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**Solid-phase extraction clean-up of ciguatoxin-contaminated coral fish extracts
for use in the mouse bioassay**

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Running title: Purification of CTX-contaminated fish extract

Abstract

Florisil solid-phase extraction (SPE) cartridges were used for purifying ciguatoxin-contaminated (CTX-contaminated) coral fish extracts, with the aim of removing extracted lipid but retaining optimal level of CTXs in the purified fractions. The CTX-containing fraction (target fraction) in fish ether extract was isolated and purified by eluting through a commercially available Florisil cartridge with hexane-acetone-methanol solvent mixtures of increasing polarity (hexane-acetone (4:1, v:v) < acetone-methanol (7:3, v:v) < 100% methanol). Application of Florisil SPE using acetone-methanol (7:3, v:v) condition facilitated the separation of 4.2 ± 0.4 mg (mean \pm S.E.M.) of purified target fraction from 20 mg ether extract with good retention of CTXs. The mouse bioassay was used to demonstrate that the average CTX recovery of the target fraction from CTX-spiked samples was $75.8 \pm 3.3\%$, which was significantly increased by $96.7 \pm 15\%$ when compared with CTX recovery from ether extracts ($44.8 \pm 5.2\%$) without performing SPE purification. Over 70% of non-target lipids were removed, in which no CTX toxicity was found. Moreover, the target fractions of both CTX-spiked and naturally CTX-contaminated samples gave more prominent toxic responses of hypothermia and/or induced more rapid death of the mice. The use of acetone-methanol (7:3, v:v) condition in the elution could significantly improve overall recovery of CTXs, while minimizing the possible interferences of lipid matrix from co-extractants on mice.

Keywords: *Ciguatera fish poisoning; Ciguatoxin recovery; Florisil; Mouse bioassay; Solid-phase extraction*

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Introduction

Ciguatera fish poisoning (CFP) is the commonest marine biotoxin illness in the world (Isbister and Kiernan 2005). The disease is due to consumption of toxic species of coral reef fish captured in the circumtropical regions. These fish bio-accumulate lipid-soluble sodium channel toxins, ciguatoxins (CTXs), in their flesh and viscera via marine food chain until reaching humans to cause food intoxication. Toxic forms of epiphytic benthic marine dinoflagellates inhabiting in the coral reef areas such as *Gambierdiscus toxicus* are believed to be the toxin-causative microalgae (Holmes et al. 1991; Lewis and Holmes 1993; Lewis 2001). CFP is characterized by gastrointestinal, neurological and cardiovascular disorders (Palafox and Buenconsejo-Lum 2001; Pearn 2001; Pottier et al. 2002; Keynan and Pottesman 2004). The mechanism of toxicity is elicited by the binding ability of CTXs to site 5 on the voltage-dependent sodium channel of excitable tissues (Lewis et al. 2000; Pearn 2001).

With the advance in global seafood business and international transportation, endemic disease of CFP has not been confined to certain tropical and subtropical areas (between 35° north and 35° south latitude) of the Pacific Ocean, western Indian Ocean and the Caribbean Sea (Lehane 1999; Goodman et al. 2003; Wong et al. 2005). The disease potentially poses large socio-economic and public health impacts in communities where fish is the major source of protein in the normal diet. CTXs are amongst the most potent toxins (LD₅₀ = 0.25 µg/kg, intraperitoneal injection in mice for Pacific-CTX-1) known in the world that cause significant health impact on humans even at the level

of 0.1 μg (Lewis et al. 1991; Lehané 1999; Lehané and Lewis 2000; Halstead 2002). The application of ordinary freezing or cooking does not destroy the toxins. In spite of the high toxicity and common morbidity of CTXs, much effort has been made to deal with toxicological and therapeutic mechanisms. Their research and development are however hindered by the lack of commercial CTX standards and surrogates, as well as the insufficient ciguateric fish for toxin extraction. There are also difficulties and complexities in laboratory synthesis of toxins by culturing CTX-producing dinoflagellates (Legrand et al. 1989; Holmes et al. 1991). The present knowledge on CTXs relied mainly on field sampling of CTX-contaminated fish for studying toxin extraction methods, mechanisms, and structures of toxin congeners. Considerable efforts has gone into development of different diagnostic methods for CTXs detection and analysis such as immunoassays and chemical analysis by LC/MS (Manger et al. 1995; Guzmán-Pérez and Park 2000; Lewis et al. 2000; Lewis 2003). The widely recommended method for CTXs testing in coral fish samples is still based on the mouse bioassay of fish ether extracts (Hoffman et al. 1983; Vernoux 1994; Ting and Brown 2001). Until establishment of a validated alternative analytical method for CTXs detection (Hungerford 2006), the mouse bioassay will still play an important role in ciguatera research and public health protection. Continuous development and improvement of the existing methodologies for this toxicity bioassay are needed for enhancing detection and accurate determination of CTXs in the fish tissues.

Florisil is a co-precipitate of silica and magnesia normal phase sorbent that has been commonly

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used to remove co-extracted interferences and contaminants in a variety of environmental and food samples (Lopez-Avila et al. 1989; Schenck et al. 1996). Its slightly basic surface may facilitate the adsorption of low to moderate polarity compounds from organic solutions. It has also been adopted to purify and isolate CTX-containing fraction from crude ether extract prior to analysis by analytical instruments such as HPLC/MS (Lucas et al. 1997; Vernoux and Lewis 1997; Hamilton et al. 2002). In the present study we have investigated the efficacy of applying a commercially available Florisil solid-phase extraction cartridge to purify and isolate CTX-containing fraction from crude fish lipid extract, with the aim of studying the potential use of adding a clean-up step in routine laboratory test for improving the purity of coral fish ether extracts.

