (Thoms et al., 2004) are such compounds.

However still the antimicrobial activities of sponges against bacterial and fungal diseases is little known and there are no antimicrobial compounds from marine sources have yet made it beyond the discovery phase into preclinical phase (Newman et al., 2016), mostly because of their toxicity (Bhakuni et al., 2005).

C. Objectives

The aim of this work is to study the antimicrobial activities of *Clathria rugosa, Clathria foliacea, Acanthella acuta* and *Antho dichotoma,* against ten human pathogens and to separate and study the active ingredients using bioauthographic TLC assay (Hamburger et al., 1987).

II. MATERIALS

A. Samples

Samples of the four sponges were collected fromDehiwala Sri Lanka.

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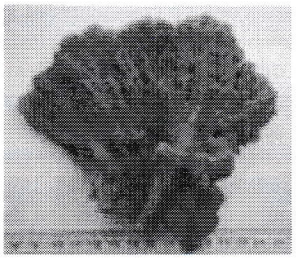

Plate 01: Clathria rugosa

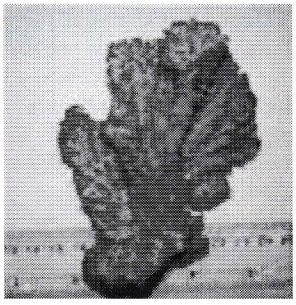

Plate 02: Clathria foliacea

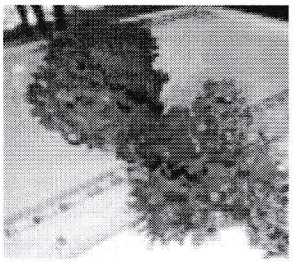

Plate 03: Acanthella acuta

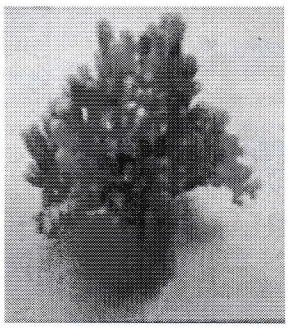

Plate 04: Antho dichotoma

B.Microbial panel

Staphylococcus aureus, Salmonella sp. and *E. coli* were obtained from National Aquatic Resources Research and Development Agency (NARA), Crow Island, Colombo 15. *Klebsiella pneumoniae, Pseudomonas aerogenosa, Proteus mirabilis, Candida albicans, Aspergillus niger,* Methicillin Resistant Staphylococcus aureus (MRSA), *Staphylococcus saprophyticus* were obtained from Faculty of Medicine, University of Peradeniya.

Bacteria were cultured in nutrient agar and fungi were cultured in potato dextrose agar. The stock cultures were maintained at 40C.

III. METHODS

A.Preparation of extracts

Cold maceration technique was used to prepare the extracts. First the samples were kept outside for several minutes for the water to drain. Approximately 20 g wet weight of each sponge sample was chopped and then separately added to 50 ml conical flasks containing 30 ml of methanol in each. Then each flask was closed with polythene. After 24 hours at room temperature the sponge tissues were squeezed by pressing it in between glass funnel and spatula to remove soaked solvent. All the solutions were filtered using Whatman No.01 filter papers separately.

Using the same method Dichloromethane extracts were also prepared using already methanol extracted, filtered, sponge tissues. The two extracts were mixed and the solvent mixture was evaporated at 400C under vacuum with the use of rotary evaporator. The resulted solid was dried in a vacuum desiccator. The solid was weighed.

Then it was re-dissolved in a known amount of methanol, dichloromethane solvent mixture to create a solution with 0.05 g cm-3 concentration.

