

Tributylamine Facilitated Separations of Fucosylated Chondroitin Sulfate (FuCS) by High Performance Liquid Chromatography (HPLC) into its Component Using 1-Phenyl-3-Methyl-5-Pyrazolone (PMP) Derivatization

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Abstract

Monosaccharide characterization study can give valuable information on newly discovered intact mucopolysaccharide. The compound fucosylated chondroitin sulfate (FuCS) is a unique mucopolysaccharide having various bioactive properties reported only in targeted holothurians species. The monosaccharide composition of FuCS from a sea cucumber, *Holothuria arenicola* was studied by comparing a modified and conventional 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization method where tributylamine addition was found to improve the chromatographic conditions. The monosaccharide proportion of FuCS from *H. arenicola* was determined to be 1:1:0.44 for glucuronic acid: N-acetylgalactosamine: fucose, respectively. Thus, the FuCS monosaccharide ratio of *H. arenicola* was first reported and this method is more useful for structurally similar compound analysis.

Keywords: Fucosylated chondroitin sulfate; Monosaccharide; 1-phenyl-3-methyl-5-pyrazolone (PMP); Tributylamine; HPLC

Introduction

Fucosylated chondroitin sulfate is an unusual compound having various physicochemical activity found in sea cucumbers [1]. The mucopolysaccharide consists of a common chondroitin sulfate chain with the β -D-glucuronic acid moiety O-substituted with sulfated fucosyl at the carbon-3 position [2]. The hydrolysis sequence of this mucopolysaccharide into its component starts by removal of sulfate esters at the vicinity of fucose branch followed by fucose release and sulfates in the chondroitin backbone. After that, the chondroitin backbone itself is hydrolyzed into glucuronic acid and acetylgalactosamine. Anticoagulation activity is one of most studied bioactivity expressed by this compound and the structural configuration is a determining factor for various physicochemical properties. Apart from that, the derived components are molecules having novelty pharmaceutical benefit. For example, glucuronic acid or its reduced form, glucuronolactone are natural antioxidant having hepatoprotective function [3], whereas, sulfated fucose are known anticancer agent [4].

Analyzing glycosaminoglycans is challenging due to their polydispersity, sequence heterogeneity and high negative charge density [5]. Monosaccharide composition is often characterized to understand the function of intact carbohydrate chain but saccharides are naturally low in UV absorbency. Therefore, derivatization is often required to label the compound with chromophore tag that allows ionic property changes to promote the desired separation conditions. 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization is particularly well documented for carbohydrates separation using HPLC since PMP derived carbohydrates are more hydrophobic and thus better retained in the reverse phase material. Separation techniques are involving the use of PMP for improving with the current knowledge that ion-pairing reagent facilitates carbohydrates derivatization. To the best of our knowledge, there is currently no reported on the use of ion-pairing techniques in the derivatization steps for determination of FuCS monosaccharide composition from sea cucumbers. Here, in this research, we were described the FuCS monosaccharide composition from a sea cucumber, *Holothuria arenicola* and the efficacy of using tributylamine facilitated derivatization strategy.

Materials and Methods

Materials

D-Glucuronic acid, N-Acetyl-D-Galactosamine, L-fucose, 1-phenyl-3-methyl-5-pyrazolone, trifluoroacetic acid were purchased from Sigma. Extraction solvents, methanol, n-hexane, acetonitrile, ethyl-acetate, chloroform and (HPLC grade) were purchased from Merck (USA). All other analytical grade reagents were available commercially.

Preparation of fucosylated chondroitin sulfate from sea cucumber

FuCS was extracted from the body wall of sea cucumber *Holothuria arenicola* harvested in Semporna, Sabah, Malaysia (mean weight 60 g) using a modified method from Mourão et al. [6]. Briefly, the sea cucumber was eviscerated and cleaned under running tap water to remove visible impurities. The body wall was carefully cut to separate from other tissue and homogenized. The homogenate was treated with acetone to dehydrate for 24 h. The dried residue was then digested with papain in a 0.1 M sodium acetate buffer solution (pH 6) containing 5 mM EDTA and 5 mM cysteine at 60°C for 24 h to remove proteins in the sample. The supernatant was collected, precipitated with 5% cetylpyridinium chloride solution and kept in room temperature. The clear precipitate was collected with centrifugation and the resultant