Materials and methods

Coral fish samples preparation

Coral fish species used for this study included *Cheilinus undulates* (humphead wrasse), *Epinephelus areolatus* (areolated grouper), *Epinephelus lanceolatus* (giant grouper), *Epinephelus polyphekadion* (camouflage grouper), *Lutjanus stellatus* (star snapper), *Plectropomus areolatus* (squaretail coralgroup), *Plectropomus leopardus* (leopard coralgroup) and *Variola albimarginata* (lyretail) (Table 1). All coral reef fish samples were collected during different periods from markets in Hong Kong, except a piece of giant grouper flesh, which was provided by a donor who had suffered from ciguatera symptoms after consumption of this fish flesh. Two retrospective coral fish samples, X and Y (CTX-positive samples), were used as positive controls for this study. All samples were kept

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3 frozen at -80°C in Biotoxin Laboratory upon collection and were then put into 4°C refrigerator one
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6 day before extraction.
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8 9 *Sample extraction*

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11 The fish flesh (50/100 g) was cooked at 70°C water bath for 15 min in a water bag. Sample was
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13 cooled in refrigerator, or put in -80°C freezer for 5 min. The cooled fish sample was minced and
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15 extracted with acetone. A lipid-soluble extract was obtained by liquid-liquid partitioning procedures
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17 as previously described (Wong et al. 2005). The final dried lipid ether extract was stored in -20°C
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19 freezer prior to testing.
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27 28 *Mouse bioassay*

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30 In order to analyze the toxicity and potential presence of CTXs in the coral fish samples, all ether
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32 extracts were first screened for CTXs using a validated mouse bioassay as previously described
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34 (Wong et al. 2005). The mice were closely monitored (1-h interval) for 6 h after injection. Raised
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36 bottom grids with white background were inserted into the bottom of the cages for monitoring the
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38 condition of diarrhea easily. Video camera was set to record the experimental setup for at least 24 h
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40 to determine the mice survival time and toxic symptoms beyond 6 h. The mice were then monitored
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42 for another 4 consecutive days. Rectal temperature and body weight were also recorded at days 1
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44 and 4, respectively. All experimental mice used in the study were sacrificed in accordance with
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46 stipulated ethical standards and guidelines.
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57 58 *Spiked coral fish samples*

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60 Among 16 coral fish samples used in this study (Table 1), two samples (humphhead wrasse (Hw3)

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3 and giant grouper (Gg)) were detected to have trace amount of CTXs at <3.6 MU/100g flesh and
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6 ~4.7–9.4 MU/100g flesh, respectively. Others were determined to be CTX free (CTX-negative)
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9 after the examination. Five pieces of raw coral fish flesh (CTX-negative) including a humphead
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12 wrasse (Hw2), two squaretail coralgroupers (Sc2 and Sc2-a) and two leopard coralgroupers (Lc2
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15 and Lc3) were spiked with pure Pacific-ciguatoxin-1 (P-CTX-1) purchased from Prof. Richard J.
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18 Lewis (University of Queensland, Australia). The P-CTX-1 was dissolved in chloroform-methanol
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21 (1:1, v:v) and evenly spiked into the raw coral fish flesh (500 ng P-CTX-1/100g flesh) carefully.
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24 The spiked flesh samples were kept in room temperature for ~15 min before storage in –80 °C
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27 freezer. The samples were defrosted in 4 °C refrigerator a day prior to sample extraction.
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34 *Purification of fish lipid extract (ether extract) with Florisil*

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37 Fish ether extracts were purified using commercially available Florisil cartridges (Waters, USA).
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40 The elution procedures were based on the method previously described by Vernoux and Lewis
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43 (1997), and Hamilton et al. (2002), with slightly modifications. The CTX-containing fraction was
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46 purified by eluting the cartridge with hexane-acetone-methanol solvent mixtures of increasing
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49 polarity. Briefly, ether extract (20 or 40 mg) was dissolved in 0.8 ml of hexane-acetone (4:1, v:v)
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52 and loaded onto a 125 µm 60Å Florisil cartridge pack (Waters, 3 ml, 500 mg, 0.8 ml bed volume)
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55 which had been pre-washed with three bed volumes (V_b) of acetone-methanol (7:3, v:v) and then
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58 five V_b of hexane-acetone (4:1, v:v). Fraction hexane-acetone (fraction HA) was eluted into a
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sample vial with four additional V_b of hexane-acetone (4:1, v:v) solvent mixture which was applied

to remove hydrophobic contaminants in the lipid extract. The target acetone-methanol (fraction AM) fraction, was subsequently collected by applying eight V_b of acetone-methanol (7:3, v:v) solvent mixture onto the cartridge. Three additional methanol (M) fractions (M1, M2 and M3) were collected separately by eluting two V_b of 100% methanol for fraction M1 and five V_b of 100% methanol twice, respectively for fractions M2 and M3. Therefore, each fraction collected was equivalent to the corresponding quantity contained in 20 or 40 mg of crude ether extract. Residues of ether extract (fraction ERC) retained inside the cartridge were discarded. Extraction of fish ether extracts through Florisil cartridge was performed by using Vacuum Manifold Processing Station (Agilent) under atmospheric gravity. All eluted fractions (fractions HA, AM, M1, M2 and M3) were collected into V-Vials (Wheaton), dried under nitrogen gas, weighed by electronic balance and then stored in -20°C freezer until testing for CTX toxicity in each fraction with validated mouse bioassay. Each dried fraction was suspended in 0.5 ml of 1% Tween 60/0.9% saline, sonicated for 5–10 min to suspend the dried fraction, heated at 37°C and injected carefully into mice by i.p. route. In order to study extraction efficiency of the Florisil cartridge and potential toxicity of CTX in each fraction, different conditions of acetone-methanol solvent mixtures (9:1 and 7:3, v:v) and lipid extract quantities (20 and 40 mg) were tested for establishing optimal condition for CTX recovery and isolation.

P-CTX-1 for calibration and toxicity study

The calibration equation $\log(\text{MU}) = 2.3 \log(1+1/T)$ was used to establish an in-house CTX

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calibration curve (Lewis and Sellin 1993; Lewis 1995, 2003). MU and T represent the number of mouse unit and survival time (hours), respectively for each mouse. The quantity of CTXs in the sample was evaluated by determining dose (log MU) versus time-to-death (log $(1+1/T)$) relationship from local mice species (female ICR mice) using pure P-CTX-1. Dosages between 0.01 and 12.5 MU (62.5 pg–70 ng) of P-CTX-1 were applied to study their toxic effects on mice and to differentiate the range of lethal and sub-lethal doses. A lethal dose calibration was established by injecting diluted standard toxins (in the range of 10–70 ng/0.5 ml of 1% Tween 60/0.9% saline) into mice through i.p. route. LD₅₀ was determined by analyzing relationship between injected dose (d) and survival time (T) using the derived equation $\log d = a \log (1+1/T) + b$, where d represents dosage in ng/kg, T is survival time in hours, a and b are minimum survival time and extrapolated smallest dose that kills mice at infinite time, respectively (Molinengo 1979; Meier and Theakston 1986; Legrand et al. 1989). A linear relationship between $\log d$ and $\log (1+1/T)$ was produced with intercept on y-axis for evaluating the value of LD₅₀.

Results

220±10.7 mg (mean±S.E.M.) of fish ether extracts, ranged between 135 and 285 mg, were obtained from each 100 g of fish samples after extraction.

