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ASSESMENT OF GENERAL TOXICITY AND ANALGESIC PROPERTY OF SEVERAL SEA CUCUMBER SPECIES

Dissertation submitted in partial fulfillment for the
Degree of Bachelor of Health Sciences in Forensic Science

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I dedicate this dissertation to:

1) All the underwater world lovers with the hope that this kind of research may bring benefit to mankind...

2) My Mum and Dad and the rest of the family for the love and the undying support in taking care of me...

CERTIFICATE OF APPROVAL

This is to certify that the dissertation entitled,

"ASSESMENT OF GENERAL TOXICITY AND ANALGESIC PROPERTY OF SEVERAL SEA CUCUMBER SPECIES"

is the record of research work done by Zury Azreen Bin Azizul Rahman during the period from October to February under my supervision.

Signature of Supervisor,

____

Prof. Syed Mohsin Syed Sahil Jamalullail,
Deputy Dean of Academic and Student Affairs,
School of Health Sciences,
Universiti Sains Malaysia,
Date: 3/3/04

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-The Author-

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1. ABSTRACT

This study was conducted with the objective of establishing the presence of toxicity, mainly on the Central Nervous System (CNS) of both the sea cucumber species namely the *Holothuria spp* and *Stichopus spp.*

Upon obtaining permission of the Fishery Department of Malaysia for sample harvesting of both the sea cucumber species, an expedition was arranged to the Perhentian Island. Sample were harvested at the Perhentian Island and transported while preserved in seawater prior to laboratory processing and experimentation. Subsequently both the sea cucumber species were extracted by organic and non-organic solvent using body fluids and body tissue samples. Body fluids were collected from the sea cucumber during the process of dissecting and washing. For body tissue, the sample was dried in hot air oven and ground into powdered form before extraction was performed with organic or non-organic solvents. The organic solvent used is petroleum ether while non-organic is distilled water. The sea cucumber was extracted and dried to form a powder. The technique used to do this is by water or ether based extraction followed by filtration, saturation and finally freeze-drying.

From the sample powder extract obtained, calculation of dosage is done based on the amount of powder extract, volume solvent and body weight of mice. The sample solution is than test on mice. The tests include behavioral changes by observation, tail flick test and hot plate test. The study was conducted on 80 male swiss albino mice. The route of administration in this test is through the intraperitoneal method.

The behavioral study was done on a 4 hours of observation period after administration of the extract. In this study, it was found that CNS disturbance occurs in mice with both the

sea cucumber species. This effect includes changes in the attitude, restlessness, sedation, tremor, paralysis, pilo-erection, passitivity and jerky movements.

In the entire research, the dosage of use is 10mg of powder extract per kg of mice body weight (mg/kg). This dose is the startup point of toxic effect observed in this experiment.

In the tail flick test, mice are checked for analgesic effect of the extract of sea cucumber. It is found that only water-based extract from the *holothuria spp* gives a positive result.

In the hot plate test, mice are tested for their analgesic effect caused by the sea cucumber extract similar as the above.

2. INTRODUCTION

Sea cucumber or holothuroid live no more than 40ft under the sea. It belongs to a large class called the Holothurioidea. It is estimated that the class consist of 900 species (Moore & Clark 1962). Its appearance is elongated, worm-like or bun-shaped with a mouth located on the anterior end and the anus at its posterior end (Fechter 1970). Sea cucumbers are invertebrate from the phylum Echinoderm, class Holothurioidae (Moore 1966). For this research, I will be studying the phylum and class mentioned above and under the order of aspidochirotida and both the family Holothuriidae and Stichopodidae will be studied. The taxonomy detail is shown in Figure 1.

In the peninsula of Malaysia, sea cucumber is well known as 'gamat' while in Sabah it is called as 'bat' or 'balat' and in Sarawak it is named as 'brunok' (Chan & Liew 1986). There are at least 23 species of sea cucumber identified in the waters of Sabah (Ridzwan 1993). The identification of sea cucumber can be done based upon the extrinsic morphology which is the size, weight and color of skin (Hyman 1955; Ridzwan 1987)

A review of the history of the sea cucumber usage showed that it has been used as a food source and for medication purposes for more over than 300 years ago. This practice has been passed down from generation to generation (Hassan et al. 1994). The main reason for this its extensive use is due to the large distribution of the species and its common appearance along shallow waters in east coast of Malaysia. It is thought that holothuroids may form the main source of food during that time (Hyman 1955; Bakus 1973; Ridzwan & Che Bashah 1985).

