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Lanostane Triol (Holothurin Group) Isolated from Triterpenic Fraction of *Holothuria floridana* Inhabiting in Shallow Waters of Cuban Archipelago †

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Abstract: Holothurians (Echinodermata, Aspirochirotida, Holothuridae, Holothuria), are marine, epibenthic organisms widely distributed on a planetary scale. Several species, especially in neotropical and subtropical regions, are used by coastal human populations as a nutritional source. These invertebrates are characterized by a great diversity of bioactive secondary metabolites, among them, the triterpene glycosides with a typical holostane (lanostane) molecular pattern. The present communication highlights the preliminary chemical-structural study (NMR-NMR-analytical drop reactions) of sea cucumbers (holothurias) that inhabit the coastal eco-zone of the Cuban archipelago, reporting as a case study, the isolation and molecular characterization of a holostane derivative isolated from the methanolic fraction of *Holothuria floridana*. After the chromatographic separation (SiO_2—Amberlite) of the fraction containing triterpenic glycosides, and the corresponding spectroscopic study, it was identified as the major component of this fraction holost-Δ9,11-en-12α,17α,22ξ triol tetraoside, a member of group A of holoturins; and the monosaccharide fraction consists of 3β-D-glucose, D-xylose, D-quinovose and 3-O-methyl-D-glucose.

Keywords: *Holothuria floridana*; triterpene glycoside; holothurin; monosaccharides; chromogenic reactions

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1. Introduction

The Sea Cucumbers, or Holothurians, (sea cucumbers, Echinodermata Aspirochirotida, Holothuridae, Holothuria), include about 2000 solitary or gregarious species and are marine epibenthic, of low mobility, invertebrates with a planetary geographical distribution, constituting more than 90% of the abyssal fauna. These organisms are abundant in coral reef regions (1.20–12 m) in calm waters, in sea bottoms where phanerogams, thalassia, and mangrove plants decompose. Several species are used for human consumption [1]. Some species inhabit depths of more than 5000–6000 m (genus *Pelagothuria*) and reach population densities of up to 70 individuals per square meter. Holothurians range in size from a few centimeters to more than 1 m in length (*Holothuria tomasi* and *Stichopus* sp.), and 15–24 cm in diameter. The edible species reach the largest sizes, between 40–50 cm. They feed on sediments and detritus and are of geological interest. They are fundamental in the marine food chain since their larvae benefit planktophages [2] and adult individuals can be eaten by starfish, crabs, lobsters and

turtles. Holothurians are important ecological agents in marine ecosystems as they recycle marine benthic substrate and contribute to sea bottom development by destroying stratification and restructuring the benthic community. Their excreta can contain up to twice as much nitrogen as the surrounding sediment, processing up to 120 g per day of sediment [3].

These echinoderms constitute a potential source of biomedical resources with unique pharmacological properties, creating additional markets for new pharmaceutical entities and their formulations. Products extracted from these organisms possess anti-fungal activity and exhibit anti-arthritic, anti-ulcer, and anti-carcinogenic action. Some of the steroidal glycosides, typical metabolites in these organisms, are used to regulate insect populations through inhibition of the Krebs cycle.

Notwithstanding their use in gastronomy and traditional medicine, and considering their chemical ecology, sea cucumbers constitute an extraordinary source of secondary metabolites [4,5] and natural products of great molecular diversity, being mostly predominant the triterpene glycosides of the holostane-lanostane type [6–8]. These holothuride saponins, which are characterized by a great molecular diversity and varied structural patterns, considering genin structures and functionalized or non-functionalized monosaccharide fractions, constitute the molecular basis of several therapeutic treatments and biological effects [9–19]. The recognized fundamental molecular patterns of these holostane-type secondary metabolites [20] are represented in Figure 1

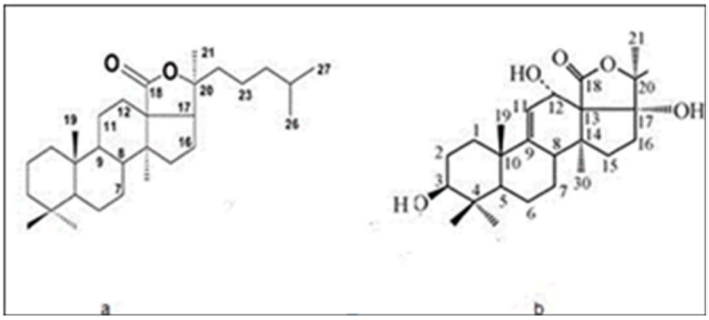

Figure 1. Fundamental molecular patters (**a**,**b**) of the holostane systems with γ-lactone unit C18–C20, and double bond C9–C11.