Extraction and isolation efficiency of Florisil solid-phase extraction cartridge

20 mg of ether extracts from CTX-negative samples

20 mg ether extracts were obtained from nine CTX-negative samples, including two humthead wrasses (Hw1 and Hw2), two camouflage groupers (Cg1 and Cg2), two areolated groupers (Ag1

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3 and Ag2), a squaretail coral grouper (Sc1), a leopard coral grouper (Lc1) and a lyretail (Lt). These
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6 were passed through Florisil cartridges for purification and fractionization under the
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9 acetone-methanol (7:3, v:v) solvent mixture condition. Different eluates (fractions HA, AM, M1,
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12 M2 and M3) were obtained after Florisil purification of 20 mg of CTX-negative extracts (n=18).
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15 Overall, $47 \pm 4.6\%$ (mean \pm S.E.M.) of 20 mg ether extract was eluted out while $53 \pm 4.6\%$ of residues
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18 (ERC) were retained inside the cartridge after purification. Among the eluates, it composed of
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21 $3.0 \pm 0.7\%$ HA, $15 \pm 2.9\%$ AM, $29 \pm 2.7\%$ M (M1, M2 and M3). The percentage of target fraction
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24 (fraction AM) isolated from the crude extracts ranged from 2.5 to 33% (0.6 to 7.8 mg). The average
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27 weight of target fraction AM isolated from the 20 mg ether extracts was 3.7 ± 0.5 mg under the
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30 acetone-methanol (7:3, v:v) solvent mixture condition. Over 80% of components from the 20 mg
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33 crude ether extracts were removed after Florisil purification. This represents the clean-up efficiency
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36 of the cartridge on the target fraction AM.
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39 40 *20 mg of ether extracts from P-CTX-1-spiked and CTX-positive samples*

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43 For P-CTX-1-spiked and CTX-positive samples, 20 mg ether extracts from six samples including
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46 three P-CTX-1-spiked samples (humphead wrasses: Hw1, squaretail coral grouper: Sc2 and leopard
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49 coral grouper: Lc2) and three CTX-positive samples (humphead wrasses: Hw3, giant grouper: Gg
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52 and sample X) were passed through Florisil cartridges for purification and fractionization under
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55 acetone-methanol (7:3, v:v) solvent mixture condition. Different eluates (fractions HA, AM, M1,
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58 M2 and M3) were obtained after Florisil purification of 20 mg of P-CTX-1-spiked (n=13) and
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CTX-positive (n=6) extracts. Overall, $48 \pm 7.8\%$ of 20 mg ether extract was eluted out while

52±7.8% of residues (ERC) were retained inside the cartridge after the purification. Among the eluates, it composed of 4.4±1.8% HA, 21±5.1% AM, 31±3.7% M (M1, M2 and M3). The percentage of target fraction (fraction AM) isolated from the crude extracts ranged from 7.1 to 42% (1.8 to 11 mg). The average weight of target fraction AM isolated from the 20 mg ether extract was 4.8±0.6 mg under the acetone-methanol (7:3, v:v) solvent mixture. By using the condition of acetone-methanol (9:1, v:v) solvent mixture instead of the (7:3, v:v) condition for performing Florisil purification on Sc2 extracts (n=4), the amount of target fraction AM (1.1±0.3%) collected was only 5.6% of those collected under the acetone-methanol (7:3, v:v) condition. The percentages of residues retained inside the column after purification were 43±3.7% and 75±3.1% under the elution of acetone-methanol (7:3, v:v) and (9:1, v:v), respectively. With regard to the clean-up efficiency of the cartridge on the target fraction AM, over 70% of components from the 20 mg crude ether extracts were removed after Florisil purification under the condition of acetone-methanol (7:3, v:v) solvent mixture.

40 mg of ether extracts from P-CTX-1-spiked and CTX-negative samples (Pilot study)

40 mg ether extracts from two P-CTX-1-spiked samples (squaretail coralgrouper: Sc2 and leopard coralgrouper: Lc3) and three CTX-negative samples (camouflage grouper: Cg2, areolated grouper: Ag2 and star snapper: Ss) were passed through Florisil cartridges for purification and fractionization under the acetone-methanol (7:3, v:v) solvent mixture condition. Different eluates (fractions HA, AM, M1, M2 and M3) were obtained after Florisil purification of 40 mg of P-CTX-1-spiked (n=6) and CTX-negative (n=3) extracts. The two eluants, acetone-methanol (9:1,

v:v) and (7:3, v:v) solvent mixtures, were used to elute target fraction AM from the 40 mg crude ether extracts. Under the condition of acetone-methanol (9:1, v:v) solvent mixture, $4.2 \pm 0.2\%$ of target fraction AM was collected from both ether extracts of spiked samples Sc2 and Lc3. By contrast, $28 \pm 6.8\%$ of such fraction could be obtained by using the condition of acetone-methanol (7:3, v:v) solvent mixture. According to the two acetone-methanol solvent mixture conditions applied, the latter one eluted more amount of fraction AM and the residues remained inside the cartridge ($39 \pm 3.2\%$) was also less than those of the acetone-methanol (9:1, v:v) elution ($59 \pm 8.2\%$). Overall, $55 \pm 6.7\%$ of 40 mg ether extract was eluted out while $45 \pm 6.7\%$ of residues (ERC) were retained inside the cartridge after the purification under the condition of acetone-methanol (7:3, v:v) solvent mixture. Among the eluates, it composed of $12 \pm 3.6\%$ HA, $15 \pm 5.9\%$ AM, $27 \pm 5.1\%$ M (M1, M2 and M3). The percentages of target fraction AM isolated from the 40 mg crude extracts ranged from 1.9 to 38% (0.9 to 20 mg). The average weight of target fraction AM isolated was 8.4 ± 3.1 mg. With regard to the clean-up efficiency of the cartridge for extracting the target fraction AM, over 70% of components from the 40 mg crude ether extracts were removed after Florisil purification under the elution of acetone-methanol (7:3, v:v) solvent mixture.

In addition to fractions HA and M3 collected from 40 mg ether extracts, there was no significant difference ($P > 0.05$; Student's *t*-test) in percentage of fractions AM, M1, M2 and ERC isolated between the application of 20 mg and 40 mg of ether extracts under the acetone-methanol (7:3, v:v) solvent mixture condition. Application of 40 mg ether extracts for Florisil purification yielded more

amount of fraction AM as compared with those yielded from 20 mg ether extracts. In general, the average weight of fractions AM collected after Florisil purification (under acetone-methanol (7:3, v:v) solvent mixture condition) of 20 mg ether extracts of CTX-negative, P-CTX-1-spiked and CTX-positive fish samples was 4.2 ± 0.4 mg ($17 \pm 1.6\%$ of 20 mg ether extracts). Percentage of other non-target components that were excluded after the purification was $83 \pm 1.6\%$. For the application of 40 mg ether extract under the same condition of solvent mixture, the average percentage of target fraction AM collected was $15 \pm 5.9\%$ and those non-target components excluded after the purification was $83 \pm 6.0\%$. As a result, no significant difference ($P > 0.05$; Student's *t*-test) in extraction efficiency of the cartridge could be found between the use of 20 mg and 40 mg ether extracts for performing such purification.

