THE EFFECTS OF *Stichopus variegatus* CRUDE EXTRACTS ON ACUTE CONTUSIVE RAT SPINAL CORD INJURY MODEL : *IN VITRO* AND *IN VIVO* STUDIES

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by

MOHD NOR AZIM AB PATAR

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DEDICATION

I dedicate this thesis to my late father, Mr Ab Patar Mohamed

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LIST OF ABBREVIATIONS

ALT	Alanine aminotransferase enzyme
AST	Aspartate aminotransferase enzyme
BBB	Basso Beattie Breshnan Locomotor Scale
BDA	Biotinylated Dextrin Amine
BMS	Basso Mouse Scale
CD47	Cell of Diffentiation 47
CNS	Central Nervous System
cSCI	contused Spinal Cord Injury
CST	Corticospinal tract
DHG	Depolarized Holothurian Glycoaminoglycan
EC_{50}	Effective Concentration 50%
EGF	Epidermal Growth Factor
ERK	Extracellular signal-Regulated Kinases
FG	Fluoro Gold
GAGs	Glycoaminoglycans
GC-MS	Gas Chromatography- Mass Spectrometry
i.t	Intrathecal
ICR	Imprinting Control Region
IH	Infinite Horizon impactor
LD_{50}	Lethal Dose 50%
LDH	Lactate dehydrogenase enzyme

MACSIS	Multicenter Animal Spinal and Injury Studies
MDA	Malodialdehyde conjugated dienes
MIC	Minimum Inhibitory Concentration
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NACSIS	National Acute Spinal Cord Injury Studies
NYU	New York University
PB	Phosphate buffer
PBS	Phosphate Buffered Saline
PCS	Polysaccharide Chondroitin Sulphate
PHA-L	*Phaseolus vulgaris*-Leucoagglutinn
RITC	Rhodamine-Isothiocyanate
SCI	Spinal Cord Injury
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOD	Superoxide dismutase
SV	*Stichopus variegatus*
SVWE	*Stichopus variegatus* water extract
T_{10}	10^{th} Thoracic level
T_{12}	12^{th} Thoracic level
T_{13}	13^{th} Thoracic level
T_9	9^{th} Thoracic level
TNF-α	Tumour Necrosis Factor-Alpha
VMN	Ventral Motor Neuron
w/w	Weight per weight

LIST OF SYMBOLS

®	Registered trademark
%	percentage
°C	degree Celsius
cm	centimeter
g	gram
mg	miligram
min.	minute/minutes
ml	mililiter
mm	milimeter
µg/ml	microgram per mililiter
kDa	kilo Dalton
h	hours
Gy	Gray
µl	microliter
v/v	volume per volume
kPa	kilo Pascal
Nm	nanometer
mg/kg	milligram per kilogram
rpm	revolution per minutes
µl/hr	microliter per hour
w/w	Weight per weight

LIST OF PRESENTATION AND PUBLICATIONS

Azim, P., Mohsin, SSJ., Hasnan, J., Abdullah, J.
Analysis of Sea Cucumber Body Wall Extracts From Perhentian *Stichopus variegatus* species (2012) *Eur J of Scientific Res,* 68(1): 54-57 (indexed in Scopus)

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The Effect of a Sea Cucumber *Stichopus variegatus* Water Extract On Spinal Astrocytes Proliferation *in vitro* (2012) *Curr Neurobiology,* 3(1): 7-12 (indexed in Scopus)

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Body Wall Extract of Stichopus variegatus promotes repair of acute contused spinal injury in rats by improving motor function and reduces intramedullary hemorrhage. (2012) *Biomedical Research,* 24(3) In press (indexed in ISI)

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Water Extraction of Body wall of *Stichopus sp*., does not affect behaviour performances of Contused Spinal Cord Injury in Rodent Model. *Proceedings of the Society for Neuroscience*, Abstract 752 (SfN 38th Annual Meeting, Washington, DC, USA, 2008) (indexed in ISI, Scopus)

Azim, P., Mohsin, SSJ., Hasnan, J., Abdullah, J.
A Step towards the development of Standard CSF Sampling Method on Laboratory Rats in Universiti Sains Malaysia. *Proceeding of the Society for Neuroscience*. Abstract 541 (SfN 36th Annual Meeting, Atlanta, GA, USA,2006) (indexed in ISI, Scopus)

KESAN EKSTRAK MENTAH *Stichopus variegatus* KE ATAS KECEDERAAN KONTUSI KORDA SPINAL TIKUS AKUT: KAJIAN *IN VITRO* DAN *IN VIVO*

ABSTRAK

Timun laut daripada Pulau Perhentian, Malaysia terkenal sebagai sumber marin yang dimakan dan mendatangkan nilai perubatan kepada masyarakat tempatan. Sungguhpun begitu dengan kehadiran industri kecil dan penggunaan dalam kalangan masyarakat dulu kala, terlalu sedikit kajian saintifik dijalankan dalam menentukan aktiviti biologi ekstrak timun laut pada sistem saraf pusat. Dalam kajian ini, persampelan, pemprosesan dan pengekstrakan *Stichopus variegatus* (SV) dijalankan di dalam makmal dan seterusnya dinilai kesan proliferatif pada sel titisan astrosit (*in vitro*) dan model tikus kajian kecederaan korda spina teraruh(*in vivo*). Analisis *in vitro* menunjukkan ekstrak SV mempamerkan aktiviti proliferasi pada sel titisan astrosit korda spinal pada kepekatan 5 µg/ml dan 10 µg/ml. Nilai EC_{50} ekstrak SV ialah 5.18 µg/ml. Analisis *in vivo* menunjukkan Methylprednisolone dan 10 µg/kg ekstrak menunjukkan kesan peningkatan skor Basso Beattie Breshnan (BBB) pada model kajian tikus kecederaan korda spinal selama 14 hari berbanding kumpulan kawalan($p=0.04$) namun tiada perubahan ketara dalam jarak yang diambil oleh setiap tikus dalam selepas 14 hari ($p>0.05$). Methylprednisolone dan ekstrak 10 µg/kg menunjukkan penurunan pendarahan intramedular berbanding kumpulan kawalan ($p<0.05$). Analisis hubungan menunjukkan korelasi negatif yang kuat di antara skor BBB dengan jisim kelabu (r^2= -0.99) dan jisim putih(r^2=-0.93). Methylprednisolone dan ekstrak 10 µg/kg memulihkan jisim putih sebanyak 74.2% dan 67.7% selepas 14 hari mengalami kecederaan berbanding kumpulan kawalan (33%). Pemulihan jisim putih berkadar terus dengan

peningkatan kelakuan tikus kecederaan korda spinal teraruh (r^2=0.91). Keputusan kajian memberikan beberapa kesimpulan : Ekstrak timun laut dengan dos 10 μg/kg dicadangkan meningkatkan fungsi motor, mengurangkan pendarahan intramedular dalam jisim kelabu dan jisim putih serta memulihkan jisim putih selepas kecederaan korda spinal.