In continuing our studies of Cuban echinoderms and preliminary characterization of secondary metabolites associated with sea cucumber inhabiting the coastal marine waters of the western insular platform zone of the Cuban archipelago, the present work reports the preliminary chemical study of *Holothuria floridana* and details some molecular considerations about the major holostane-type component isolated from the methanolic fraction of *Holothuria floridana* [21–23].

2. Materials, Equipment and Methodologies

The general operational flow is depicted in Figure 2.

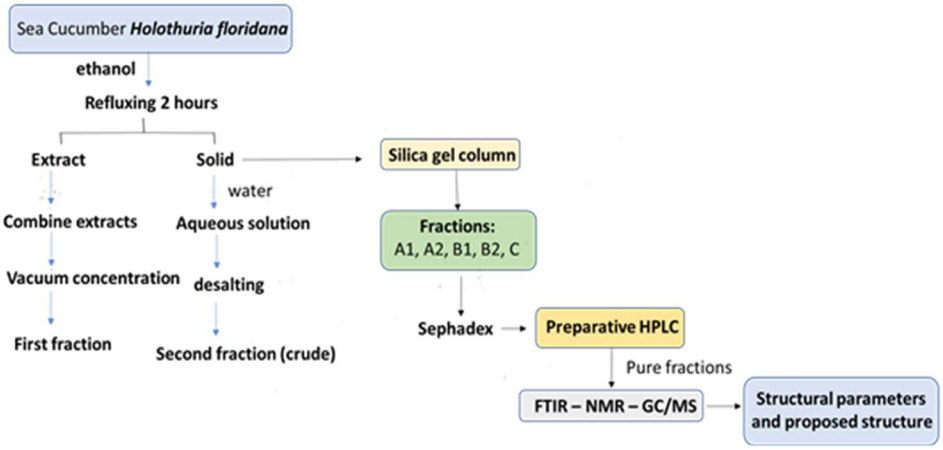

Figure 2. Operational flow for processing *Holothuria floridana* from raw material to fractions.

2.1. Collection of Holothuria floridana Specimens

Individuals of *Holothuria floridana*, collected in the western insular shelf region of the Cuban archipelago (Gulf of Batabanó, manually, during August 2020) were cut into small pieces and preserved under refrigeration prior to molecular characterization studies. Sea cucumbers were identified by Prof. Irma Alfonso; voucher specimens are preserved in Center for Engineering and Chemcal Researches, CIIQ, La Habana, Cuba and in the GIM-Laboratory at Technical University of Esmeraldas, Ecuador.

2.2. General Experimental Procedures

The melting point was determined by using, without correction, in open capillaries, an Electrothermal equipment IA6304, USA. The specific rotation, [α] D_{25}-value was determined by using a Perkin-Elmer Polarimeter Model 341 LC-California, USA (C 0,003 in ethanol). For the recording of infrared signals with analytical value, a FTIR Philips Analytical PU 960 (ν_{max}.-KBr tablets, cm^{-1}, Tokyo, Japan) spectrophotometer equipment, in the range 450–4500 cm^{-1}, at 25 °C equipment was used. To corroborate the presence of triterpene glycosides by means of *smart signals* associated to specific molecular fragments and their corresponding chemical shifts (ppm (δ), lactones, genin, double bonds position, hydroxy functionalities, as well as anomeric carbons in monosaccharide chain), the Nuclear Magnetic Resonance was used. The corresponding spectra, (^{13}C-^{1}H) were recorded in a Brucker ACF-250 MHz (Germany) instrument, at 25 °C, using TMS as an internal standard, and 0.3 M solutions in $CDCl_3$. For the separation and identification of major components of the fractions under study and the characterization of peracetates of monosaccharides present in the triterpene glycosides, a GC-MS equipment was used (HP-GCD Plus-California, capillary columns HP-5MS-30m, 70eV mode, USA). All results were compared with authentic reference samples and by using publicly available reported identification bases.

The extended flow chart of procedures is described in Figure 3.