Mouse bioassay analysis

In this study, 5.6 ng of pure P-CTX-1 was determined as LD_{50} for 20 g mice according to the dose versus time to death relationship curve of standard P-CTX-1. Intraperitoneal injection of 20 mg ether extract of CTX-negative samples to each mouse did not elicit any typical toxic symptoms of CTXs. Rectal temperatures of the treated mice were at 37.3 ± 0.0 °C (mean \pm S.E.M.) ($n=33$) and their body weight showed average 0.0 ± 0.1 g (mean \pm S.E.M.) and 2.1 ± 0.2 g increase at days 1 and 4, respectively. Mice injected with Tween 60/0.9% saline showed rectal temperature of mice maintained at 37.3 ± 0.0 °C ($n=32$) and their body weight increased 0.2 ± 0.1 g and 2.2 ± 0.1 g at days 1 and 4, respectively, during the experimental period. Body weight gain at days 1 and 4 were

comparable to those with CTX-negative samples ($P > 0.05$; Student's t -test).

Recovery of spiked P-CTX-1 from coral fish flesh before and after Florisil extraction

Recovery of spiked P-CTX-1 from coral fish flesh before Florisil extraction

For mice injected with ether extracts containing spiked P-CTX-1 (500 ng/100g flesh), they all exhibited typical signs of CTX poisoning including hypothermia (rectal temperature $< 33^{\circ}\text{C}$ within 2 h), severe diarrhoea, significant reduced locomotor activity and breathing difficulty during the course of experiment. No recovery from hypothermia was noted after injection and all mice experienced terminal convulsions before death.

Recovery of spiked P-CTX-1 ranged from 32.6 to 69.4% (29.1 to 62.0 MU/100g flesh) for six spiked P-CTX-1 coral fish samples including a humphead wrasse (Hw2), three squaretail coralgroupers (Sc2 Pt1, Sc2 Pt2 and Sc2-a), and two leopard coralgroupers (Lc2 and Lc3) (Table 2).

Analysis of the results revealed that recovery of spiked P-CTX-1 for the six fish samples averaged $44.8 \pm 5.2\%$. Overall, the degree of P-CTX-1 recovery also varied between the tested samples. By comparing P-CTX-1 recovery between three samples of squaretail coralgroupers (Sc2 Pt1, Sc2 Pt2 and Sc2-a), no significant difference ($P > 0.05$; Student's t -test) could be found between the samples from the same species of fish and between the samples taken from the same piece of fish flesh. Significant difference in P-CTX-1 recovery ($P < 0.05$; Student's t -test), however, was found between the two leopard coralgrouper samples (Lc2 and Lc3) of same species. All mice injected

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with 20 mg ether extracts of the spiked P-CTX-1 samples (Hw2, Sc2 Pt1, Sc2 Pt2, Sc2-a, Lc2 and Lc3) experienced significant hypothermia within an hour without recovery and died within 6 h.

Recovery of spiked P-CTX-1 from coral fish flesh after Florisil extraction

For 20 mg ether extracts that passed through Florisil cartridge, P-CTX-1 was retrieved in fraction AM by applying the acetone-methanol (7:3, v:v) solvent condition. P-CTX-1 recovery for all tested samples was improved (overall recovery increased by more than 90%) as compared with those without Florisil purification (Table 2). The recovery percentage of spiked P-CTX-1 in samples Hw2, Sc2 Pt2, Sc2-a and Lc2 increased from 32.6 to 76.3%, 41.3 to 84.8%, 43.1 to 72.5% and 38.9 to 69.7%, respectively. The average recovery percentage of the tested samples after Florisil extraction was 75.8±3.3%. On the other hand, the average recovery percentage of P-CTX-1 for two squaretail coralgrouper (Sc2 Pt2 and Sc2-a) under the elution of acetone-methanol (9:1, v:v) solvent mixture maintained at 43.4%, which was comparable to that without performing purification through Florisil cartridge. In addition, there was no significant difference ($P > 0.05$; Student's t -test-Paired) in recovery of spiked P-CTX-1 between samples Sc2 Pt2 and Sc2-a of same species. Further application of 40 mg ether extract (twofold dosage) for Florisil purification showed that no significant improvement could be found even under the acetone-methanol (7:3, v:v) condition. Similar results were also found by application of 40 mg ether extract of sample Lc3 to pass through Florisil cartridge under both conditions (7:3 and 9:1, v:v) of acetone-methanol solvent mixture separately.

Figure 1 shows the rectal temperature patterns in mice injected with both crude ether (non-Florisil purified) extracts and fraction AM (Florisil purified) of the P-CTX-1-spiked samples (Sc2 Pt2, Sc2-a, Lc2 and Hw2). There was a similar pattern and trend of hypothermia in both sets of mice. In general, mice survival times showed characteristic difference between the toxicity of crude ether (non-Florisil purified) extracts and AM fractions (Florisil purified). Mice receiving AM fractions experienced a much more rapid death than those receiving non-Florisil purified ether extracts.

Other fractions of eluates including HA, M1, M2 and M3 were also collected by applying 20 or 40 mg ether extracts of CTX-negative, CTX-positive and P-CTX-1-spiked samples to pass through Florisil cartridges under acetone-methanol (7:3, v:v) solvent mixture condition. Mouse bioassay analysis showed no significant toxic effect in mice injected with those fractions of eluates. The average rectal temperature of mice was 37.5 ± 0.0 °C during the course of experiment while the body weight increased 0.0 ± 0.0 g and 2.2 ± 0.1 g, respectively at days 1 and 4, respectively.

Toxicity of CTXs from CTX-positive samples before and after Florisil purification

Toxicity of CTXs from CTX-positive samples before Florisil purification

Mouse bioassay results from two CTX-positive and two retrospective field samples (CTX-positive) are summarized in Table 3. For mice injected with 20 mg ether extracts of samples Hw3 (Humphead wrasse) and Gg (Giant grouper), sub-lethal doses of CTXs, <3.6 MU/100g flesh and 4.7–9.4 MU/100g flesh respectively, were determined by using validated mouse bioassay. Signs of

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CTX poisoning including hypothermia, mild diarrhoea, reduced locomotor activity were observed in all mice after i.p. injection of the ether extracts.

