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Chemical fingerprinting and phylogenetic mapping of saponin congeners from three tropical holothurian sea cucumbers

Karen Grace V. Bondoc [a,*,1], Hyeyoung Lee [b,1], Lourdes J. Cruz [a], Carlito B. Lebrilla [c], Marie Antonette Juinio-Meñez [a]

[a] The Marine Science Institute, University of the Philippines, Diliman, Quezon City, Philippines
[b] Department of Food Science and Technology, University of California, Davis, United States
[c] Department of Chemistry, University of California, Davis, United States

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ABSTRACT

Holothurians are sedentary marine organisms known to produce saponins (triterpene glycosides), secondary metabolites exhibiting a wide range of biological activities. In this paper, we investigated the saponin contents of semi-purified and membranolytic HPLC fractionated extracts from the body wall of three species of Holothuriidae as an attempt to examine its chemical diversity in relation to phylogenetic data. MALDI–FTICR MS and nano-HPLC-chip Q-TOF MS were used for mass profiling and isomer separation, respectively giving a unique chemical saponin fingerprint. Moreover, the methods used yield the highest number of congeners. However, saponin concentration, bioactivity and chemical diversity had no apparent relationship. MS fingerprint showed the presence of holothurinosides, which was observed for the first time in other *Holothuria* genera besides the basally positioned *Holothuria forskali*. This congener is proposed to be a primitive character that could be used for taxonomic purposes. The phylogenetic mapping also showed that the glycone part of the compound evolved from non-sulfated hexaosides to sulfated tetraosides, which have higher membranolytic activity and hydrophilicity, the two factors affecting the total ecological activity (i.e. chemical defense) of these compounds. This might be an adaptation to increase the fitness of the organism.

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1. Introduction

Sea cucumbers (Class Holothuroidea, Phylum Echinodermata) are slow-moving marine animals preyed upon by fishes, sea stars, gastropods, and crustaceans (Francour, 1997). To counter them, holothurians possess biologically active metabolites called saponins in their body wall, viscera, Cuvierian tubules (Bakus, 1968; Bakus, 1974), and gonads (Matsuno and Ishida, 1969). Saponins possess a triterpene lanosterol aglycone, which has an 18(20)-lactone called holostane and carbohydrate chain containing up to six sugar units of xylose (Xyl), glucose (Glc), quinovose (Qui), and 3-O-methylglucose (MeGlc), and in some cases sulfate groups (Kalinin et al., 1996; Stonik et al., 1999; Kalinin et al., 2005; Kalinin et al., 2008). These compounds have membranolytic action on cellular membranes with (5)-6 unsaturated sterols. They interact to the membrane by forming complexes to modify their structural organization and properties leading to the formation of cellular pores, eventually causing lysis (Kalinin et al., 1996; Popov, 2002, Stonik et al., 1999).

This is the main reason for the wide array of biological activities of saponins including ichthyotoxicity. Sea cucumber extracts can damage the capillaries of fish leading to death (Bakus, 1968; Nigrelli, 1952), thus increasing their overall fitness (Kalinin, 2000). The molecular structure of these compounds was preserved as it also provides internal (breeding regulation) and external (defense against predators, fouling organisms, or space competitors) advantages (Kalinin, 2000). These molecules are widely distributed and are very diverse among holothurians. The diversity is due to the variation on the aglycone and glycone moieties. These include the position of double bonds and the presence of different functional groups (i.e. $-OH$, $-COOH$, $-CH_3$) and lateral groups (i.e. acetoxy, keto groups) in the aglycone and the number of sugar chains and the number and position of sulfate groups in the glycone. Even if the diversity is great, saponins from closely related species still retain the same molecular motif (Kalinin et al., 1996; Stonik et al., 1999). In Holothuriidae alone, 59 types of saponins in 41 species were discovered and chemically described with each species having a specific congener mixture (Caulier et al., 2011). Some congeners are shared within this family (e.g., holothurins A and B). On the one hand, some congeners are very specific to each species, genera or even supergenera which imply that these compounds can be potential chemical taxonomic markers (Moraes et al., 2004; Kalinin et al., 2008; Caulier et al., 2011).

In this study, we determined and compared the mass spectrometric profiles of the body wall of three tropical holothurians species (*Holothuria scabra*, *Holothuria impatiens*, and *Holothuria fuscocinerea*) through matrix-assisted laser desorption/ionization (MALDI)–Fourier

* Corresponding author at: Institute of Inorganic and Analytical Chemistry, Friedrich Schiller University Jena, Germany.
E-mail address: karen.bondoc@uni-jena.de (K.G.V. Bondoc).
[1] Authors who equally contributed to this paper.

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Table 1
Species from Holothuriidae that were included in the phylogenetic analysis.

Species	GenBank Accession Number	
	16s	cox1
Actinopyga agassizi	JN207496	JN207565
Actinopyga echinites	EU822454	EU848216
Bohadschia argus	AY574870	AY574878
Bohadschia bivittata	AY574873	AY574880
Bohadschia marmorata	AY574877	AY574883
Holothuria arguinensis	GQ214735	GQ214755
Holothuria atra	EU220799	EU220820
Holothuria austrinabassa	EU220797	EU220818
Holothuria cinerascens	JN207554	JN207584
Holothuria dakarensis	EU191979	GQ214752
Holothuria edulis	EU220811	EU220830
Holothuria excellens	EU220796	EU220817
Holothuria floridana	EU220803	EU220822
Holothuria forskali (1)	GQ214740	GQ214761
Holothuria forskali (2)	EU220798	EU220819
Holothuria fuscocinerea	JN207560	JN207618
Holothuria hilla	JN207515	JN207616
Holothuria impatiens (1)	GQ214739	GQ214760
Holothuria impatiens (2)	JN207526	JN207632
Holothuria lentiginosa lentigenosa	GQ214733	GQ214753
Holothuria leucospilota	JN207541	JN207617
Holothuria lubrica	JN207497	JN207566
Holothuria mammata	EU191949	GQ214743
Holothuria mexicana	EU220802	EU220821
Holothuria nigralutea	EU220805	EU220824
Holothuria nobilis	EU822441	EU848246
Holothuria polii	EU191981	GQ214759
Holothuria portovallartensis	JN207558	JN207574
Holothuria sanctori	GQ214741	GQ214763
Holothuria scabra	EU822456	FJ971395
Holothuria signata	EU220812	EU220831
Holothuria tubulosa	FJ231192	GQ214748
Holothuria whitmaei	AY509147	EU848245
Pearsonothuria graeffei	EU822440	EU848285
Stichopus chloronotus	EU856692	EU856620
Stichopus herrmanni	EU822451	EU848281
Stichopus horrens	EU822434	EU848282
Stichopus ocellatus	EU220793	EU220814

transform ion cyclotron resonance (FTICR) mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analysis. In addition, nano-high performance liquid chromatography (HPLC)-chip quadropole-time of flight (Q-TOF) MS was used to completely analyze the diversity of isomers. Quantitative assays were also performed to compare the natural volumetric concentrations (NVCs) and its membranolytic activity amongst each other. Insights on the evolution and adaptive role of these compounds in the Holothuriidae were also derived by mapping the saponin types on a constructed phylogenetic tree.

2. Materials and methods

2.1. Sample collection

Three species from Holothuriidae (*H. scabra* Jaeger 1833, *H. fuscocinerea* Jaeger 1833, and *H. impatiens* Forskål 1775) were collected from Bolinao, Pangasinan, Philippines. The organisms were placed together in re-circulating water tanks with sediment. Prior to body wall collection, the sea cucumbers were killed by freezing.

2.2. Extraction and purification of saponins (triterpene glycosides) from the body wall of three holothurians

For each species, five to seven samples were dried using paper towels and these were pooled together to negate any interspecies difference in saponin content. Afterwards, pooled samples for each species were divided into six technical replicates and the volumes of each were determined by water displacement. Considering that marine organisms are highly hydrated, concentrations of chemical compounds produced by marine organisms in natural systems are well reflected by volumetric measurements. Thus, the differences in tissue volume can be controlled (Harvell et al., 1988; O'Neal and Pawlik, 2002).

The extraction methods were modified from those by Van Dyck et al. (2009). The body wall was homogenized and extracted twice with 70% ethanol. The extract was filtered, evaporated under reduced pressure, and lyophilized to obtain a dry extract. The dried extract was redissolved in 90% methanol and partitioned with n-hexane (v/v). The water content of the hydromethanolic portion was adjusted to 20% and partitioned against dicholoromethane (v/v). The same phase was adjusted to 40% water content and partitioned against chloroform (v/v). The hydromethanolic phase was then dried and desalted via methanol precipitation. The methanolic portion was evaporated, diluted with water, and partitioned against iso-butanol (v/v). The butanolic portion contained the semi-pure saponins.