Table 01: Volume of the solvent mixture added to prepare 0.05 g cm^{-3} concentrated extracts

Sample	Wet drained weight (g)	Weight of the solid after evaporating the solvent (mg)	Volume of the solvent mixture added to prepare 0.05 g cm^{-3} (cm^3)
Clathria rugosa	20.85	399.6	08.0
Clathria foliacea	20.20	646.9	12.9
Acanthella acuta	18.22	285.4	05.7
Antho dichotoma	20.17	273.3	05.5

B. Antimicrobial assay

Antibacterial activity was tested using the standard disc diffusion method.

1)Preparation of 0.5 McFarland Standards:

Aqueous solutions of $BaCl_2$(0.5 mL of 0.048 mol dm^{-3}) and 9.95 mL of 0.18 mol dm^{-3} H_2SO_4 (aq) was mixed to prepare the McFarland standard.T 70 UV/VIS Spectrometer was used to measure theoptical density. Optical density (OD) of a 0.5 McFarland standard at 530 nm (OD_{530}) ranges between 0.11 and 0.14.The prepared McFarland standard was stored at 4^0C.

2)Preparation of inoculums:

At least four morphologically similar colonies were transferred into 5mL of sterile distilled water. Then the visible turbidity was adjusted by adding distilled water or adding more colonies until it is equal to0.5 McFarland standard (0.5 McFarland standard ≈1.5×108 CFU/mL).

3) Preparation of agar plates:

S. aureus, Salmonella sp., E. coli, K. pneumoniae, P. aerogenosa, P. mirabilis, C. albicans, Methicillin Resistant *S. aureus* (MRSA), *S. saprophyticus* lawns were prepared by spreading the bacterial suspension separately (correspondent of 0.5McFarland Standard) on Nutrient Agar plates using a sterilized cotton swab. Aspergillus niger was streaked evenly on Potato Dextrose Agar plates. The distance between streaks is about 7 mm.

4) Application of sponge extracts:

Dried sterile (Whatman No 01) filter paper discs with 5mm diameter were prepared using approximately 10 µL of 0.1 g cm^{-3} sponge extracts separately. Five different discs were placed on each plate. Entire assay was carried out in triplicate.

5) Control discs:

Dried sterile paper discs were prepared using the two solvents, methanol and dichloromethane separately to determine whether there could be any effect of residues of the solvents with antimicrobial activity.

6) Incubation conditions:

The subjective microorganisms are human pathogens. Therefore, all the plates were incubated at 370C for about 24 hours.

7) Selection of sponges with antimicrobial activity:

Sponges that resulted in inhibition zones were noted and the diameter of the inhibition zones were measured and tabulated.

C. TLC analysis of sponge extracts

Pre-coated (MERCK) analytical silica gel G60 F254 A1 sheets with a florescence indicator were used for Thin Layer Chromatography.

1) Selection of a proper mobile phase for TLC analysis:

Toluene, hexane, diethyl ether, methanol, dichloromethane and water were mixed in different ratios to find a proper mobile phase.

2) Application of Extracts:

4 µL of 0.1 g cm^{-3} sponge extracts which resulted antimicrobial activity in previous assay was applied on TLC plates. The TLC plates were pre-heated at 1800C for 1 hour to sterilize.

3) Development of Chromatograms:

The plates were developed in a pre-saturated chamber using Hexane, Toluene, Dichloromethane, Diethyl Ether,

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Methanol and Water (Mixed in a ratio of 2: 0.05: 0.6: 0.3: 0.1: 0.002) solvent system.

4) Visualization:

Separated compounds were visualized under (254 nm and 365 nm) UV light. The separated compounds were marked and the Rf values were calculated and tabulated.

$$\text{Retardation factor } (R_f) = \frac{\text{Distance from starting front to the compound}}{\text{Distance from starting front to solvent front}}$$

5) Selection of compounds with antimicrobial activity:

The developed chromatograms were placed facing up in 16 cm diameter Petri dishes. Nutrient agar medium mixed with the susceptible microorganisms were poured on the TLC plates. Plates were incubated at 37°C for about 24 hours. Inhibition zones were visualized by adding hydrogen peroxide reagent. Marked the compounds with inhibitory activities and their R_f values were noted. Entire assay was carried out in triplicate.

