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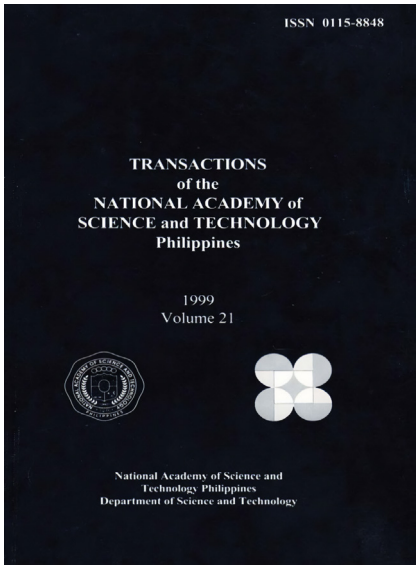

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THE CIGUATERIC POTENTIAL OF SOME PHILIPPINE RABBITFISHES (FAMILY SIGANIDAE)

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ABSTRACT

Extraction and bioassay for polar lipid toxins in *Siganus spinus* (Linnaeus), implicated in a human poisoning case in La Union, and other members of Family Siganidae namely *S. canaliculatus* (Mungo Park), *S. virgatus* (Cuvier & Valenciennes), *S. guttatus* (Bloch), and *S. punctatissimus* Fowler & Bean from several locales found toxins in the fish viscera that could amount to 10 MU/100g. One Mouse Unit (MU) was defined as the amount of toxin that can kill a 20g mouse within 24 hours. Histopathological analysis in experimental mice revealed damages in the ventricular myocardium and other tissues which could be caused by maitotoxin most possibly present in the extracts. Kymograms of frog neuromuscular and heart activity suggested both ciguatoxin-like and maitotoxin-like effects. The gut contents of Siganid fish as well as the La Union reef included algae which are known preferred substrates of ciguateric poison-elaborating dinoflagellates.

Key Words: Ciguatera, ciguatoxin, maitotoxin, ichthyosarcotoxism, Siganidae, rabbitfish, fish poisoning, neurotoxin, cardioactive toxin, ion channel activator

INTRODUCTION

Ciguatera is a human illness chiefly characterized by gastrointestinal, neurological, and cardiovascular disorders that may result from the ingestion of seafood. Originally, the term ciguatera referred to poisoning caused by "cigua", the Caribbean marine snail *Livona pica,* but is now applied to the similar disorders following the intake of various normally edible fishes. Within two to thirty hours after consumption of toxic fish, the following symptoms appear: nausea, often

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followed by watery diarrhea, sometimes vomiting, sensitivity disturbances such as the reversal of temperature sensations where in cold feels hot and hot feels cold, intense itching, numbness with tingling in the limbs, slow or accelerated pulse rates, slightly modified heart beats, general weakness, joint pains, and dizziness (SPC Health Programme Staff, 1991). The illness is an established phenomenon, and is well-recognized for centuries (Randall, 1958). It is separate and distinct from other illnesses such as botulism and scombroid poisoning (Ragelis, 1984). It may have existed longer than the 500 years documented by historical records as proven by the fishes that are not affected by the toxins that they bear (Lewis, 1984). Mann (1978) wrote of mankind being aware of it for some 6,000 years. It is widespread within the geographical belt between 35°N and 35°S (Mann,1978). Particularly in many islands of the Pacific, ciguatera is a serious problem, gravely affecting health and economies (Glaziou *et al.* , 1995). It is reported that some 20,000 people are affected by ciguatera annually (Yasumoto and Satake, 1996).

The clinical signs of ciguatera are diverse. Prevailing symptoms are determined by the kind of toxins involved. Ciguatera poisons are polyether compounds of two principal classes: ciguatoxin and maitotoxin. The ciguatoxin family includes ciguatoxin (CTX) and several congeners. The major ciguatoxin, $C_{60}H_{86}O_{19}$ (Murata *et al.*, 1989; Murata *et al.*, 1990) is a Na^+-channel activator. Maitotoxin (MTX), $C_{164}H_{256}O_{68}S_2Na_2$ (Murata *et al.*, 1994) on the other hand could affect a receptor-mediated affect Ca^{++}-channel (Yasumoto and Murata, 1993). Understandably , because of their modes of action, these are very potent toxins. Maitotoxin is the most potent marine biotoxin (Ohizumi and Yasumoto, 1983; Ohizumi *et al.*, 1983; Takahashi *et al.*, 1983). The fact, however, that the ciguatera syndrome is only occasionally fatal in humans (SPC Health Programme, 1991) may perhaps be due to individual sensitivities and not very particularly high levels of toxins in fish.

