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Original Research

Capsular Polysaccharide Biosynthesis from Recombinant *E. coli* and Chondroitin Sülfate Production

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Abstract: Chondroitin sulfate (CS) is an important biomedical product. CS is the basic structural component of the mammalian extracellular matrix and is widely used in many applications in the fields of medicine, veterinary medicine, pharmaceuticals and cosmetics. For CS production, mainly animal sources are used. However, in today's conditions, due to various risks and artificial synthesis, there has been an increase in alternative sources of production methods for CS, instead of using animal resources. In this study as a powerful alternative microbial production of CS has been targeted. By using recombinant *E. coli* strains to integrate VHb /*vgb*$^+$ and *kfo*$^+$ systems, the aim was to obtain high purity CS from reliable biotechnological processes. Plasmid pUC8:15 bearing the *vgb* gene region, and plasmid pETM6-PACF carrying the *kfoA, kfoC* and *kfoF* genes responsible for chondroitin synthesis, were transferred to *E. coli* bacteria. Microbial CS was obtained by adding sulfate groups to chondroitin acquired after the treatments. The results were confirmed by HPLC and NMR analyses. The product, compared to its counterparts, was found to be an effective drug, potentially with a low molecular weight value.

Key words: Chondroitin; Nonfructosylated chondroitin; Chondroitin sulfate; Vitreoscilla hemoglobin; *vgb* gene.

Introduction

Chondroitin is a component of human connective tissues found in cartilage and bone. In supplements, chondroitin sulfate (CS) usually comes from animal cartilage. Animal sources such as cattle shrubs, swine nose septum, caruncle and dogfish are usually used for CS production. However, in today's conditions, there has been an increase in CS production methods based on artificial synthesis and alternative sources instead of animal resources. This is largely due to ecological risks such as bird flu, mad cow disease, extinction of shark species and animal rights (1). In our study, microbial CS production has been achieved with a newly generated recombinant strain using a safe biotechnological application.

In Figure 1, the cell wall structure of a typical Gram-negative bacteria is shown (2). The purified part is the capsular polysaccharide part, which is the outermost layer. Bacterial capsules are protective coatings on the outer surface of bacteria that form an immune response (3). The capsular polysaccharide possessed by the pathogenic *Escherichia coli (E. coli)* K4 strain shows a very similar structure to chondroitin, and therefore chondroitin can be obtained from this structure (2,4).

It is also possible to transfer this property to new hosts using the plasmid pETM6-PACF, which carries genes responsible for capsular chondroitin synthesis using the *E. coli* K4 strain [*kfA, kfoC* and *kfoF* genes]. Bacteria carry this plasmid synthesized chondroitin without the fructose unit, which means that an additional post-fermentation process will not be required, because the pETM6-PACF plasmid we use in our study does not transfer the fructosylation-inducing gene [*kfo*E] (pETM6-PACF plasmid was obtained from Mattheos Koffas, Rensselaer Polytechnic Institute, Troy, NY). In our study, the microbial chondroitin was obtained primarily by chondroitin synthesis and subsequent purification protocols after transformation. Finally, CS production was completed by the addition of sulfate. It has been noted that VHb synthesis increases the growth and development of the organisms, and occurs at high rates in low oxygen environments both in its natural hosts and in cloned organisms due to the oxygen-sensitive *vgb* promoter (5, 6). Various metabolites and recombinant proteins, which are produced at a certain level of oxygen, have also shown significant increases in organisms carrying the VHb/*vgb* system (7). Based on the

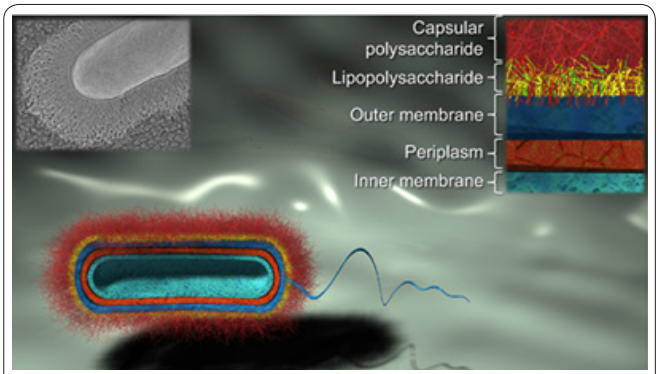

Figure 1. Cell wall structure of a typical Gram-negative bacteria (2).