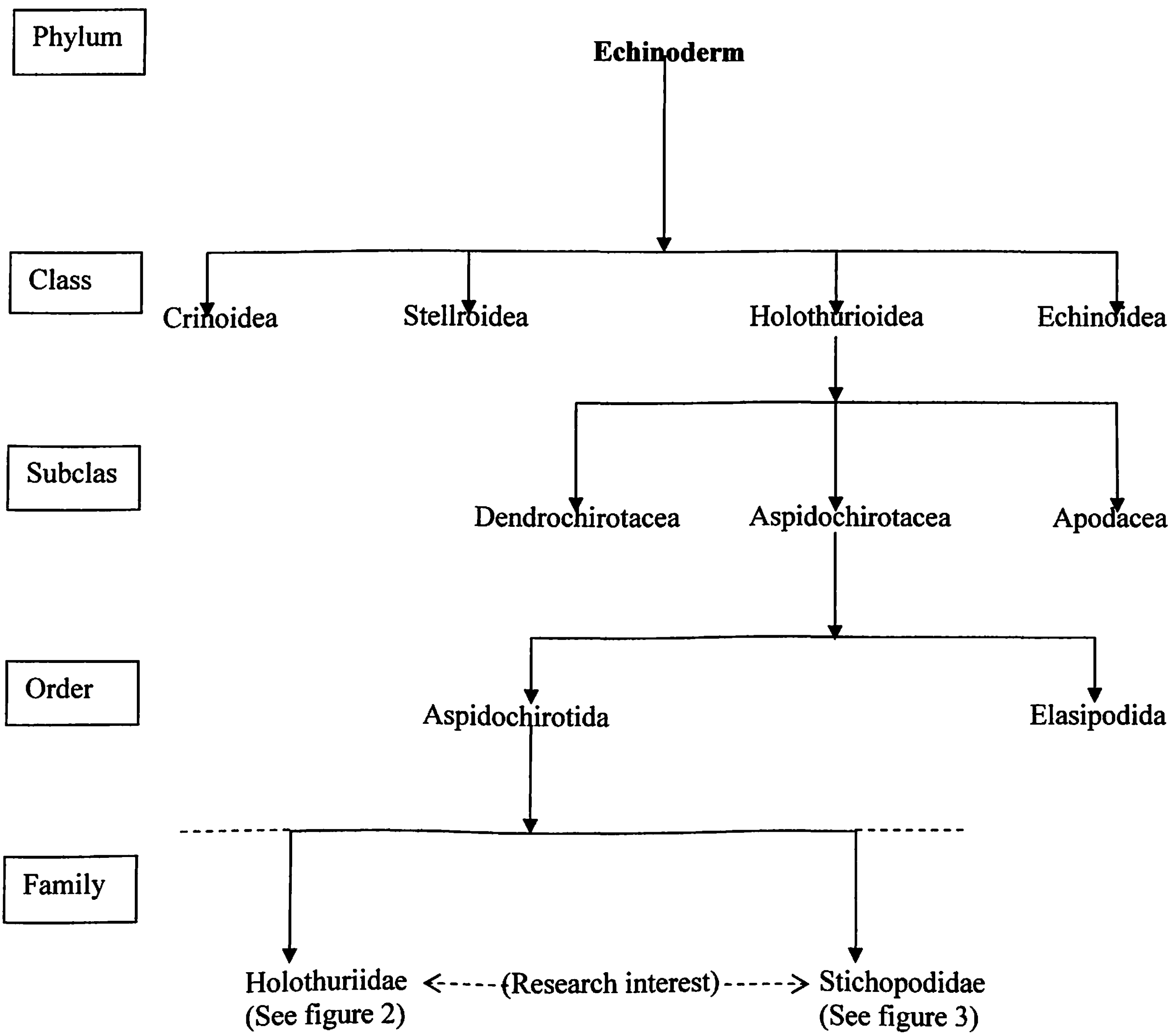


Figure 1: Taxonomy of Sea Cucumber (Holothuroid)