Hypothermia was detected within an hour in mice (n=4) injected with 20 mg ether extracts of sample Hw3 (Figure 2), but there was subsequent recovery within 5 hours at day 0. Rectal temperatures of treated mice during the 6 hours ranged between 32.1–37.3 °C (averaged at 35.1±0.3 °C). Rectal temperature of mice returned to normal at both days 1 (37.5±0.4 °C) and 4 (37.4±0.2 °C). It should be noted, however, that significant body weight changes (2.2±0.3 g decrease) and growth depression (0.0±0.9 g increase) were observed at days 1 and 4, respectively. Sublethal dose of CTXs present in sample Hw3 was then estimated to be less than 3.6 MU/100g flesh.

Intraperitoneal injection of 20 mg ether extracts of Gg into mice (n=7), during 6-hour observation period at day 0, caused a decrease in rectal temperature (Figure 2), which ranged between 30.7 and 35.6 °C (averaged at 33.4±0.2 °C). Hypothermia (rectal temperature below 33 °C) of treated mice was more conspicuous after 3 h observation. Lower rectal temperature (34.2±0.9 °C) in mice was also detected at day 1, which returned to normal at day 4 (37.1±0.1 °C). It should be noted that significant body weight change (2.5±0.4 g decrease) and growth depression (0.5±0.7 g increase) were also observed at days 1 and 4, respectively. Sublethal dose of CTXs in sample Gg was estimated to be 4.7–9.4 MU/100g flesh.

Toxicity of CTXs from CTX-positive samples after Florisil purification

Five fractions of eluates including HA, AM, M1, M2 and M3 were collected by applying 20 mg ether extracts of Hw3 and Gg to pass through Florisil under the condition of acetone-methanol (7:3, v:v) solvent mixture. Injection of Florisil purified fraction AM (target analyte) of sample Hw3 into mice (n=2) induced significant decrease in rectal temperature during 6-hour observation period at day 0 (Figure 2). The rectal temperatures ranged between 27.1 and 35.7 °C, averaged at 31.5 ± 0.6 °C. Hypothermia in mice during the course of experiment was more prominent than those obtained after injection with 20 mg ether extract of Hw3 (non-Florisil purified). In addition, hypothermia persisted (32.1 ± 3.5 °C) together with significant body weight decrease (1.5 ± 0.7 g decrease) at day 1. One of the mice died at day 4 while the one that survived still experienced persistent lower rectal temperature (35.7 °C) and a significant (3.0 g) decrease in body weight. On the other hand, injection of fraction AM of sample Gg into mice (n=2) induced significant hypothermia within an hour (Figure 2). The rectal temperature of the mice during 6-hour period ranged between 24.2 and 32.8 °C (averaged at 29.7 ± 0.6 °C). Hypothermia in mice during the course of experiment was also more prominent than those injected with 20 mg ether extract of Gg (non-Florisil purified). The treated mice died at 6.6 ± 0.2 h with terminal convulsion. The amount of CTXs was estimated to be at sublethal dose ranging from 6.5 to 12.9 MU/100 g flesh for Hw3 and lethal dose of 25.4 MU/100g flesh for Gg, respectively.

For mice (n=2 for each set) injected with each fraction of other eluates (HA, M1, M2 and M3) from

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samples Hw3 and Gg, there was no conspicuous sign of toxic effect of CTX poisoning after injection of each fraction from both samples. Mice rectal temperature during the whole course of experiment, ranged between 36.7 and 38.3 °C (averaged at 37.7±0.0 °C) for sample Hw3, and between 36.6 and 38.4 °C (averaged at 37.5±0.0 °C) for sample Gg. No prominent body weight depression was detected in mice at days 1 (0.0 ±0.1 g increase and 0.3. ±0.2 g decrease for Hw3 and Gg, respectively) and 4 (2.0 ±0.2 g increase and 2.1 ±0.2 g increase for Hw3 and Gg, respectively) for both samples.

For the two retrospective samples *X* and *Y* (positive control samples), there was significant enhancement of CTXs toxicity (22.2 to 33.6 MU/100g flesh) ($P < 0.05$; Student's *t*-test-Paired) on mice after injecting Florisil-purified fraction AM of sample *X* (Table 3). No prominent toxicity change, however, was found in mice injected with Florisil-purified fraction AM of sample *Y*. Nevertheless, the agreement in rectal temperature patterns between mice injected with fraction AM and crude ether extract of sample *Y* showed that their toxic responses were comparable to each other (data not shown). These results confirmed that CTXs could mostly be isolated in fraction AM from 20 mg ether extract of both samples *X* and *Y* by applying a Florisil purification step under the condition of acetone-methanol (7:3, v:v) solvent mixture. On the other hand, no conspicuous sign of toxic effect or CTX poisoning symptom was found in mice injected with each fraction of the other eluates (HA, M1, M2 and M3) from both retrospective samples.

Discussion

Since reliable rapid kits are lacking (Pearn 2001) for only ciguatoxins (CTXs) detection, nor are there validated extraction and clean-up procedures for chemical analysis, information on the mouse bioassay is still central to the toxicological study and detection of CTXs in coral fish. In order to prevent micro-epidemic outbreak of ciguatera, surveillance and detection of CTXs in coral fish samples is one of the better means to prevent people from suffering ciguatera intoxication (Johnson and Schantz 2006). Most of ciguateric fish that had been implicated in clinical ciguatera or sporadic outbreak cases, have very often been eaten or not easily available for further laboratory analysis. Therefore, fish samples collected from these cases are vital for checking the presence and toxicity of CTXs using traditional mouse bioassay. However, results of the bioassay often depended on toxicity responses of mice injected with crude ether extracts, which are partially purified from standard extraction of coral fish flesh. Both quantity and quality of lipid residues or components in the ether extracts may vary between different fish species and sizes. The previously described standard extraction method has a CTX recovery at ~63%, ranging between 49 and 71%, from studies using CTX-spiked samples (Lewis and Sellin 1993; Lewis 1995). This method has also been documented to be useful in extracting CTXs from ciguateric fishes (Lewis 1995, 2003). Lewis and Sellin (1993) revealed that there was no significant interference of lipids on mice if standard toxins were added to 20 mg ether extracts of unspiked coral fish. The present study showed that such matrix interference might exist by spiking the standard Pacific-CTX-1 (P-CTX-1) into raw flesh of certain coral fish. From the results of recovery test on P-CTX-1-spiked samples, the average