IV. RESULTS AND DISCUSSION

Indiscriminate use of antibiotics leads to drug resistance in microorganisms. Discovery of new molecules to challenge the drug resistance is critically important. Oceans which cover more than 70% of the earths' surface still comprise unexplored mysteries as it is a hard attempt to discover them. Sponges are such mysterious organisms where many of their defense mechanisms are still unknown. Bioactivity of marine sponges against bacterial and fungal diseases is little known. Therefore, aim of this work is to study natural antibiotics from marine sponges.

Prior to extraction, it is essential to freeze the dead sponges to prevent possible contaminations. If the contaminating microorganism has an antimicrobial activity, it would result false positives. And it is not advisable to wash sponges using distilled water. Some compounds dissolve even in salt saturated water when the sponge is dead. Washing with distilled water will remove valuable secondary metabolites which are in very small quantities. Cold maceration technique is advisable to extract compounds which are unknown, as they may be intolerant to higher temperatures. Methanol is a polar solvent with the polarity index of 5.1. Polar compounds show higher affinity to polar solvents whereas non-polar compounds show higher affinity to non-polar solvents like dichloromethane which has the polarity index of 3.1.

One of the biggest problems with these extracts is that they contain a significant amount of water. The boiling point of water is 1000C, where methanol and dichloromethane has lower boiling points, 650C and 400C respectively. Bringing the temperature of the solvents to a higher temperature in order to evaporate the solvent would damage the thermo labile compounds. Rotary evaporator is used to bring down the boiling temperatures of the solvents under vacuum. This also will reduce the possible damages to the thermo labile compounds.

In the in vitro antimicrobial assay of methanol, dichloromethane extracts of four sponges were tested against *S. aureus, Salmonella sp., E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis, C. albicans, A. niger,* MRSA and *S. saprophyticus* and their potency was measured as diameter of the inhibition zone (Table 02).

A. Antimicrobial activities

Salmonella sp., E. coli, K. pneumoniae, P. aeruginosa, P. mirabilis, S. saprophyticus and A. niger were resistant to extracts of all sponges. In the study of Biological Activities of Aqueous and Organic Extracts from Tropical Marine Sponges, Sepčić and colleagues, (2010) found that many inhibited the growth of Gram positive bacteria, but a few were active against Gram negative bacteria. The only extracts that showed considerable activity towards Gram negative strain in their study were those from Topsentia ophiraphidites and Aplysina archeri.

Table 02: Average diameters of inhibition zones in millimeters

Microorganism / Test material	*C. rugosa* extract	*C. foliacea* extract	*A. acuta* extract	*A. dichotoma* extract	CH_3OH added disc	CH_2Cl_2 added disc
S. aureus	0.0	11.7	0.0	9.0	0.0	0.0
MRSA	0.0	9.0	0.0	10.0	0.0	0.0
C. albicans	0.0	0.0	0.0	13.0	0.0	0.0

Clathria rugosa and *Acanthella acuta* were not effective against any of the subjective microorganism. *Clathria foliacea* extract was active against *S. aureus* and MRSA. Antho dichotoma has shown the highest antimicrobial activity and it was active against *C. albicans, S. aureus* and MRSA. However in a certain study Clathria indica which is an Indian sponge has shown antibacterial activity against common and multi drug resistant Salmonella typhiand anti-fungal activity against *C. albicans and C. neoformans.* And it was ineffective against *E. coil, P. aeruginosa, Streptococcus* pyogenes and S. aureus (Ravichandran et al., 2011). In a similar study eleven Kalihinol compounds, which are multifunctional diterpenoid antibiotics, from two *Acanthella sp.* has shown antimicrobial activity against *S. aureus, Bacillus subtilis* and *C. albicans* (Clifford et al., 1987).