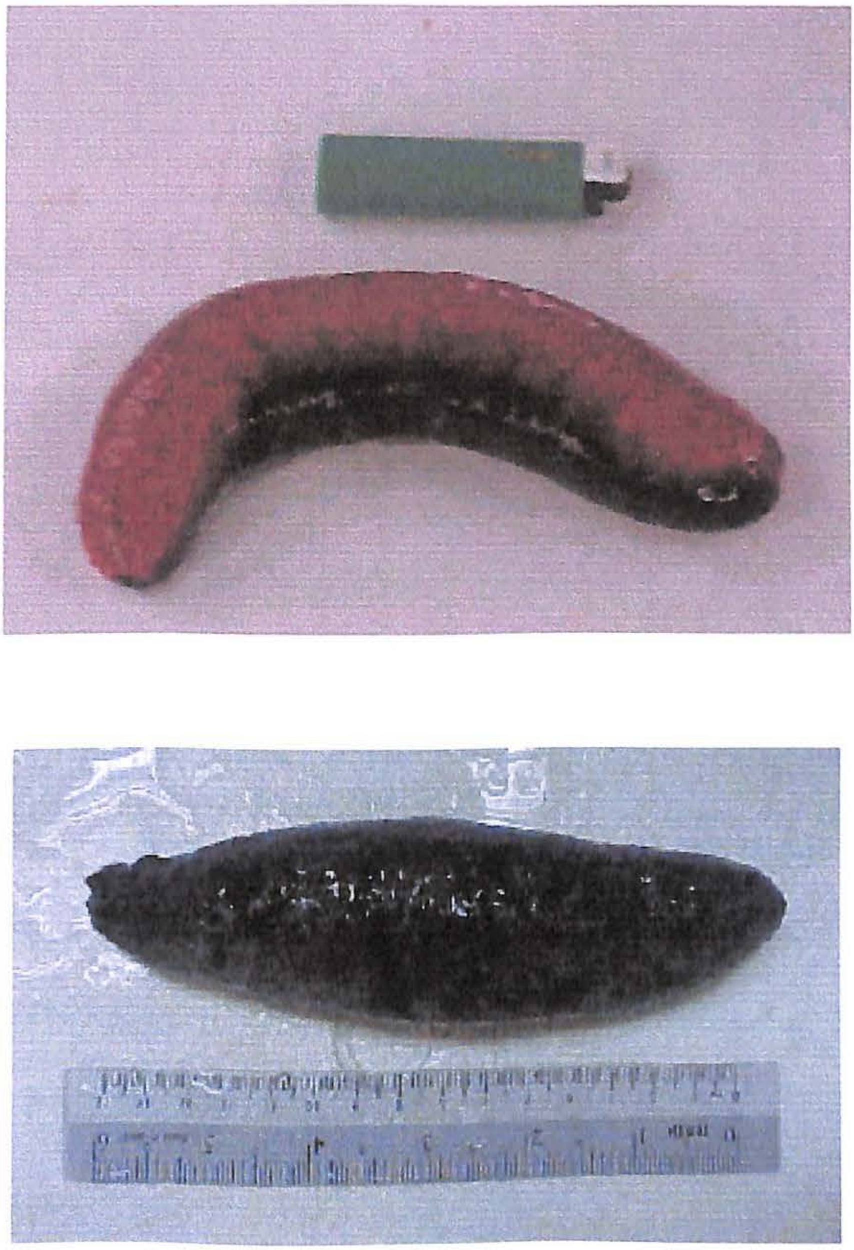


Figure 2: Sea Cucumber [family Holothuriidae]