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pellet was further precipitate first with 95% ethanol and washed twice with 80% isopropanol. The final precipitate was dialyzed against distilled water at room temperature using Spectra/Por membranes (MWCO 1000) for 3 buffer changes (2h; 6h; overnight) and finally lyophilize. The crude extract was fractionated on a FPLC systems (AkTAPRime) equipped with a DEAE Sephadex A-50 column with elution gradient from 0.2 to 2 M NaCl buffer (pH 7). Aliquots of each collected fractions were analyzed by phenol-sulfuric assay in microplate format adapted from Masuko et al. [7]. Positive fractions were lyophilized and afforded 5.7% FuCS extract.

Monosaccharide analysis

Purified FuCS (~1 mg) was hydrolyzed with 2 M TFA (1 ml) at 110°C for 4 h. The hydrolyzate was dried under a stream of dry nitrogen gas to remove TFA and was derivatized with PMP according to the following conventional and modified method.

Conventional PMP derivatization method

The dried hydrolyzate was derivatized with PMP based on Honda et al. [8]. Briefly, 1 mg of TFA hydrolyzed samples were added into a 2 ml reaction vial containing 100 µl of 0.3 mol/L methanolic PMP solution and subsequently added 50 µl of 0.3 mol/L NaOH. The mixture was left to react at 60°C for 60 min. After cooling to ambient temperature, the mixture was neutralized with 50 µl of 0.3 mol/L of HCl and diluted to a final volume of 1 ml. Excess PMP reagent was removed with three times 1 ml chloroform extraction. The final aqueous layer was diluted with deionized HPLC grade water prior to HPLC analysis.

Modified PMP derivatization method

Derivatization procedure was adapted from Zhang et al. [9] and replacement used NaOH with 50 µl tributylamine as the catalyst. Without NaOH, the neutralization step with HCl was omitted. The mixture (sample + tributylamine + methanolic PMP) was vortexed and left to react in a thermomixer for 30 min at 60°C with 300 rpm agitation. The mixture was then centrifuged at 6000 rpm for 10 min upon completely reaction and formed two-layers. The aqueous, lower portion was made to 1 ml. The upper organic layer consisted of tributylamine with excess PMP reagent removing by extraction with three times of equal volume of chloroform. The final aqueous layer was diluted for final concentration prior to HPLC analysis.

HPLC analysis

The PMP derivatized samples ($n=3$), individual sugar standards ($n=3$) and mix sugar standards ($n=3$) were carried out on Waters HPLC system equipped with a model 1515 quaternary pump, a model 2717W autosampler and a 2487 dual UV wavelength detector. The column (Agilent, Zorbax Eclipse XDB-C18, 250 × 4.5 mm) was optimized for PMP separations on Breeze I HPLC system software. The flow rate was set constant at 1 ml/min. Mobile phase A and B were consisted of water and acetonitrile; used with a gradient run of 10 to 90% B in 18 min and hold for another 3 min during run. The injection volume was set to 20 µl. Single wavelength at 245 nm was used for data acquisition with a sampling rate of 2, 2.0 AUFS and 0.1 times constant. Each samples analysis was done in triplicates.

Sample preparation for GCMS analysis

A confirmation study was done using Gas Chromatography Mass Spectrometry (GCMS) for identification of monosaccharide composition using the *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatization method. The hydrolyzed syrup was subjected to

silica gel (60-200 µm mesh size) column chromatography purification using hexane (20 ml), methanol (20 ml) and acetonitrile:water (20 ml, 1:1, v/v) fractions. Fractions were collected and dried using reduced pressure and redissolve with 50 µl of BSTFA reagent. The mixture was incubated at 60°C for 30 min in a heating block and subsequently purge with a constant nitrogen stream to remove excess reagent. The derivatives were made for the final dilutions using acetonitrile before injecting into GCMS.