recovery was ~76% after injecting the Florisil-purified target fractions (fraction AM) of 20 mg ether extracts. The P-CTX-1 recovery was significantly enhanced by 97% as compared with that (~45%) from only ether extracts without performing such purification. It is of interest to note that mice injected with the target fractions induced a more rapid death than those injected with non-Florisil purified ether extracts. It is most likely that the Florisil clean-up step simply removed crucial lipid component(s) or residue(s) in the ether extracts which hampered the toxic action of P-CTX-1 on the target site(s) (e.g. sodium channels of excitable membranes) of mice. The degree of such interference effects by the lipid matrix might vary widely depending on fish species as well as the extraction procedures. In the CTX-positive field samples Hw3 (Humphead wrasse) and Gg (Giant grouper), prominent difference in pattern and trend of hypothermia was noted between mice injected with Florisil-purified fraction AM extracts and non Florisil-purified ether extracts. All mice exhibited a more serious hypothermia shortly after intraperitoneal injection of the Florisil-purified extracts. Based on the degree of toxic responses of mice in hypothermia during the course of experiment, it can be seen that the presence of unnecessary lipid matrix has a substantial masking effect on the toxic response of mice to CTXs. Although it is still speculative to confirm the possible existence of any chemical interaction or biochemical mechanism between CTXs and other lipid component(s) of the field coral fishes, the results from this study showed that these unnecessary lipid component(s) might suppress or retard the toxicological actions of CTXs on mice. Eliminating such component(s) in the extracts might facilitate the mechanistic action of the toxins on the target sites, and thus enhance the toxic responses of mice to the toxins. From the results of CTX-positive

control sample Y, there was no significant enhancement of toxic responses of mice to CTXs even injecting the Florisil-purified target fraction isolated from its ether extract. It is suggested that the bioassay may not sufficiently sensitive to differentiate such low levels of CTXs by observing the patterns of hypothermia in mice alone.

The extraction efficiency of Florisil cartridges varies with the specified gradient of solvent mixtures. Increasing polarity (hexane-acetone (4:1, v:v) < acetone-methanol (7:3, v:v) < 100% methanol) optimized the overall recovery of CTXs in the target fraction AM. The application of 20 mg fish ether extract facilitated the separation of ~17% (~4.2 mg) of target fraction AM with good retention of standard toxin. Although lipid extraction efficiency of Florisil cartridges (such as percentages of the target fraction AM collected and residues remained inside the cartridge) on both 20 mg and 40 mg ether extracts were comparable to each other, the recovery efficiency for CTXs showed a better result by using 20 mg ether extract for performing this Florisil extraction. In addition, there was significant difference in toxin recovery by using different conditions of acetone-methanol solvent mixture (9:1, v:v) and (7:3, v:v). The latter condition optimized the overall recovery of CTXs from the ether extracts. The recovery of standard toxin from spiked samples under the condition of acetone-methanol (9:1, v:v), the eluting solvent condition previously used with Florisil by Lucas et al. (1997) and Hamilton et al. (2002), was less efficient than that under the condition of acetone-methanol (7:3, v:v) because most of elements and CTXs in the ether extracts were still trapped inside the cartridge. Preliminary examination of the Florisil cartridges by

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applying 40 mg (twofold dose) ether extracts for such purification, however, showed that no significant improvement on CTX recovery could be demonstrated in mouse bioassay by injecting with the target fractions. This may be due to overloading of the cartridge by the 40 mg ether extract, or some CTXs were still trapped or retained on the adsorbent surfaces or lipid matrix without eluting out together with the target fractions. Poor elution efficiency and toxin recovery found by using acetone-methanol (9:1, v:v) condition could also be explained by the fact that most of CTXs were still retained on Florisil adsorbent surface before complete elution of the target analytes from the cartridge.

In order to eliminate the possibility of interferences, the maximum amount of extract used for CTX test in mouse bioassay is set at 20 mg for a 20 g mouse by intraperitoneal route (Lewis and Sellin 1993). Extraction through Florisil cartridge using the condition of acetone-methanol (7:3, v:v) solvent mixture facilitated the separation of 4.2 ± 0.4 mg target fraction AM from 20 mg ether extracts (CTX-positive, CTX-negative and CTX-spiked samples). The target fraction collected was acceptably below the maximum level of extract required for the bioassay. Therefore, if necessary, an amount of pooled target fractions below the maximum level requirement may be administered into mice for eliciting more conspicuous toxic responses of CTXs, especially for borderline cases.

In summary, a Florisil solid-phase extraction step using acetone-methanol (7:3, v:v) condition demonstrated an efficient way to purify and isolate ciguatoxins containing fraction from ciguateric

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3 coral fish ether extract. Though small sample sizes were used in this study, the results showed that
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6 the modified condition in the elution would optimize overall recovery of CTXs while minimizing
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9 the possible interferences of lipid matrix from co-extractants on mice. Further work would be
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12 necessary to evaluate the application of this extraction step in suspect cases and borderline case
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15 samples, and to examine its use in routine tests for various species of fish samples.
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Declaration

All commercial products and trade names mentioned in this article do not represent any suggestion or endorsement for use by our department.

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Table 1
Summary of 16 coral fish and 2 retrospective coral fish samples used in this study

Sample	Species names	Common names
Hw1	<i>Cheilinus undulatus</i>	Humphead wrasse ^a
Hw2	<i>Cheilinus undulatus</i>	Humphead wrasse ^{a, *}
Hw3	<i>Cheilinus undulatus</i>	Humphead wrasse ^b
Ag1	<i>Epinephelus areolatus</i>	Areolated grouper ^a
Ag2	<i>Epinephelus areolatus</i>	Areolated grouper ^a
Gg	<i>Epinephelus lanceolatus</i>	Giant grouper ^{b, #}
Cg1	<i>Epinephelus polyphekadion</i>	Camouflage grouper ^a
Cg2	<i>Epinephelus polyphekadion</i>	Camouflage grouper ^a
Ss	<i>Lutjanus stellatus</i>	Star snapper ^a
Sc1	<i>Plectropomus areolatus</i>	Squairetail coralgrouper ^a
Sc2	<i>Plectropomus areolatus</i>	Squairetail coralgrouper ^{a, *}
Sc2-a	<i>Plectropomus areolatus</i>	Squairetail coralgrouper ^{a, *}
Lc1	<i>Plectropomus leopardus</i>	Leopard coralgrouper ^a
Lc2	<i>Plectropomus leopardus</i>	Leopard coralgrouper ^{a, *}
Lc3	<i>Plectropomus leopardus</i>	Leopard coralgrouper ^{a, *}
Lt	<i>Variola albimarginata</i>	Lyretail ^a
X	Retrospective sample ^b	
Y	Retrospective sample ^b	

