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Holothuria atra: An Underutilized Marine Resource for Nutritional and Collagen Benefits

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Authors' contributions

This work was carried out in collaboration among all authors. Authors NJJ and NDW designed the study, performed the study and wrote the first draft of the manuscript. Authors IW and VKG supervised the study and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To assess the nutritional content in the body wall of an underutilized sea cucumber species: *Holothuria atra* and to extract and characterize the potential collagen types from the body wall
Study Design: The specimens of *H.atra* were identified based on morphology and the nutritionally analyzed with respect to lipid profile, vitamins, minerals, and carbohydrates. *H. atra* body wall was used to extract collagen and later characterized.
Place and Duration of Study: Samples were collected at Mannar, Sri Lanka. Study was conducted at the Department of Zoology, University of Sri Jayewardenepura, Sri Lanka.

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Methodology: Nutritional content in the body wall (proximate analysis, lipid profile, vitamins, minerals, and carbohydrates) was analyzed using biochemical assays. Moreover, *H.atra* body wall was used to extract collagen, by acid soluble collagen extraction and characterized by physico-chemical methods.
Results: The results showed that body wall tissues of *H.atra* contained high moisture level (83.2%), proteins (10.2%) and low levels of fat (2.0%) and carbohydrates (2.1%). Further, flesh contains, 0.4% saturated fatty acids, 0.4% unsaturated fatty acids, considerable amount of Calcium, Magnesium and Sodium. Collagen yield from the body wall was recorded as 0.95% and fibrils observed as irregular and dense with a loose and porous structure. Attenuate Total Reflectance-Fourier Transform Infrared Spectroscopy and Ultra Violet Vis spectroscopy indicated the collagen was Type I.
Conclusion: Results suggest that underutilized, non-commercial *H.atra* is a potential nutritional source and it contains type I collagen. Further studies are underway to increase the collagen yield from *H.atra* and to develop a collagen membrane which can be used in future industrial applications.

Keywords: Holothuria atra; marine collagen; marine genetic resources.

1. INTRODUCTION

Holothuria atra (Lolly fish) is a non-commercial sea cucumber species available in Sri Lankan coast, especially along the North-West region [1]. It has long been consumed in many regions of the world as a potential biological source against aging, oxidation, inflammation, microbial activity, wounds etc [2]. *H.atra* flesh is high in protein and further, it contains significant levels of essential and non-essential amino acids [3]. Sea cucumbers are known to offer remarkable nutritional profile and have attracted the attention in both culinary and health perspective. Therefore, it is worthwhile to investigate full nutritional profile of non-commercial, underutilized sea cucumber species. Recent research related to isolating bioactive compounds such as collagen, flavonoids, phenolic components, terpenoids, saponins, alkaloids, acid mucopolysaccharide and triterpene glycoside from *H. atra* has shown sufficient evidence for unique industrial values of this species [2].

Being a promising option among "blue materials" which can substitute mammal-derived collagen, marine collagen has many applications in the fields of medicine, cosmetics, pharmaceuticals and food industries [4]. Further, they are rich in antioxidant properties, environmentally friendly extraction procedures, low molecular weight, minor regulatory and quality control problems, a negligible number of biological contaminants and toxins, low inflammatory response and excellent metabolic compatibility, free of infectious diseases, free of allergies and avoiding religious concerns [4]. Due to its unique structural properties, marine collagen is being used in food industry as additives, packaging material, dietary supplement, functional food, confectionery and desserts while used as a multifunctional biomaterial in tissue regeneration, wound dressing, etc [5]. Bioactive properties such as anti-aging and anti-wrinkling activities enable the use of collagen to formulate lotions and gels with high moisturizing action with UV protective properties that are important in cosmetic industry [5].

Sea cucumber-derived collagen fibrils frequently show symmetrical spindle shape and short length [6]. They are bipolar molecules with a surface associated with proteoglycans [6]. Covalent internal crosslinks are very similar to mammalian collagens yet permanent crosslinks are absent in the structure which facilitates isolation of collagen, avoiding mechanical damage. Moreover, absence of permanent cross links help to slide pass one another in shortening and lengthening process among fibrils [6]. Type I collagen is the most common and abundant collagen type in marine invertebrates with two equivalent α1 and one α2 polypeptide chains which composed of 1.1 × 300 nm size collagen molecules [7].