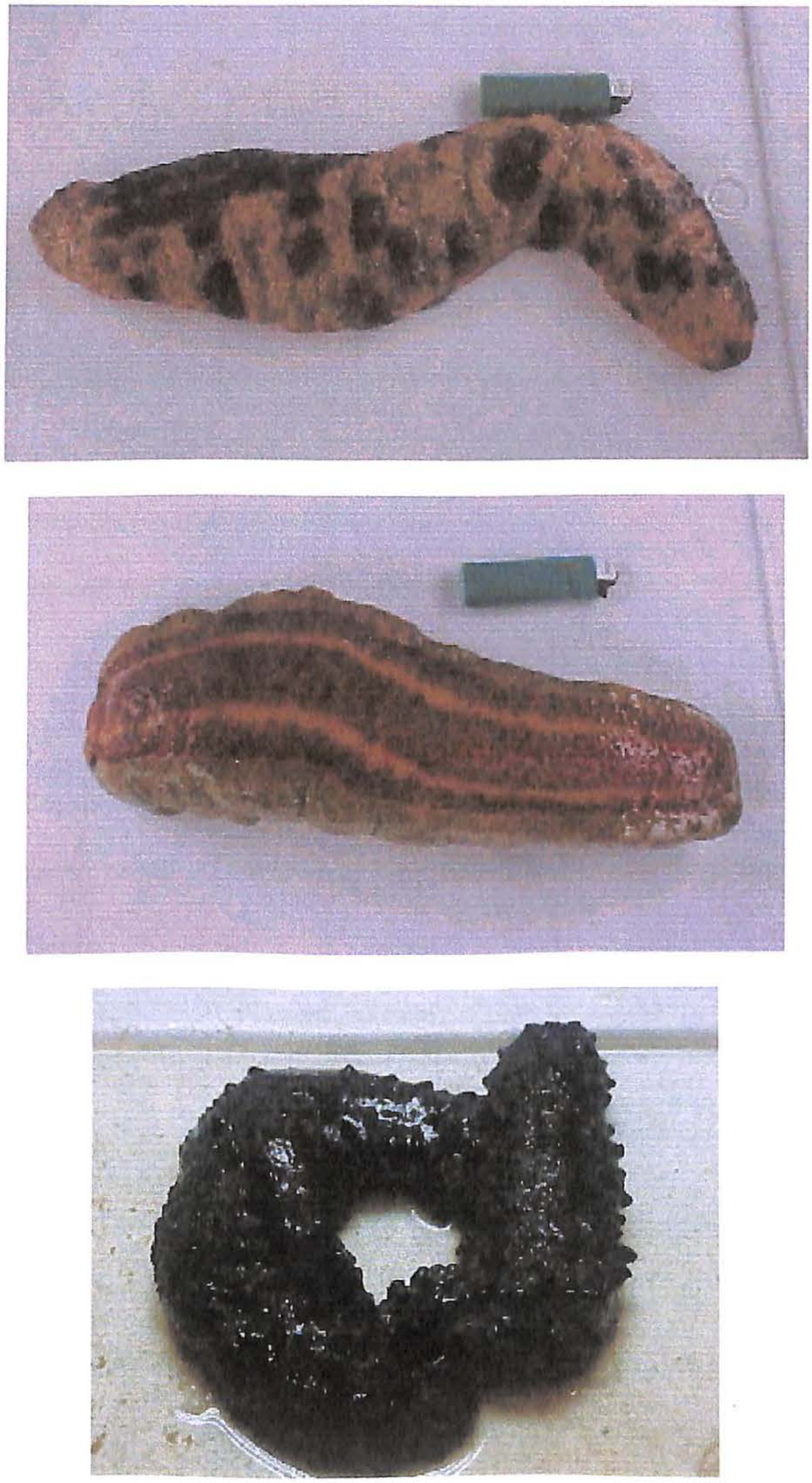


Figure 3: Sea Cucumber [family Stichopodidae]

Sea cucumber is said to be a potential food source due to its high protein content (Hyman 1955; Ridzwan & Che Bashah 1985; Ridzwan 1987). Approximately twenty five to thirty species from the family Holothuriidae are made into *trepang* and are eaten. Trepang is a Malay word, and it refers specifically to cooked, dried, and smoked sea cucumber, which is considered as delicacies (Fechter 1970). In addition, sea cucumber are also said to have medicinal value based on traditional method of preparation (Chan & Liew 1986). It is well known for its use in wound healing. They are also used for the healing of wound in mothers who had delivered babies (Zainudin et al. 1986).

This research will study the toxic effect of the sea cucumber. It is well known now that sea cucumbers are used as embrocating oil (Tarlochan 1980) and also as a food source (Hassan et al.1994). This research is set out to determine if sea cucumber are toxic to the body. This is important to establish due to the fact that it might be a source of toxicant to the body. The outcome might be negative or poor based on the fact that from day to day people are still using it.

The type of toxicity in this study is generally on the central nervous system (CNS) and specifically on the behavioral changes and analgesic effect. There are quite a number of changes in the behavior and a slight effect on the analgesic.

An important research protocol to be followed is that before harvesting sea cucumbers from the waters of Terengganu a letter of permission to harvest is sent to Fishery Department for approval (Figure 41). This process takes around 6 weeks. This is an important research protocol because the sea marine animals are protected by law especially in marine parks like those surrounding Terengganu's islands. After being granted permission, samples are harvested according to the conditions given by the Fishery Department under Act 317, section

43(2) of Fishery Act 1985. The Island of interest is Perhentian Island (Figure 13), Redang Island (Figure 16) and Lang Tengah Island (Figure 11).

Another research protocol that needs to be followed is when handling the mice. Here issues of ethics must be followed and maintained. The animal must be treated humanely, without being put under undue stress and pain. (http://research.uiowa.edu/animal)

Besides that, good laboratory practice is crucial in preparing and conducting the experiment. Small techniques and procedures that we might have thought irrelevant or important may play a big role in the long run. It is vital that previous researchers are consulted.