* 500 ng of Pacific-ciguatoxin-1 (P-CTX-1) was evenly spiked into the flesh
Sample was implicated in ciguatera fish poisoning and was donated by patient from overseas
^a CTX-negative sample, ^b CTX-positive sample after checking with validated mouse bioassay

Table 2 Recovery of spiked Pacific-ciguatoxin-1 (P-CTX-1, 500 ng) from the flesh of different coral fish samples

Sample	Quantity of ether extract obtained (mg)	Amount of ether extract injected (mg)	No. of mice used for bioassay	Acetone-methanol condition applied (v:v)	Survival time (h) (Mean±SEM)	Calculated amount of P-CTX-1 in 100 g flesh (ng)	Mouse unit of P-CTX-1 in 100 g flesh (MU)	Recovery (%)
Hw2	210.7	20	2	-	4.8±0.1	163	29.1	32.6
		20	2	(7:3)	3.6±0.0	381	68.1	76.3
Sc2 Pt1	182.9	20	3	-	3.3±0.1	219	39.1	43.8
Sc2 Pt2	241.2	20	4	-	4.3±0.2	206	36.8	41.3
Sc2-a	284.9	20	2	-	4.9±0.1	216	38.5	43.1
Sc2 Pt2	241.2	20	2	(7:3)	2.5±0.2	424	75.7	84.8
Sc2-a	284.9	20	2	(7:3)	3.1±0.2	307	54.8	72.5
Sc2 Pt2	241.2	20	2	(9:1)	4.0±0.1	224	39.9	44.5
Sc2 Pt2	241.2	20	2	(9:1)	3.9±0.3	230	41.1	46.0
Sc2-a	284.9	20	2	(9:1)	5.4±0.7	198	35.4	39.6
Lc2	230.9	20	2	-	4.4±0.4	194	34.7	38.9
		20	2	(7:3)	2.8±0.1	348	62.2	69.7
Lc3	252.6	20	5	-	2.9±0.1	347	62.0	69.4
		40	1	(9:1)	2.0	331	59.2	66.3
		40	1	(7:3)	1.9	352	62.9	70.5
Sc2 Pt2	241.2	40	1	(7:3)	5.3	170	30.4	34.0
Sc2-a	284.9	40	1	(7:3)	5.2	204	36.4	40.7

Key: Pt1 and Pt2 represent two different parts of flesh cut from the same coral fish Sc2

Table 3 Summary of mouse bioassay results from CTX-positive field coral fish samples.

Sample	Quantity of ether extract obtained Mean (mg/100g flesh) ±SEM	Amount of ether extract used (mg)	No. of mice used for bioassay	Acetone-methanol condition applied (v:v)	Survival time (h) (Mean±SEM)	Calculated amount of CTXs in 100 g flesh (ng)	Mouse unit of CTXs in 100 g flesh (MU)
Hw3	145.0±10.3	20	2	-	-	<20.3	<3.6
		20	2	(7:3)	>24 h [#]	~36.2–72.5	~6.5–12.9
Gg	210.0±16.3	20	7	-	-	~26.3–52.5	~4.7–9.4
		20	2	(7:3)	6.6±0.2	142	25.4
X ^{**}	-	20	2	-	4.3±0.4	124	22.2
		20	2	(7:3)	3.1±0.1	188	33.6
Y ^{**}	-	20	6	-	-	~12.8–32.1	~2.3–5.7
		20	2	(7:3)	-	~12.8–32.1	~2.3–5.7

Keys: [#] One of the mice was dead after 24 hours. (The rectal temperature of the mouse was 28.6 °C at 24-hour while another one was 35.7 °C, the body weight depression (1.5±0.7 decrease) was also observed at 24-hour)

^{**} Samples X and Y , retrospective ciguateric fish samples collected in Hong Kong, were used as positive control in this study.

Figure legends:

Figure 1 Changes of rectal temperature in mice after i.p. injection of (A) 20 mg ether extracts of P-CTX-1 spiked samples Sc2 Pt2 and Sc2-a, and their target fractions AM; (B) 20 mg ether extract of samples Hw2 and Lc2, and their target fractions AM. Data are represented by mean \pm SEM.

Figure 2 Changes of rectal temperature in mice after i.p. injection of (A) 20 mg ether extracts of sample Hw3, and their target fractions AM; (B) 20 mg ether extracts of sample Gg, and their target fractions AM. Data are represented by mean \pm SEM.

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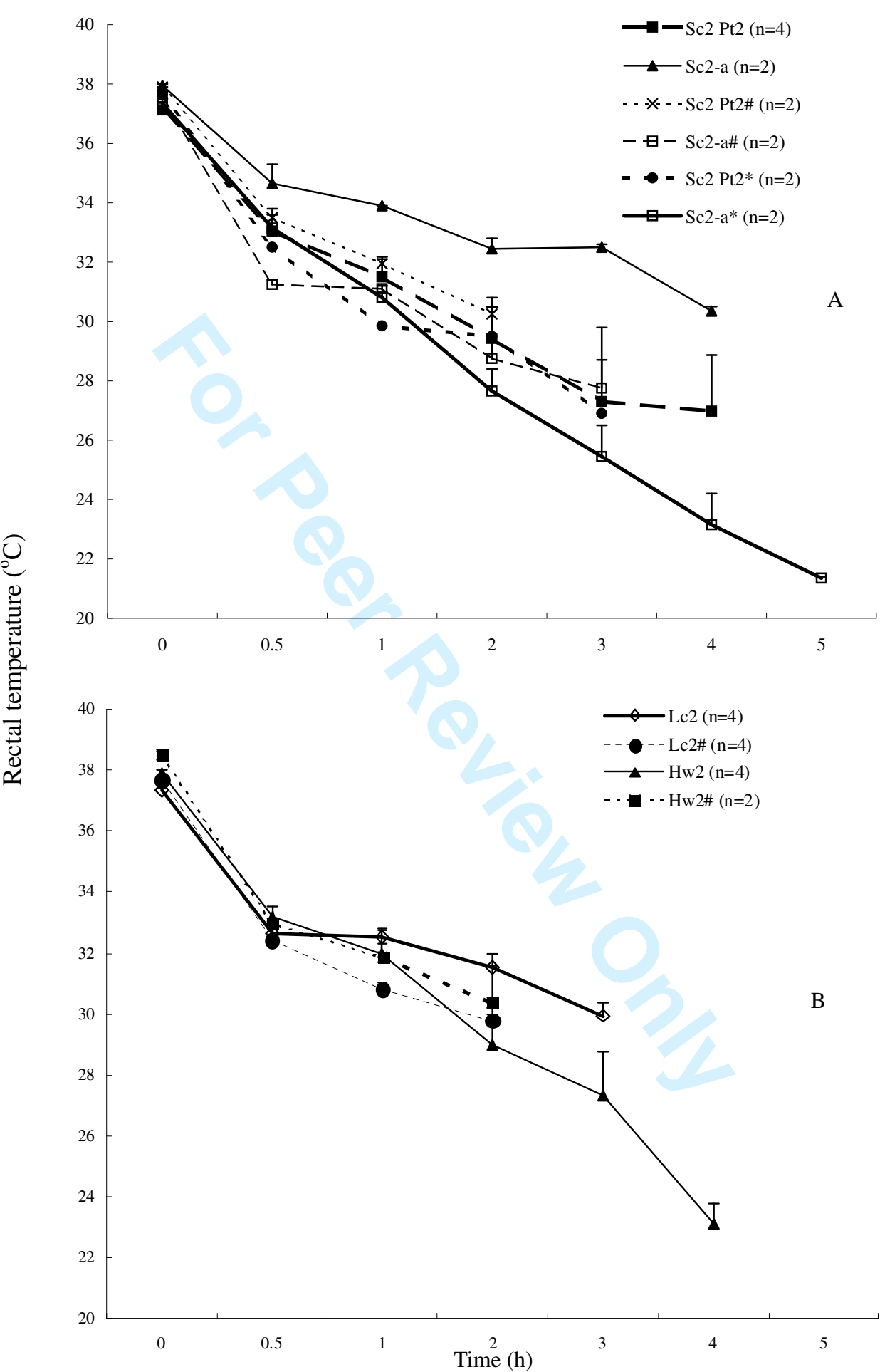