Full nutritional profile of sea cucumbers can be investigated using well established protocols. Collagen which is a potential protein of sea cucumbers can be extracted using conventional and novel methods. Characterization of extracted collagen is essential prior to investigating on commercial applications. However, present work aimed

to analyze the full nutritional profile of *H.atra,* underutilized sea cucumber species in Sri Lanka and obtain collagen to examine their physicochemical characteristics and structure. Investigations on collagens extracted from the sea cucumber are scanty, compared to other marine species. There is growing interest on the non-commercial marine species with respect to bio prospecting. Accordingly, the present study provides the basis for nutritional quality, extraction and characterization of collagens from *H.atra*.

2. MATERIALS AND METHODS

2.1 Sample Collection

Live specimen of *H.atra* were obtained commercially from Mannar, Sri Lanka and stored in ice. The viscera of sea cucumber samples were immediately removed and the body wall was diced and kept at −20^{O}C until further use.

2.2 Nutritional Composition Analysis of *H.atra*

2.2.1 Proximate composition

The diced tissue samples of *H.atra* were used to test proximate composition. The moisture, ash, protein and crude fiber content were determined according to the Pearson's composition and analysis of foods [8].

2.2.1.1 Moisture content:

Accurately, 1g of samples were incubated in pre-heated oven at 105 °C. The weight was measured after 3 hour of drying and the weight difference was calculated to obtain the moisture content.

2.2.1.2 Ash content:

Accurately, 1g of sample was incinerated in a muffle furnace at 550-600 ˚C for 24 hours. The resulted ash was weighed and the percentage was calculated.

2.2.1.3 Total protein content

The Kjeldahl method was performed according to method 981.10 of the AOAC [9]. Approximately 50mg of raw material was hydrolysed with 15 mL concentrated sulfuric acid (H_2SO_4) containing two copper catalyst tablets in a heat block (UDK 149 Automatic Kjeldahl Nitrogen Protein Analyzer) at 420◦C for 2h. After cooling, distilled water was added to the hydrolysates before neutralization and titration. The amount of total nitrogen in the raw materials were multiplied with the traditional conversion factor of 6.25.

Nitrogen (%) = [(standard acid volume - ml blank) x N of acid x 1.4007] / weight of sample (g)

Crude Protein (%) = Total N (%) x Conversion factor (6.25)

2.2.1.4 Total fat content:

Total fat content was determined using the Werner-Schmid method by Hill [10]. Briefly, 10g portion of the sample underwent digestion with HCl to facilitate the release of fat. Fat was subsequently extracted using petroleum ether. Following the evaporation of the ether, the remaining residue was weighed and calculate the fat content by using following equation.

% Fat in sample = (Weight of fat in sample / Weight of sample taken (g)) x 100

2.2.1.5 Carbohydrate content

Carbohydrate content was calculated by the difference of all other components measured [8].

%Total Carbohydrate = [100 − % (Protein + Fat + Moisture + Ash + Fiber)]

2.2.2 Lipid composition analysis

Determination of saturated, Monosaturated, and Poly Unsaturated Fatty Acids (PUFA) with Eicosapentaenoic acid (EPA), and Docosahexaenoic acid (DHA), content of the *H.atra* flesh was analyzed with a Gas chromatograph [11] (GCMS-TQ GC Headspace/Autosampler Trace 1300 Thermo scientific, Industrial Technology institute, Sri Lanka). Cholesterol content was measured by following the method of Association of Official Analytical Chemists (AOAC) 994.10 [12] with a Gas chromatograph (GCMS-TQ GC Headspace/Autosampler Trace 1300 Thermo scientific, Industrial Technology Institute, Sri Lanka).