THE EFFECTS OF *Stichopus variegatus* CRUDE EXTRACTS ON ACUTE CONTUSIVE RAT SPINAL CORD INJURY MODEL: *IN VITRO* AND *IN VIVO* STUDIES

ABSTRACT

Sea cucumbers from Pulau Perhentian, Malaysia are well known as potential marine edible food sources and possess medicinal value to the Malaysian community. Despite the presence of small industries and its usage amongst local folks, there is little work done on its biological activity, especially on the central nervous system. In the present study, *Stichopus variegatus* (SV) were harvested, processed and extracted in the laboratory and were evaluated on their proliferative activity on rat astrocytes cell lines (*in vitro*), as well as contusive spinal cord injury in a rat model (*in vivo*). *In vitro* analysis of SV extract showed proliferative activity of rat astrocytes cell line at 5 µg/ml and 10 µg/ml. The SV extracts showed a dose dependent effect. The EC_{50} of the Perhentian SV extract was 5.18 µg/ml. *In vivo* analysis of SV extracts demonstrated that Methylprednisolone and 10 µg/kg showed an improvement of the Basso Beattie Breshnan (BBB) score of a rat contused spinal cord injury at day 14 ($p=0.04$), however there were no differences on the distance travelled by each of the rats on day 14 ($p>0.01$). Methylprednisolone and 10 µg/kg of SV extract reduced intramedullary hemorrhage compared to the control group ($p<0.05$). Correlation analysis demonstrated a significant negative correlation between the BBB Locomotor scores and gray matter hemorrhage ($r^2= -0.99$) and white matter hemorrhage ($r^2= -0.93$). Methylprednisolone and 10 µg/kg of SV extract were also found to spare white matter (74.2% and 67.7% respectively) after 14 days of injury compared to the control group (33%). The white

matter sparing was directly proportional to behavior improvement (r^2= 0.91). These findings lead to several conclusions; SV extract with 10 µg/kg are suggested to promote repair of an acute contused spinal cord injury in rats by improving motor function, reducing gray matter and white matter intramedullary hemorrhage, and sparing white matter after the spinal injury.

CHAPTER 1

INTRODUCTION

1.1 Sea Cucumber

Sea cucumbers are soft bodied, tubular sea organisms related to sea fish, sea lilies, brittle stars and sea urchins. Sea cucumbers are dioecious, although some of the holothurians are hermaphrodites. Sea cucumbers are found in every marine environment, but are most diverse in tropical shallow-water coral reefs (Reseck, 1979). They come in a variety of sizes, shapes, and colours depending upon their species (Ridzwan & Che Bashah, 1985).

1.1.1 Taxonomy

Sea cucumbers belonging to the phylum Echinodermata under the class Holothuroideae (Hyman, 1955, Arnold & Birtles, 1989) are further divided into three subclasses which are Dendrochirotacea, Aspidochirotacea and Apodacea. It comprises 25 families, 200 genera, and 1400 species worldwide (Forbes *et al.*, 1999). Under these subclasses, there are six orders : Aspidochirotidae, Apodidae, Dactylochirotidae, Dendrochirotidae, Elasipodidae and Molpadiidae (Forbes & Baine, 1998). Sea cucumbers which have five calcareous plates can be found in the surrounding of its mouth. These calcareous plates make up a calcareous ring and protect the ring of vital tissues (Figure 1.1). The distinctive shape of the calcareous plates is used as a taxonomic feature to separate genera within the family (Cannon & Silver, 1986). The classification of the orders into genus and species was achieved using identification of microscopic spicules found in the body wall. The spicules were varied from simple rods and tables in genus *Stichopus* species. Figure 1.2 shows the common spicules found in the sea cucumber (Stichopodidae family).

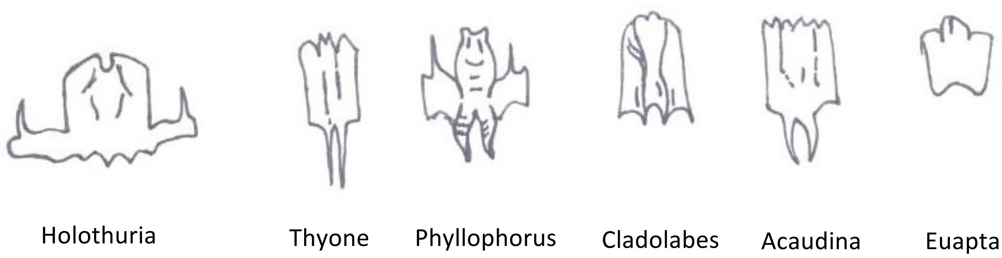

Figure 1.1: Calcareous rings found in Holothurians [adapted from Forbes *et al*., (1999)]