The butanolic portions of each species were further purified using reversed-phase HPLC using an HPLC Prominence system equipped with an ultraviolet–visible spectrophotometric detector (Shimadzu, Japan) and a C-18 column (4.6 mm × 150 mm, 5 μm, Waters, Milford, MA, USA). The mobile phase was a nonlinear gradient of 10% methanol (eluent A) and 100% methanol (eluent B). The gradient program was as follows: 0% eluent A at start, 10% to 50% eluent A from 5 min to 20 min, 50% to 85% eluent A from 20 min to 30 min, and back to 10% eluent A from 30 min to 40 min. Fractions were collected every 5 min. The collected fractions were pooled and used for subsequent analysis.

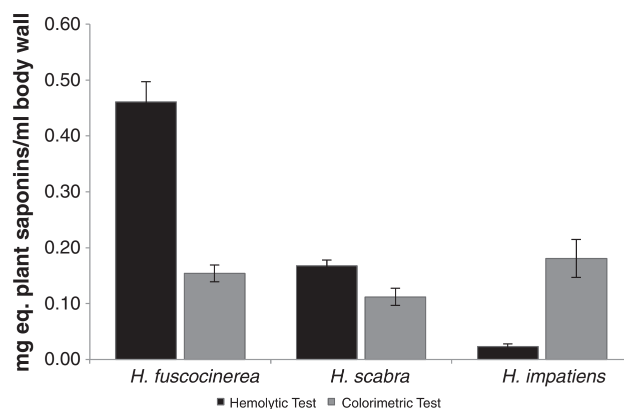


Fig. 1. Quantification of NVC of saponins in the body wall of three holothurian species (n = 6 technical replicates). Values are presented as means ± s.d. and expressed as milligram equivalents of plant saponins per milliliter of body wall.

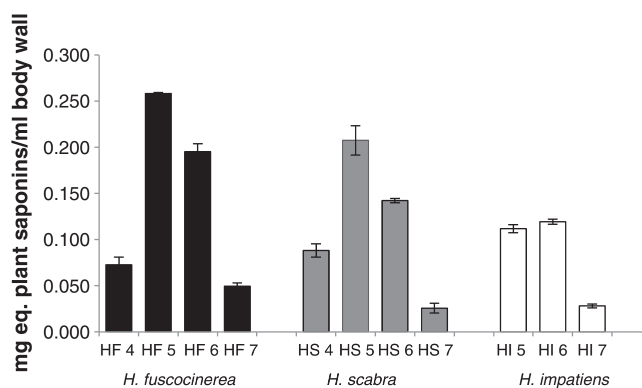
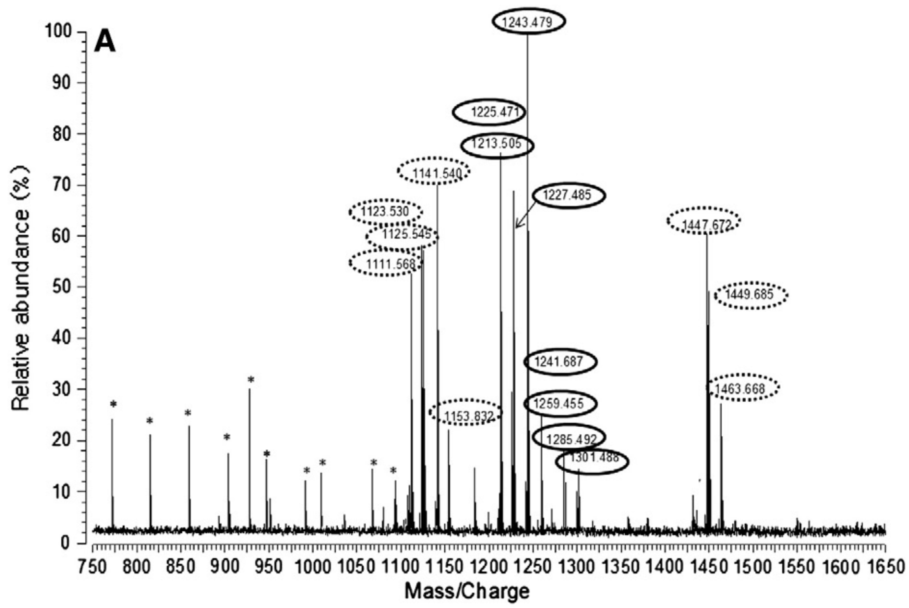
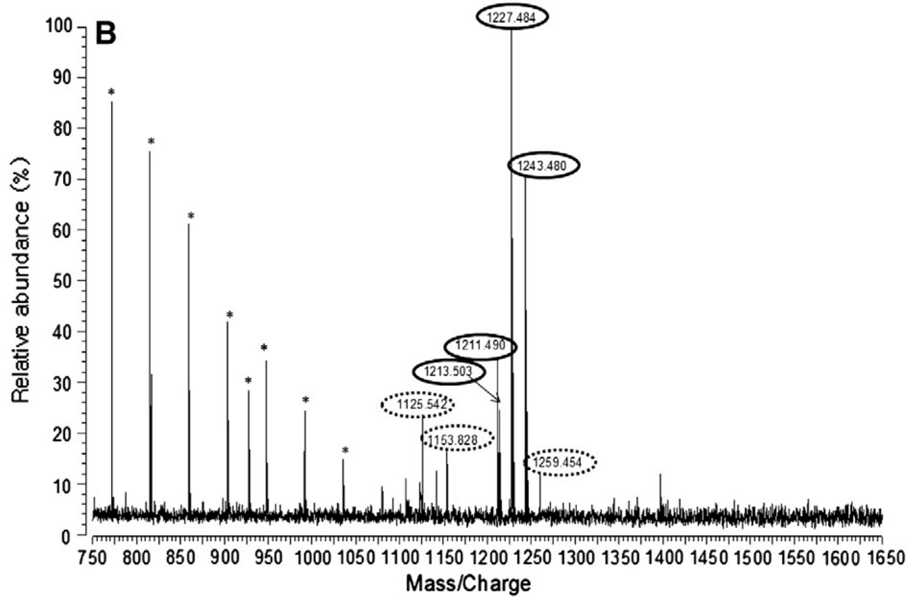
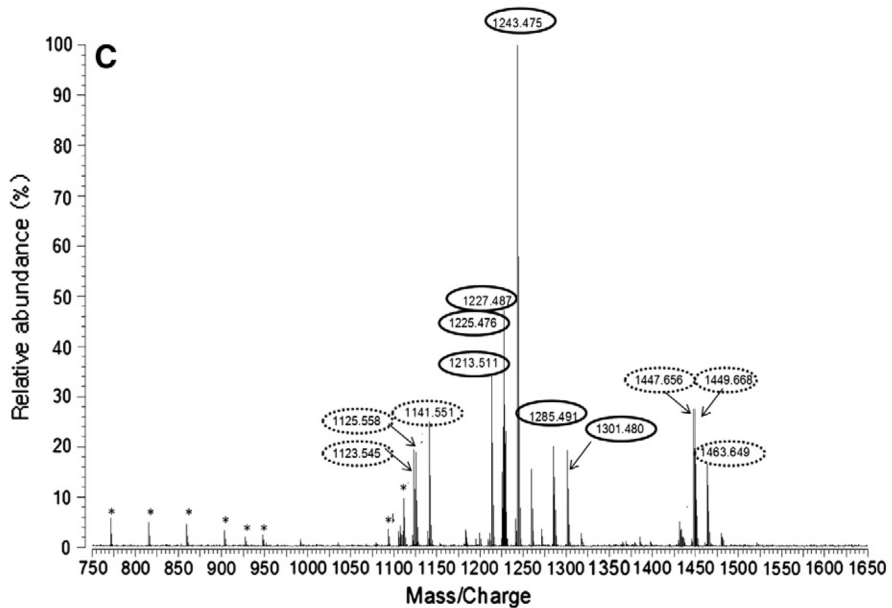


Fig. 2. NVC of HPLC fractions as determined via the hemolytic test (n = 3 technical replicates). Only the active fractions are included in the figure. Values are presented as means ± s.d. and expressed as milligram equivalents of plant saponins per milliliter of body wall.


A
1243.479
1225.471
1213.505
1141.540
1227.485
1123.530
1125.545
1111.568
1447.672
1449.685
1241.687
1463.668
1153.832
1259.455
1285.492
1301.488
Relative abundance (%)
Mass/Charge


B
1227.484
1243.480
1211.490
1213.503
1125.542
1153.828
1259.454
Relative abundance (%)
Mass/Charge


C
1243.475
1227.487
1225.476
1213.511
1447.656
1449.668
1125.558
1141.551
1285.491
1301.480
1123.545
1463.649
Relative abundance (%)
Mass/Charge

The semi-pure butanolic samples and HPLC-collected fractions were further analyzed via quantitative assays (hemolytic and vanillin–sulfuric acid colorimetric tests). Saponin identification was conducted via mass spectrometric analyses.