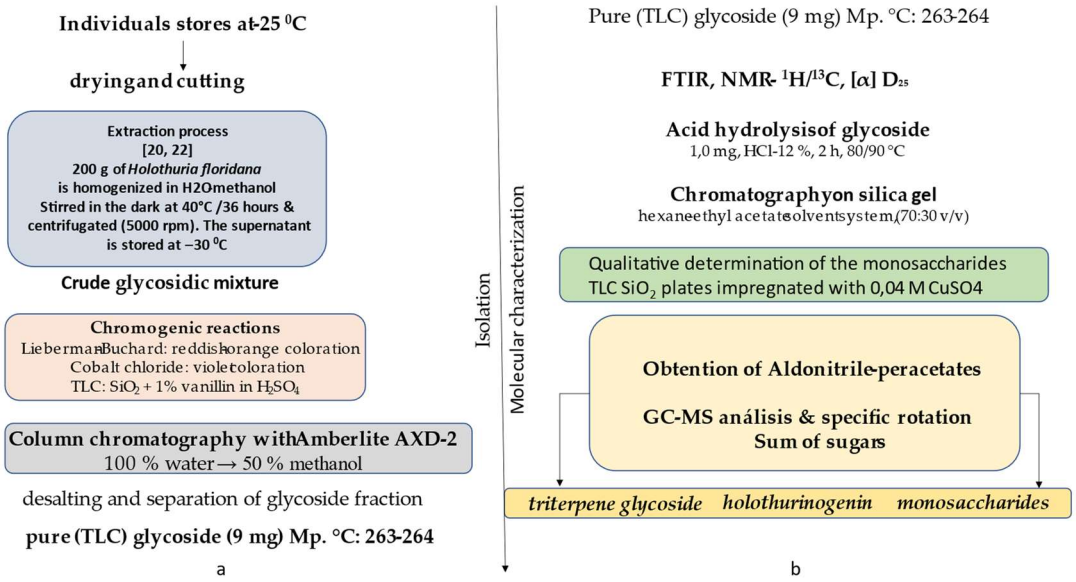

Figure 3. Flow chart of procedures used from raw *Holothuria floridana* to isolation and characterization of main components of extracts. (**a**) obtention of pure glycoside. (**b**) Obtention of genin and sum of monosaccharides.

2.3. Preparation of Aldonitrile-Peracetates for Monosaccharide Components Identification

The prepration of aldonitrile-peracetates derivatives for monosaccharide components characterization is carried out according to [24]. In general, the previously obtained and concentrated aqueous phase is treated with pyridine (1 mL) and hydroxylamine chloride (2 mg) for 30 min at 90 0C, and then acetic anhydride (0.8 mL) is added and heating is continued for 1 h.

3. Results and Discussion

The raw glycosidic mixture obtained as describes in Figure 3a, is processed using chromogenic reactions via Lieberman-Buchard and with Cobalt chloride protocols, showing the characteristic orange-reddish and violet colors, respectively, typical in the presence of triterpenes.

The study via FTIR of the obtained chromatographically pure glycoside with a melting point of 263–264 °C, revealed the presence of bands in the regions of functional groups: hydroxyl, lactone, and olefinic system, corroborating the glycosidic nature of isolated compound, specifically, 3445 cm^{-1}, 1745 cm^{-1}, 1648 cm^{-1}, 1590 cm^{-1}, 825 cm^{-1}.

In Figure 4, the fundamental ^{13}C-NMR assignments are represented, with analytical character, as well as the structure of the proposed triterpenic glycoside, to corroborate the presence of a lactone fragment (C-18/C-20), an olefinic bond (C-9/C-11), the presence of hydroxyl functional groups (C-12, C-17 y C-22) and a monosaccharide chain, via glycosidic bond in C3 composed of 4 unsubstituted components [25,26]. The -OH functional group in C-12 carries α configuration (RMN-^{1}H, δ 4,70 ppm, 1H J = 11–12 Hz).

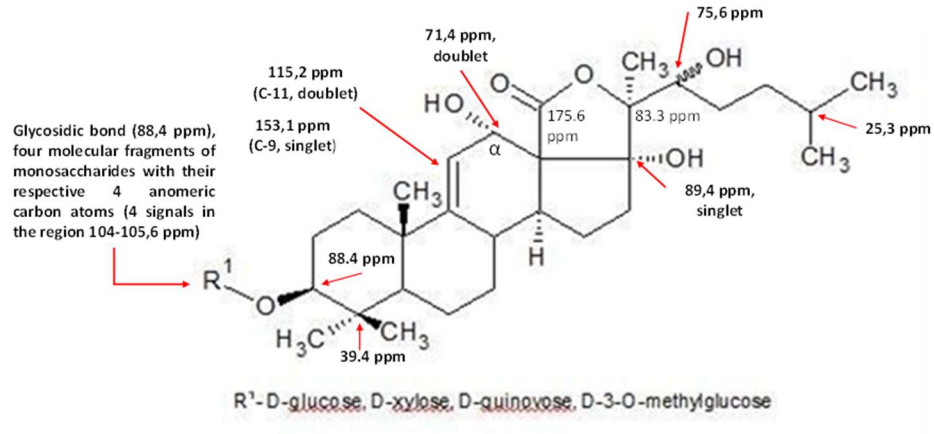

Figure 4. Triterpenic glycoside I isolated from *Holothuria floridana* inhabiting the Cuban archipelago.