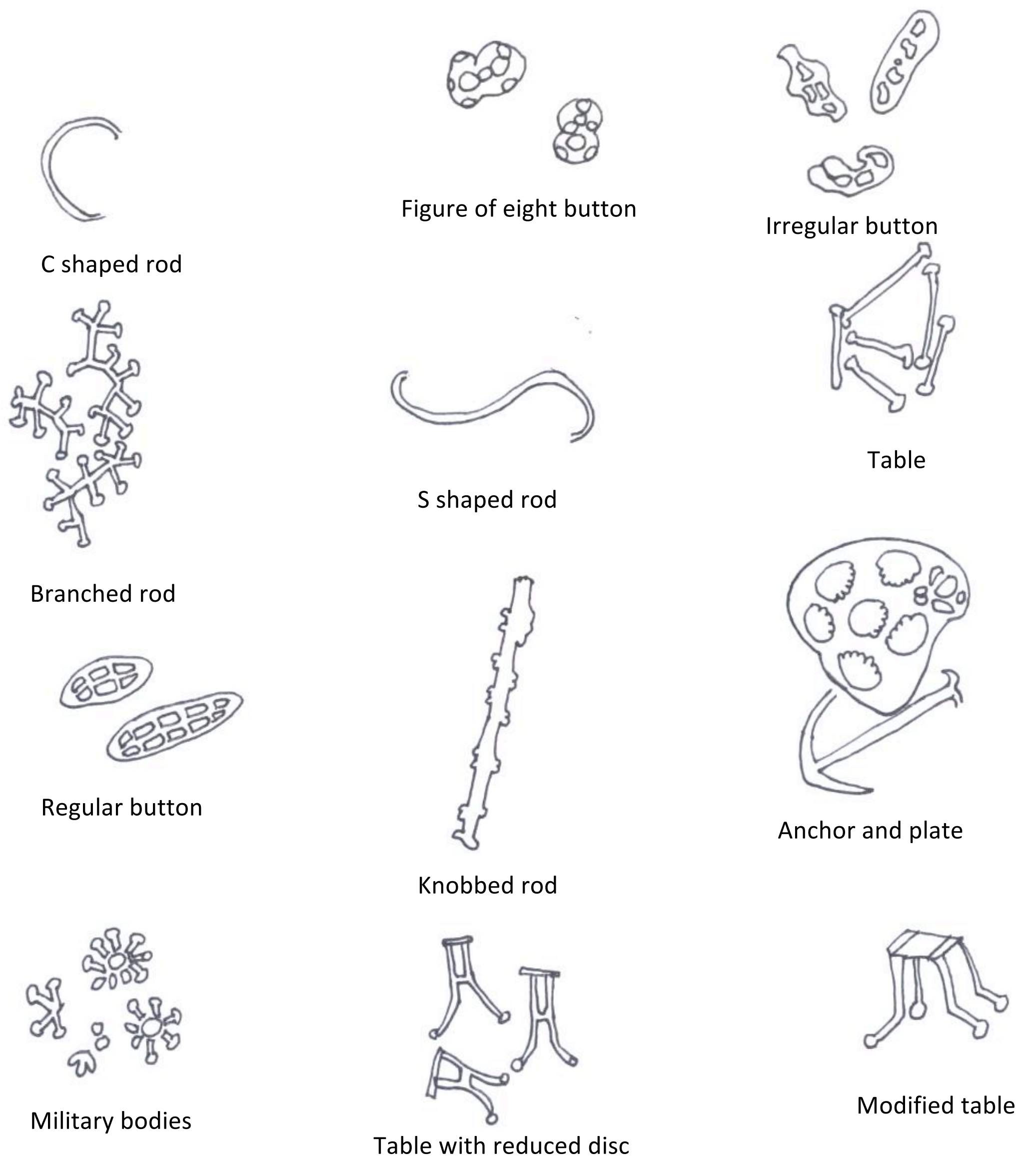

Figure 1.2: Types of spicules found in the tegument of Holothurians according to Forbes *et al*., (1999)

1.1.2 Species of interests

The family of Stichopodidae comprises of eight genera found in both tropical and temperate seas. Two genera and eight species were recorded from Malaysian waters (Figure 1.3). *Stichopus variegatus* was reported to have medicinal values and benefited human welfare by local researchers. *S. variegatu*s was well known by other common names amongst locals for example *curry fish* (English), *Trepang*, *Kasur* (Indonesia), *Gamat pasir*, *Kebasik laut* (Malaysia) and *Yush*en (Mandarin) (Forbes *et al.*, 1999). Since the identification of *S. variegatus* largely relies on the examination of morphology and types of spicules by marine biologists for the last decade, this method has been a source of confusion when identifying between this species and *S. horrens*.

S. variegatus was chosen in this study due to the number and distribution of this species which was high at that time of sampling. It was also chosen due to its popularity amongst the local traditional medicinal use. Morever, the usefulness of the *S. variegatus* remedy has been inherited as a source of medicine amongst the local Malay medicinal practitioners of Peninsula Malaysia and Sabah.

In Malaysia, *S. variegatus* is identified as roughly square in cross section with the upper surface convex. The flattened ventral surface possesses three distinct rows of tube feet and is orange-pink in colour. The body is stout, thick and firm with a moderate rough tegument with low papillae. The body of Malaysian *S. variegatus* is yellow in colour with white circles around the papillae. The body colour is variable within the species and it is geographically dependent. It grows to be approximately 40 cm in length. It consists of mixed C shapes of spicules and tables. The body wall has strong radial and longitunal muscles. The distribution of *S. variegatus* in Malaysia was found from Pulau Payar on the west coast and from Pulau Tinggi, Pulau Tioman, Pulau Redang and Pulau Perhentian groups on the east coast of Peninsular Malaysia. It was also reported from Pulau Bohey Dalang in Sabah (Forbes *et al.*, 1999, Ridzwan, 2007).