2.3. Determination of saponin concentration

The saponin concentration of the butanolic portion was determined using two complementary semi-quantitative methods, namely, hemolytic and vanillin–sulfuric acid colorimetric tests. For the HPLC-collected fractions, only the hemolytic test was used because it is more sensitive in detecting bioactive saponins. NVC, which is the concentration at which the saponins were observed in the organism, was computed for the quantification.

The hemolytic and vanillin–sulfuric acid colorimetric tests are based on the structure-related bioactivity and structural property of saponins, respectively. The vanillin–sulfuric acid colorimetric assay is based on the structure of the sapogenin or agylcone of the compound. Agylcones with an –OH group at C-3 position react with vanillin in an acidic environment to form a chromogen that has the same absorption pattern whether the aglycone is free or bound to a sugar (Hiai et al., 1976). Therefore, a more conservative estimate of the total saponin concentration in the extract can be obtained. The hemolytic test is based on the effective lysis of erythrocytes resulting from the membranolytic activity of saponins. Thus, this method can be used as a proxy for determining the concentration of bioactive saponins in the fractions or the degree of the membranolytic activity (Kalinin et al., 1996).

2.3.1. Hemolytic test

The hemolytic method used was modified from that by Van Dyck et al. (2010). Human blood was drawn in ethylenediaminetetraacetic acid tubes and centrifuged at 800 ×*g* for 15 min. Then, the plasma and buffy coat were removed. The pelleted erythrocytes were washed twice and suspended with cold phosphate buffered saline (PBS) at pH 7.4 in a final concentration of 2%. For the quantification of the semi-pure samples, 25 μL of test solution was mixed with 3 mL of the 2% red blood cell suspension. For the HPLC fractions, 5 μL of sample was added to 150 μL of blood suspension in 96-well plates. The mixture was incubated for 1 h at 37 °C and centrifuged at 800 ×*g* for 10 min. Then, the absorbance of the supernatant was measured at 540 nm. A 5 mg/mL solution of plant saponins from Quillaja bark (Calbiochem Cat No. 558255, Merck, Darmstadt, Germany) was serially diluted in PBS to construct a standard curve.

2.3.2. Vanillin–sulfuric acid colorimetric test

A colorimetric method modified from that by Jeong and Park (2006) was also used to determine saponin concentration. The test substance (100 μL) was added to 5% vanillin in 250 μL of ethanol. Then, 2.5 mL of 72% sulfuric acid was added. The solution was incubated at 60 °C for 10 min in a water bath and cooled in cold water. After vigorous vortexing, the absorbance was measured at 540 nm. A 5 mg/mL solution of plant saponins from Quillaja bark (Calbiochem Cat No. 558255) was serially diluted in distilled water to construct a standard curve.

2.3.3. Data analysis

The NVCs were expressed in mg equivalents of plant saponins by volume of body wall. All values were presented as mean ± s.d. of the replicates.

2.4. Mass spectrometric analyses

MALDI–FTICR MS was performed using an IonSpec Pro MALDI–FTICR MS instrument (IonSpec, Irvine, CA) equipped with a 7.0 Tesla superconducting magnet. A pulsed neodymium-doped yttrium aluminum garnet laser (355 nm) was used as the external MALDI ionization source. The saponin samples (1 mg/mL in 50% methanol in water) were spotted on a stainless steel probe with a 2,5-dihydroxybenzoic acid (DHB) matrix to produce gas-phase ions in either the positive or negative ion mode. The matrix solution contained 50 mg/mL DHB in 50% acetonitrile in water. For the positive ion mode, 0.01 M NaCl was added as a cation dopant. An aliquot of the sample (1 μL) was first applied to the probe tip, followed by 0.5 μL of NaCl solution in 50% acetonitrile in water and 1 μL of matrix. The samples were mixed on the probe surface and dried under vacuum prior to analysis. Ions were desorbed from the sample target plate by laser shots. In the ion cyclotron resonance (ICR) cell, the ions were excited and detected in the *m/z* range of 220 to 2500. Transients were acquired using the IonSpec OMEGA software. Tandem MS (MS/MS) analysis was conducted via collision-induced dissociation (CID). During the CID process, the ion of interest was isolated in the ICR cell. Then, a pulse of nitrogen gas was introduced into the ICR cell to collisionally activate and fragment the isolated ions.

For isomer separation, the saponin samples were analyzed using an Agilent 1200 series microwell-plate autosampler, capillary pump, nano pump, HPLC-chip interface, and 6520 Q-TOF MS (Agilent Technologies, Inc., Santa Clara, CA). A C18 microfluidic chip with a 40 nL enrichment column and 43 mm × 0.075 mm internal diameter analytical column was used. The mobile phases used were 10% methanol in water (eluant A) and 100% methanol (eluant B). Both phases contained 0.1% formic acid. A nanoliter pump gradient was delivered at 300 nL/min. At 2 min, the gradient was increased from 0% solvent (B) to 50% solvent B over 12 min. The gradient was further increased linearly to 100% solvent (B) over 35 min and maintained for 5 min. Afterwards, the amount of solvent (B) was decreased to 0% and maintained until completion of the 45 min run. The recorded mass ranged from *m/z* 400 to 2000 for MS only. Reference masses were chosen at *m/z* 680.035 and 1279.995. Data analysis was performed using the MassHunter qualitative analysis software ver. 03.01 (Agilent Technologies, Inc., Santa Clara, CA).

2.5. Phylogenetic analysis

A multigene phylogenetic tree was constructed using maximum likelihood (ML) analysis. A total of 32 species from Holothuriidae, including *H. scabra*, *H. fuscocinerea*, and *H. impatiens*, with available mitochondrial genes from subunit 1 of cytochrome oxidase (cox1) and 16s ribosomal RNA (16s) from GenBank were included (Table 1). Four species of *Stichopus* were used as outgroup. Sequences were aligned via multiple sequence comparison by log-expectation (Edgar, 2004) using default parameters and concatenated through Data Analysis in Molecular Biology and Evolution software (http://dambe.bio.uottawa.ca) (Xia and Xie, 2001). The analysis was conducted using the TN93 + G + I model. The parameters were fixed according to the results of the model test, with node support assessment by 1000 bootstrap replicates. Sequences with gaps were treated as characters or deleted partially or completely in the analyses. All runs were performed using Molecular Evolutionary Genetics Analysis version 5.05 (http://www.megasoftware.net). Based on the ML trees, the saponin types were mapped onto each species, depending on whether they were purely non-sulfated, sulfated, or mixed types. Genetic distances were also computed within species and between group types.

Fig. 3. Representative MALDI–MS spectrum of saponins from three species belonging to Holothuriidae. Positive ion mode spectrum of (A) *H. scabra*, (B) *H. impatiens*, and (C) *H. fuscocinerea*. Full circles are sulfated ion peaks, and dashed circles are non-sulfated ion peaks. Unidentified contaminants are indicated with asterisks (*).

Table 2
Summary of (A) non-sulfated saponins and (B) sulfated saponins detected in the semi-pure (SP) and HPLC fractionated samples of *H. scabra*, *H. impatiens*, and *H. fuscocinerea*.