2.2.3 Vitamin analysis

The concentration of vitamin A was determined using analytical high-performance liquid chromatography (HPLC) (Methanol: acetonitrile 50:50 phase) following AOAC 2001.13 [13] method and Vitamin C was detected following the method AOAC 2012.22 [14] at the Industrial Technology Institute (ITI), Colombo, Sri Lanka.

2.2.4 Mineral composition

Minerals; Calcium (Ca), Magnesium (Mg), Potassium (K), Sodium (Na), Zinc (Zn), Selenium (Se), Copper (Cu), Iron (Fe), and Manganese (Mn) were analysed by Inductively Coupled Plasma Mass Spectroscopy (ICP-MS). Briefly, 0.5g of samples were digested with conc. HNO_3 (analytical grade) at 200^0C for 20 minutes followed by filtering and detection by ICP- Mass Spectrometer (Agilent 7900 ICP-MS, Residual analysis laboratory, Industrial Technology Institute, Sri Lanka).

2.3 Extraction of Collagen *H. atra* Body Wall

Collagen extraction was carried out according to the method described by Yuniati and Sulardiano [15] with slight modifications. Briefly, coarsely ground sea cucumber samples (Panasonic AC300, Japan) incubated in distilled water (1: 10 w /v) for 30 minutes with stirring. In the pre-treatment process, samples were mixed and stirred with 50% alcohol (1: 2 w/v) for 30 minutes, washed with distilled water until the pH was neutral. Samples were immersed in HCl 0.1 M and 4 mM EDTA (1:10 w/v) for 24 hours stirring continuously to maintain stable pH and reduce minerals. Followed by washing with distilled water, to eliminate non-collagenous proteins, samples were added to 0.1M NaOH solution (1:10 w/v) and kept for 2 days, changing the NaOH solution in every 24 hours. Samples were thoroughly rinsed with cold distilled water until the rinsed water become neutral (pH 7.0). Remaining pellets were subjected to isolation of collagen by incubating in 0.5 M acetic acid (1:10 w/v) for 48 hours with continuous string. Supernatant was separated by centrifugation (HERMLE Z306) at 6000 rpm for 20 minutes. The pellets were re-extracted with 0.5 M acetic acid for 24 hours, both supernatants were pooled, later NaCl was added to salt out collagen until the NaCl final concentration of the supernatant reaches 1 M. Precipitated collagen were was separated by centrifugation at 2000g for 15 minutes and freeze-dried (ilShin BioBase FDS8512 freeze dryer).

2.4 Calculation of Yield of ACID Soluble Collagen (ASC)

The yield of extracted ASC was calculated based on the dry weight of starting material as per following equation,

Yield (%) = (Weight of lyophilized collagen (g) / Weight of initial dry sample (g)) x 100

2.5 Physico-Chemical Characterization of Collagen

2.5.1 Moisture content

The moisture content was determined as per the methodology by the Association of Official Analytical Chemists [16] by drying 1g of ASC at 105 °C until a constant weight was obtained. The moisture content was calculated based on the weight difference.

2.5.2 pH

Approximately 1g of collagen was dissolved in 70ml of double distilled water and the pH was measured using a pH meter (Consort C6010 Multi-parameter analyzer) [15].

2.5.3 Attenuate Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ASC samples were subjected to ATR-FTIR spectroscopy using FTIR spectrometer (Bruker Alpha FT-IR spectrometer, Department of Material Science, University of Moratuwa). The spectra in the range of 600-4000 cm^{-1} with automatic signal gain were collected in 16 scans at a resolution of $4cm^{-1}$ and were rationed against a background spectrum recorded from the clean empty cell at 25 °C. Analysis of spectral data was carried out using OMNIC Specta^{TM} data collection software program.

2.5.4 Ultra Violet (UV) –Vis absorption spectra analysis

Collagen solution (5mg/mL) was prepared by dissolving collagen in 0.5 M acetic acid. Baseline was set with 0.5 M acetic acid. UV-Vis absorption spectra of ASC samples (Model Thermo Scientific GENESYS 10S Series UV-Vis spectrophotometer, Instrument Centre, University of Sri Jayewardenepura,Sri Lanka) in the range of 190-600 nm.

