UNIVERSITI PUTRA MALAYSIA

SCREENING AND ISOLATION OF CYTOTOXIC COMPOUNDS FROM LOCAL MARINE AAPTOS SPECIES

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SCREENING AND ISOLATION OF CYTOTOXIC COMPOUNDS FROM LOCAL MARINE *AAPTOS* SPECIES

By

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March 2006

Chairman: **Professor Abdul Manaf Ali, PhD**

Institute: **Bioscience**

Over the recent years, marine sponges have been the target of research as a source of new chemicals with therapeutic potential. They have been proven to have a high strike rate especially for cytotoxic compounds. Thirteen species of marine sponges collected from Pulau Kapas, Pulau Bidong and Pulau Redang were preliminarily identified as *Aaptos* sp. (1), *Xestospongia exigua* (2), unidentified sp. (3), *Xestospongia* sp. (4), *Xestospongia testudinaria* (5), *Callyspongia* sp. (6), *Theonella* sp. (7), *Theonella* sp. (8), *Sigmaducia ambolnenuris* (9), *Ircinia* sp. (10), *Dysedia* sp. (11), unidentified sp. (12) and *Ircinia Halisarca* (13). The crude methanol extracts of these samples were screened for cytotoxic activities against a panel of cell lines, namely HL-60 (promyelocytic leukemia), CEM-SS (T-lymphoblastic leukemia), MCF-7 (breast cancer), HeLa (cervical cancer), HT-29 (colon cancer) and L929 (murine fibrosarcoma from mouse) using a colorimetric tetrazolium (MTT) assay. Crude extracts from 1 and 10 were active against all six cell lines with CD_{50} values ranging from 1.05 to 24.1 μg/ml whereas extracts 2, 3 and 8 showed activity only against HL-60, CEM-SS and HT-29 with CD_{50} values ranging from 12.95 to 29.5

μg/ml. *Aaptos* sp. (1) was chosen for further investigations due to its abundance and strong cytotoxic activity. Bioassay guided isolation and purification of compounds afforded three cytotoxic alkaloids. H19 was identified as the previously isolated aaptamine [1] and the two orange compounds were established as the new aaptaminoid alkaloids, O1 and O2. All three compounds exhibited significant cytotoxic activity against CEM-SS cells with respective CD_{50} values of 15.0, 5.3 and 6.7 μg/ml. When tested against 3T3 (normal mouse fibroblast), all three compounds displayed weak cytotoxicities. The CD_{50} of compound O1 and O2 were determined as 21.2 and 21.0 μg/ml respectively. On the other hand, compound H19 did not achieve a CD_{50}. Phase contrast microscopic analysis showed that compound H19, O1 and O2 induced apoptosis in CEM-SS cells. The apoptotic features observed include cell shrinkage, condensation of chromatin material, membrane blebbing and the formation of apoptotic bodies. Due to insufficient quantity of O1 and O2, only H19 was subjected to subsequent evaluation using fluorescence microscopy. These results further supported that H19 induce apoptosis in CEM-SS as exemplified by the morphological changes observed.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENYARINGAN DAN PEMENCILAN SEBATIAN SITOTOKSIK DARIPADA SPESIS MARIN TEMPATAN *AAPTOS* SPESIES

Oleh

KEE CHENG LING

Mac 2006

Pengerusi: **Profesor Abdul Manaf Ali, PhD**

Institut: **Biosains**

Kebelakangan ini, sepan marin telah menjadi tumpuan perhatian para pennyelidik sebagai sumber kajian untuk penemuan sebatian yang berpotensi terapeutik. Sepan marin ini telah terbukti mempunyai kadar penemuan yang tinggi terutamanya sebatian sitotoksik. Tiga belas spesies sepan marin telah dikutip dari Pulau Kapas, Pulau Bidong serta Pulau Redang dan dikenalpasti sebagai *Aaptos* sp. (1), *Xestospongia exigua* (2), sp. tidak dikenalpasti (3), *Xestospongia* sp. (4), *Xestospongia testudinaria* (5), *Callyspongia* sp. (6), *Theonella* sp. (7), *Theonella* sp. (8), *Sigmaducia ambolnenuris* (9), *Ircinia* sp. (10), *Dysedia* sp. (11), sp. tidak dikenalpasti (12) dan *Ircinia halisarca* (13). Tiga belas ekstrak mentah metanol daripada sepan-sepan ini telah disaring untuk aktiviti sitotoksik melalui kaedah kolorimetri tetrazolium (MTT) ke atas satu panel sel kanser, antaranya HL-60 (leukemia promielositik), CEM-SS (leukemia T-limfoblastik), MCF-7 (kanser payu dara), HeLa (kanser serviks), HT-29 (kanser kolon) dan L929 (fibrosarkoma murin dari tikus). Ekstrak mentah 1 dan 10 telah menunjukkan aktiviti sitotoksik ke atas keenam-enam jenis sel dengan CD_{50} daripada 1.05 hingga 24.1 μg/ml manakala