Observed m/z	MW	MF	*H. scabra* (SP HS4 HS5 HS6 HS7)	*H. impatiens* (SP HI5 HI6 HI7)	*H. fuscocinerea* (SP HF4 HF5 HF6 HF7)	Saponin name	No. of isomers (retention time, min) *H. scabra*	*H. impatiens*	*H. fuscocinerea*	Ref.
A. Non-sulfated saponins										
1111.568	1088.578	$C_{54}H_{88}O_{22}$				Unidentified	1 (17.6)	–	–	–
1123.530	1100.54	$C_{54}H_{84}O_{23}$				Unidentified	4 (14.7/ 16.3/ 17.5/ 18.2)	–	4 (15.9/ 17.5/ 18.4/ 19.3)	–
1125.545	1102.555	$C_{54}H_{86}O_{23}$				Holothurinoside C	1 (16.3)	2 (15.1/ 17.8)	4 (14.4/ 16.7/ 18.4/ 20.5)	7
1141.540	1118.55	$C_{54}H_{86}O_{24}$				Desholothurin A (Nobiliside 2A)	N.D.	–	1 (14.5)	7
1153.832	1130.842	$C_{63}H_{118}O_{16}$				Unidentified	N.D.	N.D.	–	–
1411.474	1388.484	$C_{55}H_{88}O_{40}$				Unidentified	N.D.	N.D.	–	–
1427.477	1404.487					Unidentified	N.D.	N.D.	N.D.	–
1431.679	1408.689	$C_{67}H_{108}O_{31}$				Unidentified	–	–	2 (21.7/ 23.1)	–
1447.674	1424.684	$C_{54}H_{84}O_{23}$				Impatienside A	1 (15.7)	N.D.	1 (20.5)	5
						Isomer				–
1449.688	1426.698	$C_{67}H_{110}O_{32}$				Bivittoside D	N.D.	N.D.	1 (21.8)	5
1463.670	1440.68	$C_{67}H_{108}O_{33}$				Holothurinoside H	2 (15.1 / 16.0)	N.D.	2 (17.4/19.4)	7
B. Sulfated saponins										
1211.490	1188.500	$C_{54}H_{86}NaO_{25}S$				Unidentified	–	4 (15.5/ 16.4/ 18.8/19.9)	–	–
1213.505	1190.515	$C_{54}H_{88}NaO_{25}S$				Pervicoside C	1 (15.5)	1 (17.5)	3 (15.0/ 21.0/ 22.1)	8
						Isomer				–
1225.471	1202.481	$C_{54}H_{84}NaO_{26}S$				Unidentified	3 (14.7/ 16.3/ 17.5)	6 (13.6/ 14.3/ 14.8/ 15.9/ 16.6/ 17.9)	3 (14.7/ 16.0/ 17.7)	–
1227.485	1204.495	$C_{54}H_{84}NaO_{26}S$				Fuscocinerosides B/C*	2 (13.6/15.2)	7 (13.1/ 13.4/ 14.4/ 14.7/ 15.3/ 17.8/ 18.7)	2 (13.4/ 15.0)	8
						24-dehydroechinoside A or Scabraside A*				2, 4
						Isomer				–
1229.497	1206.507	$C_{54}H_{88}NaO_{26}S$				Holothurin A2 (Echinoside A)	1 (14.2)	–	– 2 (13.4/ 15.0)	2, 6
1241.872	1218.882	$C_{58}H_{132}NaO_{21}S$				Unidentified	3 (12.8/ 13.3/ 14.0)	–	1 (12.6)	–
1243.480	1220.490	$C_{54}H_{86}NaO_{27}S$				Holothurin A or Scabraside B*	1 (13.6)	1 (13.8)	2 (13.5/15.1)	2, 6, 8
1259.455	1236.465	$C_{54}H_{86}NaO_{28}S$				Holothurin A3	N.D.	N.D.	N.D.	1
						Isomer				–
1269.482	1246.492	$C_{52}H_{88}NaO_{30}S$				Unidentified	3 (14.9/ 16.1/ 17.2)	1 (15.9)	1 (14.7)	–
1285.496	1262.506	$C_{54}H_{86}NaO_{28}S$				Fuscocineroside A	1 (14.3)	1 (12.5)	1 (14.2)	8
						Isomer				–
1301.486	1278.496	$C_{56}H_{88}NaO_{29}S$				Unidentified	1 (15.1)	N.D.	3 (12.3/ 14.9/ 17.9)	–

The relative abundances of congeners are indicated in different colors: solid = 90%–100%; grid = 50%–90%; and dotted pattern = 10%–50%.
*isomeric saponins.
N.D. = Not detectable.
Ref: (1) Dang et al., 2007; (2) Hua et al., 2009a; (3) Hua et al., 2009b; (4) Kobayashi et al., 1991; (5) Sun et al., 2007; (6) Thanh et al., 2006; (7) Van Dyck et al., 2009; (8) Zhang et al., 2006.

3. Results

3.1. Determination of saponin concentration

For the semi-pure extracts, both quantitative assays revealed differences with different trends in the NVCs of the three species as shown in Fig. 1. The conservative estimation of colorimetric test showed *H. fuscocinerea* and *H. impatiens* having almost the same concentration with *H. scabra* being lower than the two. However, *H. fuscocinerea* showed a very high membranolytic activity equivalent to 0.46 mg eq. plant saponins/mL body wall. This is approximately 3-fold and 20-fold higher than those in *H. scabra* and *H. impatiens*, respectively.

Each semi-pure sample was further purified via HPLC. Fractions were collected every 5 min. Each fraction was assayed for their NVC using hemolytic test. The active fractions were eluted at high methanol concentrations (ca. ~70% to 86%). Four active fractions were collected from *H. scabra* (HS 4–HS 7) and *H. fuscocinerea* (HF 4–HF 7), and three active fractions were collected from *H. impatiens* (HI 5–HI 7) (Fig. 2). The results showed that the fractions HS 5, HI 5, HI 6, and HF 5 had the highest saponin concentrations in *H. scabra*, *H. impatiens*, and *H. fuscocinerea*, respectively. Furthermore, HF 5 had the highest NVC (0.258 ± 0.001 mg/mL) among all of the active fractions.

3.2. Mass spectrometric analysis of saponins from *H. scabra*, *H. impatiens*, and *H. fuscocinerea*

The saponins obtained from semi-pure samples of the three sea cucumbers with different degrees of deterrence were profiled via MALDI–FTICR MS (Fig. 3). In the positive ion mode, all observed ions were sodium-coordinated species such as $[M-H + 2Na]^+$ and $[M + Na]^+$ corresponding to sulfated and non-sulfated saponins, respectively. The negative ion mode spectra showed sulfated saponins as deprotonated ions ($[M-H]^-$). The accurate mass determination provided by FTICR MS identified the saponin peaks, and the molecular formulae were assigned as shown in Table 2.

Saponin ion peaks were further identified using MS/MS by building the fragmentation patterns of their respective glycan structures. Distinct patterns were observed from sulfated and non-sulfated saponins as illustrated in Fig. 4 as Holothurin A and Impatienside A as representative examples. MS/MS of Holothurin A precursor ion at *m/z* 1243.479 showed possible fragmentation pathways by the ion peaks observed (Fig. 4A).

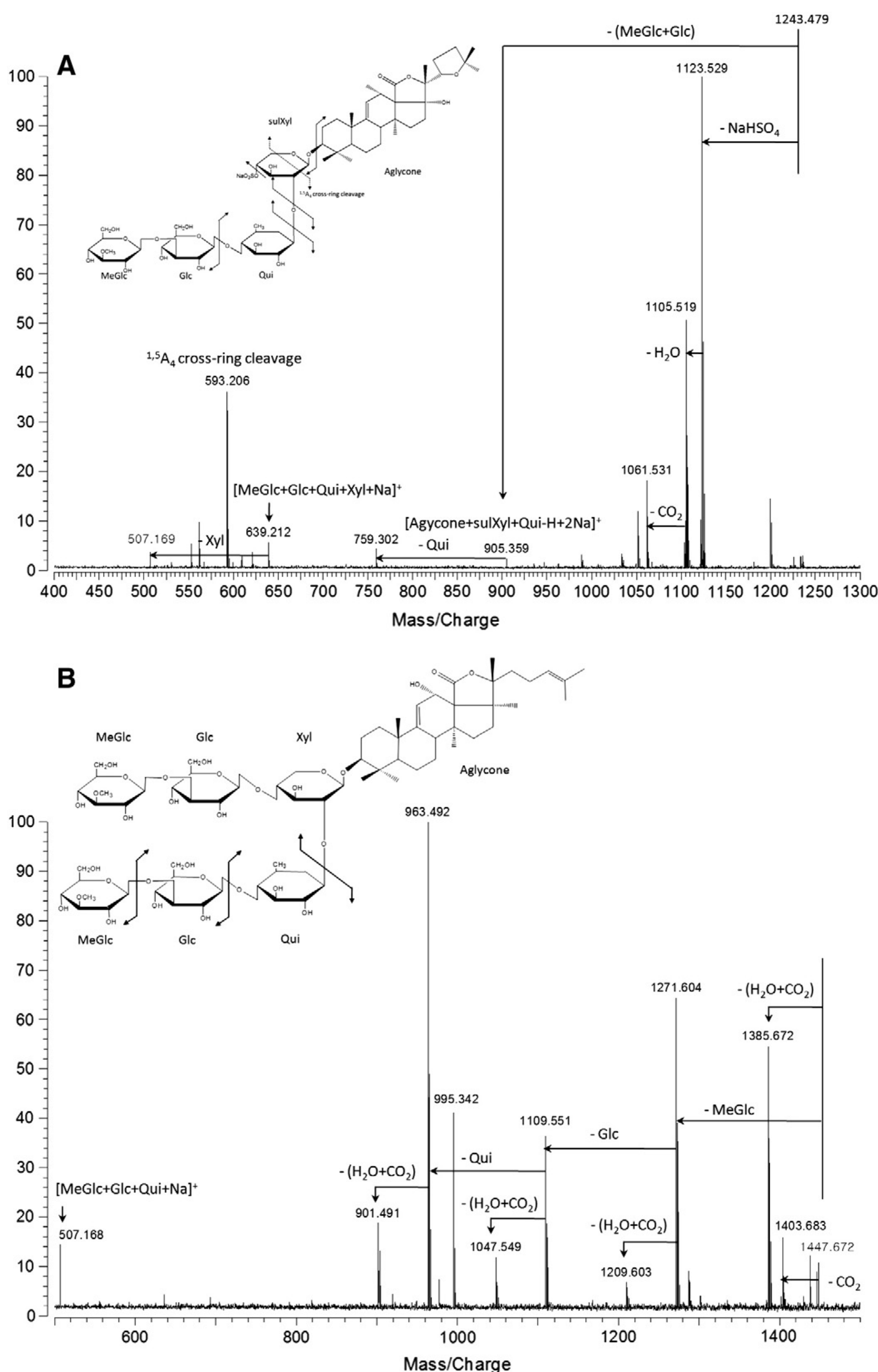


Fig. 4. Tandem mass spectra of (A) HolothurinA (m/z 1243.479) and (B) Impatienside A (m/z 1447.672) in the positive ion mode CID.