3. REVIEW OF LITERATURE

3.1 GENERAL

Sea cucumbers are well known for having medical value (Chan & Liew 1986). But not much research has been done on this aspect. As through the review, research is more on the morphology (Nicholas & Cooke 1979; Hyman 1955; Ridzwan 1993) distribution (Hyman 1955; Bakus 1973), food source (Ridzwan & Che Bashah 1985), composition content (Ridzwan 1987) and its use (Tarlochan 1980; Chan & Liew 1986). There are also other research on the sea cucumber self defense mechanism. Here it is stated that holothuroidae and Asteriodae are said to produce a toxin called Saponin when threatened (Yasumoto at el. 1967). It is also reported that sea cucumber does have some of holotoxin (Shimada 1969). In a recent study, the reproductive cycle of the sea cucumber was studied (Guzman at el. 2003; Ramofafia at el. 2003). In medical fields, the research is not that detailed yet. Researches in the medical field are more on physiological and ecological aspect (Bakus 1973; Sloan & Von Bodungen 1980; Ridzwan & Che Bashah 1985). Antifungus and also antitumor research has also been conducted (Shimada 1969). Recently, antibacterial study was done (Saruddin 1997; Zaki 2000)

3.2 MORPHOLOGY

Sea cucumber or holothuroids are lower class invertebrates. (Arbain 1990) from the class holothurioidea, phylum echinoderm (see Figure 1). Holothuroids show a variation in their shape, size and color of skin. Its color varies from black, gray or maroon is said to be characteristic to differentiate its species. The surface of its skin shows a thin layer but its body tissue is very stiff. It shows a bentonic character and moves quite slowly (Hyman 1955; Ridzwan & Che Bashah 1985; Chan & Liew 1986). The shortest ever species noted was 2cm from species H. atra and the longest was 500cm from species Synapta maculata (Bakus 1973; Benhard 1974). The absence of a rigid skeleton is compensated for by a highly developed body musculature. Under the epidermis is a layer of well-developed circular muscles, which are interrupted by five bands of longitudinal muscles that run along the ambulacra areas. The longitudinal muscles insert anteriorly to a ring shaped internal skeleton, which usually consists of a ring of ten calcareous plates surrounding the pharynx. The inner surface of the muscle is covered by the peritoneum (Fechter 1970).

3.3 DISTRIBUTION

Order Aspidochirotida in the sea cucumber is dominant in shallow tropical water especially in the Indo-Pacific waters (Hyman 1955; Bakus 1973). This was further supported by Ridzwan (1993), who showed that the order Aspidochirotida is mostly present in shallow water in Sabah. 23 species were found in this shallow water and from this, 21 were aspidochirotida, 1 Apodida and 1 Molpadiida. In the peninsula area, sea cucumber or holothuroid can be found around Islands in the east and west coast. Among those islands are Langkawi Island (Chan & Liew 1986), Kendi Island in Penang (Ibrahim et al. 1994), Redang Island and Tioman Island (Rahmad & Zulfigar 1993; Salehudin & Zulfigar 1993). Comparatively, the peninsula has less number of species compared to the Sabah waters, that is only 8 species compared to 23 species in Sabah (Kaswandi 2003: Personal Interview)

3.4 COMPOSITION CONTENT

The sea cucumber is mainly made from water, protein and fat, the composition of which varies among species. Percentage of water is 83-95%, protein is 44-45% and fat is 0.4-2.7%. The water content in the holothuroid is proportionate to the elasticity of the body tissue (Ridzwan 1987). Earlier, Hyman (1955) noted that the protein content in holothuroids in Indo-Pacific waters were 35-52%. This was later supported by Ridzwan (1987), which stated that holothuroid in Sabah composed of 44% of protein. Generally all holothuroid have fat content less than 1%. Starch is also reported to be present with a low value that is 1.26-5.36%. The amount of calorie is stated to be lower than 200 calorie.

3.5 FOOD SOURCE

Thus having high protein content, 35-52% it is surely a good protein and food source. It has a good potential of being an alternative protein source. This alternative food source is a good means to the Sabahan, who do take holothuroid as their meal (Ridzwan & Kaswandi 1995). Researchers found that almost all ethnic group in Sabah and immigrants from the Philippines, take holothuroid raw as a meal (Ridzwan 1993). In fact, Sabah also does imports 40.61 tonnes of holothuroids from foreign countries (Jabatan Perikanan Sabah 1986). The locals in Sabah also collect holothuroids to gain pocket money. One holothuroid is said to be sold for 40-50cents. There is also an industry which processes holothuroids to be exported to Sarawak, Peninsula Malaysia and also Hong Kong. The price for 60kg is between RM500-RM700 (Ridzwan 1993)

3.6 OTHER USES

Besides being a food source, holothuroids are also said to have good traditional value. Its extract is made into ointment for curing pain in lungs, wound, body muscles, parasite infection, back pain and others. The Chinese eat this to cure disease such as high blood pressure, asthma, internal bleeding, body weakness, impotence and wound healing after giving birth or having an operation (Tarlochan 1980; Chan & Liew 1986). A recent study showed that holothuria could heal wounds even faster than controlled petroleum jelly (Ridzwan 1990a at el.). This type of research was further verified by Hassan and co (1994) with their conclusion that Holothuroids do indeed have a positive effect on the healing process resulting in faster healing.