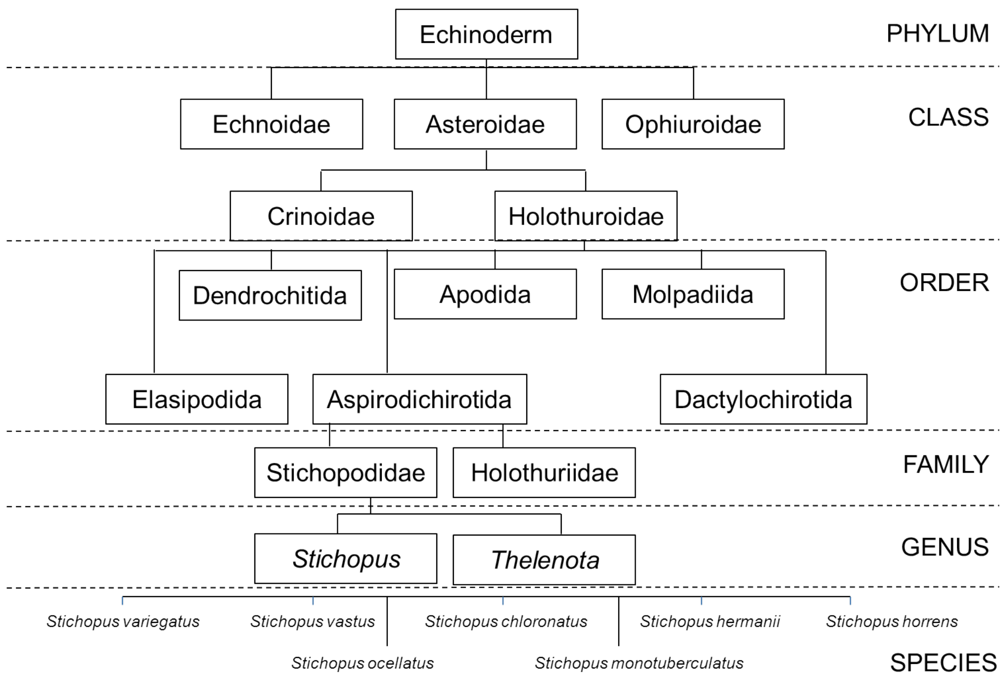

Figure 1.3: A taxonomy of sea cucumber [adapted from Forbes *et al*.,1999; Ridzwan *et al*.,2007]

1.1.3 Previous studies

S. variegatus is also known as ‘Gamat Emas’ by local folks (Figure 1.4). The extraction of its body wall, coelomic fluid and other parts of *S. variegatus* were extensively used by Malaysian local practitioners with its usage spreading within the community today as a health supplement product. It is only recently that local researcher begun to evaluate sea cucumber extracts scientifically. Research on sea cucumber extracts are divided into five broad groups including wound healing properties and associated cell growth studies, anti-microbial, anti-oxidant, anti-thrombotic properties, and arthritis remedy. The other group was much more focused on the sea cucumber itself and studied aspects such as anatomy, life cycles, preservation and identification of bioactive compounds within sea cucumber extraction.

Yaacob *et al.*, (1994) reported the effects of sea cucumbers on wound healing guinea-pigs induced wounds. The results demonstrated positive effects on healing processes with a faster rate. In another study by Ridzwan *et al*., (1990) they studied the rate of healing using four species of holothurian, namely *Holothuria atra, Bohadchia marmorata, S. badionatus* and *Actinopyga* sp. The rate of healing was compared to that controlled petroleum jelly. Holothurian species showed a faster rate of healing compared to standard petroleum jelly. This research opened up the research towards the use of sea cucumber extracts on increasing the rate for wound healing in animal models.

Gonad

Respiratory tree

hagus

careous rings

Figure 1.4: *S. variegatus* known as 'Gamat Emas'

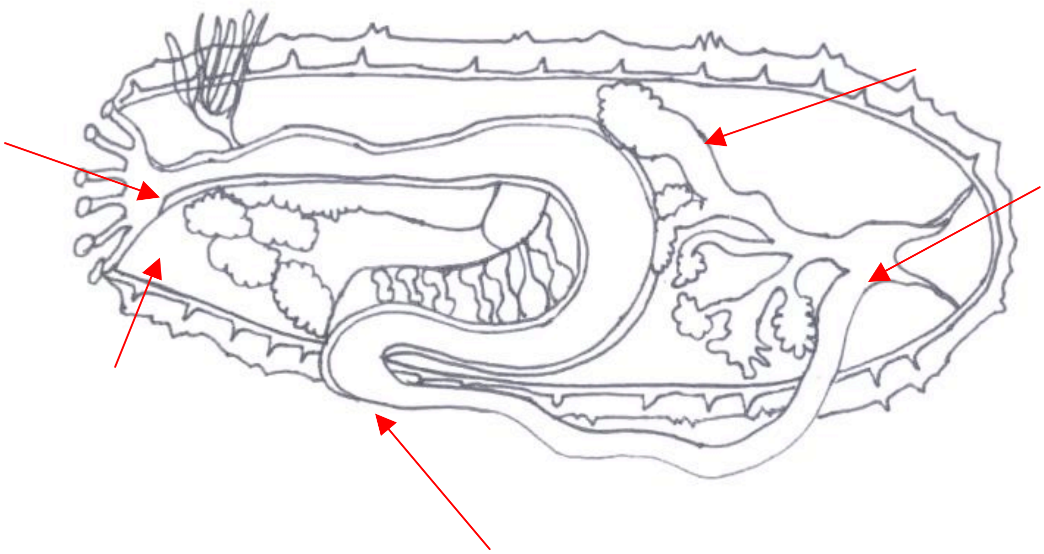

Figure 1.5: Schematic diagram of general anatomy of Stichopodidae family.

Ibrahim *et al*., (2000) demonstrated there was an increased healing rate after the treatment of *S. variegatus* and *H. atra* methanol extracts on cutaneous wounds in guinea pigs (Ibrahim *et al*.,2000). It resulted in a good quality of the scar formation via histological studies using light microscope and electron microscopy analysis. The other study looked at the effect of *S. variegatus* extracts on the healing of gastric ulcers in laboratory rats. Nihayah *et al*., (2000) investigated the methanol extract of *S. variegatus* and it showed accelerated re-epithelisation and increased gastric mucosa production (Nihayah *et al.*, 2000). The similar study demonstrated crude extract of *S. variegatus* could accelerate the gastric ulcer healing (Yanti *et al.*, 2000). The authors tested the extracts on an animal induced gastric ulcer. The histological study using a light microscope showed enhanced healing as compared to the group that was assigned without intervention.

Ibrahim *et al*., (1995) looked at the effect of crude extract of *S. variegatus* on the keloid healing model. The extracts were observed to help in repair of the damaged epithelial and connective tissues, fibroblast and endothelial cells (Ibrahim *et al.*, 1995). However, the extracts showed negative results, particularly on collagen presentation and orientation of keloid morphology. Later, a study documenting products derived from sea cucumber formulation and extraction was published (Taiyeb-Ali *et al.*, 2003). The 10% w/w of sea cucumber extracts was mixed together to formulate Gamadent® toothpaste. This formulation of toothpaste was tested on gingival wounds in patients with chronic gingivitis. The results showed the formation of plaque was controlled in the group of

Gamadent® intervention because there were increased rates of healing in the first month of the therapy group. However, the study was limited due to small sample size.