From the precursor ion, there is subsequent loss of the methyl glucose and glucose (*m/z* 905.359) and quinovose (*m/z* 759.302) leading to the peak at *m/z* 759.302 which corresponds to [Aglycone + sulXyl-H + 2Na]$^+$. Glycan fragments were generated when the precursor ion consecutively loses the $NaHSO_4$ (*m/z* 1123.529), aglycone unit (*m/z* 639.212), and xylose (*m/z* 507.169). There is a very prominent peak at *m/z* 593.206 corresponding to a $^{1,5}A_4$ ring cleavage of the xylose residue. For Impatienside A (*m/z* 1447.672), the precursor ion sequentially lost methyl glucose (*m/z* 1271.604), glucose (*m/z* 1109.551) and quinovose (*m/z* 963.492) (Fig. 4B). The MS/MS spectrum also showed the observed characteristic peak at *m/z* 507.168 which further confirmed that this compound contains the same MeGlc-Glc-Qui residue. For both spectra,

some of the peaks were also designated as water (−18 Da) and/or carbon dioxide (−44 Da) loss. When the sulfate group ($NaHSO_4$) exists in the saponin compound, such as in the case of HolothurinA, a loss of 120 Da was observed during the MS/MS.

By the combination of accurate mass and MS/MS information, saponins were categorized into four distinct carbohydrate structural types: (A) MeGlc-Glc-Qui-Xyl-Aglycone; (B) MeGlc-Glc-Qui-sulXyl-Aglycone; (C) MeGlc-Glc-Qui-(Qui-Glc)-Xyl-Aglycone; and (D) MeGlc-Glc-Qui-(MeGlc-Glc)-Xyl-Aglycone. Non-sulfated saponins had four to six monosaccharide units and three distinct structural types. Non-sulfated saponins at *m/z* 1111.568 to 1153.832, *m/z* 1411.474 to 1449.688, and *m/z* 1449.688 as well as 1463.670 had structures (A), (D), and (C), respectively. All sulfated saponins ranging from *m/z* 1211.490 to 1301.486 had structure (B) in which xylose was sulfated.

Saponin isomers were further separated via nano-liquid chromatography separation whenever possible. ESI mass spectra of the saponins are dominated by $[M + H]^+$ and $[M + Na]^+$, therefore those ions are used for extracted ion chromatograms (EICs). For example, EICs of the saponin at *m/z* 1183.520 corresponding to $[C_{54}H_{86}O_{26}S + H]^+$ showed several peaks (Fig. 5). The peaks were found in all of the three species and could correspond to 24-dehydroechinoside A or scabraside A (for *H. scabra*), Fuscocinerosides B or C (for *H. fuscocinerea*), or other isomers, differing only in the lateral side chain of their aglycone units. Retention times of saponin isomers were reported in Table 2. There were some instances where peaks previously observed from MALDI MS were not seen from the isomer separation done in LC ESI MS and vice versa. In this case, the total number of isomers seen from the latter method was reported. These techniques confirm the existence of saponins reported in literature and allowed the discovery of new saponin congeners in the species examined.

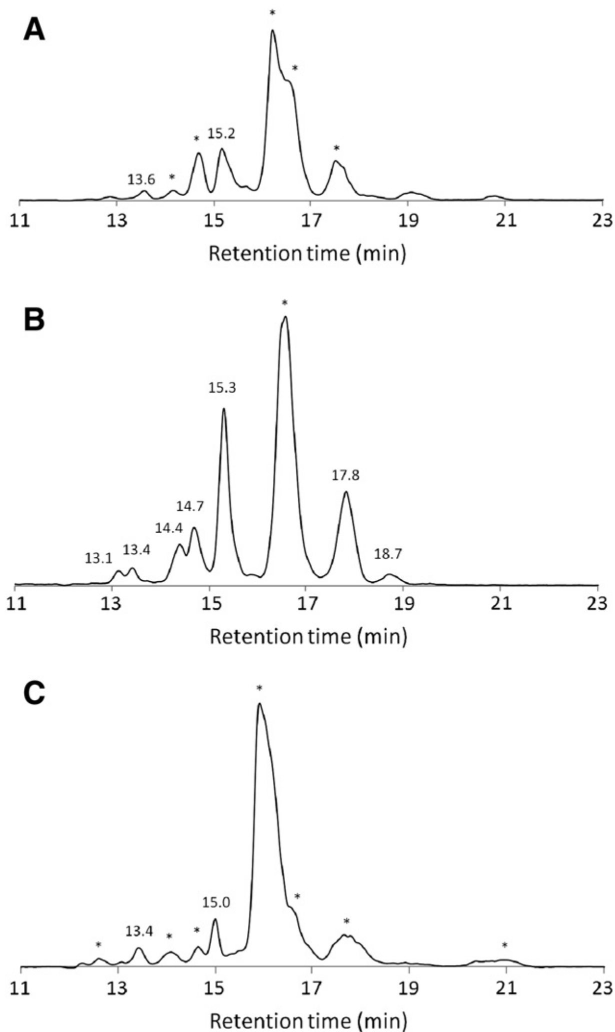


Fig. 5. Extracted ion chromatogram (EIC) of m/z 1183.520 as [M + H] in ESI from *H. scabra* (A), *H. impatiens* (B), and *H. fuscocinerea* (C). This m/z could correspond to 24-dehydroechinoside, scabraside A, fuscocinerosides B or C or other isomers except the ones indicated by an asterisk (*). Retention times are written on the top of each peak.

The MS analyses showed that the three sea cucumber species produce a mixture of common and unique saponin types, as exhibited by the diverse profiles. Some saponins were shared among species (e.g., Holothurin A), whereas others were unique to each species (e.g., Impatienside A in *H. impatiens*), as Caulier et al. (2011) have also indicated. The different relative intensities of the peaks suggest that saponins were present in the body walls of the organisms at different proportions. The mass spectra of the semi-pure samples showed the profile of all saponins in the body wall, whereas the HPLC-fractionated samples showed the bioactive saponin types only (Fig. 3 and Table 2). Not all of the congeners detected in the semi-pure samples were present in the HPLC-fractionated samples, and vice-versa. The MS profiles of the HPLC fractionated samples showed that most of the sulfated saponins in fractions 4 and 5 were eluted within 20 min to 30 min, whereas most of the non-sulfated saponins in fractions 6 and 7 were eluted after 30 min to 40 min. Most saponin ion peaks were detected in multiple fractions, indicating that HPLC fractionation along with our procedure did not effectively separate the saponins. However, coupling the MS analysis with bioactivity testing of each fraction gave an opportunity to determine what combinations of saponin types had higher bioactivity. Table 2 summarizes all of the analyses conducted on the semi-pure and HPLC fractionated samples. The molecular structures of the identified compounds are presented in Fig. 6.

H. scabra had the highest saponin diversity based on the MALDI mass profiling. The semi-pure and HPLC fractionated samples showed ten sulfated and 10 non-sulfated saponin peaks. NanoLC revealed 32 total isomers from these 20 saponin ion peaks. The positive ion mode peaks at *m/z* 1227.485, 1229.497, 1243.480, and 1259.455 corresponded to 24-dehydroechinoside A or Scabraside A (functional group isomers), Holothurin A_2 (synonymous with Echinoside A), Holothurin A, and Holothurin A_3 (Kobayashi et al., 1991; Thanh et al., 2006; Dang et al., 2007; Hua et al., 2009a; Hua et al., 2009b), respectively. The ion peaks of the non-sulfated saponins at *m/z* 1125.545, 1141.540, 1449.688, and 1463.670 corresponded to Holothurinoside C, DesholothurinA (synonymous with Nobiliside 2A), Bivittoside D, and Holothurinoside H, respectively (Van Dyck et al., 2009). This study is the first to report the presence of these non-sulfated saponins in *H. scabra*. Previous reports indicated five other saponins including four sulfated (Holothurins A_1, A_2, A_4, and B) and one non-sulfated (Holothurinogenin B) congeners. However, these types were not detected in this study.