The toxins are derived from a fish diet, primarily being produced by epiphytic toxic dinoflagellates that are ingested by the herbivore with the toxins then being passed to carnivores (Yasumoto *et al.*, 1979; Bagnis *et al.*, 1980). The most highly ciguateric fishes would be the larger carnivores that would have bioaccumulated and oxidized (Yasumoto and Murata,1993) the toxins to a greater extent within their lifetimes.

Almost any reef fish can become ciguatoxic, under the right conditions (SPC Health Programme Staff, 1991), hence, ciguatera was easy suspect for the poisoning episode in La Union in 1996 which incriminated *Siganus spinus* (Linnaeus). The investigation on the ichthyosarcotoxism case (Pocsidio and Cabrera, 1999) had established toxin levels believed to be of ciguatera poisons in the internal organs of *S. spinus*. An assessment was then made of the ciguateric potential of the other members of the Family Siganidae, especially those being vended in fish markets in La Union and elsewhere.

This present report embodies the results of the extraction, mouse bioassays and histopathological and physiological observations with partially purified toxins

from the Siganid fishes as well as gut content analysis and survey of algal community in the Lingsat Reef in La Union for the occurrences of known preferred algal substrates of ciguatera poison-producing dinoflagellates. Ciguatera toxins have been reported to affect such organs as the heart and stomach (Terao et al., 1988; Terao *et al.*, 1992) and the analysis of these particular effects could contribute to the identification of the toxins in the absence of complete chemical elucidation.

MATERIALS AND METHODS

Siganid Fish

Fish were obtained from various fish markets. The vendors provided the information about specific sources. *S. guttatus* and *S. virgatus* from Calatagan, Batangaas were definitely feral, i.e., obtained from the wild. The same were true for *S. punctatissimus* from Palawan, *S. canaliculatus* from the difference locales and *S. spinus* and *S. virgatus* from San Fernando City. Except for the *S. spinus* from La Union which were freshly caught and not kept in cold storage, all the fish had been previously packed in ice.

Visceral organs were taken from fresh and frozen fish, processed for ciguateric poisons. Samples also from fish flesh, with and without bones, as well as separate flesh and head parts were used in later extractions.

The fishes were of the following approximate total lengths: *S. spinus* (12 cm), *S. canaliculatus* (13.5 cm), *S. guttatus* (15 cm), *S. virgatus* (17 cm). The single large *S. punctatissimus* had total length of 21cm. Smaller *S. punctatissimus* were about 13.5 cm in total length.

The scientific, English , and local names of the fishes (Rau and Rau, 1980; Schroeder, 1980; Lieske and Myers, 1994) are as follows:

1. *Siganus spinus* (Linnaeus). English names: Black trevally, Scribbled rabbitfish. Local name: Danggit (Visayan)
2. *Siganus canaliculatus* (Mungo Park). English names: White-spotted rabbitfish, White-dotted rabbitfish. Local names: Danggit (Visayan), Samaral (Tagalog), Bararawan (Cuyonin)
3. *Siganus virgatus* (Cuvier & Valenciennes). English names: Barhead rabbitfish, Virgate rabbitfish. Local names: Tagbago (Visayan), Samaral (Tagalog), Mandalada (Cuyonin)
4. *Siganus guttatus* (Bloch). English names: Golden spinefoot, Golden rabbitfish. Local names: Ketong, Kitung (Visayan), Barangen (Cuyonin), Samaral (Tagalog)
5. *Siganus punctatissimus* Fowler & Bean. English names: Pearly-dotted rabbitfish, Peppered rabbitfish. Local names: Danggit (Visayan), Mandalada (Cuyonin), Samaral (Tagalog).

Extraction and Assay Methods for Toxicity

The procedure for extraction and bioassay according to Yasumoto *et al.* (1984) was adopted.

Primary extraction from the tissue samples was done through acetone homogenization (JTBaker, AR) using a Nikon blender. The homogenate was filtered by suctioning through with an Eyela Aspirator on a Buchner funnel. Afterwards, extraction from acetone-suspended sample was done with diethylether (JT Baker, AR). Subsequently, partitioning was done in Hexane (Ajax, AR) and aqueous methanol (Merck, AR). The methanol layer was dried in *vacuo* using a Buchi Rotary Evaporator. The residue was then suspended and, emulsified, in 1% Tween 60 (Sigma). I ml and 0.5 ml of diluted and undiluted suspension were injected intraperitoneally to laboratory white mice that weighed 20g on the average.

According to this procedure, one mouse unit (MU) was defined as an amount of toxin that can kill a 20g mouse within 24 hours. From this relation, a lethal 40g sample would yield toxin level of 2.5 MU/l 00g or from a 20g sample, 5.0 MU/100g. Lethality is established with the death of two mice out of three.