idea that this protein can be used as an effective agent for some microaerobic fermentations in our study, chondroitin synthesis and purification have been performed for the first time in this study with *kfo*$^+$ bacteria carrying a Vitreoscilla hemoglobin gene (*vgb*) and chondroitin synthesis genes. The bacteria carrying the pETM6-PACF plasmid synthetic chondroitin is used as a capsular polysaccharide, while the pUC8:15 plasmid carries the *vgb* gene that provides vital support for bacterial growth during prolonged culture periods (7-9). An efficient process for producing chondroitin and chondroitin sulfate has been carried out by the common expression of two gene regions.

Materials and Methods

Media, strains and plasmids

Luria-Bertani (LB) medium, with or without ampicillin (80 mg/ml), is used for the cell growth and transformation screening. LB medium consists of 10 g/L of tryptone, 5 g/L of yeast extract and 10 g/L of NaCl. The E. coli C2987 was purchased from NEB (New England Biolabs) and used as a production strain after transformation with pUC8:15. The plasmid source (pUC8:15) used in this study was *Enterobacter aerogenes* (NRRL B-427), and was obtained from the USDA culture collection in Peoria, IL. Plasmid pUC8:15, carrying *vgb* on a 2.4-kb Vitreoscilla genomic fragment, has previously been described by Dikshit (10). The plasmid pETM6-PACF transformed into the *E. coli* K4 strain responsible for capsular chondroitin synthesis [*kfA*, *kfoC* and *kfoF* genes] was obtained from Mattheos Koffas, Rensselaer Polytechnic Institute, Troy, NY.

Shake flask experiments

Shake flask experiments were conducted to produce chondroitin. For each construct in *E. coli* (C2987), cells from a 15% glycerol stock were streaked on petri dishes containing agar with 80 mg/ml of ampicillin and incubated overnight. From each petri dish, two colonies were taken as samples, and these pre-cultures were left to incubate overnight at 37ºC. Then, the samples were diluted to 25 ml at an optical density (OD) of 0.05 and transferred to a 250 ml erlenmeyer flask and left to incubate at 37ºC under agitation at 220 rpm. A sample at an optical density of 1.0 and the cultures were left to incubate under the same conditions for another 18 to 24 hours. Information on the media used for these analyses is given in the Media, strains and plasmids section above, and the media were prepared with 80 mg/ml of ampicillin supplementation.

Plasmid construction and transformation

Firstly, plasmid pUC8:15 and plasmid pETM6-PACF were transformed into a non-pathogenic *E. coli* strain, and the presence in both plasmids was then confirmed by the colony PCR method and documented by gel photographs. The method adopted for the transformation of plasmid DNA into *E. coli* using heat shock is commonly used in molecular biology (11). This method comprises the insertion of a foreign plasmid or ligation product into the bacteria. Then the bacteria are incubated for a short time in ice and the mixture of bacteria and plasmid DNA is exposed to 42ºC for 45 seconds (heat shock) and then placed back in ice. The transformed cells are inoculated into the LB-amp media and incubated at 37ºC for 30 minutes under agitation. To ensure the isolation of colonies, irrespective of transformation efficiency, two quantities of transformed bacteria are incubated.