2.5.5 Visualization of collagen under Scanning Electron Microscopy (SEM)

The internal fibre architecture of extracted collagen was observed under SEM (CARL ZEISS EVO 18 Scanning Electron Microscope, Department of Material Sciences, University of Moratuwa, Sri Lanka).

3. RESULTS AND DISCUSSION

3.1 Nutritional Composition of *H.atra*

3.1.1 Proximate analysis

According to this study, proximate analysis was carried out for fresh sea cucumber samples collected from Mannar, Sri Lanka which recorded for the first time. The moisture amount of food component which is a known parameter is an index of its water activity [17]. Fresh body walls of *H.atra* resulted higher moisture as 83.2% and found to be in accordance with the previous work with several sea cucumber species, such as *Holothuria scabra* (81.66%), *Holothuria spinifera* (80.48%) *Bohadschia sp.* (86.48%), *Bohadschia marmorata* (84.65%) (Nishanthan *et al.*, 2018) and *Holothuria leucospilota* (84.52%) [18]. The moisture content can be influenced with environmental, geographical variations, behaviour, feeding and the collection time of the year [3]. However, relatively large amount of moisture indicates the potentiality of concentrating the nutrients through loss of water or dehydration which is important to increase the shelf-life when commercializing [19].

Ash content may depend on the mineral deposit and other organic matter in flesh which get influenced by the environment and species [20]. The ash content of *H.atra* flesh was measured as 2.5% indicating relatively low ash content than other sea cucumber species reported such as, *Holothuria tubulosa* (5.13%), *Holothuria polii* (7.85%) and *Holothuria mammata* (5.13%) by Aydın et al. [21].

The protein content is important for quality and texture of the muscle of aquatic animals. The protein content of the *H.atra* flesh was recorded as 10.2% based on the weight indicating a higher value compared to other species as *H. polii* (8.66±1.2%), *H. tubulosa* (8.82±0.30%) and *H. mammata* (7.88±0.3%) by Aydın et al. [21].

Table 1. Proximate analysis (% wet weight basis) of flesh of *H. atra.*

Parameter	Weight (%)
Moisture Content	83.2
Ash content	2.5
Protein Content	10.2
Crude Fibre Content	0.2
Fat Content	2.0
Carbohydrate Content	2.1

It was observed that body wall of *H.atra* has 0.2% crude fibre and 2.0% fat content shows that sea cucumbers tend to have low content of lipid. The fat content of sea cucumbers might be influenced with several factors such as species, reproduction, food availability, feeding pattern and environmental conditions while it tends to be higher in constant temperatures than fluctuating temperatures. However, fat is an important factor to be considered nutritionally due to its importance as an energy source and relevance for many other important functions in the human body [22]. Reported Carbohydrate content (2.1%) of the study was higher than several previous studies (0.86% in *Paracaudina australis*) [23], while the reported energy value of *H. atra* was 67 kcal/100g. This suggests that *H.atra* is a good source of energy.

3.1.2 Lipid profile analysis of *H.atra*

Sea cucumbers are believed to contain bioactive substances that assist in many physiological processes such as wound healing as they feed on bottom sediments enriched with branched chain fatty acids [24]. Therefore, the fatty acids composition of sea cucumbers, especially PUFA will be of huge concerned. Based on the research, total of Saturated Fatty Acid (SFA) is moreover in similar range with the total of Monosaturated Fatty Acid (MUFA) and total of Poly Unsaturated Fatty Acid (PUFA). As per the previous studies, all most of sea cucumber have shown higher amount of saturated fatty acid and Monosaturated fatty acid and lower content of Poly Unsaturated Fatty Acid [25]. This results fit with Ridzwan et al. [25] which showed that saturated fatty acids were found dominated in *H. scabra, H. leucospilota* and *H. atra* as well. Further, the recorded low EPA value (0.05g/100g) and absence of DHA are similarly recorded in the previous study of 4 sea cucumber species; *S. horrens, H. leucospilota, H. atra and H. scabra* [25].