Histopathological Studies

Sublethal doses of 0.5 MU and 0.25 MU *S. guttatus* visceral toxin in three single doses 24 hours apart were injected intraperitoneally to laboratory white mice. On the fourth day the mice were killed. The heart, stomach, and intestine were excised, fixed in 10% neutral formalin (Chemline, Tech.), then processed by paraffin method. Tissues were sliced for light microscopy examination and stained in eosin (Fluka) and haematoxylin (BDH). The stomach, intestine, liver, lungs, kidney, muscle, and spinal cord of mice that died immediately upon injection of a lethal dose (4 MU) were also processed similarly and prepared for light microscopy. Examination was done on a Carl Zeiss microscope.

Physiological Studies

Cardiac actions in the frog of the visceral toxins were recorded on kymograph system (Phipps and Bird). Brain-pithed adult frogs *(Rana catesbiana)* averaging 12 cm in head-to-toe length were administered by gavage 2 single doses of 1 MU *S. canaliculatus* visceral toxin 10 minutes apart. Kymograph recordings of ventricular beats were done immediately prior, immediately after, 5 minutes after first treatment, and immediately after and 10 minutes after second treatment.

Actions on gastrocnemius muscle-sciatic nerve preparations from frogs were also studied. Kymograph recordings were made of the responses of the skeletal muscle to the following: (1) nerve stimulation with electrical stimulus (Phipps and Bird Student's Inductorium) immediately prior, 5 minutes after, and 10 minutes after topical application of 0.4 MU *S.canaliculatus* visceral toxin on nerve and 5 minutes after and 10 minutes after topical application of additional 0.4MU, (2) direct electrical stimulation immediately prior and 5 minutes after muscle was

injected with 0.4 MU, (3) direct electrical stimulation immediately prior and 10 minutes after immersion of muscle in 0.4 MU, and (4) direct electrical stimulation after washing previously toxin-immersed muscle with Ringer's solution.

Examination of Gut Contents

S. canaliculatus collected from Palawan were obtained through the Navotas Fishing Port. Guts were removed and preserved in 10% formalin until use. Contents of each gut were emptied into separate glass petri dishes and examined under stereozoom microscope (American Optical). Food items were scored for their occurrences in 10 fishes. Chlorophyll content of the gut contents was measured photometrically according to the method by MacKinney (1941).

Survey of Algal Community in La Union Reef

Macroalgae were collected from intertidal zone in Lingsat Coast, La Union. The algae preserved in 10% formalin were later identified using a local field guide as major reference (Calumpong and Menes, 1997; Trono, 1997).

RESULTS

The various Siganid fishes showed toxicity levels that ranged from non-detectable levels to 10 MU/100g (Table 1). Only three out of the 12 batches of the Siganid fish did not yield toxic viscera. These were *S. guttatus* and *S. virgatus* from Lucena, Quezon, and small-sized *S. punctatissimus* from Palawan.

Toxic manifestations in mice were motor ataxia, tremors, convulsion, respiratory distress, hindleg paralysis, and jumping before death.

Most significant histopathological effects of sublethal *S. guttatus* toxin were some swollen and disrupted myocardial fibers in mice (Figs.1 and 2) and erosions in gastric mucosa. Mice administered lethal *S. canaliculatus* toxin exhibited erosions in gastric mucosa (Fig. 3), disruptions in intestinal villi. (Fig. 4), intra-alveolar edema (Fig. 5), loss of nuclear bags inside the muscle spindle (Fig. 6), loss of interconnecting processes in the spinal cord (Figs. 7 and 8), disintegrating cells and increased sinusoidal spaces in the liver (Fig. 9), and hemorrhage in the kidney (Fig.10).

Topical application of 0.4 MU *S. caniculatus* toxin on the sciatic nerve initially increased the duration of the gastrocnemius muscle twitch. Afterwards, with additional 0.4 MU of toxin on nerve, skeletal muscle activity declined. Immersion of skeletal muscle in 0.4 MU toxin abolished contraction. The muscle recovered little with washing. Injection of 0.4 MU toxin into the muscle also abolished contractility. The kymograph record of these events are shown in Figures 11 and 12.

An initial positive inotropic effect followed by diminished activity of the heart was observed in the frog which was fed 2 single doses of 1 MU *S. canaliculatus* visceral toxin (Fig. 13).