The MALDI mass spectra of the semi-pure and HPLC fractionated samples of *H. impatiens* showed only 16 ions (nine sulfated and seven non-sulfated) in which a total of 32 isomers were found. Only five congeners were identified, including the sulfated Holothurin A (*m/z* 1243.480), non-sulfated Holothurinoside C (*m/z* 1125.545), Impatienside A (*m/z* 1447.674), Bivittoside D (*m/z* 1449.688), and Holothurinoside H (*m/z* 1463.670). Impatienside A and Bivittoside D were detected only in the HPLC fractionated samples. This study is the first to report the presence of Holothurinosides C and H in *H. impatiens*.

Nine sulfated and eight non-sulfated peaks were observed in *H. fuscocinerea*. Nine of these saponins have been previously reported. A total of 33 isomers were also found in this species. All of the sulfated saponins that were previously reported were detected in this species, including Pervicoside C (*m/z* 1213.505); Fuscocinerosides B or C, which are functional group isomers (*m/z* 1227.485); Holothurin A (*m/z* 1243.480); and Fuscocineroside A (*m/z* 1285.496) (Zhang et al., 2006). Peaks at *m/z* 1125.545, 1141.540, 1449.688, and 1463.670 may correspond to Holothurinoside C, Desholothurin A, Bivittoside D and

Holothurinoside H, respectively (Van Dyck et al., 2009). These compounds were also observed in *H. fuscocinerea* for the first time.

Some saponins in the semi-pure and HPLC-fractionated samples were common in the three species. Shared non-sulfated types include Holothurinoside C (*m/z* 1125.545), Holothurinoside H (*m/z* 1463.670), and a saponin with an unidentified ion peak at *m/z* 1427.477. Holothurinosides C and H, Desholothurin A (*m/z* 1141.540), and Bivittoside D (*m/z* 1449.688) were detected in *H. scabra* and *H. fuscocinerea* for the first time. Previous reports indicated that these compounds are absent in other *Holothuria* sp. except *H. forskali*. The common sulfated congeners among the sea cucumbers were Holothurin A (*m/z* 1243.480) and three unidentified saponins with peaks at *m/z* 1225.471, 1269.482, and 1301.486. Among these saponins, Holothurin A is the only known major congener with a relative abundance of greater than 60% in all the three species.

Unique saponin types were also observed when the mass spectra of the three species were compared with one another. The unique saponins of *H. scabra* were 24-dehydroechinoside A, Holothurins A_2 and A_3, Scabraside A, and an unidentified saponin with an ion peak at *m/z* 1111.568. All ion peaks are major congeners except Holothurin A_3. *H. impatiens* had three unique saponins, the non-sulfated Impatienside A and two unidentified sulfated ion peaks (*m/z* 1211.490 and 1227.485). Impatienside A and the saponin with an ion peak at *m/z* 1227.485 are major congeners. The unique congeners of *H. fuscocinerea* include Fuscocinerosides A, B, and C; Pervicoside C; and a saponin with an unidentified peak at *m/z* 1431.679. The first four types are major

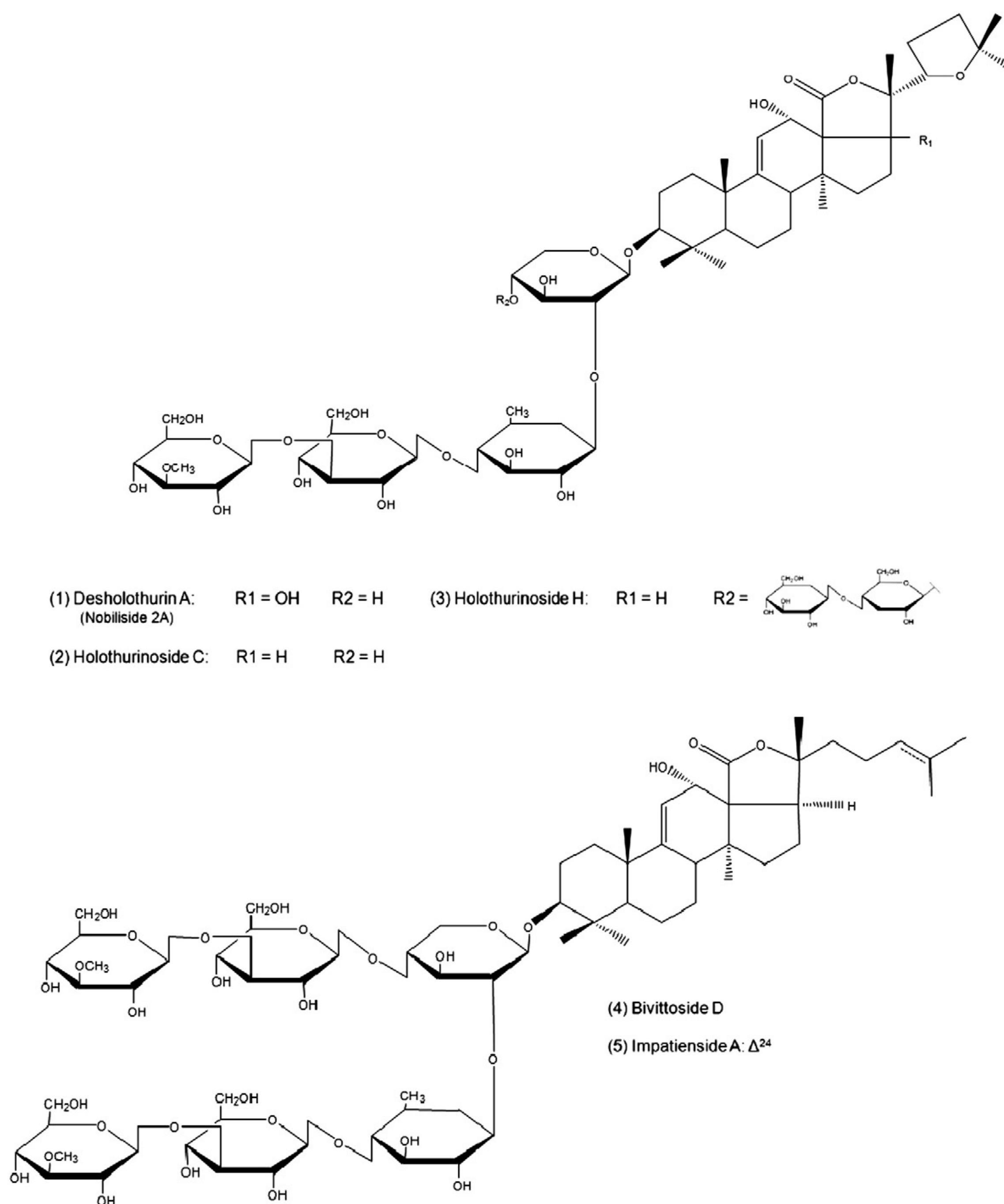


Fig. 6. Molecular structures of known saponins detected from the body walls of three species from Family Holothuriidae.