Table 2. Fatty acid profile of flesh of *H. atra*

Parameter	Amount
Saturated fatty acid (g/100g)	0.4
Monosaturated fatty acid (g/100g)	0.3
Polyunsaturated fatty acid (g/100g)	0.1
Eicosapentaenoic acid (EPA) (g/100g)	0.05
Docosahexaenoic acid (DHA) (g/100g)	Not detected
Cholesterol content (mg/100g)	1.1

3.1.3 Vitamin (Vitamin A and C) analysis

Vitamin A and C were not detected in *H.atra.* Nevertheless, Vitamin C (3.19 mg/100g) has been detected in *H. scabra* which belong to the same genus [26]. Nutrient level solely depends on environmental factors, season, location, type and size of the sea cucumber species.

3.1.4 Mineral analysis

Evaluation of mineral composition of *H.atra* is presented in Table 3. It has been reported in various studies that sea cucumbers are also a source of minerals, in general, composition is influenced by a number of factors such as physiological factors, environmental conditions, habitat and life cycle [27]. Sodium (Na) was the largest in the composition followed by Calcium (Ca), Magnesium (Mg), Potassium (K), Zinc (Zn), Iron (Fe), Copper (Cu), Selenium (Se) and Manganese (Mn).

Table 3. Mineral composition of *H. atra* flesh

Mineral	Amount (mg/kg)
Calcium (Ca)	1200
Magnesium (Mg)	1100
Sodium (Na)	4200
Potassium (K)	629
Zinc (Zn)	5.9
Selenium (Se)	2.1
Copper (Cu)	4.0
Iron (Fe)	5.3
Manganese (Mn)	1.7

3.2 Yield of ASC

Acid-Solubilized Collagen (ASC) extraction for the body wall of *H.atra,* resulted a white colour cotton wool like solid substance with a yield of 0.95% based on the dry weight. This is higher than the yield recorded (0.88%) in the previous study conducted on the same species by Yuniati and Sulardiono [15]. A lower yield can be resulted due to covalent crosslinking in the peptide region, thus reducing the collagen solubility, while the level of collagen yield can be increased after peptide digestion using pepsin enzyme while improving the collagen extraction ability [28].

3.3 Physico-Chemical Characterization of Collagen

3.3.1 Moisture content and pH

The moisture content of the collagen from *H.atra* was measured as 7.246%. The affinity of collagen and water is highly affected by collagen organization [29]. Further, the pH value plays a crucial role in formulation of collagen, especially for commercialization purposes [30]. The pH of the *H. atra* in the current study was measured as 3.95. This might be influenced by the acid concentration used in the extraction. A similar result has been reported in a previous study by [31], which used PSC Pepsin Soluble Collagen) extraction method. As per previous studies, the best pH range of commercial collagen for cosmetics was reported between 3.8 to 4.7 [30].

3.3.2 Attenuate total reflectance-fourier transform infrared spectroscopy (ATR-FTIR)

The FTIR spectra for extracted collagen sample from *H.atra* is illustrated in the Fig. 1. Major functional groups of collagens were identified in the extracted *H. atra* collagen sample and confirmed by the presence of characteristic bands, amide A (3282.60cm^{-1}), amide B (2900-3080cm^{-1}), amide I (1631.75cm^{-1}), amide II (1330-1545cm^{-1}), amide III (1075-1240cm^{-1}). Amide A band is resulted from stretching vibrations of N–H group. The range 3400 to 3440 cm^{-1} is commonly attributed to the N–H stretching vibration and when the N–H stretching is involved with hydrogen bonds, the amide A peak is shifted to lower wavenumber [32]. Therefore, the resulted peak of amide A close to 3300 cm^{-1} in this study indicates presence of H-bonds in the N–H stretching of the extracted collagen sample. The asymmetrical shape of the amide A peak