Table 1. The toxicity of some Philippine Siganid fishes

Species	Place of collection	Date of collection	Date of mouse bioassay	Sample	Toxin level MU/100g
S. canaliculatus	Lucena, Quezon (Nepa-Q-Mart)	3-7-97	3-28-97	viscera	2.5
S. canaliculatus	Bicol (Navotas Fish Port)	3-13-98	3-20-98	viscera	10
S. canaliculatus	Canaoay, San Fernando City Mart	8-23-98	2-27-99	viscera body body plus head	10 10 10 10
S. canaliculatus	Pagdalagan, San Fernando City	7-16-98	2-27-99	viscera body body plus head	5 10 ND ND
S. guttatus	Lucena, Quezon (Nepa-Q-Mart)	3-7-97	3-28-97	viscera	ND
*S. guttatus**	Calatagan, Batangas	2-3-98	2-20-98	viscera	2.5
*S. punctatissimus***	Palawan (Malabon Fish Market)	3-3-98	3-22-98	viscera flesh	5.0 2.5
S. punctatissimus **	Palawan (Malabon Fish Market)	3-3-98	3-22-98	viscera flesh	ND ND
S. spinus	Poro Point, San Fermando City Mart	8-23-98	2-27-99	viscera flesh with bone deboned flesh body plus head	5 5 5 5
*S. virgatus**	Calatagan, Batangas (coastal market)	2-3-98	2-20-98	viscera	2.5
S. virgatus	Lucena, Quezon (Nepa-Q-Mart)	3-7-97	3-28-97	viscera	ND
S. virgatus	Canaaoay, San Fernando City Mart	7-16-98	2-27-99	viscera body body plus Head	2.5 5 10

*definitely feral **large-sized ***small-sized
ND-nondetectable

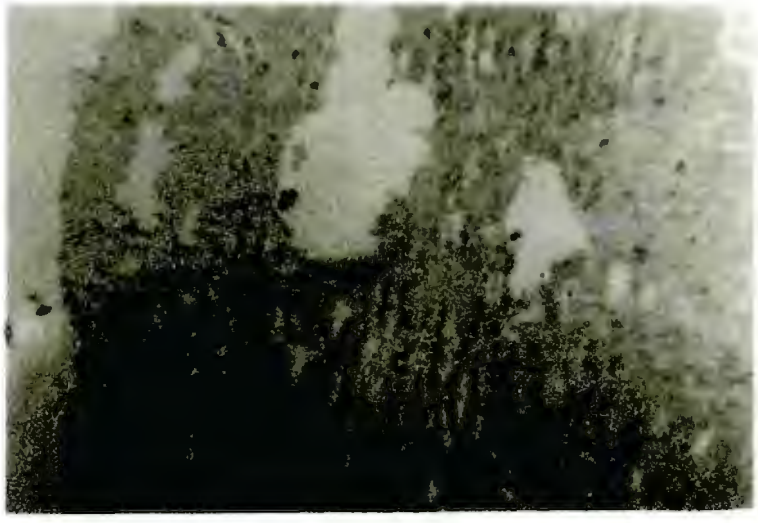

Figure 1. Photomicrograph of heart of albino Swiss mouse injected i.p. 0.5 MU *S. guttatus* visceral toxin. (c) Swollen ventricular myocardial fibers. Haematoxylin-eosin. X450.

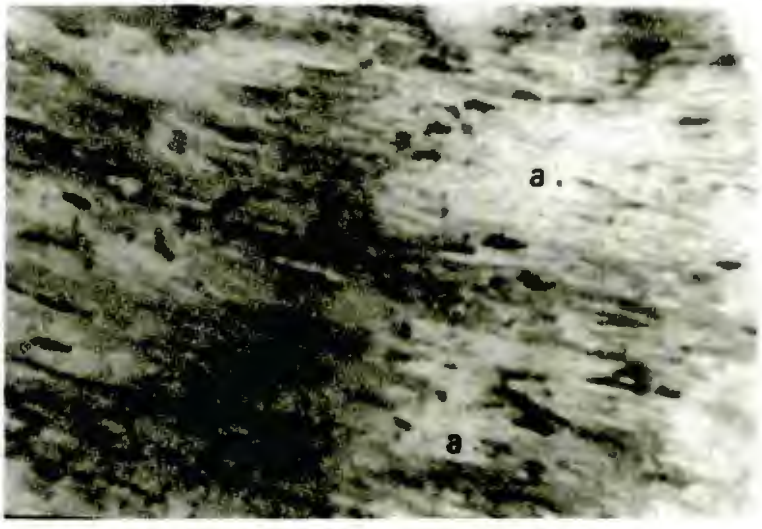

Figure 2. Photomicrograph of heart of albino Swiss mouse injected i.p. 0.5 MU *S. guttatus* visceral toxin. (a) Disrupted ventricular fibers. Haematoxylin-eosin. X1000.