The use of crude extract seemed very popular among local researchers until Fredalina *et al*., (1999) worked to elucidate the possible bioactive compounds amongst Malaysian holothurian. In this study, fatty acid compositions of holothurian (*S. chloronatus*) were identified and isolated with different solvents extraction. Gas chromatography analysis confirmed the composition of myristic acid, palmitic acid, stearic, linoleic acid, arachidic acid, eicosapentaenoic acid were detected. These were important potential bioactive materials that contribute to tissue repair (Cardoso *et al*., 2011).

The sea cucumber extracts, especially of the Stichopodidae family, were reported to have anti-microbial effects. Several studies looked into the effect of extracts on microbial activity (Ridzwan *et al.*, 1995, Ibrahim *et al.*, 1995, Fredalina *et al.*, 2004) however, to a certain extent, the crude extract showed haemolytic ability when they reacted with red blood cells *in vitro*, unless the concentration of crude extracts were below lethal dose LD_{50}=120 mg/ml (Hadzir, 2005).

Zainal Abidin *et al*., (2000) investigated the inhibitory effect of water soluble extracts of *S. badionatus, S. chloronatus, S. variegatus, H. edulis* and *H. atra.* The crude extracts were tested on *Lactobacillus* sp.*,Nocardia* sp.*, Staphylococcus aureus., Listeria monocytogenes, Bacillus subtilis, Salmaonella typhi, Salmonella enterichilitis, E.coli*

0157, Shigella sp and *Proteus* sp (Zainal Abidin *et al.*, 2000). It was demonstrated that only *S. variegatus* and *H. atra* crude extracts had an inhibitory effect on *Nocardia* sp. at 10 mg/ml fixed concentration.

S. variegatus crude extract also showed antioxidant properties by increasing in enzymatic antioxidant defense when the cells were exposed to oxygen. Hawa *et al.*, (1999) studied the potential of coelemic fluid from *S. variegatus*, *B. mamurata, Vitiensis* sp and *S.badionatus* originating from the Perhentian Islands. In this study, the crude protein, superoxide dismutase (SOD) activity, malodialdehyde conjugated dienes (MDA) and total activity of antioxidant were measured. The results showed the significant increasing activity of the antioxidant enzyme, SOD compared to the control group ($p>0.05$). These indicated the coelemic fluids from *S. variegatus* and *Vitiensis* sp contained possible antioxidant activities.

S. variegatus has a vascular system filled with crystal fluid that is reported to contain bioactive substances important for wound healing (Perchenik, 1996). Earlier, Shimada, (1969) suggested in his inaugural report on sea cucumbers back in 1969, holothuroid and the products driven could cure certain ailments. Madhavan, (1998) reported that a sea cucumber (species specific) has been described to contain vitamin E for wound healing effects.

The body wall extraction from sea cucumbers contained various elements that were reported to show important effects on the human body. The body wall of holothurian including that of *S. variegatus* was reported to contain glycoaminoglycan in the form of Depolarized Holothurian Glycoaminoglycan (DHG) by Minamiguchi *et al.*, 2004). It was documented that *S.variegatus* extracts possess anticoagulant properties and an anti-thrombotic effect. In this experiment, the DHG does not induce any major bleeding during three hours of hemodialysis in the animal renal failure model. Another compound that was found on the body wall of the sea cucumber was fucosylated chondroitin sulphates that showed an anti-thrombotic effect. Tan *et al*., (2005) studied the arterial thrombosis in the rat model which demonstrated that body wall extraction of sea cucumbers showed reduced time in bleeding compared to the untreated group (Tan *et al.*, 2005).

The sea cucumber is often associated with its ability to reduce the blood glucose level in patients suffering from diabetes mellitus. This disease is also associated with reduction in the rate of wound healing. Arwina (2000) performed a study to investigate the effect of phosphate buffered saline of *S. hermanii* extracts on diabetic induced rats with streptozosin (45 mg/kg). The results showed a significant decrease in blood glucose level with a dosage of 150 mg/kg body weight (Arwina, 2000). However, the blood glucose level increased dramatically at the fourth week after oral administration of the extracts which indicated the effect of extracts in reducing blood glucose was time dependent. In another experiment, Shila Arwina (2000) also performed the same experimental settings using the coelemic fluid of *S. hermanii*. The coelomic fluid extract of *S. hermanii* (150 mg/kg/) did not reduce the blood glucose level of a diabetic induced rat.

The body extraction of holothurian contains polysaccharide chondroitin sulphate (PCS). A group of researchers (Idid *et al.*, 2001) studied PCS in an animal model of pain where it was demonstrated that polysaccharide chondroitin sulphate was able to reduce arthritic pain. It was also documented that this body extraction benefited the treatment of osteoarthritis (Idid *et al.*, 2001) and was positive in inhibiting the replication of the HIV virus at an earlier stage (Anjaneyulu *et al.*, 1998).

The other important compartment of holothurian is the toxin. The toxin was produced spontaneously after its body had been threatened or touched. The toxin serves as a defence mechanism in the sea cucumber. Ibrahim *et al*., (1995) reported that one of the toxins like antratoxin A1, B1, B2 that were isolated from *H. atra* exhibited significant activities against various species of dermatophytes like *Microsporium canis* and *Trichophyton rubrum*. The level of dermatophytes growth were fell down by serial increment of the concentration of B1 antratoxin until it reached the Minimun Inhibitory Concentration (MIC) value of 4.28 mg/ml (Ibrahim *et al.*, 1995).

The holothurians (*S. chloronatus*) were documented to exhibit a good inhibition towards *Aspergilus fumigatus* that causes fungal keratitis (Shaharuddin *et al.*, 2006). The methanol extract of *S. chloronatus* was tested at a MIC value of 1.56 mg/ml and it showed an inhibition to the *A. fumigatus* growth and could be suggested as a replacement drug to the current treatment-amphoterin B.