ekstrak mentah 2, 3 dan 8 menunjukkan aktiviti sitotoksik hanya ke atas HL-60, CEM-SS dan HT-29 dengan nilai CD_{50} antara 12.95 dengan 29.5 μg/ml. *Aaptos* sp. telah dipilih untuk kajian seterusnya disebabkan aktiviti sitotoksiknya yang tinggi dan juga kuantitinya yang mencukupi. Teknik pengasingan biocerakinan berpandu telah menghasilkan tiga sebatian alkaloid. H19 telah dipencilkan sebagai aaptamina [**1**] yang pernah dipencilkan dahulu dan dua lagi sebatian jingga dikenalpasti sebagai alkaloid aaptaminoid yang baru iaitu O1 dan O2. Ketiga-tiga sebatian ini telah menunjukkan sifat ketoksikan yang kuat terhadap sel CEM-SS masing-masing dengan nilai CD_{50} 15.0, 5.3 dan 6.7 μg/ml. Apabila dikaji pada sel 3T3 (fibroblast tikus biasa), ketiga-tiga sebatian ini mempamerkan aktiviti sitotoksik yang lemah. Sebatian O1 dan O2 telah memberikan nilai CD_{50} 21.2 dan 21.0 μg/ml manakala sebatian H19 tidak mencapai satu nilai CD_{50}. Analisis daripada mikroskopi fasa kontras menunjukkan bahawa ketiga-tiga sebatian H19, O1 dan O2, memberi kesan aruhan apoptosis pada sel CEM-SS yang dikaji. Morfologi apoptosis yang diperhatikan termasuk pengecutan sel, kondensasi kromatin serta penghasilan tompok-tompok membran dan jasad-jasad apoptotik. Oleh sebab kekurangan sebatian O1 dan O2, hanya H19 telah dikaji seterusnya dengan menggunakan mikrosop fluoresen. Pemerhatian ke atas penukaran morfologi sel telah membuktikan lagi bahawa H19 memberi kesan aruhan apoptosis ke atas sel CEM-SS.

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LIST OF ABBREVIATIONS

AO	Acridine orange
ATCC	American type culture collection
atm	Atmospheres
CD_{50}	Cytotoxic dose at 50%
CNS	Central nervous system
COSY	Correlation spectroscopy
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
EGFR	Epidermal growth factor receptor
EIMS	Electron impact mass spectrometry
ESIMS	Electron spray impact mass spectrometry
FBS	Fetal bovine serum
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography mass spectrometry
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
HSV-1	Herpes simplex virus type 1
IR	Infrared
J	Coupling constant
LCMS	Liquid chromatography mass spectrometry
m/z	Mass-to-charge-ratio
MAPK	Mitogen activated protein kinase

mg	Miligram
ml	Mililiter
MTT	3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National cancer institute
NF-κB	Nuclear factor κB
nm	Nanometer
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffered saline
PI	Propidium iodide
PKC	Protein kinase C
rpm	Round per minute
TLC	Thin layer chromatography
TNFα	Tumor necrosis factor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
%	Percentage
μg	Microgram
λ_{max}	Wavelength at which maximum absorption occurs in UV spectroscopy
° C	Celsius

CHAPTER 1

INTRODUCTION

For decades, natural products have been the targets of researchers as an important source of new pharmaceutical compounds. "Natural products" are known as organic compounds of natural origin that are unique to one living organism including plants, animals or microorganisms. They are also known as secondary metabolites, which are synthesized by secondary metabolic pathways (Williams *et al.*, 1989). These natural products, be it plant, marine or microorganism origin, are sources that could lead to discovery of new and unique chemical entities with pharmaceutical potential.