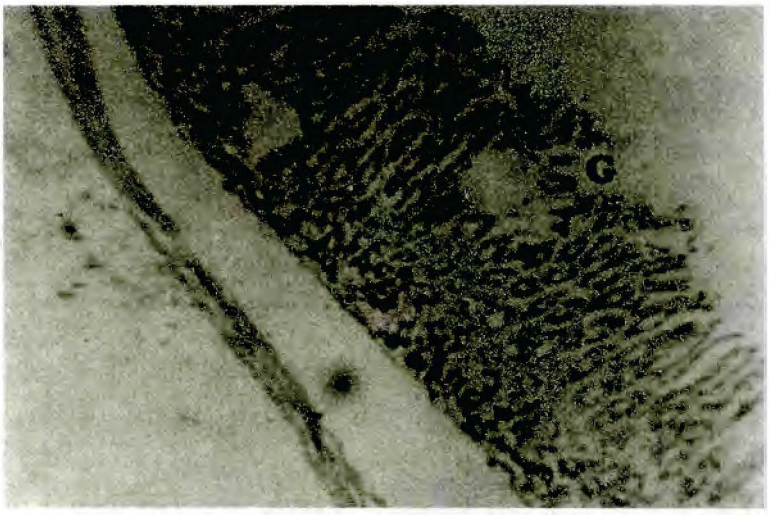

Figure 3. Photomicrograph of stomach of albino Swiss mouse injected i.p. 4.0 MU *S. canaliculatus* visceral toxin. (G) Erosion in gastric mucosa. Haematoxylin-eosin. X450.

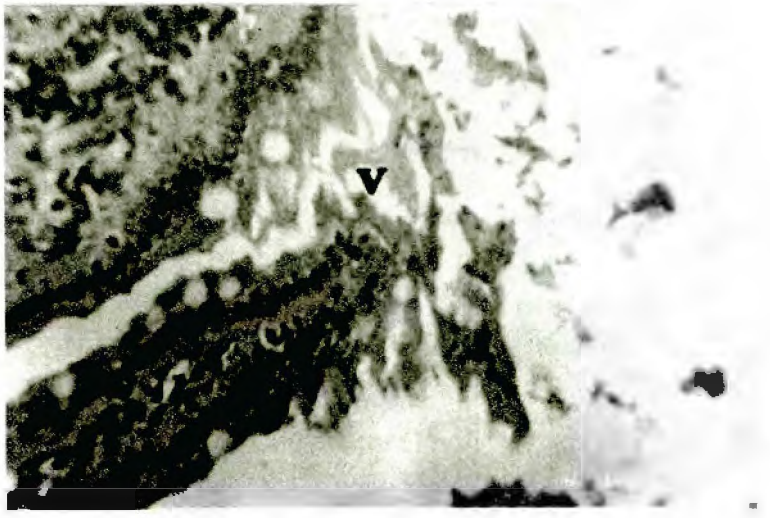

Figure 4. Photomicrograph of small intestine of albino Swiss mouse injected i.p. 4 MU *S. canaliculatus* visceral toxin. (V) Disruptions in intestinal mucosa. Haematoxylin-eosin. X450.

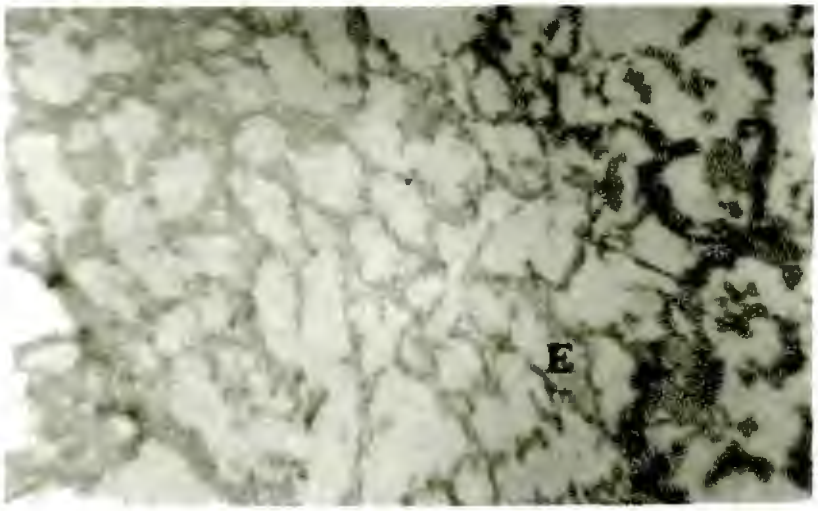

Figure 5. Photomicrograph of lung of albino Swiss mouse injected i.p. 4 MU *S. canaliculatus* visceral toxin. (E) Intra-alveolar edema. Haematoxylin-eosin. X450.