observed further reveals presence of some amount of water in the collagen sample. As per Susi and Ard [33], absorption band for bound water should be overlapped with amide A illustrating a low-frequency shift of ~100 cm^{-1} for the H_2O absorption band. The observed peaks of Amide B were identified and attributed at 3074.49 cm^{-1} for CH_2 asymmetric stretching and at 2931.85 cm^{-1} for CH_2 symmetric stretching which were reported by Abe and Krimm [34] in the similar range. The peak appearing at 1631.75 cm^{-1} was assigned to amide I band which is associated with stretching vibrations of the carbonyl group (C = O bond) along the backbone of the polypeptide chain. The amide I peak is usually found in the range from 1600 to 1700 cm^{-1} and the lower side shifted frequency observed was due to the high number of H-bonds of the carbonyls of an amide group [32].

Amide II peak was observed at 1545.04 cm^{-1} was attributed to the N-H bending vibration strongly coupled to the C-N stretching vibration of protein amide groups which are usually observed in the range 1530-1550 cm^{-1} [35]. Amide II minor bands at lower frequencies were also resulted in the study. The peak at 1449.81cm^{-1} was attributed to CH_2 bending and the peak at 1385.50 cm^{-1} was derived from COO-symmetrical stretching. The peak at 1337.94 cm^{-1} was observed was attributed to the wagging vibration of the proline side chains found in the type I collagen of body tissues [35]. Amide III peaks attributed to the N-H bending coupled with C-N stretching and C-O stretching were detected at 1237.00 cm^{-1} and 1075.82 cm^{-1} respectively. Moreover, functional group analysis with FTIR confirm the preserved triple helix structure in the extracted collagen sample from *H.atra* with amide III/II ratio of 0.85 which is approximately equal to 1.0. The similar results were reported previously for *Stichopus japonicas* and *Holothuria parva* [28].