The marine environment is a rich source of both biological and chemical diversity. This diversity has been the source of structurally unique chemical compounds for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agrochemicals (Kijjoa and Sawangwong, 2004). In the last 25 years, natural products derived from marine organisms have been the focus of many investigations. Since the initial discovery of Ara-C by Bergman in 1951, more than 12,000 compounds have been isolated from marine microorganisms, algae, sponges, soft corals, and marine invertebrates such as bryozoans, echinoderms, molluscs and ascidians (Faulkner, 2001).

These marine derived compounds are known to have various bioactivities such as antibacterial, antifungal, antiinflammatory, antiplatelet, antiprotozoa, as well as antiviral activities (Mayer, 2000). Nevertheless, they are also well known for their anticancer or cytotoxic properties (Mayer, 1999). To date, there are approximately 300 patents for marine metabolites and more than 10 of these are in various stages of clinical trials (Faulkner, 2001).

Over the years, it appears that pharmaceutical investigations of marine natural products have tended to focus on the anticancer therapeutics. These efforts have yielded a considerable number of drug candidates, which are in various stages of clinical trials including bryostatin-1, dolastatin-10, ecteinascidin-743, aplidine and kahalalide F. Clinical results from these investigations have proven the compounds to be promising anticancer agents. These results clearly anticipated the potential of the marine ecosystem in cancer therapy. During the last decade about 2500 new metabolites with antiproliferative activity have been reported (Jimeno *et al.*, 2004).

Macroorganisms such as sponges and tunicates are important sources of bioactive marine metabolites. These sessile, soft-bodied marine invertebrates that lack obvious physical defences are prime candidates to possess bioactive metabolites (Faulkner, 2000). A screening program done by the US National Cancer Institute (NCI) has shown that sponges contain the widest range of secondary metabolites and most of these natural products show biological activity, which are often applicable for medical use (Garson, 1994). Therefore, the present study was carried out to evaluate the cytotoxicity of crude extracts and isolated bioactive compounds from sponges,

collected from the east-coastal waters of Malaysia, mainly Pulau Bidong, Pulau Perhentian and Pulau Redang.

The objectives of this study are:

1. To screen for cytotoxic compounds from marine sponges using a colorimetric tetrazolium (MTT) assay against various cancer cell lines.
2. To isolate the active compounds from selected sponge through bioassay guided fractionation technique.
3. To elucidate the structure of the isolated active compounds.
4. To study the effect of the isolated compounds on CEM-SS cells in terms of morphological changes and mode of cell death induced by the compounds.

CHAPTER 2

LITERATURE REVIEW

2.1 Natural Product

The term 'natural product' is commonly known as organic compounds derived from natural sources including plants, animals as well as microorganisms. They are also known as secondary metabolites, previously regarded as waste products, which have no apparent function (Verpoorte, 1998) and appear to be non-essential to the organisms that produce them. These secondary metabolites are synthesized by secondary metabolic pathways (Williams *et al.*, 1989) and probably only activated during periods of stress caused by nutritional shortage or predator attack (Mann, 1986).

The role of natural products as a source for remedies has been recognized since ancient times. Natural products have provided mankind essential materials for shelter, furniture, food, clothing, writing and colouring materials, weapons, gifts, and most importantly, for the treatment of numerous diseases. This 'gift' from nature continues to contribute a great deal in the discovery and development of novel bioactive compounds, especially those with medicinal value. According to Martin and Bohlin (2004), natural products have been the most powerful source of drugs and it is also the single most successful strategy for the discovery of new medicines. Over 60% of drugs used today are of natural product origin (Newman, 2003) or are

based on natural product models and the world wide market in these plant-derived drugs alone is currently amounted to US$22 billion per annum (Jaspars, 2001).

2.2 Marine Natural Product

Historically, plants have served as the major source of medicinally useful natural products, developed from a legacy of folk medicine based on herbal remedies (Carte, 1993; Cordell, 2000). A prominent example of plant-derived drug is paclitaxel (taxol), isolated from the plant, *Taxus brevifolia*. Taxol was first discovered in 1967 and it took 20 years to reach its first real clinical responses observed with ovarian cancer in 1987. Today, it is undoubtedly the best selling anticancer drug, famous for its anticancer activity and used for the effective treatment against refractory breast and ovarian cancer (Mann, 2002).