1.1.4 Toxicity study of *S. variegatus* extracts

Traditional medicinal practitioners treat their patients using crude extract preparations like sea cucumber embrocating oil and at other times, they use coelomic fluids extraction. The method of use depends upon the disease that is being treated. They may overlook the possible side effects of sea cucumber extracts in the long run. At present, there is no scientific study that looks into the long-term effects of these preparations. There has been no recorded history of sea cucumber intoxication either. The traditional preparation of sea cucumber extracts was prepared by cooking their body walls in a pot heated with wood fire (Ridzwan, 2007). The resultant solutions of cooked sea cucumbers were kept in large, lidded containers for about 6 months to reduce the strong odours and turbidity of the solution. Thereafter, the traditional practitioners filtered the solutions for homemade use or for selling purposes (Ridzwan, 1995). In short, the sea cucumber based industrial products are available in the local market without proper documentation.

In fact, the toxicity of Malaysian sea cucumber species was not extensively reviewed. However, Ridzwan (2007) reported there was a toxicity concerned of *S. variegatus* where it was previously shown to have a toxic effect with an increasing concentration of the methanol extracts starting from 20 mg/kg up to 1000 mg/kg body weight of mice. Wan Mohd Zaidi (1999) had tested different concentrations of methanol extracts of *S. variegatus* on Imprinting Control Region (ICR) mice by intraperitoneal injection. They evaluated the level of serum alanine aminotransferase enzyme (ALT), aspartate aminotransferase (AST) and lactate dihydrogenase (LDH). The levels of the three enzymes showed a significant increase with the increasing dose of the methanol extracts upto 900mg/kg body weight (Wan Mohd Zaidi, 1999). The toxic dose was found to be in between the concentration of 10 mg/kg and 1000 mg/kg of the methanolic extracts (Ridzwan 2007, Wan Mohd Zaidi,1999).

In the same year, Noorharisan (1999) determined the value of Lethal Dose (LD_{50}) in several extracts of *S. variegatus*. Five different concentrations of *S. variegatus* methanol and phosphate buffered saline (PBS) extracts was tested on mice. The LD_{50} values were determined for methanolic extract and PBS extract and they were 50.07 mg/kg and 136.55 mg/kg respectively (Noorharisan, 1999). Further study concluded that *S. variegatus* methanol and PBS extracts exhibited the hepato-toxicity where hepatic tissues degenerate, necrosis and bleeding were observed with increasing concentration of methanol extracts. It concurred with previous studies by Ridzwan (2007) and Wan Mohd Zaidi (1999).

Mohd Ariffin (2000) showed interesting findings in his toxicity studies of *S. variegatus* extract. The coelomic fluid and methanol extracts were given oral administration with the dose of up to 1600 mg/kg and the ALT, AST and LDH enzymes activity was measured. Serum levels treated with coelomic fluid and methanol extract of *S. variegatus* did not show any increasing enzyme activities compared to the control groups. The author also mentioned that the LD_{50} values were impossible to determine because there was no mortality reported in the study (Mohd Arifin, 2000). They therefore concluded that the administration of methanol extract of *S. variegatus* orally was not toxic to hepatic cells at a concentration of up to 1600 mg/kg.

To the best of our knowledge, there were no toxicity studies reported on the use of crude water extracts of *S. variegatus*. The use of such extracts or preparation by local practitioners has been going on for decades. However, there have been no anecdotal or official reports of possible toxicity of their use amongst the local populace. The reason of using crude water extracts in this study was due to its natural preparation and to mimic daily treatment that have been practised by local people.

1.2 Spinal cord injury (SCI)

Acute injuries to the spinal column and spinal cord are a major cause of disability. Young healthy males between 15 and 35 years old are the predominant groups that were affected (Shackelford *et al.*, 1998, Celani *et al.*, 2001, Jackson *et al.*, 2004, O' Connor, 2005, O' Connor and Murray, 2006, Cosar *et al.*, 2010, Ramakrishnan *et al.*, 2011). This prevalence has important socioeconomic consequences and the cost of lifetime care and rehabilitation are increasingly high.

1.2.1 An overview of demographics

Spinal cord injuries can be classified into several classes based upon types of injury and severity of the impact. The main cause of spinal injuries in humans is blunt trauma due to motor vehicle accidents (48%) followed by falls (21%) and sports injuries (14%) (Jackson *et al.*, 2004). The assault and penetrating trauma contributed to 10-20% of the cases (Jackson *et al.*, 2004, Cosar *et al.*, 2010). Most of the spinal injuries result in neurologic deficits, sometimes fatal. The survival of the patients is inversely related to the age of the patients. Mortality rate during the admission to the hospital was reported as 10% (Celani *et al.*, 2001).

Blunt trauma to the cervical and thoracic level was the main interest to researchers and clinicians alike in an effort to find cures. Spinal fraction and dislocation at the cervical and thoracic level contributed to 10-14% of all injuries to the spinal column (Shackelford *et al.*, 1998, O' Connor and Murray, 2006). Most injuries to the spinal cord occur at the time of trauma (85%), whereas 5-10% of spinal cord injuries present themselves in the immediate post-injury period (O' Connor, 2005). Thoracic spinal cord injury patients suffered from loss of function in the legs and bowel control, as well as bladder and sexual functions. The thoracic injuries cause the disability to move the lower limbs at a certain period of time and this condition is also known as paraplegia. The quality of life of paraplegic patients is greater than quadriplegic patients whose upper limb function is lost (Cosar *et al.*, 2010). The 57.1 % of SCI patients with paraplegia in Malaysia return to work after having experienced an SCI in their lifetimes

(Ramakrishnan *et al.*, 2011). The prognosis of a spinal cord injury patient is dependent upon the level of spinal injury. Injury at the higher levels results in more severe presentations with the additional loss of upper limb, pulmonary and respiratory function (Shackelford *et al.*, 1998). Other increasing matters of concern include neuropathic pain, autonomic dysreflexia and spasticity (O' Connor, 2005).

Therefore, scientists have been working with animal models to mimic human thoracic spinal injuries, and most of the models are rat spinal injuries. Also, the increasing numbers of experimental neuroprotection therapies as well as the improvement of the development of spinal cord injury devices in animal models have contributed to a greater understanding of the pathophysiology and the effects of therapies. The usage of rats as an animal model has been popular for the past decades until today (Kwon *et al.*, 2004). Apart from being cheap, friendly with latter transgenic potential, the anatomy of a rat spine and spinal function is relatively well understood.