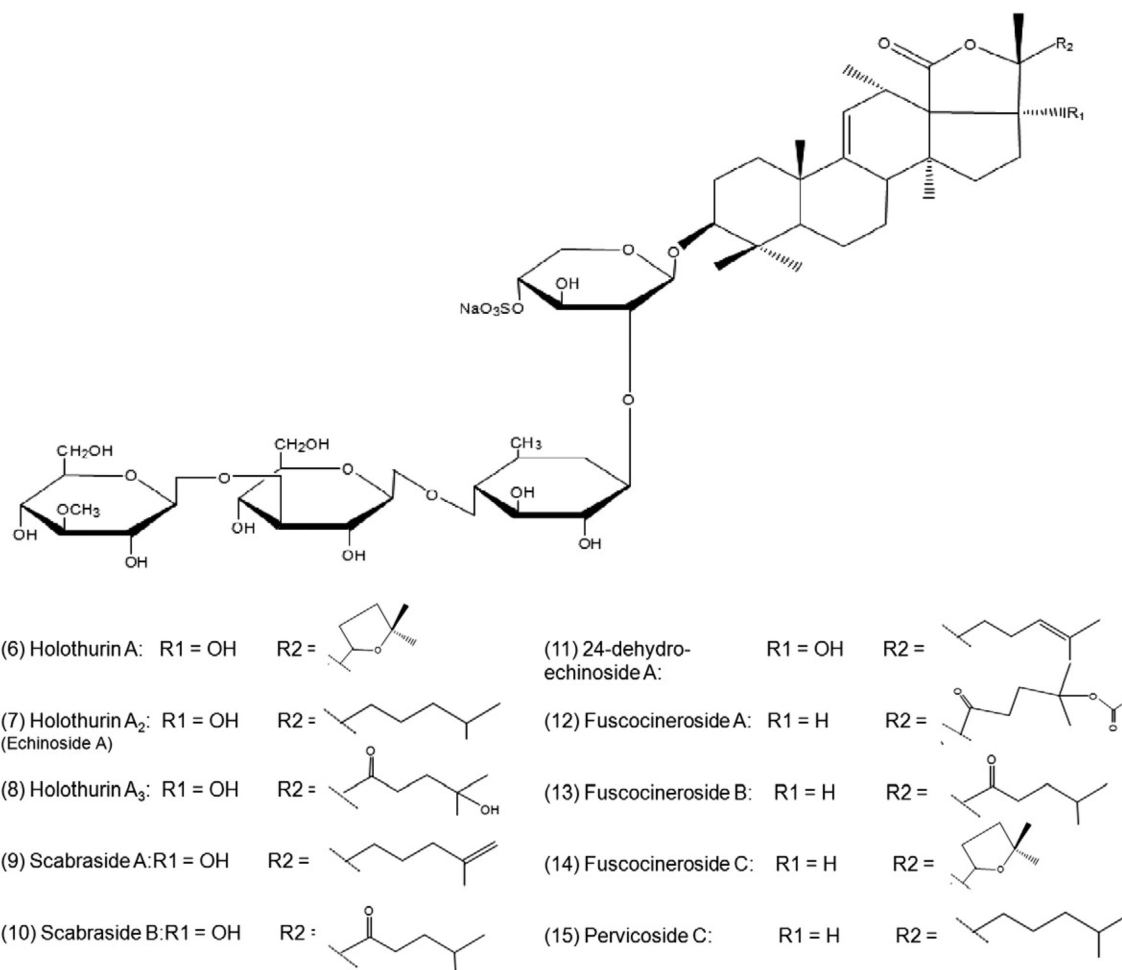


Fig. 6 (*continued*).

sulfated congeners, whereas the saponin with an unidentified peak is a minor non-sulfated congener.

3.3. Phylogenetic analysis

The mtDNA alignments of 16s rRNA and cox1 included 434 bases (including 28 gap positions) and 441 bases (including one gap position), respectively. Concatenated data showed 846 nucleotide positions when gaps and missing data were eliminated. ML analyses generated trees with −ln scores of 9737.30, 11170.25, and 10107.99 when gaps were completely deleted, partially deleted, or used as a character, respectively. The tree reconstruction methods produced poorly resolved trees with polytomies. Completely deleting the gaps produced trees with lower numerical scores and less polytomies, indicating that this tree was the most likely topology (Fig. 7).

Mapping the known saponin congeners, based on whether the saponins were purely non-sulfated, sulfated, or mixed, onto the generated tree revealed the evolutionary significance of saponins. The genetic distances between these groups (Table 3) showed that species containing purely non-sulfated saponins were basal to those containing purely sulfated (0.251) and mixed (0.229) congeners. The sulfated group was more likely derived from and more closely related to the mixed group (0.191). In the genus *Holothuria* non-sulfated saponins arose first (in *H. forskali*, node F), followed by a mixture of sulfated and non-sulfated types (*Holothuria nobilis*, *H. impatiens*, *H. scabra*, *Holothuria leucospilota*, and *H. fuscocinerea*). The last were purely sulfated types (*Holothuria mexicana*, *Holothuria floridana*, *Holothuria atra*, *Holothuria edulis*, *Holothuria cinerascens*, *Holothuria polii*, and *Holothuria tubulosa*). *Holothuria hilla* (node L), a sister species to *H. leucospilota*, was clustered together with those that have mixed saponin types. This species reportedly has sulfated saponins only. However, its saponin profile is not yet complete given that the studies that isolated compounds from this species targeted the bioactive compounds only (Wu et al., 2006). The trend of saponin evolution was also very evident on the monophyletic *Actinopyga* + *Bohadschia* + *Pearsonothuria* clade (node H). *Bohadschia* spp., *Pearsonothuria graeffei*, and *Actinopyga* spp. have purely non-sulfated, mixed, and sulfated congeners, respectively.

Interestingly, all of the species used in this study were concentrated on one major clade. *H. impatiens* was the most basal (node J), with a genetic distance of 0.184 to 0.220 (Appendix A) from the rest. *H. scabra* separated earlier from the other *Holothuria* sp. (node K). *H. fuscocinerea* (node N) diverged more recently, with a genetic distance of 0.191 to 0.210 (Appendix A) from the other two species tested.

4. Discussion

Saponin profiles of three tropical holothurians species from Holothuriidae were determined by mass spectrometric techniques. Congeners detected were very diverse among the three species examined with almost equal numbers of isomers (32 to 33). Excluding the number of isomers, *H. scabra* had the highest number of saponin ion peaks (20), followed by *H. fuscocinerea* (17) and lastly *H. impatiens* (16). To date, this paper reports the highest number of saponin types detected in the body wall of sea cucumbers. In contrast with the diversity, significant differences were observed in the saponin concentration and membranolytic activity of the three species. *H. fuscocinerea* and

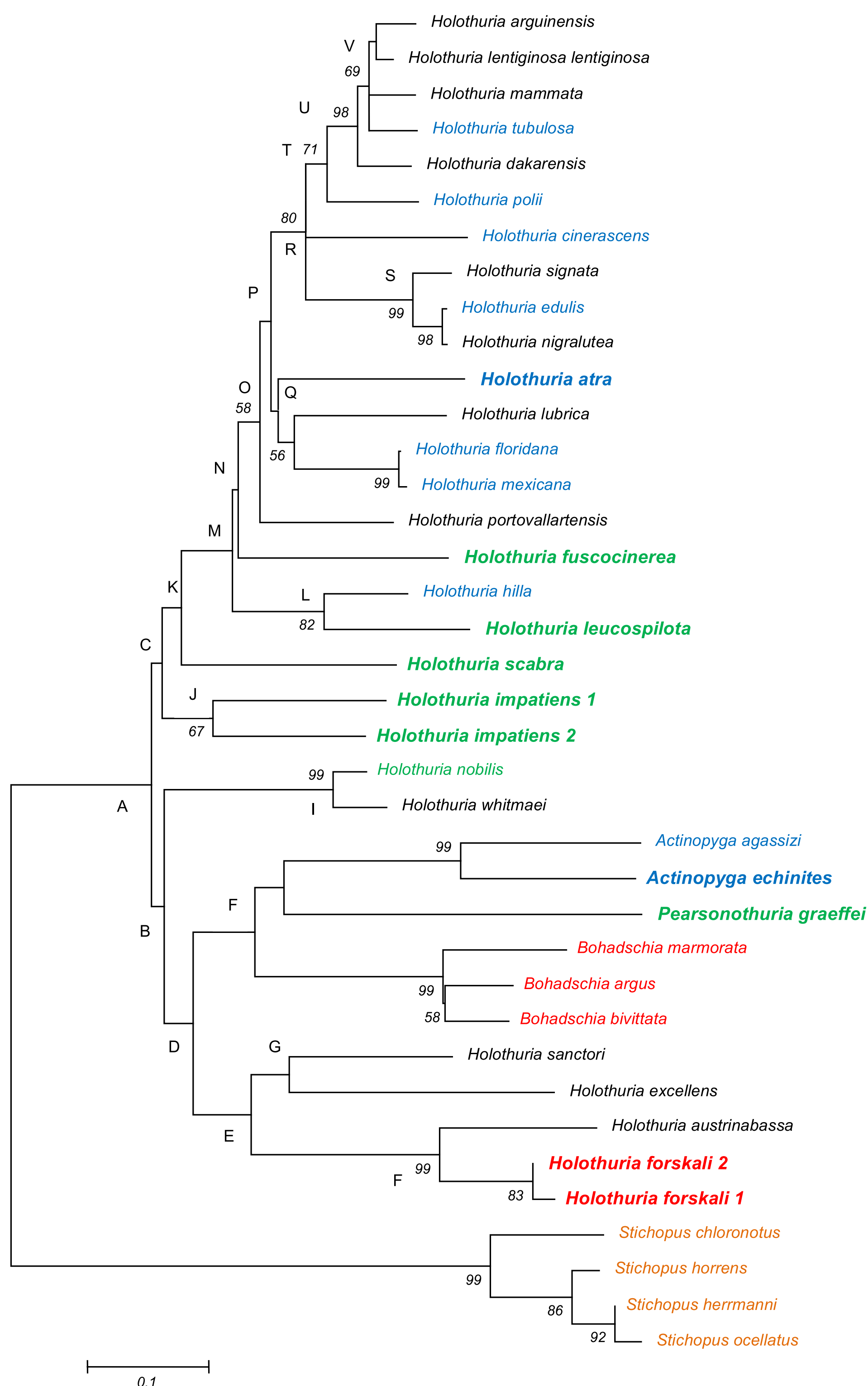


Fig. 7. Optimal phylogenetic tree of Family Holothuriidae from maximum likelihood analysis. Bootstrap values >50% are shown. Clades mentioned in the text are identified using letters. Species are colored based on their saponin types: red, non-sulfated; blue, sulfated; green, mixed; orange, outgroup; and black, no available data. Species in bold have comprehensive saponin profiles.