GCMS analysis

The prepared polar fractions (methanol and ACN:water, $n=3$) were injected into GCMS system consisting of an Agilent 7890A gas chromatograph system tandem with an Agilent 5975C mass spectrometry detector. Separation was done using capillary column HP-5MS (30 m × 0.25 mm) of 0.25 µm coated film thickness in splitless mode. Injector temperature was adjusted at 250°C and the oven temperature ramp settings were as follow: initial temperature was held constant for 3 min at 156°C and ramp to 180°C in 25 min at a rate of 1°C/min. The flow rate used was a constant 1 ml/min of high purity helium gas (99.9% pure) as the carrier gas. Compound identification was done with matching scan spectral from National Institute of Standards and Technology (NIST) library and the compositions were computed with reference to the abundance of the compounds in chromatogram.

Results and Discussion

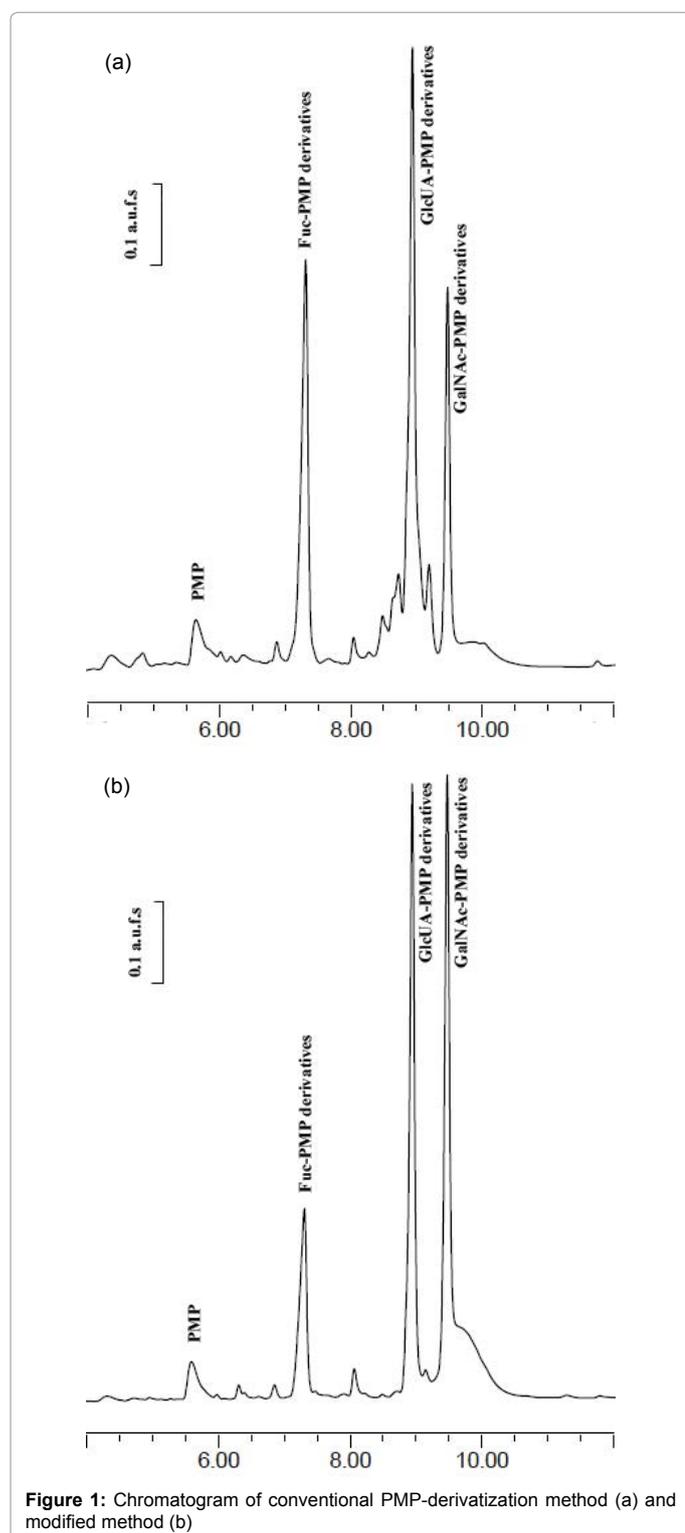
HPLC analysis of monosaccharide composition

The study objective was to investigate the influence of tributylamine on separation of FuCS monosaccharide composition and thus conventional and modified derivatization methods were compared with mix sugar standards. Figure 1a and b were the chromatograms for conventional derivatization method and modified method respectively on derived FuCS monosaccharide composition. The three main monosaccharide retention time were successfully separated among each other as expected. Each of the derivatization peaks were matched with standard monosaccharide derived using the same derivatization manner (Figure 2) and the order of separations were fucose (Fuc), glucuronic acid (GlcUA) and *N*-acetyl-galactosamine (GalNAc).