B. Thin Layer Chromatography

Most appropriate mobile phase for Thin Layer Chromatography was a mixture of Hexane, Toluene, Dichloromethane, Diethyl Ether, Methanol and Water mixed in a ratio of 2: 0.05: 0.6: 0.3: 0.1: 0.002. In the antimicrobial assay of separated compounds it is better to use preparative grade TLC plates, where compound loading capacity is high, for a better detection of inhibition zones.

1) Developed Chromatograms:

Twelve different compounds of *A. dichotoma*, eight different compounds of *C. foliacea* were separated. The developed chromatograms were observed under 254 nm and 365 nm UV wave lengths. (Plate 06)

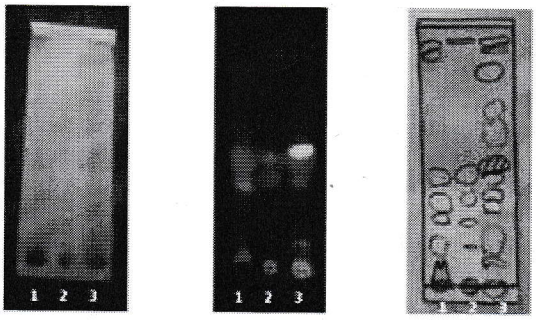


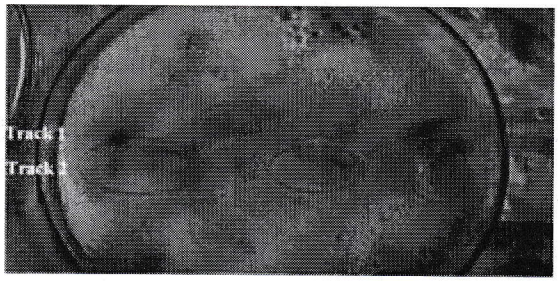
Plate 06: TLC of methanol, dichloromethane extract of C. foliacea (Track 01), *A. dichotoma* (Track 03) developed with Hexane, Toluene, Dichloromethane, Diethyl Ether, Methanol and Water (Mixed in a ratio of 2: 0.05: 0.6: 0.3: 0.1: 0.002) solvent system and visualized under (A) 254nm and (B) 365nm UV radiation (C) Diagram showing the separated compounds.

2) Antimicrobial activity of separated compounds:

Bio autography is a method to localize antimicrobial activity on a chromatogram in order to find new antibiotics. Here the antimicrobial compounds are transferred from the chromatographic layer to an inoculated agar layer through a diffusion process. But the diffusion rates of the compounds may be different. To minimize the problem it is recommended to mix the susceptible microorganisms with the growing medium. Any inhibition was not observed when the bacterial lawns are prepared on the surface of the medium. Inhibition zones were visualized by adding hydrogen peroxide reagent. Quickly, the compounds with inhibitory activities were marked before the foam cover the entire plate. The R_f values of the compounds were noted.

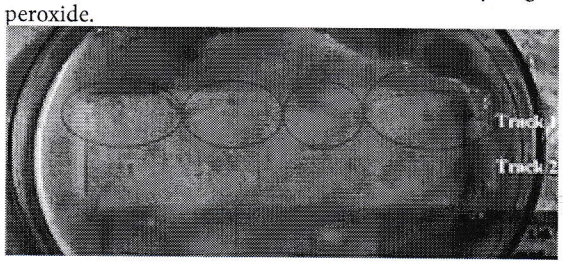
After the addition of Hydrogen peroxide, the inhibition zones on the TLC plates were clearly visualized. Three out of eight compounds (Table 04) of *C. foliaceaand* six out of twelve compounds (Table 03) of *A. dichotomawere* active against MRSA (Plate 07). All the separated compounds (Table 03) of *A. dichotomahave* shown antimicrobial activity against *Candida albicans* (Plate 08).

Plate 07: Visualization of inhibition zones on MRSA cultured plates after the addition of Hydrogen peroxide.



