AN INVESTIGATION OF BIOACTIVE CONSTITUENTS IN TROPICAL SPONGES

TOH HAN LOONG

THIS DISSERTATION WAS WRITTEN TO FULFILL A PART OF THE REQUIREMENT TO OBTAIN A DEGREE IN BACHELOR OF SCIENCE WITH HONORS

MARINE SCIENCE PROGRAM
SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITI MALAYSIA SABAH

March 2007

UNIVERSITI MALAYSIA SABAH

BORANG PENGESAHAN STATUS TESIS@

JUDUL: AN INVESTIGATION OF BIOACTIVE CONSTITUENTS IN TROPICAL SPONGES

Ijazah: SARJANA MUDA SAINS DENGAN KEPUJIAN (SAINS MARIN)

SESI PENGAJIAN: 2004 / 2007

Saya TOH HAN LOONG

(HURUF BESAR)

mengaku membenarkan tesis (LPS/Sarjana/Doktor Falsafah)* ini disimpan di Perpustakaan Universiti Malaysia Sabah dengan syarat-syarat kegunaan seperti berikut:

1. Tesis adalah hakmilik Universiti Malaysia Sabah.
2. Perpustakaan Universiti Malaysia Sabah dibenarkan membuat salinan untuk tujuan pengajian sahaja.
3. Perpustakaan dibenarkan membuat salinan tesis ini sebagai bahan pertukaran antara institusi pengajian tinggi.
4. **Sila tandakan (/)

[ ] SULIT (Mengandungi maklumat yang berdarjah keselamatan atau kepentingan Malaysia seperti yang termaktub di dalam AKTA RAHSIA RASMI 1972)

[ ] TERHAD (Mengandungi maklumat TERHAD yang telah ditentukan oleh organisasi/badan di mana penyelidikan dijalankan)

[/] TIDAK TERHAD

Disahkan oleh

Tohhanloong.
(TANDATANGAN PENULIS)

(TANDATANGAN PUSTAKAWAN)

Alamat Tetap: 32, Tmn Sg Chua, 43000 Kajang, Selangor Darul Ehsan

DR. CHARLES S. VAIRAPPAN
Nama Penyelia

Tarikh: 16 April 2007

Tarikh: 16 April 2007

CATATAN: * Potong yang tidak berkenaan.

** Jika tesis ini SULIT atau TERHAD, sila lampirkan surat daripada pihak berkuasa/organisasi berkenaan dengan menyatakan sekali sebab dan tempoh tesis ini perlu dikelaskan sebagai SULIT dan TERHAD.

@ Tesis dimaksudkan sebagai tesis bagi Ijazah Doktor Falsafah dan Sarjana secara penyelidikan, atau disertasi bagi pengajian secara kerja kursus dan penyelidikan, atau Laporan Projek Sarjana Muda (LPSM).

DECLARATION

I declare that this is the product of my own work except for the excerpts and summaries which the sources have been stated.

16 March 2007

TOH HAN LOONG

HS2004 - 2751

APPROVED BY

Signature

1. SUPERVISOR

(DR. CHARLES S. VAIRAPPAN) ____________

2. CO-SUPERVISOR

(MISS ZARINAH WAHEED) ____________

3. EXAMINER 1

(DR. SUJJAT AL AZAD) ____________

4. EXAMINER 2

(DR. NORMAWATY MD. NOOR) ____________

5. DEAN

(DR. SHARIFF A. KADIR S. OMANG) ____________

ACKNOWLEDGEMENT

The author wishes to express appreciation to Dr. Charles for his contribution in the research of this project and the opportunity he provided to widen the intellectual capacity of the author. In addition, special thanks to Ms. Zarinah for her assistance during sampling and lab assistants in the Institute for Tropical Biology and Conservation (ITBC) whose help is vital during critical times. I would also want to thank all the post graduate students of Borneo Marine Research Institute (BMRI) and Marine Science program seniors whose familiarity with the needs and ideas was helpful during the early laboratory work and writings. Also, remembered always, my friends for their valuable inputs. As usual, the author takes full responsibility for the information and opinions contained in this project. The errors? Mine. All mine.