Conventional derivatization method gave ubiquitous spurious peaks probably due to the organic salt formed by addition of sodium hydroxide and during hydrochloric acid neutralization. Zhang et al. [9] demonstrated the addition of triethylamine can facilitate reaction to completeness in a mixture of sugar standards. In our conventional method, GalNAc-PMP derivative had lower peak intensity than GlcUA-PMP. We noticed that the concentration of NaOH was not sufficiently high to facilitate complete reaction and thus have the most obvious decrease in average peak area of 28.13% relative to GlcUA-PMP. The relative standard deviation for reproducibility was calculated to be 2.83% which was acceptable range while stability of derivatized samples when monitored within a 48 h frame at 8 h interval. Peak area was satisfactory with a decrease of 3.87% and 11.14% at 24 h and 48 h, respectively.

The FuCS structure is common backbone of chondroitin sulfate reported by several authors [6,10-12]. In fact, previous studies on similar compound found almost same amounts of GlcUA and GalNAc with different molar range of Fuc and sulfate [13]. Hence, we were presented the monosaccharide proportion of *H. arenicola* FuCS to be 1.0:1.0: 0.44 (Table 1) based on the modified method.

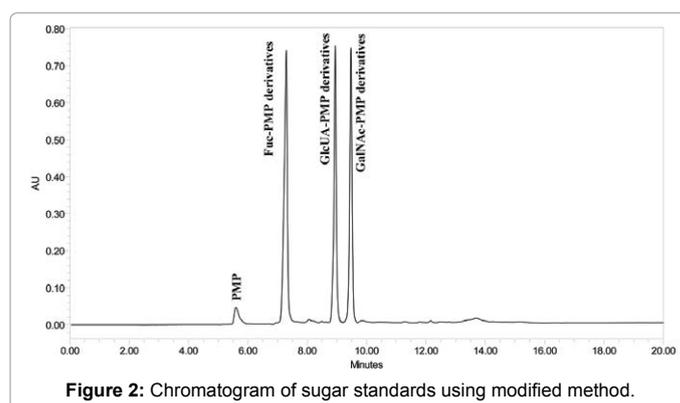
Zhang et al. had successfully determined several types of



monosaccharides using triethylamine as a catalyst during PMP derivatization [9]. The basis of choosing tributylamine in this experiment is that longer alkyl chain length showed better compatibility with on-column retention of saccharides for a typical C18 type stationary phase [5]. Furthermore, longer alkyl chain was presumably better catalyst during PMP derivatization since longer alkyl chain length can give a higher pKa at 10.89 compared to 10.78

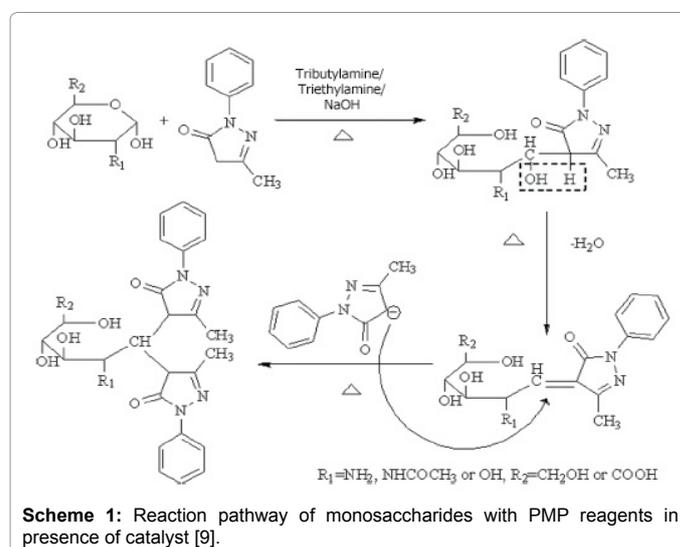
for triethylamine which eventually affects by charge delocalization. Therefore, tributylamine is more readily to accept the protons from the deprotonated monosaccharide species. The ability of the deprotonated hydronium ions was stabilized by tributylamine result in a rapid formation of ducing region which is essential for the PMP reagent to bind (Scheme 1).