1.2.2 Normal rat cord anatomy

The rat spinal cord is short; it is thin with tubular bundles of nerves extend from the brain. It is enclosed in and protected by the vertebral column (Narita & Kuratani, 2005). The rat spinal cord is 119.9 mm long (Greene, 1968) compared to the human spinal cord which is 45 cm in males and 42-43 cm in females (Watson & Kayalioglu, 2009). Rat spinal cords are made up of 34 segments: 8 cervical (named C_1 to C_8), 13 thoracic (T_1 to T_{13}), 6 lumbar (L_1 to L_6), 4 sacral (S_1 to S_4) and 3 coccygeal (Co_1 to Co_3). The largest of the rat cervical vertebrae are the atlas and the axis. The typical rat vertebra has a body, a neural arch and seven processes like that found in humans. The length of the thoracic vertebrae increases caudally from 2 to 7 mm (Greene, 1968). The human spinal cord differs from the rat spinal cord in having 12 thoracic segments, 5 lumbar segments, 5 sacral segments and 1 coccygeal segment, making a total of 31 segments. The cervical enlargement in a rat and human extends from C_5 to T_1. The lumbasacral enlargement extends from L_2 to L_6 in rats and from L_2 to S_2 in humans (Watson & Kayalioglu, 2009).

Pairs of spinal nerves arise from the rat spinal cord and leave the vertebral column through the intervertebral foramina. A rat spinal cord ends at the level of L_3, whereby in a cat, it ends at the L_5 vertebrate (Padmanabhan & Singh, 1979). Filum terminale in rats is traceable into the tail beyond the third caudal nerves. The cauda equina is made up of all the lumbar, sacral, and caudal nerves concealed in the extend of the rat cord (Padmanabhan & Singh, 1979). Each segment of the rat spinal cord possesses about 15 dorsal rootlets and about 15 ventral rootlets on each side. Humans have only 6-8 dorsal and ventral rootlets (Watson & Kayalioglu, 2009). The dorsal rootlets are bundled together to form the dorsal root of spinal nerve, whereby ventral rootlets form the ventral root.

The rat spinal cord is composed of white and gray matter. The cross bar of the 'H', called the gray matter, encloses the central canal. The dorsal projecting arms of the gray matter are called the dorsal horns and the ventrally projecting arms are called the ventral horns. The white matter consists of longitunally running axons and also glial cells (Watson & Kayalioglu, 2009). The ventral most part of the dorsal column contains a tract that is made up of small diameter fibers found in rat and non-primate spinal cord. This group constitutes the dorsal corticospinal tract (Padmanabhan & Singh, 1979). This tract diminishes in size from rostral to caudal levels. The dorsal corticospinal tract fibers terminate in the dorsal horn up to the interneuron of the ventral horn. The spinal gray matter is made up of neuronal cell bodies, dendrites, axons and glial cells. It is characterized by layers of cells from the dorsal to ventral sections. These layers were described by Rexed (1952) and they divided the spinal gray matter into 10 regions on the basis of cytoarchitecture as seen in transverse sections (Watson & Kayalioglu, 2009).

The rat spinal cord is supplied by one ventral spinal artery and two dorsal spinal arteries. In addition to that, lateral spinal arteries are also found to supply the blood to the cord on the lateral surface of the cord. The ventral and dorsal spinal veins are found on the ventral and dorsal surface of the cord respectively and these veins span the entire length of the cord. Interestingly, rat spinal cords do not contain a lymph vessel (Brierley & Field, 1948)(Figure 1.6).

B

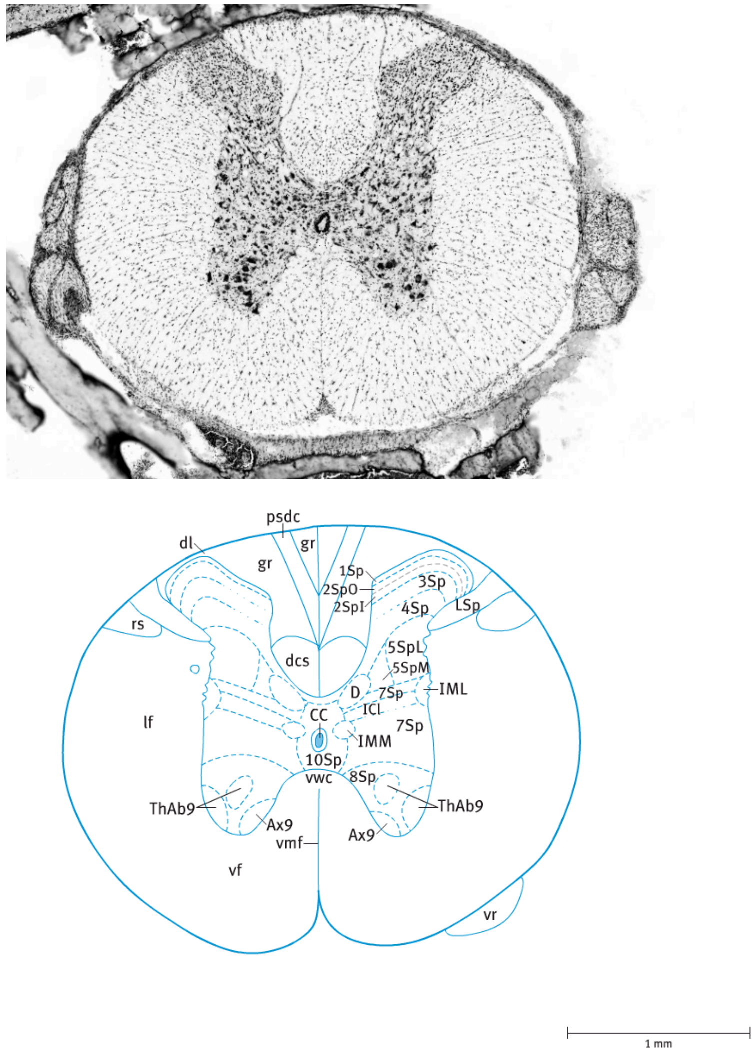

Figure 1.6 : Thoracic T9 of rat spinal cord section (A&B) (Watson & Kayalioglu, 2009). Abbreviation : gr=gracile fasciculus, dcs= dorsal corticospinal tract, CC= central canal, rs=rubrospinal tract, vf= ventral funiculus, dl=dorsolateral fasciculus, D= dorsal nucleus (Clarke), lf= lateral funiculus, 1Sp= lamina 1 of spinal gray, 2SpI= lamina 2 of spinal gray,inner part, Ax9= axial muscle of motoneurons of lamina 9, vmf= ventral median fissure, vr= ventral root, ThAb9= thoracicabdominal wall muscle motoneuron of lamina 9, LSp = lateral spinal nucleus.