ABSTRAK

Kewujudan sebatian bioaktif secara semulajadi pada span laut meyokong teori evolusi dalam adaptasi pertahanan kimia. Sesetengah sebatian kimia ini menunjukkan kesan positif kepada manusia dari segi perubatan. Hatta, tujuan kajian ini adalah untuk menyiasat potensi span laut tropika dalam penghasilan sebatian bioaktif. Estrak mentah untuk 3 spesis span laut diekstrak dan kromatografi lapisan nipis serta bioesei digunakan untuk menyiasat potensi antibakteria. Pemisahan selanjutnya ekstrak mentah dilakukan mengunakan kromatografi lapisan nipis preparative untuk mendapatkan sebatian major. Sebatian bioaktif dicirikan berdasarkan nilai R_f, pencahayaan UV dan penyembur kimia khas. Kajian menunjukkan spesis 3 menghasilkan peratusan ekstrak mentah tertinggi dengan 1.1315 peratus diikuti spesis 2 dan spesis 1 masing-masing dengan peratusan 0.1944 % dan 0.0843 %. Kebanyakkan sebatian spesis 1 adalah tidak berkutub manakala sebatian spesis 2 dan spesis 3 mempunyai kekutuban yang tinggi. Ekstak mentah heksana bagi spesis 1 dan metanol bagi spesis 3 mempunyai aktiviti terhadap bakteria marin *Clostridium cellabioparum, Clostridium novyi* dan *Vibrio parahaemolyticus*. Pencirian sebatian bioaktif dalam spesis 1 berkemungkinan sterol dengan nilai R_f 0.87 dalam larutan HE (3:1 v/v) manakala sebatian aktif dalam spesis 3 pula dikenalpasti berkemungkinan glikolipid dengan nilai R_f 0.24 dalam larutan CMW (6.5:3.5:0.4 v/v/v).

ABSTRACT

The common occurrence of biologically active natural products in sponges reflects an important ecological adaptation as chemical defense. Some of these chemicals have been found to have beneficial pharmaceutical effect for human. As such, the purpose of this research is to investigate the potential of tropical sponges for bioactive properties. Crude extract for 3 tropical sponges obtained using extraction while TLC and PTLC Bioassay were used to screen the crude extracts for bioactive properties. Further separation was done using PTLC to obtain major compound. Bioactive compounds in crude extract is then characterise using R_f values, UV illumination and TLC spray reagents. Results indicate that species 3 produced the highest crude extract yield of 1.1315 percent followed by species 2 with 0.1944 percent and species 1-lowest with 0.0843 percent. Most of the compounds in species 1 are non polar whereas compounds in species 2 and 3 are of high polarity. Hexane crude extract for species 1 and methanol crude extract for species 3 shown activities against marine bacteria *Clostridium cellabioparum, Clostridium novyi* and *Vibrio parahaemolyticus.* TLC characterisation conclude that bioactive compound in species 1 is probably sterol with R_f value of 0.87 in HE (3:1 v/v) solvent while bioactive compound in species 3 could be glicolipid with R_f value of 0.24 in CMW (6.5:3.5:0.4 v/v/v) solvent.

CONTENTS

LIST OF TABLES

LIST OF DIAGRAMS

LIST OF ABBREVIATIONS

TLC	Thin Layer Chromatography
PTLC	Preparative Thin Layer Chromatography
HE	Hexane: Ethyl acetate (3:1 v/v)
CMW	Chloroform: Methanol: Water (7.5:2.5:0.4 v/v)
UV	Ultra Violet
R_f	Mobility relative to front
Molybdo	Molybdo (VI) phosphoric acid reagent
Orcinol	Orcinol Monohydrate reagent
g	Gram
mg	Miligram
L	Litre
ml	Milliliter
ºC	Degree Celsius

CHAPTER 1

INTRODUCTION

1.1 Realising Natural Product Potentials

The potential of natural products and secondary metabolites has fascinated the human race for centuries. The drugs that were used by ancient civilization were extracts from plants and animal products with significant therapeutic effects (Oleszek, 2000). Natural products such as morphine, codeine and penicillin are still widely in use today. And with the dawn of new century, scientists are on the brink of discovering new application for the use of natural products. The understanding that nature could yield novel drug template was a reality rather than a dream.