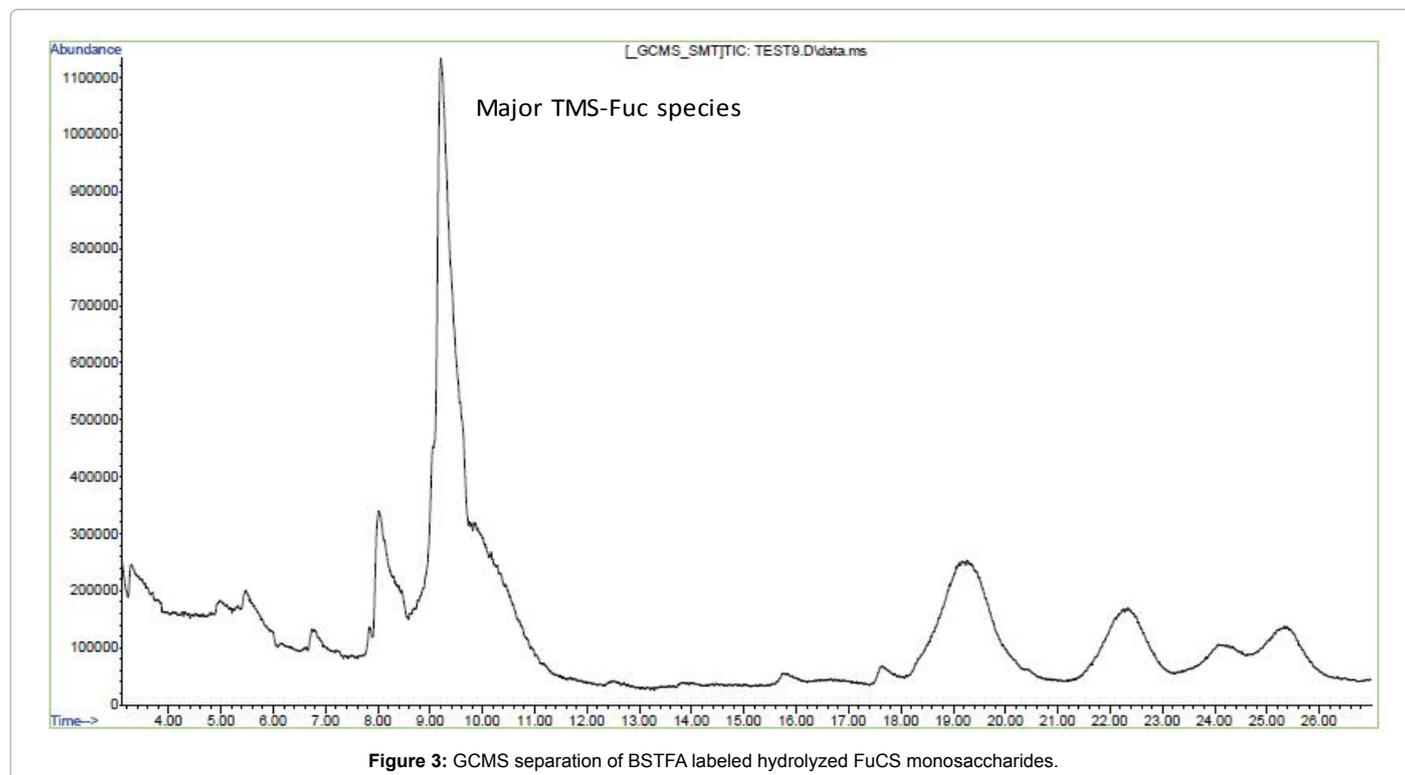
To give a better confidence on the monosaccharide composition of purified FuCS, our research group determined the monosaccharide composition using GCMS. Methanol fraction afforded most of the sugar component while the ACN:water fraction has little to trace amount of other sugar species. The hexane fraction consisting of non-polar compounds in trace amount is presumably impurities and byproducts produced during TFA hydrolysis. The main monosaccharide component was identified from HPLC analysis and also presence in the methanol fraction, however, not clear separation (Figure 3) when the use of a semi-polar stationary phase column, HP-5MS. The column used is not specific to carbohydrate analysis and the separation is solely based on the oven temperature, where the volatility of the compounds is reflected in the retention time. Failing the distinct separation by using non-specific column is due to the compounds nature, as the interested