In the NMR-1H spectrum, are observed signals, with analytical value, that corroborate the proposed structure for compound I represented in Figure 4. The signals (chemical shift and significance) are detailed in Table 1.

Table 1. 1H-NMR signals (ppm) and their analytical significance of the triterpenic glycoside I isolated from *Holothuria floridana* inhabiting the Cuban archipelago.

Chemical Shift (ppm)	Characteristic Proton
0.39	s, 3H, C-19
0.88	d, 6H, C-26/C-27
0.97	d, 1H, C-5, J = 10.2 Hz
1.07 y 1.26	s, 6H, C-30/C-31,
1.39	m, 1H, C-1
1.49	m, 2H, C-7
1.74	s, 3H, C-32
1.89	s, 3H, C-21
3.37	d, 1H, C-8
4.70	d, CH-O
5.63	m, 1H, =C-11

The glycoside obtained was subjected to a hydrolysis process in the presence of 12% hydrochloric acid, generating a sum of holothurinogenins which, subsequently, was subjected to a preparative column chromatography process, yielding 0.37 mg (1.85×10^{-4}%) of a compound with a melting point of 285–286 °C, which was identified via comparison with spectral data reported as a genin as represented in Figure 5 [27,28].

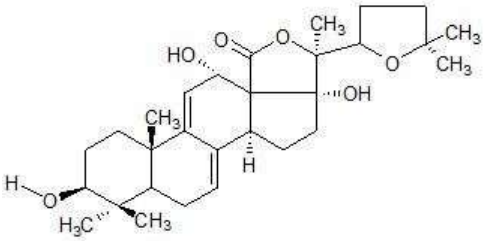

Figure 5. Genin II, obtained after treatment with 12% hydrochloric acid of compound I.

For corroborating the obtention of this genin derivative after hydrolysis in the presence of 12% hydrochloric acid, the data obtained from the ^{1}H-NMR study were used. The values of the signals and their significance are reported in Table 2.

Table 2. ^{1}H-NMR signals of genin II isolated from hydrolisis of triterpenic glycoside I isolated from *Holothuria floridana* inhabiting the Cuban archipelago.

Chemical Shift (ppm)	Characteristic Proton
0.90 and 1.02	s, 6H, C-30/C-31
0.94	d, 6H, C-26/C-27
1.09	s, 3H, C-19
1.15	m, 1H, C-5
1.19	s, 3H, C-32
1.36	s, 3H, C-21
1.49	m, 2H, C-7
2.06	m, 2H, C-2
2.81	m, 2H, C-1
3.23	m, 1H, C-3
3.26 y 3.86	m, CH-O
5.29 y 5.51	m, = C-7 y = C-11

During the acid hydrolysis of I, a mixture of monosaccharides is generated, which were analyzed by TLC with Silicagel plates impregnated with 0.04 M $CuSO_4$ and revealed with naphthoresorcin in an alcohol-sulfuric acid mixture, and by gas chromatography in the form of aldonitrile-peracetates [29,30].

The obtained data from specific rotation for a mixture of monosaccharides revealed a value of $[\alpha]\ D_{25}$ +39.0 indicating a D configuration for the described monosaccharide chain composed of D-glucose, D-xylose, D-quinovose, and 3-O-methyl-D-glucose in a ratio of 1:1:1:1

In conclusion, in the present communication, we report the preliminary study of the fraction enriched with triterpene glycosides isolated from the ethanol treatment of *Holothuria floridana* inhabiting the Cuban archipelago, recognition of the presence of triterpenic structure via chromogenic reactions, desalting by passing this fraction through Amberlite column, the subsequent elution with solvents of increasing polarity, and the isolation of its major component, whose aglycone is holost-Δ9,11-en-12α,17α,22ξ-triol and the monosaccharide fragment, obtained after treatment of the glycoside with 12% hydrochloric acid, is characterized by the presence of 4 components: 3β-D-glucose, D-xylose, D-quinovose and 3-O-methyl-D-glucose in its side chain. The compound II, is a genin of the holostanic series with a heteroannular dienic system $\Delta^{7,9(11)}$

A previous study on the chemical composition of triterpene glycosides of Holothuridae inhabiting the Caribbean Sea reveals the existence of similar molecular patterns for Compound I, which corroborates the results obtained for *Holothuria floridana*. In this context, it should be noted that in a report on Vietnamese holothurids, a genin, similar to compound **2**, is described under natural environmental conditions. [31–33]. More advanced structural studies (MS, NMR-ROESY, NOESY and HMBC) are currently being developed to corroborate the proposed structures and evaluation of biological activity.

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performance of the research, and in the interpretation of the results. In addition, they have critically reviewed the manuscript, approved the final version and consent to its publication

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships and do not have any potential conflict of interest. The Universities had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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