With natural products featuring heavily among the most widely used, and best selling pharmaceutical of today, either in their original form or closely related derivatives, its evident that they are the logical choice as new drug leads for the future (Oleszek, 2000). Marine natural products are gaining reputation as the major source of useful compounds although its development is still in its infancy. Researchers throughout the world have recognised the problem of diminishing supply of terrestrial's natural products and has shifted their attention to harvest them from marine resources.

1.1.1 Marine Natural Products

Marine organisms, as a whole, represent a vast, basically untapped source of new and potentially biologically active natural products (Gabriele, 1999). More than 2 million species of marine animal and microorganisms that are available for investigation is enormous and can not be ignored. Containing approximately half of the global species, the marine environment with 70 % of Earth's surface and 95 % of its tropical biosphere possesses a biodiversity of all the rain forests combined (Khalid, 1999).

In addition to the open ocean ecosystem, there are also many diverse areas as mangroves, coral reefs, hydrothermal vents and deep-sea sediments, providing dynamic areas to search for microbes (Knight, 2003). This natural product is accumulated in slow moving and sessile marine invertebrates such as sponges, tunicates, and mollusks. Although there are a number of interesting marine organisms, I choose to emphasise on the potential of sponges due to its dynamic ability in producing novel compounds.

1.1.2 Sponges

Sponges have generated interest in natural product more than any other phylum due to their propensity to produce bioactive metabolites (Faulkner, 2002). Sponges are classified in the phylum Porifera. They live attached to solid substrates and are effective filter feeders. 5000 species of sponges has been known across the world. Sponges are primarily marine, but around 150 species live in fresh water (Castro and Huber, 2005). Sponges play important roles in many marine habitats but we still know very little about their diversity and ecology as compared to other marine animals.

The Porifera are subdivided into three classes, the Hexaclinellida, the Demospongia and the Calcarea. This cellular-level organism have specialised cells to perform different functions, thus the simplest kind of cellular organisation found in animalia. Sponges possess a system of pores creating complex network of water conducting canals (J. F. Imhoff and R. Stohr, 2003). Sponges, through its pores capture detritus particles and bacteria that is brought close to it by water currents created by choanocytes's flagellum, a specialised cell to assist feeding.

1.1.3 Tropical Sponges

Chemical defenses by tropical sponges may be most important in tropical environments due to greater predation pressure given the complexity of tropical reef ecosystem (Mikel *et al.*, 2003). Some of these chemicals have been found to have beneficial pharmaceutical effects for humans, including compounds with respiratory, cardiovascular, gastrointestinal, anti-inflammatory, antitumor, and antibiotic activities (Yoo, K. L. *et al.,* 2001). Majority of drugs from sea were isolated from tropical sponges (Cooper, 2004).

1.1.4 Sponge-Associated Bacteria

Bacteria in the surrounding water are actively swirled in by the sponges. Most of the bacteria are retained within the sponge body (J. F. Imhoff and R. Stohr, 2003). They grow, divide and appear to have reached a balanced state within the sponge. Increasing evidence is highlighting the important role of bacteria in bacteria- sponge associations. However, these bacteria are often addressed as symbionts, although their function is not fully or sufficiently understood.

The exact source of the bioactive compounds extracted from sponges is a controversial issue. It has been demonstrated that microsymbionts living in sponges may indeed be the source of bioactive compounds, but that sponge cells themselves also appear to produce them (Yoo, K. L. *et al.*, 2001). Many of the novel metabolites found in sponges are microbial in origin and result from the activities of microbes, particularly bacteria and microalgae.

It remains to be conclusively proved; that endosymbionts living inside the sponge cells are the true source. Although clear hints for such symbiotic interaction exist in some special cases, the specific interrelationship of great many bacteria with the sponge has not been demonstrated (J. F. Imhoff and R. Stohr, 2003). However, the purpose of this study is limited by not to scrutinise the source of bioactive metabolites whether it's from the sponges or symbionts but only to investigate the presence, if any and characterisation of such bioactive compound.