Chondroitin purification

The chondroitin purification procedure for the capsular polysaccharide was performed according to He Wenqin et al. (4). Accordingly, the cell pellet was first resuspended in water and autoclaved using a liquid cycle for 15 minutes. The supernatant obtained was taken and the pellet was discarded. The autoclaved supernatant from the cell pellet and the cell culture supernatant were precipitated using 80% by volume of cold ethanol. Then the samples were stored at −20ºC overnight allowing the recovery of both intracellular and extracellular chondroitin.Following the precipitation, the pellet was collected and re-suspended in digestion buffer (100 mM Tris, pH 7.5, 50 mM $MgCl_2$ and 10 mM $CaCl_2$). Following the addition of DNAse (1 mg/L, Sigma), the sample was left to incubate for 1 hour at 37ºC. At the end of the incubation period, Protease K (2.5 mg/ml, Sigma) was added and left to incubate for 2 hours at 56ºC. After the second cold ethanol precipitation (80%), the dry pellet was collected, re-dissolved in water (1 ml) and then filtered through a 10 KDa spin column to remove residual small peptides and salt. The sample obtained was lyophilized for future analysis using nuclear magnetic resonance (NMR) spectroscopy.

NMR analysis and quantifying chondroitin using HPLC–MS

The purified chondroitin samples were analyzed by one-dimensional 1H NMR (12). A Bruker Avance III 600 MHz spectrometer (Bruker BioSpin, Bill-Erica) with Topsin 2.1.6 software was used for the NMR measurements. Each sample was dissolved in 0.5 ml of deuterium oxide (D_2O) and freeze-dried repeatedly to remove the exchangeable protons. The samples were redissolved in 0.4 ml of D_2O and transferred to NMR microtubes. The conditions for one-dimensional 1H NMR spectra are a wobble sweep width of 20,0276 ppm, an acquisition time of 2.72 seconds, a relaxation delay of 4.00 seconds and a temperature of 296 K (12). Using an HPLC–MS offers a structurally specific assay for quantifying chondroitin (13). Chondroitin was depolymerized using chondroitinase ABC. A previously proven method was adopted for the analysis. The use of colorimetric assays, such as carbazole (14) for quantifying GAGs derived from bacteria fermentation is limited by interference from media and cellular debris.

Chondroitin sulfate production method

Firstly, 4 to 6 grams of the synthesized microbial chondroitin was dissolved in 80 to 120 ml of aqueous DMF (Dimethylformamide) and the solution cooled to between 0 and 5ºC. Secondly, 12 to 15 grams of pyridine sulfur trioxide was added to the solution. The solution, at room temperature, was precipitated by the addition of 400 to 800 ml of NaCl-saturated acetic acid. The mixture was filtered. The resulting solid was dissolved in 200 to 400 mL of demineralized water and the solution produced was neutralized with 1 N of NaOH. After

neutralization, the solution was heated to between 30 to 50°C and 0.2 to 0.3 N 60 mL of NaOH was added. After waiting for 1 to 3 hours at this temperature the solution was neutralized with 1 N of HCl acid. This solution was then filtered through a membrane with a conductivity of less than 10 µS. Finally, the solution was lyophilized and dried in vacuum conditions.

NMR analysis and quantifying chondroitin sulfate using HPLC–MS

The methods adopted for the NMR analysis and quantification of chondroitin were also used for NMR analysis and quantifying chondroitin sulfate.

Results

Plasmid transformation

Satisfactory cell formation was performed using the standard $CaCl_2$ method. The transformation of plasmids pETM6-PACF and pUC8:15 into *E. coli* was performed according to the standard heat-shock protocol in published literature (11). Then the presence of both plasmids was confirmed by the colony PCR method, and documented by gel photographs (Fig. 2 a, b).

As shown in the gel pictures, colonies with numbers 29 to 35 consist of both plasmids, which have the same electrophoretic mobility as their positive controls. At the end of the procedures, a new recombinant *E. coli* strain was generated by transforming both the plasmids pETM6-PACF and pUC8:15 (Fig. 3). The *kfoA, kfoC, kfoF* genes responsible for chondroitin synthesis and the *vgb* gene are commonly expressed in the strain formed.