	GlcUA	GalNAc	Fuc
conventional	1.00	0.48	0.69
modified	1.00	1.00	0.44

Table 1: Molar ratio of FuCS monosaccharide component from conventional and modified derivatization method





compounds (GlcUA, GalNAc, and Fuc) are homological similar. Thus, the obtained total ion chromatograms from GCMS were further tested by using deconvolution strategy for determination its separation integrity. Derivatized fucose with trimethylsilyl was seen clustering in unresolved peaks within retention time 7.846 to 9.314 while derivatized galactosamine signal appear at later retention time within 12.578 to 13.452. Derivatized glucuronic was not observed in methanol fraction but appear within a peak range of retention time 15.770 to 16.312.

Conclusions

The present study using a modified PMP-derivatization with addition of tributylamine is a robust method to determine hydrolyzed FuCS component. The improved chromatographic properties are allowed good separation on main monosaccharide composition of FuCS from *H. arenicola*. Nevertheless, it is tentatively proposed that non-commercial sea cucumber species can be served as a source of FuCS compound for potential pharmaceutical industry.

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References

1. Yamada S, Sugahara K, Ozbek S (2011) Evolution of glycosaminoglycan. *Communicative and Integrative Biology* 4: 150–158.
2. Pomin V (2014) Holothurian fucosylated chondroitin sulfate. *Marine Drugs* 12: 232–254.
3. Vīna I, Linde R, Patetko A, Semjonovs P (2013) Glucuronic acid from fermented beverages: biochemical functions in humans and its role in health protection. *IJRRAS* 14: 217-230.
4. Hung LD, Sato Y, Hori K (2011) High-mannose N-glycan-specific lectin from the red alga *Kappaphycus striatum* (Carrageenophyte). *Phytochemistry* 72: 855-861.
5. Volpi N, Linhardt R (2010) High-performance liquid chromatography- mass spectrometry for mapping and sequencing glycosaminoglycan-derived oligosaccharides. *Nat Protoc* 5: 993-1004
6. Mourão P, Pereira M, Pavão M, Mulloy B, Tollefsen D, et al. (1996) Structure and anticoagulant activity of fucosylated chondroitin sulfate from echinoderm. *Journal of Biological Chemistry* 271: 23973–23984.
7. Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, et al. (2005) Carbohydrate analysis by phenol-sulphuric acid method in microplate format. *Anal Biochem* 339: 69-72.
8. Honda S, Akao E, Suzuki S, Okuda M, Kakehi K, et al. (1989) High-Performance Liquid Chromatography of Reducing Carbohydrates as Strongly Ultraviolet-Absorbing and Electrochemically Sensitive 1-phenyl-3-methyl-5-pyrazolone Derivatives. *Anal Biochem* 180: 351–357.
9. Zhang Y, Zhang P, Wang Z, Huang L (2011) An innovative derivatization method for simultaneous determination of uronic acids and neutral and amino sugars in coexisting samples by HPLC-ESI-MS/MS². *Journal of Liquid Chromatography & Related Technologies* 34: 1754-1771.
10. Kariya Y, Watabe S, Kyogashima M, Ishihara M, Ishii T (1997) Structure of fucose branches in the glycosaminoglycan from the body wall of the sea cucumber *Stichopus japonicus*. *Carbohydr Res* 297: 273-279.
11. Chen S, Xue C, Yin L, Tang Q, Yu G et al. (2011) Comparison of structures and anticoagulant activities of fucosylated chondroitin sulfate from different sea cucumbers. *Carbohydrate Polymers* 83: 688-696.
12. Wu M, Huang R, Wen D, Gao N, He J et al. (2012) Structure and effect of sulfated fucose branches on anticoagulant activity of the fucosylated chondroitin sulfate from sea cucumber *Thelenotaaanas*. *Carbohydrate Polymers* 87: 862-868.
13. Myron P, Siddiquee S, Al-Azad S (2014) Fucosylated chondroitin sulfate diversity in sea cucumbers: a review. *Carbohydrate Polymers* 112: 173-178.