1.1.5 Biomarkers Characterisation

Biomarkers are used to identify the same species of sponges which do not have the same physical characteristics but having certain distinct compound in its metabolism. Biomarkers investigation recently has embarked upon DNA genomics study, providing more information regarding sponge characteristics and taxonomic relationship. In this study however, simple sponge's characteristics like its compounds polarity, UV absorbing ability and the percentage of crude extract yield will be undertaken.

1.2 Research Objectives

The purpose of this research is to study the potential of tropical sponges as a source of bioactive properties. Nevertheless, in order to accomplish this purpose, specific objectives have been set to navigate the course of this research. The objectives are:

1. To **extract and perform chemical profiling** of tropical sponges. This is a fundamental step in investigation of bioactive compounds in tropical sponges.

2. To **determine biological activities of crude extracts** of tropical sponges. These extracts will be tested against bacteria to detect biological activities.

3. To **investigate the presence of biomarkers** in tropical sponges by means of TLC characterisation.

1.2.1 Significance Statement

Tropical sponges have unusual biochemical properties and they are worth investigating especially in the rich species biodiversity of Sabah. Furthermore, the lack of comprehensive research pertaining to the sponge's chemistry in Malaysia can be addressed- to investigate tropical sponge's bioactive potential.

CHAPTER 2

LITERATURE REVIEW

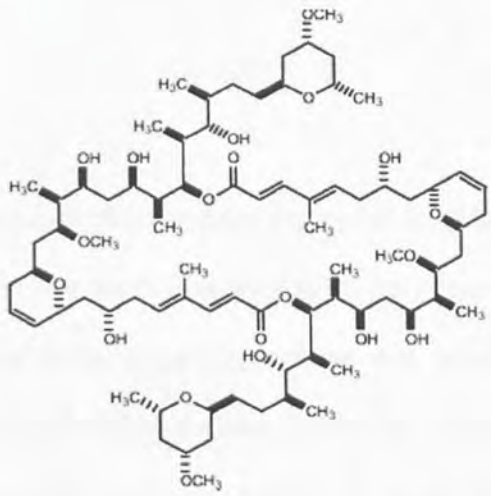
2.1 Overview of Sponges Potential

Pioneering research by Emerson and Taft (1945) proved that marine organisms produced secondary metabolites which were rare and usually absent in the terrestrial environment (Abas et al, 1999). Marine natural products chemistry began to focus on the discovery of new potential drugs in 1951 when Bergmann and Feeney reported on the isolation of the unusual nucleosides spongouridin and spongothymidin from the sponge *Cryptotethya crypta*, which served as lead structures for antiviral drugs. Subsequently, compounds from marine sponges have been identified for their anti-cancer (Kato *et al,* 1986), anti-bacterial (Chang *et al,* 1987), anti-fungal (Carmely *et al,* 1989) and anti-viral (Gunawardana *et al,* 1988) properties ((Yoo, K. L. *et al.,* 2001).

Research about the future potential of marine natural products by König in 1996 revealed that sponges have interesting bio-medical potential, pharmaceutical relevance and diverse applications. As such, this chapter will look into numerous interesting species of sponges particularly tropical ones and will provide elaborated information on its active properties. Whenever possible, brief discussion regarding the chemical structures and application will also be given.

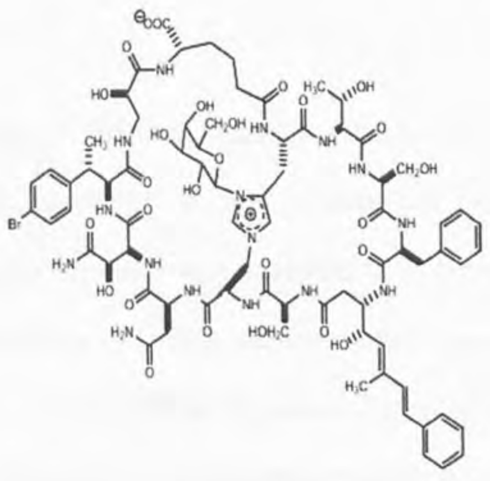
2.1.1 *Theonella swinhoei*

The sponge *Theonella swinhoei* collected in the Philippines contains the macrolide swinholide A **(1)**. It is proven to have a significant **antimalarial activity** against the D6 and W2 clones of *P. falciparum* and demonstrates in vitro cytotoxic activity.