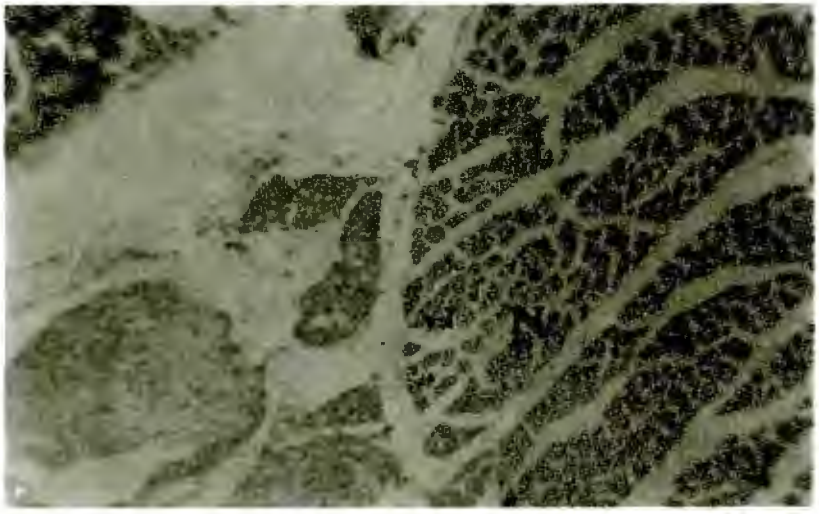

Figure 6. Photomicrograph of skeletal musle of albino Swiss mouse injected i.p. 4 MU *S. canaliculatus* visceral toxin. (N) Loss of nuclear bag inside the muscle spindle. Haematoxylin-eosin. X450.

Figure 7. Photomicrograph of spinal cord of albino Swiss mouse injected i.p. 4 MU *S. canaliculatus* visceral toxin. (C) Loss of interconnecting processes. Haematoxylin-eosin. X100.

Figure 8. Photomicrograph of spinal cord of albino Swiss mouse injected i.p. 4 MU *S. canaliculatus* visceral toxin. (C) Loss of interconnecting processes. Haematoxylin-eosin. X450.

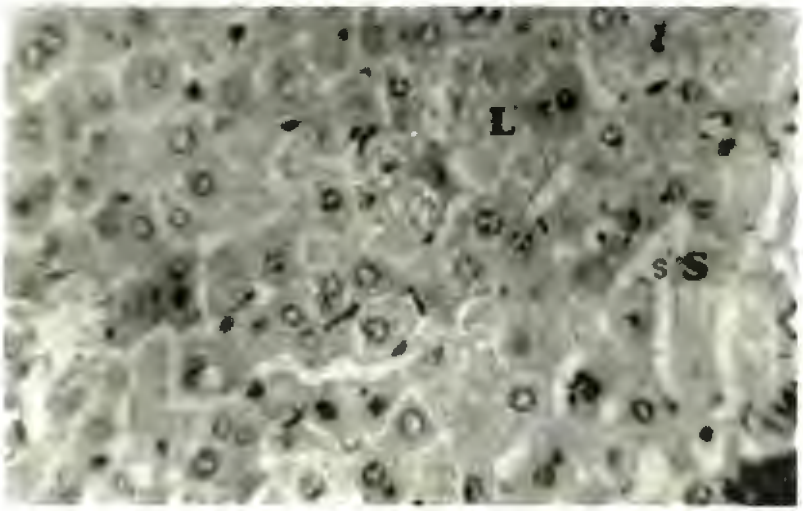

Figure 9. Photomicrograph of liver of albino Swiss mouse injected i.p. 4 MU *S. canaliculatus* visceral toxin. (L) Disintegrating cells. (S) Increased sinusoidal spaces. Haematoxylin-eosin. X450.

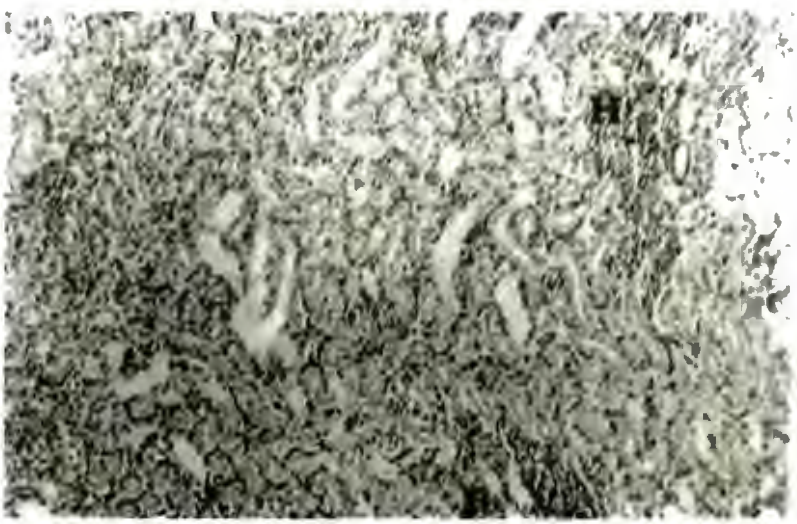

Figure 10. Photomicrograph of kidney of albino Swiss mouse injected i.p. 4 MU *S. canaliculatus* visceral toxin. (H) Hemorrhage. Haematoxylin-eosin. X450.