With regards to bioactive compounds of pharmaceutical potential, how does the marine environment fare in comparison to the more traditional areas such as terrestrial microorganisms and plants? In 1999, Munro and co-workers cited in their paper that, based on a cytotoxic screening done by the US National Cancer Institute (NCI), marine invertebrates were found to have a much higher incidence of significant cytotoxic activity. The data in Figure 2.1 shows that almost 2% of the 6,540 screened marine animals showed cytotoxic activity compared to less than 1% of 18,293 terrestrial plants and over 8000 microorganisms. This clearly indicated that marine organisms are a preferred source for study.

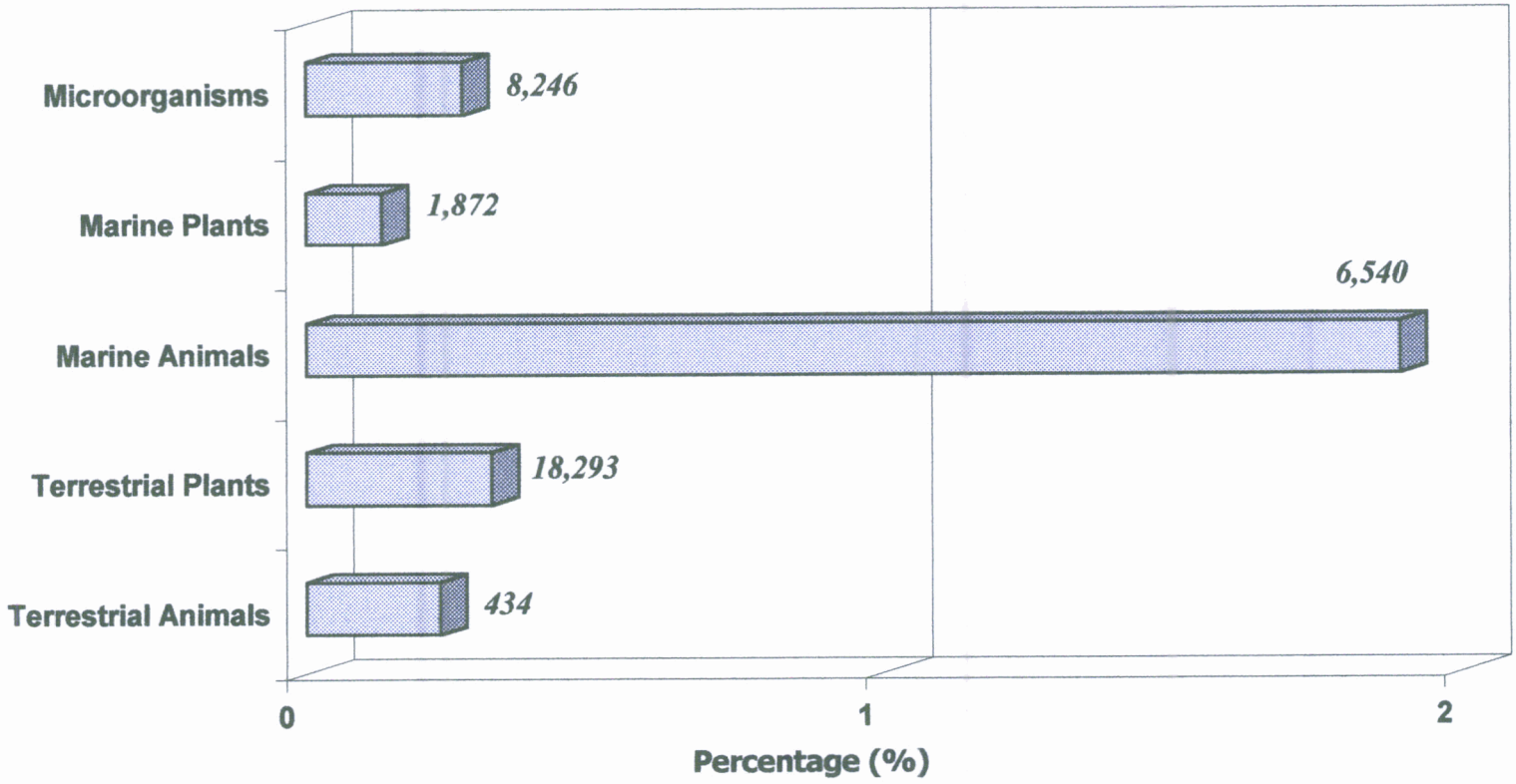

Figure 2.1: Distribution of samples with significant cytotoxicity in the NCI's preclinical screen (Garson, 1994)