Characterization of the chondroitin structure with NMR and HPLC

The fraction of chondroitin obtained was lyophilized and sent for NMR analysis for verification. The sample for analysis was dissolved in 0.5 ml of deuterium oxide (D_2O) and measured at a wavelength of 600 MHz by 1 H NMR at 12.3 kHz and 25°C. NMR analysis was carried out in IBTAM (Inonu University Scientific and Technological Research Center).

The chondroitin structure synthesized by recombinant bacterium has been checked by previous studies (14,15). NMR spectra (Fig. 4) were compared to the corresponding control, and the spectral structures of the samples were observed to be identical. In both spectra, the H4 proton at 4.74 ppm and the H6 proton at 4.21 ppm are evidence of the presence of chondroitin. At 4.15 ppm, the NH amide proton is present. Overall, we also see aliphatic methyl hydrogens at 1.98 ppm and etheric CH at 3.61 ppm. According to these results, we can say that the molecule we produce is correctly confirmed by the NMR results for chondroitin. HPLC analysis of chondroitin samples obtained from the recombinant *E. coli* strain was performed. According to HPLC analysis (Fig. 5), we obtained microbial chondroitin with a molecular weight of 203 Daltons at the observed peak.

Characterization of the chondroitin sulfate structure using NMR and HPLC

The microbial chondroitin we obtained was sulfated at the 6-position. Cutting with the chondroitinase ABC enzyme was performed for HPLC analysis of the chondroitin 6-sulfate. In this way it is aimed to analyze the sulfated units by freeing disaccharide units. As indica-

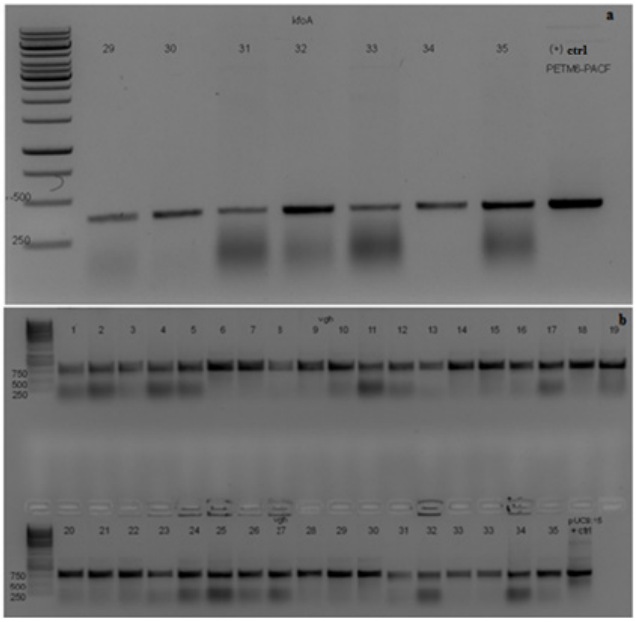

Figure 2. pETM6-PACF plasmid gel photographs **b.** pUC8:15 plasmid gel photographs.

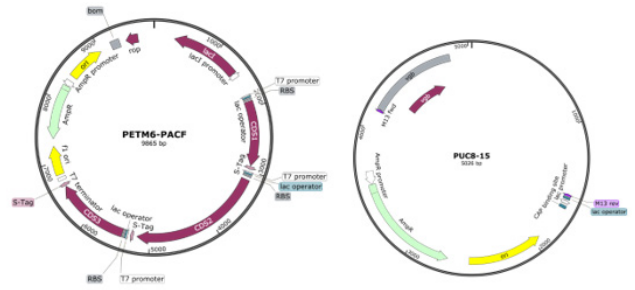

Figure 3. The pETM6-PACF and pUC8:15 plasmid maps.