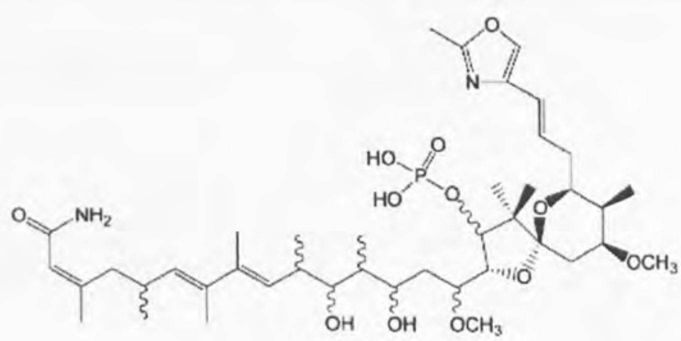
(1)

The extract of *T. swinhoei* collected at Karkar Island aroused interest due to its exceptionally strong **insecticidal activity**. High-performance liquid chromatography (HPLC) analysis revealed only one major peak, which subsequently proved to be the active principle that we called swinhoeiamide A. **(2)** (Proksch et al, 2003). In addition to its insecticidal activity, swinhoeiamide A exhibited **fungicidal activity** against the human pathogenic fungi *Candida albicans* and *Aspergillus fumigatus*.



(2)

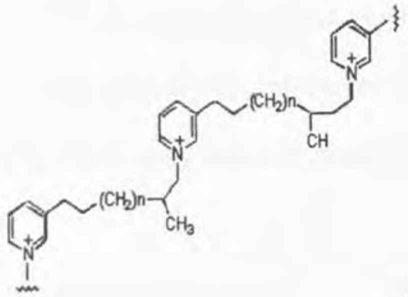
Theonella swinhoei also produce the cyclic peptide theopalauamide **(3)** and shows **anti-fungal activity** while *Theonella* specimens from the Great Barrier Reef in Australia contain the sesquiterpenes spirodysin and herbadysidolide. This sponge supports unicellular heterotrophic bacteria, unicellular cyanobacteria and filamentous heterotrophic bacteria at the same time (Yoo K. L. et al, 2001). Studies on *Theonella swinhoei* resulted in the discovery that complex bicyclic peptides were produced by filamentous bacteria and that the cytotoxic metabolite swinholide A **(1)** was found in a fraction containing many unicellular heterotrophic bacteria- suggesting that the sponge appears to produce no useful chemicals.



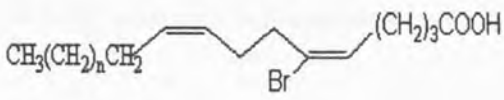
(3)

2.1.2 *Amphimedon sp.*

According to Berlinck, (1996) the halitoxin complex **(4)** from the marine sponge Amphimedon viridis, collected in the Southeastern Brazilian coast, contained **pharmacological properties** and were evaluated in terms of its lethality, antimitosis, hemolysis and neurotoxicity. Another tropical marine sponge from the same genus, *Amphimedon terpenensis* metabolises an unusual fatty acid with a common scheme of following structure **(5)** with outstanding reaction against free radicals.



(4)

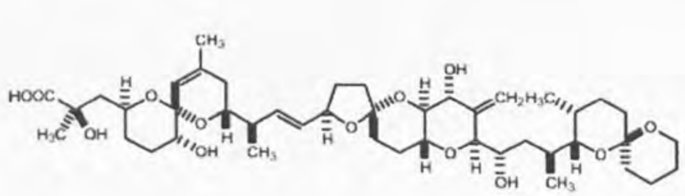


n = 12, 13 or 14

(5)

2.1.3 *Halichondria sp.*

Sponges of the genus *Halichondria* such as *H. okadai* or *H. melanodocia* provide a well-known example of the importance of microalgae for the typical natural products. Both *Halichondria* species contain the protein **phosphatase inhibitor** okadaic acid **(5)** (T.L. Simmons et al, 2005).