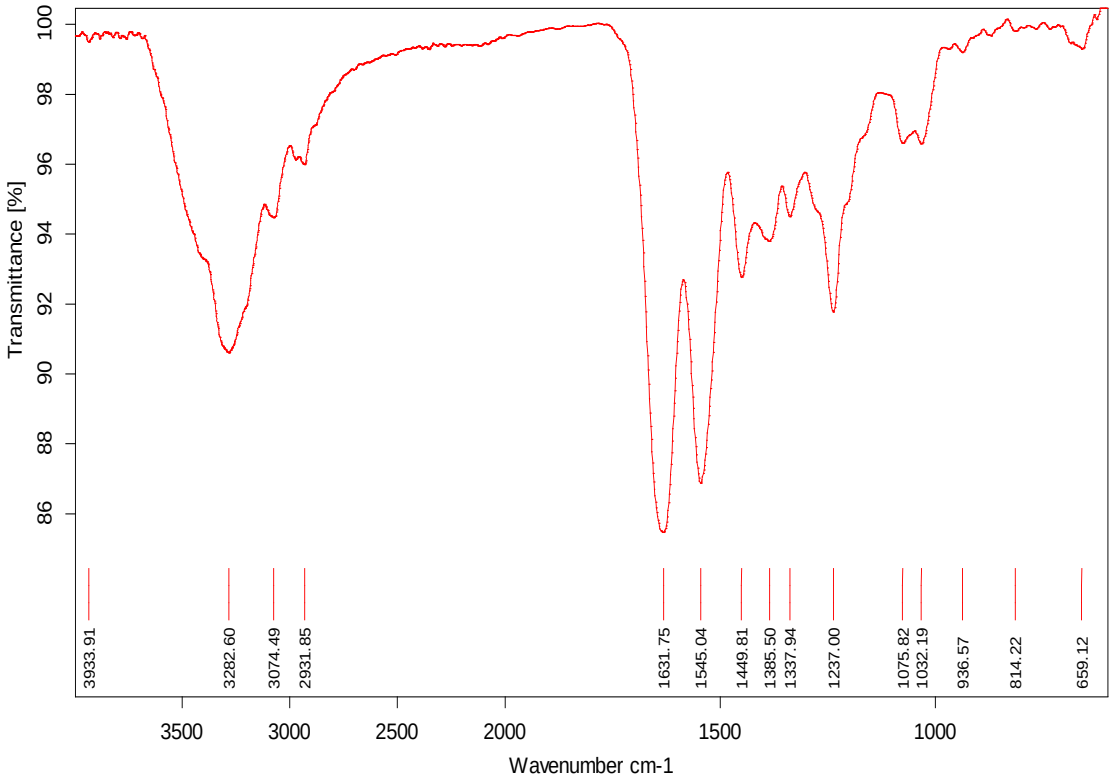

Fig. 1. The FTIR spectrum of acid soluble collagens from *H.atra* (Model Bruker Alpha FT-IR spectrometer, Department of Material Science, University of Moratuwa, Sri Lanka)

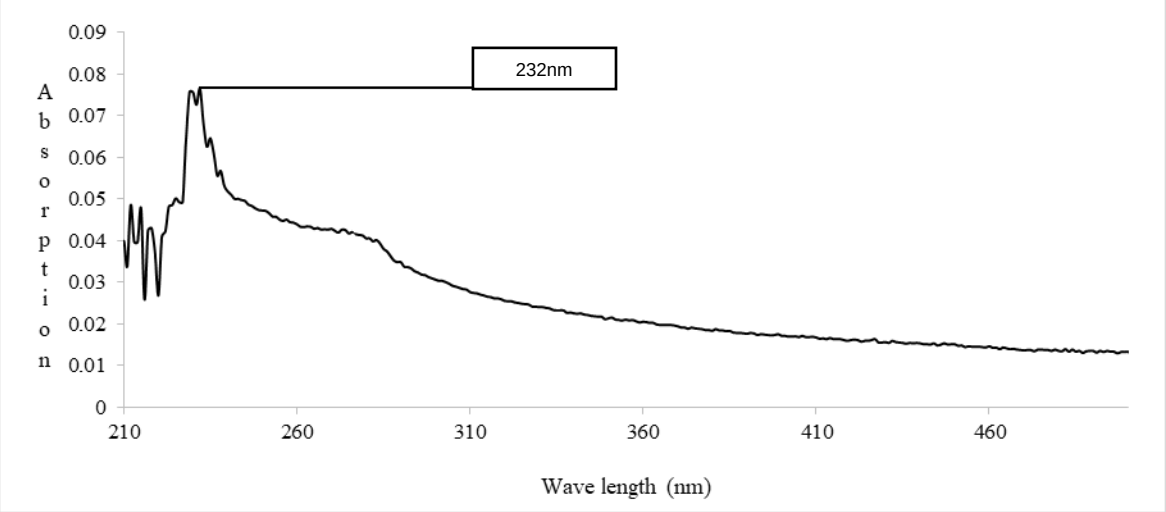

Fig. 2. The ultraviolet absorption spectrum of ACS from *H.atra* (Model Thermo Scientific GENESYS 10S Series UV-Vis spectrophotometer, Instrumental Centre, University of Sri Jayewardenepura, Sri Lanka)