In a more recent study, Noor Ibrahim and Lim (2000) studied the effects of methanol extracts of *Stichopus variegatus* and *Holothuria atra* on cutaneous wound healing in guinea pigs. Histological studies using both light and scanning electron microscopes were performed. Both the extracts were found to enhance the rate of healing as well as the quality of the resultant scar.

3.7 MEDICAL RELATED

The research interest in sea cucumbers as a source of pharmacological and medical agents was initiated since S. variegatus was widely utilized as a traditional remedy for asthma, hypertension, rheumatism, sinus, cuts, and burns. This long period of widely community practice needs to be properly proven by scientifically based evidence (Mohsin at el. 2003).

Through the review, sea cucumber in related to medical research started way back where Shimada (1969) had found antifungus and antitumor in Stichopus japonicus Selenka. But this research was conducted abroad. In Malaysia, research on the antifungus and microbial in the holothuria started by Ibrahim & Khow (1990), followed by Ibrahim et al. (1994), Ridzwan et al. (1990b), Ridzwan et al. (1995a), Mastura (1999) and many more. Recently, Mohsin SSJ & Philip Rajan indicated that gamat has antimicrobial and anti-pseudomonal activity against *S.aureus, E.c*oli and *P.aeruginosa* from the lowest concentration of 10 mg tested (Mohsin at el. 2003). Indeed, there are many researches on the antifungus, antibacterial and antitumor.

Besides those mentioned above, there is also a research on the antiasthma done by Zainuddin et al. (1986) whereby he did not find any substance that resembles β-agonist or depress the phosphodiesterase and gives effect to antiasthma.

3.8 TOXIC EFFECT

The uniqueness of phylum echinoderms is its ability to produce toxin to other species. This toxin is caused by saponin (Hashimoto 1977). Saponin is a natural steroid compound, which is water soluble, methanol soluble and has a complex structure with glycosides of monoxides, aglikon steroid or triterpenoid and sulphuric acid (Nigrelli et al. 1955; Chanley 1959). Even though it is possible to get saponin from a lot of plant species, in the animal kingdom only phylum Echinoderm from family Holothuroidea and Asteroidae have this saponin or known as holothurin for the holothuria (Yasumoto et al. 1967).

The parts of the body, which contains this holothurin, is the egg, testis, body wall surface and visceral fluid, This is a kind of defense mechanism to the holothuroid (Nigrelli 1952; Yamanouchi 1955). Based on the bubble test and Liebermann-Buchard test, Ridzwan (1993) found that all the sea cucumber species in Sabah water have saponin. This was further supported by Kaswandi at el. (1990)

Earlier on, it was shown that holothuria is a good food source. But isn't it dangerous with the saponin content in all the holothurians? According to Baslow (1977) the holothurin does not give any effect to humans due to the fact that our enzymes deactivate this toxin in our gastrointestinal system. This was later supported by a local researcher, Norhayati(1987)

This research is to verify this finding.

4. OBJECTIVE OF THE STUDY

The main objective of this study is to verify toxicity of sea cucumber or holothuroid from the family Holothuriidae and Stichopodidae on central nervous system of the mice. The toxicity evaluation of this plant could be divided as the following:

4.1 To identify possible toxic species of sea cucumber.

4.2 To establish type of toxicity if any.

4.3 To determine the most toxic extract from both the type of extract and both the Sea Cucumber family.

4.4 To observe the behavioral changes and CNS toxicological signs during acute poisoning.

4.5 To determine any analgesic activity from either the extract of both the Sea Cucumber family.

5 MATERIALS AND METHODS

5.1 EQUIPMENT

Below is a list of the equipment that was used during conducting my research. The equipment can be found at the Pharmacology lab and at the Lab Facility Unit (UKM- Unit Kemudahan Makmal).

1. Hot air oven (Figure 4)
2. Grinder (Figure 5)
3. Weighing Scale (Figure 6)
4. Refrigerator (Figure 7)
5. Freeze dry (Figure 8)
6. Hot plate analgesic meter (Figure 9)
7. Tail flip analgesia meter (Figure 10)

5.2 MATERIALS

5.2.1 Sea Cucumber

Sea cucumber was harvested freshly from Perhentian Island, Redang Island and Lang Tengah Island. 5 species were identified from 2 families, which are the Holothuriidae and Stichopodidae (Figure 2,3 & 20-24). From each of this species, two types of extraction were conducted to its body tissue and body fluids that is the organic base extraction and the non-organic base extraction.