H. impatiens have the highest NVC but only the former has a high membranotropic activity. *H. scabra* has a high membranotropic activity but low NVC. There was no correlation between saponin diversity, membranolytic activity and saponin concentration. This finding was consistent with the study by Van Dyck et al. (2010) on five tropical holothurians (*Actinopyga echinites*, *Bohadschia subrubra*, *H. atra*, *H. leucospilota*, and *P. graeffei*).

The techniques used in this study were able to highlight the diversity of saponin types as well as unique profile of saponins for each holothurian with no bias towards biologically active congeners. To date, only nine species including those that were used in this study have comprehensive saponin profiles which can be used as a “chemical fingerprint”. This has potential use not only in determining possible species that has bioactive congeners but also in correct taxonomic classification.

Table 3
Distance matrix from the pair-wise distance calculation of the species grouped into three saponin types (purely sulfated, non-sulfated, and mixed congeners) and used for phylogenetic analysis. The Tamura–Nei model with gamma shape distribution of 5 was used. Standard error estimates are shown above the diagonal. All positions containing gaps and missing data were eliminated.

	Sulfated	Non-sulfated	Mixed	Outgroup
Sulfated		0.017	0.011	0.020
Non-sulfated	0.251		0.015	0.019
Mixed	0.191	0.229		0.018
Outgroup	0.288	0.267	0.274	

For instance, *Stichopus mollis* (Stichopodidae, Aspidochirotida) has neothyonidioside, a saponin that has a sulfate group on its xylose residue which is uncommon in the genera. Based on this finding and in combination with morphological data, this species was then re-classified as the new genus *Australostichopus* (Moraes et al., 2004).

The high diversity was also linked to ecological functions; the most important of it is chemical defense (Kalinin, 2000; Van Dyck et al., 2011). When the saponin types were mapped into the phylogenetic tree, it was apparent that non-sulfated congeners are basal and the sulfated ones are more derived. The sugar units of saponins evolved from non-sulfated hexaosides to sulfated tetraosides. Furthermore, the evolution is parallel to those in the families belonging to Order Aspidochirotida, which includes Holothuriidae (Kalinin and Stonik, 1996; Kalinin et al., 1996, 2005). This trend was observed in the genus *Holothuria* and the monophyletic *Actinopyga* + *Bohadschia* + *Pearsonothuria* clade. *H. forskali*, which was the most basal in the genus *Holothuria*, exclusively contained non-sulfated tetraosides and hexaosides. The more derived species had purely sulfated tetraoside saponin types. Between these groups containing exclusively sulfated or non-sulfated saponins, some species contained mixed types of congeners, which are proposed to belong to the transition group. This group includes the three species used in the study, namely, *H. impatiens*, *H. scabra*, and *H. fuscocinerea*, which were found to have distinct saponin profiles and natural concentrations.

H. forskali and *H. impatiens* have a long evolutionary history, diverging at 102 mya and 170.3 mya, respectively (Borrero-Pérez et al., 2010). The former has been proposed to be checked for its taxonomic position since it contains holothurinosides instead of the usual holothurins (Stonik et al., 1999). From the present study, through chemical fingerprinting, it was observed for the first time that holothurinosides were common in the three species. In morphofunctional analysis, character traits that are found in common from the ancestral to the derived species are the most primitive trait on a monophyletic tree (Crisci and Stuessy, 1980; Kalinin and Stonik, 1996). This shows that holothurinosides could be primitive congeners and that *H. forskali* is still in the correct taxonomic position. Furthermore, these saponin types have a specific role in chemical defense on *H. forskali*. The organism released holothurinosides C and F, and desholothurin A in prolonged stress while continually producing holothurinoside G even on relaxed state. Desholothurin A and holothurinoside C are also converted into holothurinosides G and H, respectively via addition of a disaccharide unit (MeGlc-Glc), increasing its hydrophilicity (Kalinin, 2000; Van Dyck et al., 2011). Hydrophilicity and membranolytic activity are the properties that affect the total ecological function of saponins. Higher solubility and mobility in seawater can facilitate rapid diffusion of the compounds in the environment deterring the predators in the vicinity of the organism-producer (Kalinin et al., 2000; Kalinin et al., 2008; Van Dyck et al., 2011). However, the distribution of these compounds across Holothuriidae remains unclear given that only a few species have complete saponin profiles. As of the moment, only *Bohadschia marmorata* and *Bohadschia subrubra* were reported to also contain Holothurinoside H, *Pearsonothuria graeffei* has Holothurinoside C and Desholothurin A and *H. nobilis* and *H. leucospilota* has Desholothurin A (Van Dyck et al., 2010; Caulier et al., 2011).

H. impatiens is the most basal in the three species used in this study. It could be possible that the divergence of this species is the start of the production of sulfated saponins. The modifications of the structural components of saponins increased the biodiversity of congeners with the biological functions enhanced or retained. This is termed as morphological degeneracy (Kalinin and Stonik, 1996: Kalinin, 2000). The presence of a linear tetrasaccharide chain and quinovose as the second monosaccharide unit increases the membranolytic activity of the compound. The sulfate group in the C-4 of the first xylose residue does not increase the activity on congeners with a linear tetrasaccharide chain but its absence in biosides can decrease their activity. Sulfation and sugar groups can increase the hydrophilicity of the compounds (Kalinin et al., 1992; Kalinin, 2000). Sulfation and the presence of a linear tetrasaccharide chain were proposed to be selected because these factors increase the membranotropic activity and hydrophilicity of saponins (Kalinin and Stonik, 1996). *H. impatiens* has a significantly lower membranolytic activity compared to the other two species. This could be due to Impatienside A and Bivittoside D which are both unique major non-sulfated congeners of this species. In comparison, *H. scabra* and *H. fuscocinerea* each have four unique major sulfated saponins, hence a higher membranolytic activity and hydrophilicity.

It is possible that the two properties of saponins also increased while the organism enhanced the first function of saponins as breeding regulators. Chemical defense then arose extrinsically as the main ecological function when predator pressure (i.e. fish) increased throughout time (Kalinin, 2000). However, there is still a need to test chemical defense through relevant feeding assays on predators. There have been numerous studies as to the ichthyotoxic activity of sea cucumber extracts (Bakus, 1968, 1974, 1981) and some palatability studies (De Vore & Brodie Jr. 1982; Bryan et al. 1997). Preliminary palatability tests of extracts from the body wall of *H. forskali* incorporated on a food matrix were palatable to the fish *Sciaenops ocellatus*, whereas those from the Cuvierian tubules were deterrent (Van Dyck et al., 2011). First feeding experiments of the body wall of the three species used in the study showed that *H. fuscocinera*, *H. impatiens* and *H. scabra* were observed to have the highest, intermediate and lowest deterrence respectively towards the specialist predator *Tonna* sp. (K.G.V.B., L.J.C. and M.A.J.M., unpublished observation). In sponges, NVCs of specific compounds (i.e., formoside from *Erylus formosus* and ectyoplaside as well as feroxoside from *Ectyoplasia ferox*) have been shown to provide chemical defense (Kubanek et al., 2000; Kubanek et al., 2002). As of the moment, there is no solid report on the specific congeners that chemically defends holothurians. Testing of feeding activity could be done by incorporating pure compounds in their natural concentration onto a food matrix, as conducted in other studies (Kubanek et al., 2000; Kubanek et al., 2002).

5. Conclusions

Chemical fingerprinting of saponins in holothurians can give insight on the correct taxonomic position of a species. The mass spectrometric techniques (MALDI–FTICR MS and nano-HPLC-chip Q-TOF MS) described in this study, allowed direct analysis and identification of complex samples (semi-purified and membranolytic HPLC extracts). Even if these techniques were not enough to structurally elucidate unknown compounds, it has provided a distinct saponin profile for each species. This study also provided the highest number of saponin types to be detected in a body wall of a holothurian. Linking the profiles to a phylogenetic tree showed support for the basality of *H. forskali* by providing evidence that holothurinosides are primitive taxonomic characters which are common in Holothuriidae. This congener has the potential to be used as a chemotaxonomic marker for the whole family instead of the usual holothurins. However, there is a need to do comprehensive saponin profiles for a species of interest, in particular for cryptic

species. The phylogenetic tree also supported the separation of the three species wherein *H. impatiens* having non-sulfated saponins as major congeners is the most basal. This further supports the observation before that holothurians evolved from having saponins with non-sulfated hexaosides to sulfated tetraosides (Kalinin and Stonik, 1996; Kalinin, 2000). This increased or did not significantly decrease the membranolytic activity and hydrophilicity, the factors that affect total ecological activity, in this case, chemical defense. Ecologically-relevant feeding assays such as using a natural prey and incorporating a pure compound into a food matrix should be done to confirm the role of saponins as chemical defense.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpb.2013.09.002.

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