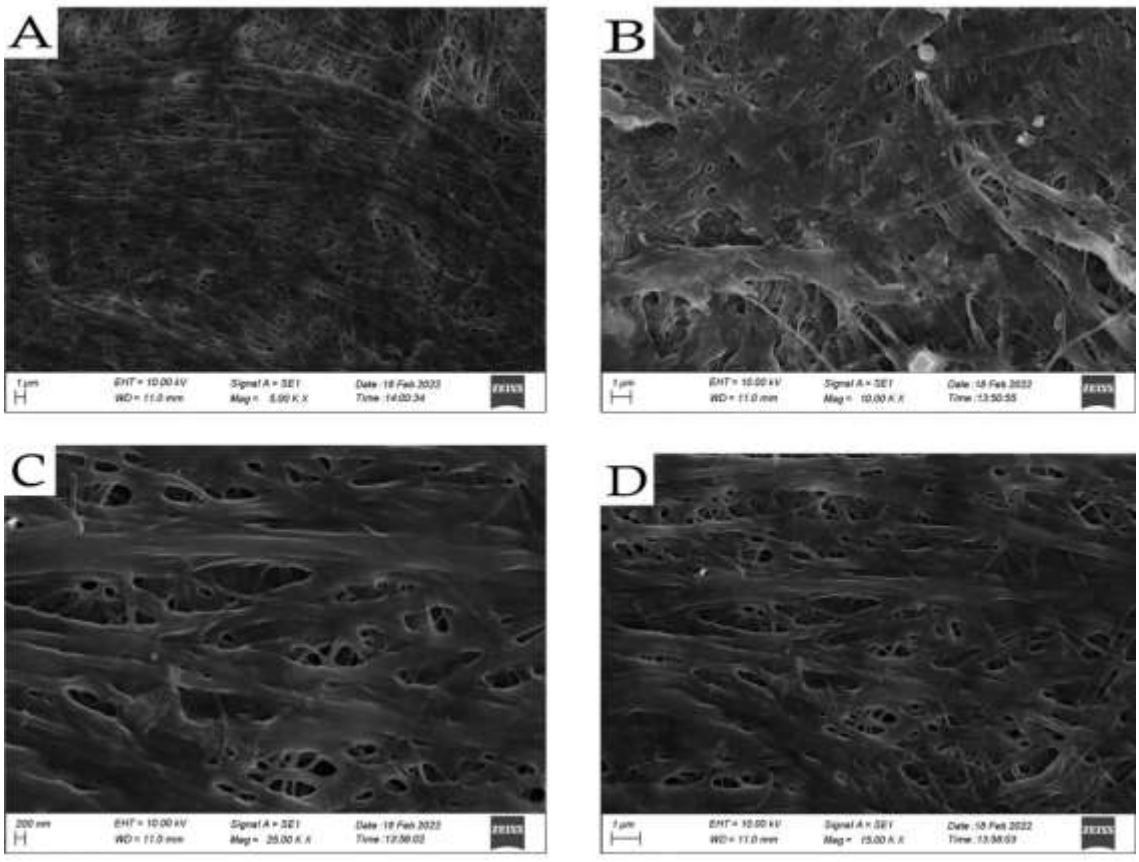

Fig. 3. SEM images of ASC from *H.atra*. (A)5k x; (B)10k x; (C)25k x (D)15k x (CARL ZEISS EVO 18Scanning Electron Microscope, Department of material sciences, University of Moratuwa, Sri Lanka)

3.3.3 UV–Vis absorption spectra analysis

The UV-Vis spectrophotometric analysis was conducted to determine collagen's type and purity based on the absorption at a pre-identified wavelength. The UV-Vis spectra of extracted collagen from *H.atra*. Figure 2 exhibited a maximum absorbance at 232 nm. As per the observed maximum absorbance at 232 nm, identified the presence of type 1 collagen in the tested sample. Further, the absorbance peaks observed between 200-220 nm were attributed to the collagen peptide bonds. The aromatic side chains contained in collagen absorb light in the 240-300 nm region resulting several peaks at 280nm (tryptophan), 275nm (tyrosine), 258nm (histidine, and phenylalanine) [31]. However, no obvious absorption observed at around 250-280 nm indicating the fact that the extracted collagen is in high purity.

3.3.4 Scanning Electron Microscopy (SEM) analysis

The scanning electron microscope (SEM) images of collagens from *H.atra* is given in Figure 3. Bundles of circumference fibrils networks, having irregular and dense pleated appearance with a loose and porous structure are prominent in collagen extracted from *H. atra* and in accordance with the description by Saallah et al. [31].

4. CONCLUSION

This study present nutrition profile and collagen characterization of *H.atra* body wall collected from North-west coast, Sri Lanka for the first time. It is rich with various nutrients. Further, Collagen was extracted from *H. atra* body wall using the acid base extraction method which can be considered as a low yield. However, the collagen was characterized as type I collagen with a unique structure. Application of collagen from *H.atra* in industry is questionable due to the low yield thus an alternative extraction method or development of a product with low yield is required in the future directions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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