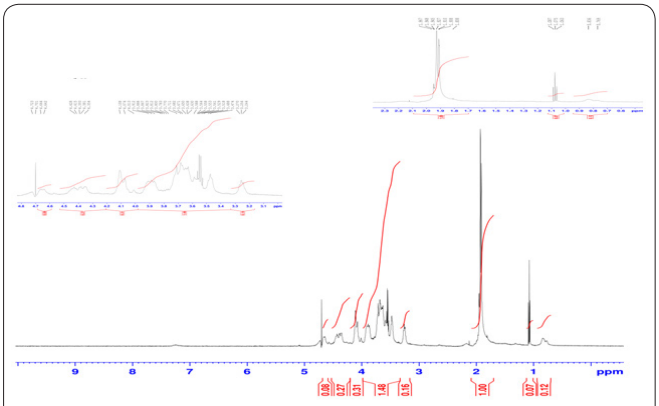

Figure 4. Chondroitin NMR results from the *E. coli* (pETM6-PACF+pUC8:15) strain.

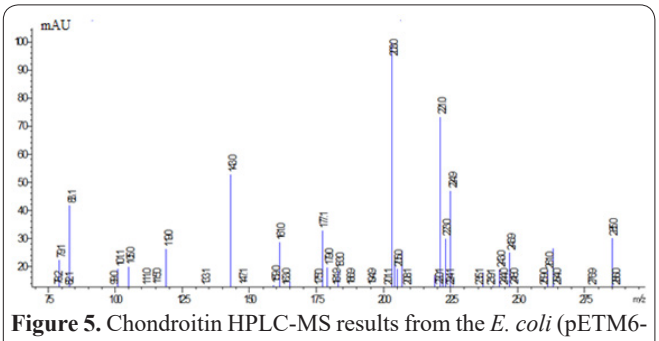

Figure 5. Chondroitin HPLC-MS results from the *E. coli* (pETM6-PACF+pUC8:15) strain.

ted by Joly J et al. (16), the commercial products have a molecular weight of between about 18,000 and 20,000 Daltons. However, microbial chondroitin sulfate has a molecular weight of between 300 and 3000 Daltons or more. According to our HPLC analysis (Fig. 6) results, the peak observation at a molecular weight of 269 Daltons confirms that we produced microbial chondroitin sulfate at a lower molecular weight than studies state in current literature.

In the NMR analysis (Fig. 7), the H4 proton in the microbial CS source at 4.74 ppm, the H6 proton at 4.21 ppm, and the NH amide proton at 4.15 ppm can be seen. Overall, aliphatic methyl hydrogen at 1.98 ppm and etheric CH at 3.61 ppm are also seen. All these structures are in agreement with the NMR data of the CS structure in reference material (4,14).

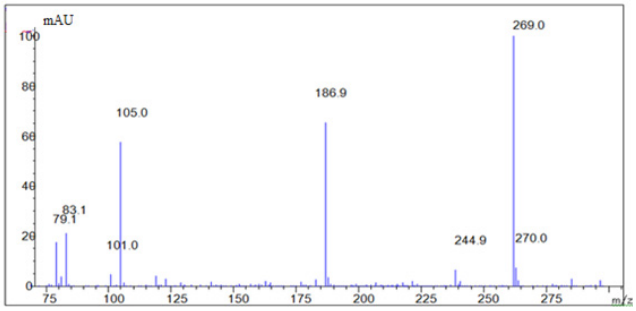

Figure 6. Chondroitin Sulfate HPLC results from the *E. coli* (pETM6-PACF+pUC8:15) strain.

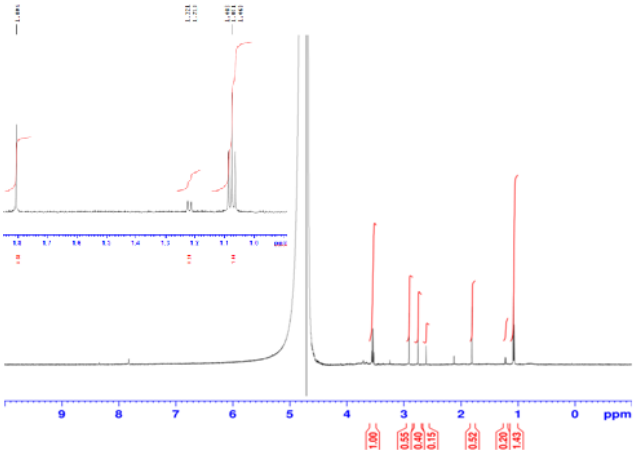

Figure 7. NMR results from the *E. coli* (pETM6-PACF+pUC8:15) strain.