1.2.3 Acute pathophysiology of Spinal Cord Injury (SCI)

It has been hypothesized that two steps are involved in the pathologic processes leading to an acute traumatic spinal cord injury: the primary and mechanical secondary injury. The primary mechanical injury induced by weight drop initiated additional damaging processes. The concept of secondary injury was postulated by Allen (1911). He demonstrated the secondary injury by removal of post traumatic hematomyelia which resulted in improvement of neurologic function in dogs. According to Zeidmann *et* al. (1996) the mechanism of hemorrhage within the cord causes the release of toxic excitatory amino acids, free radicals and the accumulation of amounts of endogenous opiates, causing the hydrolysis of lipid and in turn resulting in ischemia as well as reperfusion (Zeidman *et al.*, 1996, Young, 2009).

After acute injury, the spinal cord underwent a sequel of pathologic changes which included hemorrhage, edema, axonal and neuronal necrosis as well as demyelination followed by cyst formation and infarction of spinal tissues (Kwon *et al.*, 2004). The changes happened over time from a few minutes of injury to several weeks after the primary insult. Petechial hemorrhages occurred in the gray matter and edema of the white matter after 15 minutes of acute injury. The hemorrhages were increased after 2 hours, 4 hours, 6 days and worsen with time leading to necrosis (Taoka & Okajima, 1998). Electron microscopy studies by Dorman *et al*., (1971) and several other groups summarized the muscular venules of gray matter that were swollen by the accumulation of erythrocytes. Axonal changes were seen after 15 to 30 minutes after the injury with the entering of erythrocytes into perivascular spaces leading to progressive axonal changes and development of necrotic zone after a few days of the injury (Dohrman *et al.*, 1971). Breshnan (1978) emphasized the trauma to the spinal cord induces granular degradation of the axoplasm and vesicular disruption of myelin in the white matter (Breshnan, 1978,Cosar *et al*.,2010).

It has been reported that the edema develops at the injury site and spreads into segments of the cords (Taoka & Okajima, 1998). The theory proposed for endothelial cell damage was suggested to support the formation of an edema. The alteration of endothelial cell function will lead to an increase in vascular permeability which in turn causes an edema to occur at the injury site (Kwon *et al.*, 2004). In addition, the necrotic processes started to appear at the central zone followed by hemorrhagic zone which exhibited the formation of cavity and advanced necrosis with worsened defined margins. A progressive change became obvious with increasing cavitation and coagulative necrosis at the injury site and adjacent areas (Taoka & Okajima, 1998). Wallace et al., (1987) described and proposed that the further necrosis indicated posttraumatic infarction (Wallace *et al.*, 1987,Cosar *et al*.,2010)

1.2.4 Rat animal models of SCI

There were several available rat models for an experimental spinal cord injury across the laboratories in the world. The models varied from transected and contusion models of the past decades. Small laboratory animals like mice, rats, and larger species of animals (dog, cat, macaque and sheep) were used in these models, thereby providing a setting for an improved evaluation procedure for neuroprotective strategies in the acute spinal injury. However, the laboratory rat remains the most popular animal model that has an approach to answer research question in experimental spinal cord injuries.

The blunt contusive injury model or contusion rat model had been developed since the first contusive injury model was introduced by Allen in 1911. The author developed a model described as the crude weight drop model. The weight drop model induces injuries resembling the pathophysiology that occur in most human spinal injuries (Berhmann *et al.*, 1994). The pattern of evolution from the beginning of the spreading of hemorrhagic necrosis and edema, partial repair and tissue reorganization reaching to chronic phase is characterized by the formation of central cystic cavities. The atrophic parenchyma and glial scars resembled most of the pathophysiology of human spinal injuries (Ma *et al.*, 2001, Inman & Steward, 2003). Thus, the contusion model provides a neuroprotective strategy in an acute phase of injury (Young, 2009).

The other important reason why the contusion model was regarded as the best model to represent most of the human spinal injuries was the observation that has been made in setting for complete paraplegia after a blunt injury (Wyndale,2010). The cord is rarely seen completely transected, but leaves a residual, normal appearing cord at the peripheral of the injury zone. Subsequently, the contusion model produced the same lesion but the neural tissues remained intact at the peripheral rim of the injured cord (Basso, 2000, Ramakrishnan *et al*.,2011). Both female and male rats are used in SCI studies worldwide where both have advantages and disadvantages. The female rats are mostly used in the SCI study due to the easier urination procedure based on the presence of a significantly shorter and straighter urethra compared to male rats (Sedy *et al.*, 2008).

1.2.5 Contusion Injury

Contusion injuries are induced by hitting the exposed cord with a dropped weight (New York University Impactor) (Basso *et al.*, 1996a) or solenoid-driven device that displaces the spinal cord by a pre-selected amount (The Ohio State University Impactor)(Popovich *et al.*, 2002). The other device is an Infinite Horizon Impactor (IH) that allows the scientist to select the force to be applied to the spinal cord (Steward *et al.*, 2007). The contusion injury made by these devices should exert reproducible and consistent neurologic injuries. The contusion injury model is the best available which mimics a human spinal injury (Berhmann *et al.*, 1994, Ma *et al.*, 2001, Inman & Steward, 2003). However, the contusion model does not have control over the exact location and the severity of the lesion (Basso, 2000). Within this scenario, therefore, the spinal cord injury researchers are involved in developing techniques and devices to better deal with