(Track 1: C. foliaceaextract and Track 2: A. dichotomaextract)

Plate 08: Visualization of inhibition zones on Candida albicans cultured plates after the addition of Hydrogen peroxide.



(Track 1 and Track 2: A. dichotoma extract)

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Table 03: Separated compounds of A. dichotomashowing antimicrobial activity.

Microorganism / Rf value	MRSA	*C. albicans*
0.00	+	+
0.10	+	+
0.16	+	+
0.30	-	+
0.34	-	+
0.41	-	+
0.45	-	+
0.59	+	+
0.68	+	+
0.84	-	+
0.91	-	+
0.94	+	+

Table 04: Separated compounds of C. foliacea showing antimicrobial activity.

Microorganism / Rf value	MRSA
0.00	+
0.05	-
0.15	-
0.25	+
0.32	+
0.43	-
0.89	-
0.94	-

Antimicrobial assay against *S. aureus* could not accomplish due to the lack of time and lack of extractions prepared.

V. CONCLUSION

Antho dichotoma is the most bioactive sponge in antimicrobial activity in terms of inhibition then number of microbial species inhibited as well as higher number of antimicrobial compounds.

None of sponges tested were active against the Gram negative bacteria in the microbial panel used. *S. aureus* was the most susceptible organism for the sponge extracts tested. Even though *S. aureus* and MRSA were susceptible for few sponge extracts, *S. saprophyticus* has shown resistance for all the sponge extracts used.

VI. FUTURE PERSPECTIVES

More complete separation of extracts can be achieved by two dimensional (2D) development of TLC. The second development should be performed using a different mobile phase. Visualization methods should be improved for a better detection of inhibition zones on chromatograms. If there is a significant compound with such activities, it is worthy to elucidate the chemical structure, in order to synthesis an economical drug.

Methicillin Resistant *Staphylococcus aureus* (MRSA) is of major concern as it expresses resistance against commonly used antibiotics such as all penicillinase stable penicillins, macrolides, lincosamide, tetracyclines etc. Here are two sponges which comprises potential chemotherapeutic compounds against MRSA. The compounds should be tested for toxicity and side effects prior clinical trials.

A study should be done to verify that the compounds with antimicrobial activities are of microbial origin or sponge origin. If the compounds are of microbial origin, a separate study should be carried out isolating the symbiotic microorganisms and testing for their metabolites.

REFERENCES

Andrews, J. M. (2001), Determination of minimum inhibitory concentrations, Journal of Antimicrobial Chemotherapy, 48, Suppl. S1, 5- 16.

Bacus, G.J. and Green, G. (1974), Toxicity in sponges and holothurians: a geographic pattern, Science, 13, pp. 951–953.

Bhakuni, D. S. and Rawat, D.S. (2005), Bioactive Marine Natural Products, ISBN 1-4020-3484-9 (e-book), Available at: http://rushim.ru/books/biochemie/bioactive-marine-natural-products.pdf, Accessed at: 28 March 2016.

Cavalieri, S. J., Rankin, I. D., Harbeck, R. J., Sautter, R. L., McCarter, Y. S., Sharp, S. E., Ortez, J. H., Spiegel, C. A. (2005), Manual of antimicrobial susceptibility testing, American Society for Microbiology, ISBN 1-55581-349-6, P.102-107.

Chang, C. W. J., Patra, A., Baker, J. A., and Scheuer, P. J.(1987),Kalihinols, Multifunctional Diterpenoid Antibiotics From Marine Sponges Acanthella ssp., J. Am. Chem. Soc. 1987.109, 6119-6123.

Gunasekera, S. P., Gunasekera, M., Longley, R. E., Schulte, G. K. (1990), Discodermolide: a new bioactive polyhydroxylated lactone from the marine sponge Discodermia dissoluta. J Org Chem.; 55; 4912–4915.