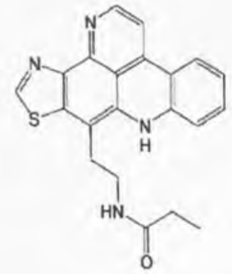
(5)

Because of their phenomenal **biological activity** in killing cancer cells and great structural complexity, the halichondrins rapidly became targets for chemical synthesis. The first total synthesis was completed in 1990 by Kishi group- focused on the synthesis of structurally simplified halichondrin analogues which retained or had enhanced biological properties, and this eventually led to the discovery of the clinical candidate E7389 (T.L. Simmons et al, 2005).

Another example of sponge contribution towards pharmaceutical development is arabinose-nucleosides with **antiviral and anticancer activity** isolated from sponge *Cryptotethya crypta*, which are used clinically; while manoalide obtained from sponge *Luffariella variabilis* is a candidate for new drugs with **anti-inflammatory activity**.

2.1.4 *Oceanapia sp.*

Sponges of the genus *Oceanapia* occur frequently in the Indopacific as well as Pacific Oceans. During bioassay fractionations, pyridoacridine alkaloids of the kuanoniamine **(6)** type, which are also responsible for the conspicuous red color of the sponges, proved to be the main **deterrent constituents** of *Oceanapia* sp (Proksch et al, 2003). The alkaloids clearly deterred feeding by fish in field feeding experiments at their respective physiological concentrations.



(6)

2.1.4 *Agelas sp.*

Given their high concentrations in sponge tissue, the conservation of alkaloids like dispacamide A and oroidin most probably serve as the primary **chemical defenses** of many *Agelas* species, while minor compounds such as keramadine are not present in high enough concentrations to contribute to chemical defense (Thomas et al, 2000).

According to McCaffrey and R. Endean in 1985, there was a negative correlation between antimicrobial activity and surface-fouling, raising the possibility of using free from surface-fouling as an indicator of antimicrobial activity. Four of five encrusting species of coral boulders showed **antimicrobial activity**.

In Malaysia however, a study by Abas to verify sponge's cytotoxic activity using the Brine Shrimp Lethality Assay were conducted in 1999. From 21 samples extracts, 9 were found to show significant **cytotoxic activity**.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

Sponges were collected at Pulau Sulug in Tunku Abdul Rahman Marine Park (TARMP) using SCUBA diving. TARMP is situated at the Northwest coast of Sabah-known for its rich species biodiversity and interesting marine organisms. A collection of 3 species were discovered. The weights of the wet samples were then taken. They were also cleaned to remove dirt, organisms and other epiphytes that were attached.

3.2 Chemical Analysis

3.2.1 Extraction of Crude extracts

The sponges were then cut into small pieces and stored into a plastic container containing methanol solvent and stored for 14 days. After the storing stage, the methanol solvent will be filtered by filter paper using the gravity filtering method.

REFERENCES

Berlinck, R.G.S., Ogawa, C.A., Almeida, A.M.P., Sanchez, M.A.A., Costa, L.V., Malpezzi, E.L.A., Freitas, J.C., Hajdu, 1996. Chemical and Pharmacological Characterization of Halitoxin from Amphimedon viridis (Porifera) from the Southeastern Brazilian Coast, *Comparative Biochemistry and Physiology*, **115**

D. J. Faulkner, (2002). *Nat. Prod. Rep.* **19**, 1

Edwin L. Cooper, 2004. Drug discovery, complimentary alternative medicine and natural products. *Evidence-based Complimentary. And Alt. Medicine*, **Vol 1**, Number 3, 215-217.

E. J. McCaffrey and R. Endean, 1985. Antimicrobial activity of tropical and subtropical sponges. *Marine Biology* **89**, 1-8.

Gabriele M. König and Anthony D. Wright, Trends in marine biotechnology, 1999. In: S. Grabley and R. Thiericke (Eds) *Drug Discovery from Nature*, Springer-Verlag, Germany, 180-185.