5.2.2 Animals for Test

The subject for this research is the Swiss albino mice. 80 male swiss albino mice were tested during the whole research. The mice body weight was between the range of 20-30g. The mice were bred and supplied by Universiti Sains Malaysia Health Campus animal house with the price of RM8 per mice. They were kept in an air-conditioned room with adequate food and water supply. Only fresh and healthy mice were used in the study. Mice were taken at random in each test.

5.2.3 Route of Administration

At first, oral and intraperitoneal (ip) route of administration were planned. But after running the test, time became a crucial matter. There was time for only one route of administration and ip was chosen as the route of administration. Generally, ip reacts faster than oral route of administration.

5.2.4 Dose and Duration of Observation

For the entire test that was conducted, the dose was set to be 1mg of powder extract per kg body weight of mice (1mg/kg). The test duration for the behavioral changes is for 4 hours. While for the hot plate test and tail flip test, no fixed time was set.

5.3 METHODS

5.3.1 Harvesting and Transportation

As mentioned earlier, permission is needed before harvesting (Figure 45). After which, harvesting is conducted (Figure 18 &19) at Perhentian Island, Redang Island and Lang Tengah Island (Figure 11). All the sea cucumbers are placed in a container filled with seawater while waiting to be transported to the lab. Frequent water exchange is required to maintain the life of the sea cucumber. This is done manually and if possible once after every few hours.

5.3.2 Processing for Extract

Upon arriving at the lab, sea cucumbers are separated according to their species. Then, their body weight is weighed individually by using a weighing scale (Figure 7) to get a mean average of its body weight.

Next, sea cucumbers are washed and left in a container to obtain its body fluid. This process will take around an hour or so. Body fluids are then collected (Figure 32) and preserved in a refrigerator (Figure 6) for extraction.

For the body tissue, the process is a little longer. From the wet body tissue after fluid extraction, the Sea Cucumbers are dissected (Figure 25-27) to exclude their internal organs. After that, these body tissues are put in a container then into the hot air oven for drying. This process will take nearly 2 weeks. The operating temperature was 58°C.

After drying in a hot air oven, the result was a piece of tight and packed body tissue (Figure 35 & 36). Extraction with this is not possible. A grinder (Figure 5) had to be used to blend the body tissue into powder form (Figure 38). Now, both the body fluid and body tissue are ready for extraction process.

5.3.3 Extraction Process

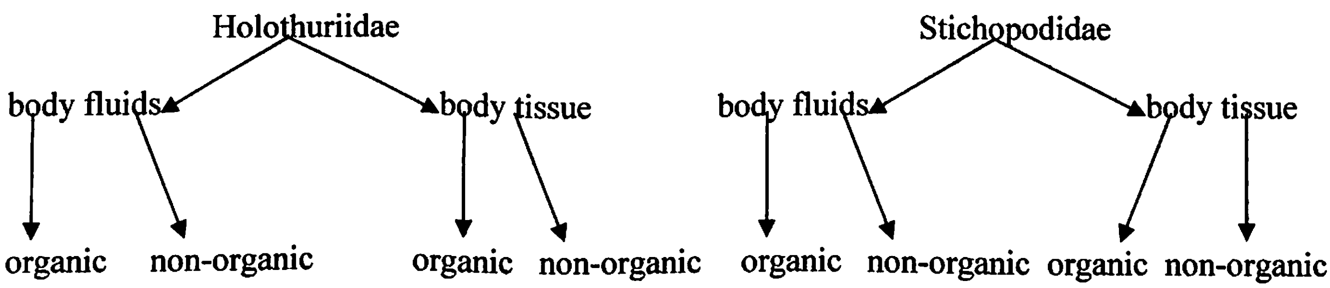
Two types of extraction were done here which are organic extract and non-organic extract. For the organic extract, petroleum ether was used as the extract solvent. Meanwhile, for the non-organic extract, distilled water was used. Both the extract solvent are present at the Lab Facility Unit (UKM) at School of Health Science, USM.

For the body fluid, the extraction process differs from the body tissue. First of all 50ml of body fluid was poured into a 100ml volumetric cylinder. 50 ml of petroleum extract was poured to make the mixture equiv. Volume and then mixed vigorously for a few minutes. Next, this mixture was poured into a separating pipette forming 2 separate layers of liquid. The upper portion is lighter and this resembles the petroleum ether portion. The lower portion is not organic soluble particles because if it were, it would have been in the upper portion. The lower portion resembles non-organic (distilled water) portion.

A beaker was placed under the separating pipette and separated the two liquid portions (organic & non-organic). The 2 extracts were labeled and transferred into a plastic test tube (Figure 33 & 34) before being placed it into a refrigerator for freezing and preserving.