Discussion

This study provides an alternative approach to producing chondroitin sulfate from a non-pathogenic recombinant strain of *E. coli* by utilizing biotechnological methods. Chondroitin sulfate (CS), due to its confirmed anti-inflammatory (17), anti-apoptotic (18), anti-oxidant (19) properties, and its efficacy in cell-signaling pathways, has the potential for use in many different products in the biomedical field. Therefore, chondroitin production and diversity of alternative resources for its production is very important. Animal tissues are mainly used as a source of chondroitin production. Its microbial production is extremely limited. Considering these points, our primary objective was to make a novel and original study in this limited area. In our study, a new and original strain was formed with effective potential for the production of CS by selecting advantageous gene groups to increase production and product quality. The *kfo* genes that provide chondroitin synthesis (4) and the *vgb* genes, which provide vital benefits to the bacteria during prolonged culture periods (7,8), were transferred to the bacteria using different plasmids (pETM6-PACF and pUC8:15 Plasmids). In chondroitin and CS studies, a double gene effect system has been tried for the first time. With the selected gene groups, it aimed to form a more advantageous source of chondroitin. It can be said that previous studies mostly focused on chondroitin genes. The idea of common expression of the *vgb* gene with chondroitin genes was performed for the first time in our study. The Vitreoscilla hemoglobin gene was expected to undertake the task of renewing the energy consumed for the production of chondroitin in such a system. The expected results were obtained in the experimental process and efficient gene expression findings were obtained. Evaluating the HPLC and NMR results (4,14), the production of microbial CS with a low molecular weight (269 Daltons) was confirmed.

It has been reported that the sources and production methods of CS production affected the degree of purity, resulting in changes in the efficacy of the treatment (4, 20). It has also been reported that different bioavailability and pharmacokinetic properties were determined depending on the source of CS, and that CS with different purity and activity levels were obtained from different bacteria (21). It was stated that microbial CS had all the functions that animal CS possess. In addition, due to its lower molecular weight, compared to that of the counterparts obtained from animal sources, a higher absorption was obtained after oral administration, and it was more effective in the treatment process. (4,20,21). This information and the results of the analyses strongly suggest that microbial CS with a low molecular weight value can be functionally more effective compared to CS obtained from animal sources and CSs produced from other microbial sources. The system created in our study promises a new and effective alternative CS resource to research literature. This approach has multiple implications in terms of animal rights, ethics and human health. The limited number of studies on microbial CS production (1) has limited the development of fermentation processes on this subject. In this manner, our study also provides an alternative. With the increase in the number of similar studies, bacteria can become the ideal source for non-animal, glycosaminoglycan-derived products.

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References

1.Schiraldi, C, Cimini D, De Rosa M. Production of chondroitin sulfate and chondroitin. Applied Microbiology and Biotechnology