Figure 1

Keys:

- # Fraction AM of the ether extract was collected by extraction through Florisil cartridge under the condition of acetone-methanol (7:3, v:v) solvent mixture.
- * Fraction AM of the ether extract was collected by extraction through Florisil cartridge under the condition of acetone-methanol (9:1, v:v) solvent mixture.

Figure 1. Change of rectal temperature in mice after i.p. injection of (A) 20 mg ether extracts of P-CTX-1 spiked samples Sc2 Pt2 and Sc2-a, and their target fractions AM; (B) 20 mg ether extract of samples Hw2 and Lc2, and their target fractions AM. Data are represented by mean \pm SEM.

Figure 1

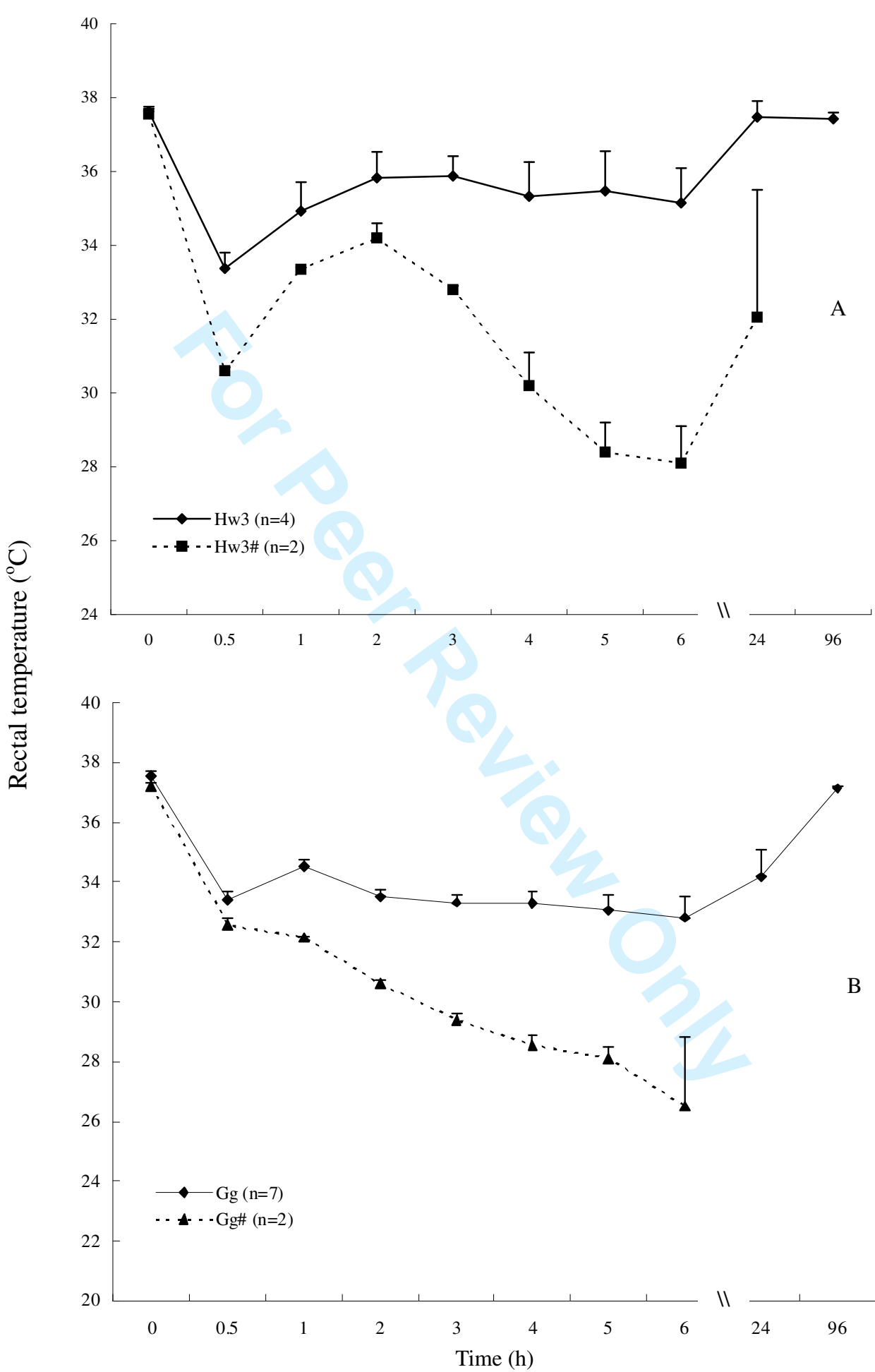


Figure 2

Keys:

Fraction AM of the ether extract was collected by extraction through Florisil cartridge under the condition of acetone-methanol (7:3, v:v) solvent mixture.

Figure 2. Change of rectal temperature in mice after i.p. injection of (A) 20 mg ether extracts of sample Hw3, and their target fractions AM; (B) 20 mg ether extracts of sample Gg, and their target fractions AM. Data are represented by mean \pm SEM.

Figure 2