For the body tissue, the body was weighed in mg before mixing with the solvent extract in a funnel. For example, if the weight of body tissue powder was 7.5mg, 7.5ml of solvent extract was used. This was to make sure that the concentration of the extract would be 1mg/ml. After mixing, the solvent extract with its soluble compound was separated into a beaker below the funnel. This process was done to both types of solvent extraction (organic & non-organic) for both the sea cucumber species. After collecting the extraction, they were labeled and transferred to a plastic test tube before being storied in a refrigerator for freezing and preserving.

The completion of these two types of extractions (organic & non-organic) to two parts of the sea cucumber (body fluids & body tissue) of the two different families (Holothuriidae & Stichopodidae), will result in a total of 8 types of extractions as shown in the diagram below:



All these samples are frozen in a refrigerator first before being transported to the Pharmacology Lab for freeze-drying.

5.3.4 Freeze Dry

At the Pharmacology Lab, samples are placed in an appropriate container for running the freeze dry process. In this process, water is sucked out by using pressure from cold air, which results a dry sample. The operating temperature and pressure is -55°F and 200atm respectively. This process took overnight to finish. When running the samples, it was made sure that bubbles were not formed from the sample so over spilling would not occur with other samples.

Powder extract is the result of this process. It was stored in a refrigerator before being used in dose preparation.

5.3.5 Dose Preparation

5.3.5.1 Materials

1. Distilled water
2. Dimethyl Sulphoxime (DMSO)
3. Spatula
4. Digital Balance
5. Extract's powder
6. Beaker (1-10ml)
7. Glass rod
8. Plastic test tubes (1-20ml)

5.3.5.2 Methods

From the powder extract formed by freeze-drying, a solution with a concentration of 1mg/ml was calculated and formed. This was done by mixing the powder extract in the same amount of number of solution. For example, if the powder is 11.3mg then the solution should be 11.3ml. The solution used here is distilled water with a few drops of Dimethyl Sulphoxime (DMSO). The DMSO is used to dissolve compounds, which are insoluble to water.

Next, the dose is calculated to result in 10mg of powder extract to 1kg body weight of mice (mg/kg). This was done by adjusting the volume (ml) of administration according to the body weight (kg) of the mice. For example, if the body weight is 0.03kg then the volume of administration should be 0.03ml. With this calculation, the dose and concentration of extract was fixed at 1mg/kg and 1mg/ml with the end result of dosage being 1mg/kg. To get a dosage of 10mg/kg, the volume of administration was increased 10 times. For the example above, the volume should be 0.03x10 = 0.3ml. The dosage needed for this experiment was 10mg/kg.

On completion of the dosage preparation, the prepared solution was stored in a refrigerator before being used on the mice.

5.3.6 Behavioral Changes Observation

Mice were weighed and examined before the test. The weight had to be in the range mentioned earlier. The mice were labeled at the tail and body to differentiate them from each other. Mice were then injected with samples of extract through intraperitoneal. The mice were placed in a large transparent container. Parameters of observing were behavioral changes, movements, reactivity to various stimuli, cerebral and spinal reflexes and muscle tone. These parameters were used to describe mice changes in response to the dose given. Any changes to the mice within 4 hours of observation were recorded in an observation form as such as below:

Observation and Examination	Common sign of toxicity	Observed Signs & Symptoms
1) Behavioral	a. Change in attitude to observer b. Unusual vocalization c. Restlessness d. Sedation	
2) Movements	a. Twitch b. Tremor c. Ataxia d. Catatonia e. Paralysis f. Convulsion g. Forced movements h. Pilo-erection	
3) Reactivity to various stimuli	a. Irritability b. Passitivity c. Anesthesia d. Hyper aesthesia	
4) Cerebral and spinal reflexes	a. Sluggishness b. Absence	
5) Muscle tone	a. Absence b. Flaccidity c. Tenseness	

Table 1: Behavioral observation and examination category form

Common sign of toxicity		1 Hour				2 Hour				3 Hour				4 Hour			
		1/4	1/2	3/4	1	¼	1/2	3/4	1	1/4	1/2	3/4	1	1/4	1/2	3/4	1
1	Change in attitude to observer																
2	Unusual vocalization																
3	Restlessness																
4	Sedation																
5	Twitch																
6	Tremor																
7	Ataxia																
8	Catatonia																
9	Paralysis																
10	Convulsion																
11	Forced movements																
12	Pilo-erection																
13	Irritability																
14	Passitivity																
15	Anesthesia																
16	Hyper aesthesia																
17	Sluggishness																
18	Absence																
19	Rigidity																
20	Flaccidity																
21	Tenseness																

Table 2: Behavioral changes observation form