2010; 87: 1209–1220.
2.Cress B F, Englaender JA, He W, Kasper D, Linhardt R J, Koffas M. Masquerading microbial pathogens: Capsular polysaccharides mimic host-tissue molecules. FEMS Microbiology 2014; Reviews 38: 660–697.
3.Martens EC, Koropatkin NM, Smith TJ, Gordon JI. Complex glycan catabolism by the human gut microbiota: the Bacteroidetes Sus-like paradigm. J Biol Chem 2009; 284 (37): 24673–24677.
4.He W, Fu L, Li G, Andrew Jones J, Linhardt RJ, Koffas M. Production of chondroitin in metabolically engineered *E. coli.* Metab Eng 2015; 27: 92-100.
5.Erenler S, Gencer S, Geçkil H, Stark B, Webster D. Cloning and expression of the Vitreoscilla hemoglobin gene in *Enterobacter aerogenes*: Effect on cell growth and oxygen uptake. Applied Biochemistry and Microbiology 2004; 40: 241-248.
6.Kaur R, Ahuja S, Anand A, Singh B, Dikshit KL. Functional implications of the proximal site hydrogen bonding network in Vitreoscilla hemoglobin (VHb): Role of Tyr95 (G5) and Tyr126 (H12). FEBS Letters 2008; 582: 3494-3500.
7.Geçkil H, Stark B. Webster D. Cell growth and oxygen uptake of Escherichia coli and Pseudomonas aeruginosa are differently effected by the genetically engineered Vitreoscilla hemoglobin gene. Journal of Biotechnology 2001; 85: 57-66.
8.Chi PY, Webster D A, Stark BC. Vitreoscilla hemoglobin aids respiration under hypoxic conditions in its native host. Microbiological Research 2009; 164: 267-275. 5.
9.Rajendra P, Dikshit KL, Yixiang L, Webster DA. The bacterial hemoglobin from Vitreoscilla can support the aerobic growth of *Escherichia coli* lacking terminal oxidases. Archives of Biochemistry and Biophysics 1992; 293: 241-245.
10.Dikshit KL, Webster DA, Cloning Characterization and expression of the bacterial globin gene from Vitreoscilla in *Escherichia coli*. Gene 1988;70: 377–386.
11.Manıatıs T, Frıtsch EF, Sambrook J. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989.
12.Fu L, Li G, Yang B, Onishi A, Li L, Sun P, Zhang F, Linhardt RJ. Structural characterization of pharmaceutical heparins prepared from different animal tissues. J. Pharm. Sci 2013; 102: 1447–1457.
13.Yang B, Chang Y, Weyers AM, Sterner E, Linhardt RJ. Disaccharide analysis of glycosaminoglycan mixtures by ultra-high-performance liquid chromatography–mass spectrometry. J.Chromatogr.A 2012; 122: 591–98.
14.Gustavo RC.S, Adriana AP, Bianca FG, Ana MFT, Mariana S P, Eduardo V, Paulo ASM. Systematic Analysis of Pharmaceutical Preparations of Chondroitin Sulfate Combined with Glucosamine. Pharmaceuticals 2017; 10(2): 38.
15.Jıng P, Yi Q, Xiaodong Z, Pazandark A, Frazier SB, Weiser P, Hog L, Lijuan Z. Oversulfated chondroitin sulfate is not the sole contaminant in heparin. Nature Biotechnology 2010; 28(3): 203-207.
16.Jolly J, Klimaszewski K, Nakanishi Y, Matsubara H, Takahashi T, Nishio K. Microbial-derived chondroitin sulphate. Official Gazette of the United States Patent and Trademark Office Patents, Patent Publication Number: US 2010/0063001 A1. 2010
17.Campo GM, Avenoso A, Campo S, D'Ascola A, Traina P, Sama D, et al.. Glycosaminoglycans modulate inflammation and apoptosis in LPS-treated chondrocytes. J Cell Biochem 2009a ;106: 83–92.
18.Campo GM, Avenoso A, Campo S, D'Ascola A, Traina P, Calatroni A. Chondroitin-4-sulphate inhibits NF-kB translocation and caspase activation in collagen-induced arthritis in mice. Osteoarthritis Cartilage 2008; 16: 1474–1483.
19.Campo GM, Avenoso A, Campo S, Ferlazzo AM, Calatroni AAntioxidant activity of chondroitin sulfate. Adv Pharmacol 2006a; 53: 417-431.
20.Cress BF, Englaender JA , He W, Kasper D, Linhardt RJ, Koffas MA. Masquerading microbial pathogens: Capsular polysaccharides mimic host-tissue molecules. FEMS Microbiol Rev 2014; 1–38.
21.Vázquez JA, Rodríguez AI, Montemayor MI, Fraguas J, González MP and Murado MA. Chondroitin Sulfate, Hyaluronic Acid and Chitin/Chitosan Production Using Marine Waste Sources: Characteristics, Applications and Eco-Friendly Processes: A Review Mar. Drugs 2013; *11*(3), 747-774.