Hamburger, M.O. and Cordell, G.A. (1987), A Direct Bioautographic TLC Assay for Compounds Possessing Antibacterial Activity, Journal of Natural Products, Vol. 50, No. I, pp. 19-22.

Hooper, J.N.A. and van Soest, R.W.M. (2004), Systema Porifera. A Guide to the Classification of Sponges. The End of a Beginning, Boll. Mus. Ist. Biol. Univ. Genova, 68, pp. 22.

International society of ethnobiology (2016) A Brief History of the Human Use of Marine Medicines, Available at: http://www.ethnobiology.net/brief-history-human-use-marine-medicines/, Accessed at: 23 March 2016.

Kato, Y., Fusetani, N., Matsunaga, S., Hashimoto, K., Fujita, S., Furuya, T. (1986), Bioactive marine metabolites, Part 16, Calyculin A, A novel antitumor metabolite from the marine sponge Discodermia calyx. J. Am. Chem. Soc.; 108: 2780–2781.

Laport, M., Santos, O., Muricy, G., 2009. Marine Sponges: Potential Source of new antimicrobial drugs. Curr Pharm Biotechnol; 10:86–105. PMID: 19149592.

Newman, D.J. and Cragg, G.M. (2016), Drugs and Drug Candidates from Marine Sources: An Assessment of the Current "State of Play", Planta Med; 82(09/10), DOI: 10.1055/s-0042-101353, pp. 784-785.

Petit, G. R., Cichacz, Z. A., Herald, C.L., Gao F, Boyd, M. R., Schmidt, J. M., Hamel, E., Bai, R. (1994), Antineoplastic agent 300. Isolation and structure of the rare human cancer inhibitory macrocyclic lactones spongistatins 8 and 9, J. Chem. Soc. ChemCommun: 1605–1606.

Ravichandran, S., Wahidullan, S., D'Souza, L., and Anbuchezhian, R. M., 2011.Antimicrobial Activity of Marine Sponge Clathriaindica.BioorgKhim.2011 Jul-Aug; 37(4): 483-9.

Riddle, J.M. (1987) Folk tradition and folk medicine: recognition of drugs in classical antiquity. In Folklore and Folk Medicine, edited by J. Scarboroough. Madison: American Institute of the History of Pharmacy.

Sepčić, K., Kauferstein, S., Mebs, D., Turk, T. (2010), Biological Activities of Aqueous and Organic Extracts from Tropical Marine Sponges. Mar Drugs; 8(5): 1550–1566. PMCID: PMC2885080, Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2885080/, Accessed at: 04/04/2016.

Spector, I., Shochet, N. R., Kashman, Y., Groweiss, A. (1983), Latrunculins-novel marine toxins that disrupt microfilament organization in cultured cells. Science; 214:493–495

Thakur, N.L., Hentschel, U., Krasko, A., Pabel, C.T., Anil, A.C., Mu¨ ller, W.E.G., (2003) Antibacterial activity of the sponge Suberitesdomuncula and its primmorphs: potential basis for epibacterial chemical defense. AquatMicrobEcol 31, 83–87.

Thoms, C., Wolff, M., Padmakumar, K., Ebel, R., Proksch, P. (2004), Chemical defense of Mediterranean sponges Aplysina cavernicola and Aplysina aero

phoba, Zeitschrift fur Naturforschung. C, Journal of biosciences, 59(1-2):113-22, PMID: 15018063, Abstract only. Available at: https://www.ncbi.nlm.nih.gov/pubmed/15018063, Accessed at: 04/04/2016.

Tudela, J. L. R., Estrella, M. C., Guerra, T. M. D., Mellado, E. (2001), Standardization of Antifungal Susceptibility Variables for a Semiautomated Methodology, American Society for microbiology, journal of clinical microbiology, doi: 10.1128/JCM.39.7.2513-2517.2001, Available at: http://jcm.asm.org/content/39/7/2513.full, Accessed at: 23/03/2016.

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