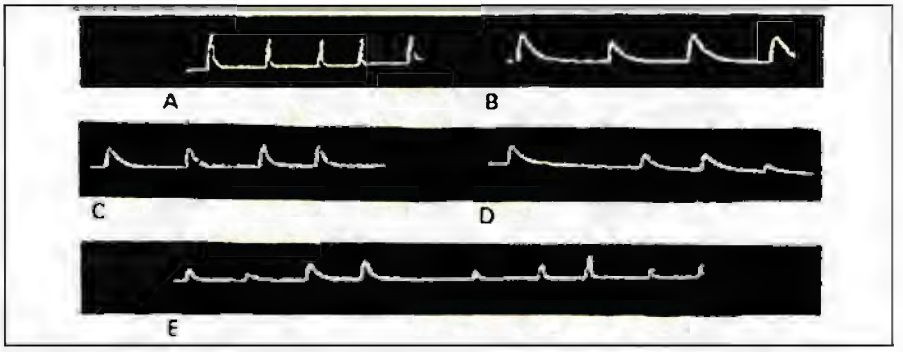

Figure 11. Responses of frog gastrocnemius muscle to electrical stimulus on sciatic nerve. Effects of topical application of 0.4 to 0.8 MU *S. canaliculatus* visceral toxin to sciatic nerve. Kymograph recording (A) immediately prior to application of 0.4 MU toxin, (B) 5 minutes after, (C) 10 minutes after, (D) 5 minutes after application of additional 0.4 MU toxin, (E) 10 minutes after.

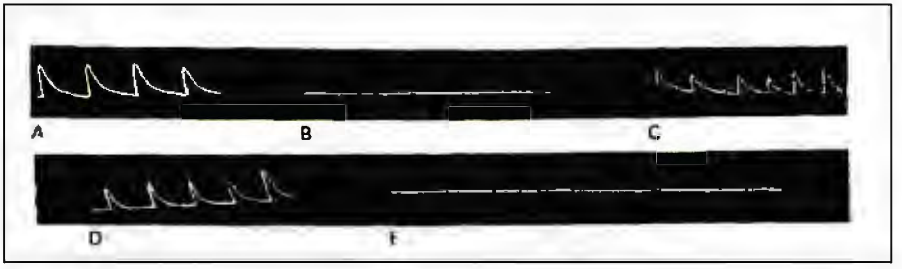

Figure 12. Responses of frog gastrocnemius muscle to direct electric stimulus. Kymograph recording (A) immediately prior to immersion of muscle in 0.4 MU S. canaliculatus visceral toxin, (B) after 10 minutes of immersion in the toxin, (C) after washing of toxin-immersed muscle with Ringer's solution, (D) immediately prior to injection of 0.4 MU toxin into muscle, (E) 5 minutes after injection.

Figure 13. Responses of frog ventricle to 1 MU S. canaliculatus visceral toxin in 2 single doses. Kymograph recording (A) immediately prior to feeding of 1 MU toxin, (B) immediately after feeding, (C) 5 minutes after feeding, (D) immediately after second feeding of 1 MU toxin, (E) 10 minutes after.

Examination of gut contents of *S. caniculatus* yielded 28.6% and 43.0% occurrences, respectively, of *Jania sp.*, and *Spyridia filamentosa* (Table 2). Both are known preferred algal substrates of the most important ciguateric poison - producing dinoflagellate, *Gambierdiscus toxicus* (Yasumoto *et al.*, 1979; Yasumoto *et al.*, 1980; Bagnis *et al.*, 1980; Shimizu *et al.*, 1992). The survey on the algal community in Lingsat Reef found the following macroalgae: Class Chlorophyceae- *Ulva sp., Halimeda macroloba, Chaetomorpha crassa, Caulerpa toxifolia, Caulerpa racemosa;* Class Phaeophyceae- *Sargassum sp, Padina sp, Hydroclathus clathratus; Class Rhodophyceae- Gracillaria verrucosa, Gracillaria salicornia, Galaxaura oblongata, Amphiroa sp., Hypnea boergesenii, Hypnea cervicornis, Jania sp, Laurencia papillosa, Acanthophora spicifera, and Mastophora rosea.*

Table 2. Gut contents of *S. canaliculatus*

Food Item*	Percent Occurrence**
Class Chlorophyceae	
Cladophora sp.	14.3
Caulerpa toxifolia	14.3
Halimeda sp.	28.6
Class Phaeophyceae	
Sphacelaria tribuloides	43.0
Dictyota cervicornish	28.6
Padina sp.	28.6
Hormophysa cuneiformes	14.3
Sargassum sp.	28.6
Dictyopteris sp.	14.3
Lobophora variegata	14.3
Class Rhodophyceae	
Grateloupia sp.	14.3
Amphiroa sp.	28.6
Cheilosporum sp.	14.3
Jania sp.	28.6
Hypnea sp.	43.0
Gracillaria sp.	43.0
Spyridia filamentosa	43.0

*The chlorophyll a content of gut contents by spectrophotometric analysis averaged 4.53 g/100g