H.H. Abas, Y. Zulfigar and K.L. Chan, 1999. Cytotoxicity and drug metabolism screening of several marine sponges from Pulau Pasir, Kedah and Pulau Aur, Johor, Malaysia, *ASEAN Review of Biodiversity and Environmental Conservation (ARBEC)*

H. Jork, W. Funk, W. Fishcer, & H. Wimmer, 1994. TLC Reagents & Detection Methods – *Physical & Chemical Detection Methods: Activation Reactions, Reagent Sequences,* **Vol 1b**, Wiley & Sons, New York.

J.C. Touchstone, 1992. *Practice of Thin Layer Chromatography*, 3rd edition, Wiley-Interscience, New York.

J. F. Imhoff, R. Stohr, Sponge-Associated Bacteria: General overview and special aspects of bacteria associated with Halicondria panicea, 2003. In: Werner E.G Muller (Ed.), *Sponges (Porifera) Marine Molecular Biotechnology*, Springer-Verlag, Germany, 35-54.

Khalid A. El Sayed, D. Chuck Dunbar, Piotr Bartyzel, Jordan K. Zjawiony, William Day, and Mark T. Hamann, 1999. Marine natural products as leads to develop new drugs and insecticides. In: Stephen J. Cutler and Horace G. Cutler (Eds) *Biologically Active Natural Products: Pharmaceuticals*, CRC Press, 233-252.

Khalid A. El Sayed, 2000. Natural products as antiviral agents. In: Atta-ur-Rahman, (Ed) Studies in Natural Product Chemistry; Elsevier Science, **24**, 473-572.

König G.M. and A.D. Wright, 1996. Marine natural products research: Current directions and future potential. *Planta Med.* **62**, 193-211.

McLaughlin, J.L., Chang, C.J. and Smith, D.L. 1991. 'Bench-top' bioassay for the discovery of bioactive natural products: Proceeding of the *8th National Seminar and UNESCO Regional Workshop on the Bioassay of Natural Products with Special Emphasis on Anticancer Agents,* 21-22 October *1991,* Kuala Lumpur, Malaysia.

Mikel A. Becerro, Robert W. Thacker, Xavier Turon, Maria J. Uriz and Valerie J. Paul, 2003. Biogeography of sponge chemical ecology: Comparisons of tropical and temperate defenses. *Oecologia*, Springer-Verlag.

W. Oleszek and A. Marston, 2000. *Saponins in Food, Feedstuff and Medicinal Plants,* Kluwer Academic Publishers, The Netherlands.

P. Proksch, R., A. Edrada and R. Ebel, 2002. Drugs from the seas- current status and microbiological implications, *Applied Microbiology and Biotechnology*, Springer-Verlag.

P. Proksch, R. Ebel, R. A. Edrada, P. Schupp, W. H. Lin, Sudarsono, V. Wray, and K. Steube, 2003. Detection of pharmacologically active natural products using ecology- selected examples from Indopacific marine invertebrates and sponge-derived fungi. *Pure Appl. Chem.*, **Vol. 75**, Nos. 2–3, 343–352.

Peter Castro and Michael E. Huber, 2005. *Marine Biology.* **5th Edition.** McGraw-Hill.

Thomas Lindel, Holger Hoffman, Matthias Hochg, Urtel and Joseph R. Pawlik, *2000.* Structure-activity relationship of inhibition of fish feeding by sponge derived and synthetic alkaloids. *Journal of Chemical Ecology, **26**, No 6.*

T. Luke Simmons, Eric Andrianasolo, Kerry McPhail, Patricia Flatt and William H. Gerwick, 2005. Marine natural products as anticancer drugs, *Molecular Cancer Therapy*, **4,** 333-342.

V. Knight, J. J. Sanglier, D. DiTullio, S. Braccili, P. Bonner, J. Waters, D. Hughes and L. Zhang, 2003. Diversifying microbial natural products for drug discovery, *Applied Microbiology and Biotechnology*, Springer-Verlag.

Yoo Kyung Lee, Jung-Hyun Lee, and Hong Kum Lee, 2001. Microbial symbiosis in marine sponges, *The Journal of Microbiology*, **39**, No. 4, 254-264.