**Percent Occurrence = No. of fishes in which food item occurs x 100 / total no. of fishes examined

Three out of ten fish examined had empty guts

DISCUSSION

The ciguateric potential of the Siganid fish, particularly those that were obtained directly from coral reefs, was evident as shown in the mouse bioassays done with several extracts from various fish samples. The histopathological and physiological analyses on some experimental animal models also revealed the potential danger of eating internal organs of the fish and even the flesh especially the large-sized members of the fish family. The toxic manifestations exhibited by the dying mice were similar in almost all instances to the lethality tests and suggested that the various Siganid fishes contain most probably the same kind of toxins. The presence of maitotoxin, one ciguateric poison, was indicated most specifically by the swelling and disruption of ventricular myocardial fibers and the erosion of gastric mucosa in the mice that were administered even sublethal doses of the Siganus visceral toxin. The kymograph recording results of the frog skeletal muscle and heart activity suggested both ciguatoxin and maitotoxin effects. Although no experiments were done to consider the possible role of commonly used fish poisons such as sodium cyanide or the local alkaloidal fruit or bark extracts of the local "bayating" or *Tinomiscium philippinensis* Diels (Menispermaceae), ciguatera, on the other hand, was indicated by the above results.

Although more selective for the fat soluble ciguatoxin, the extraction procedure used in this study could abstract the water-soluble maitotoxin with the use of the aqueous methanol in the later part of the extraction. Considering that maitotoxin is largely contained in ciguateric herbivorous fish (Yasumoto *et al.*, 1995), more of the substantial changes that were observed in the present study could perhaps be attributed to this poison.

Maitotoxin was demonstrated to target the heart and stomach (Terao *et al.*, 1988; Terao *et al.*, 1992). The destructive effects were attributed to the ability of the toxin to activate Ca^{++} channels. It has been suggested that maitotoxin could probably be binding to receptor-mediated Ca^{++} channels (Yasumoto and Murata, 1993), hence, there are the many possible pathways, metabolic or otherwise, which could ultimately cause cell death. Heart beat vigor depends much on the amount of calcium ions made available for the contractile mechanism (Guyton, 1986), so that the initial positive inotropic effect observed in this study could be explained by the increased inward conductance of calcium ions from the extracellular fluid . Nevertheless, the resultant accumulation of calcium would have likely caused the subsequent structural damages and diminution or even abolition of functional abilities such as what were observed in the skeletal muscles that were either injected or immersed in the Siganus toxin.

Ciguatoxin, though it may not be dominant in the extract, could also have affected the heart. Lewis (1988) reported that while low doses of ciguatoxin could induce positive inotropic response in the guinea-pig atria and papillary muscles by opening myocardial voltage-gated Na^{+} channels, high doses could cause negative inotropy associated with cell depolarization and signs of calcium overload.

In the initial experiment, whereby electrical stimulation was applied to the frog's sciatic nerve which was previously administered with the toxins, there was a resultant increase in the strength and duration of the gastrocnemius muscle twitch. This could be due to a prolonged presynaptic depolarization and subsequent increased Ca^{++} influx, increased amount of transmitter release, greater amplitude and duration of the muscle action potential, greater amount of Ca^{++} to effect the sliding of the filaments, and longer duration of the relaxation phase of the twitch. Capra (1992) reported that during electrical stimulation of the ventral coccygeal nerve in the rat tail, the administration of sulblethal dose of ciguatoxin increased the refractory period and extended the magnitude and duration of the supernormal period of the compound action potential. These changes were explained by the increased ease in the opening of the Na^+ channel and the time course of Na^+ channel opening that was caused by the toxin. Based on structure, ciguatoxin could undergo slow conformational change while binding to the voltage-sensitive sodium channel and alter the gating or inactivation mechanism of the channel (Yasumoto and Murata,1993). The physiologic activity of ciguatoxin , that of increasing sodium ion influx, has been described as similar to that of brevetoxin which binds to receptor site 5 on the α–subunit of voltage-gated sodium channel (Baden, 1995). Again, the paralysis that ensued after treatments by topical application on the nerve, immersion or injection into muscle would have to be attributed to the combined actions of both maitotoxin and ciguatoxin. Another possible action of ciguatera toxins that has been suggested is the antagonism of the nicotinic cholinergic receptor (Escalona de Motta *et al.*, 1992).

Furthermore, it may be worthy to note that macroalgae constitute the bulk of the diet of the Siganid fish. This was suggested by the high chlorophyll content in the gut contents. The occurrences of some algae that are known to harbor ciguatera poison-producing dinoflagellate in the food of the Siganid fish could be associated with the ciguateric potential. That the algae were also found in the algal community in the Lingsat Reef in La Union could also be significant. One important future study would be to actually obtain the toxic dinoflagellates and establish the reef as ciguateric.

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