J Young Pharm, 2020;12(2):135-140
A multifaceted peer reviewed journal in the field of Pharmacy
www.jyoungpharm.org | www.phcog.net

Original Article

Production and Characterization of Melon Sulfated Pectin (*Cucumis melo* var. acidulus) Nanoparticles with Anticoagulant Potential

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ABSTRACT

Objective: Unfractionated heparin and low molecular weight heparin are the drugs most used today in coagulopathy. Although effective, these drugs have limitations in their usage, which aroused the interest of the scientific community in researching other substances with a similar effect. This study aims to analyze the anticoagulant activity of isolated and chemically modified pectin extracted from Caipira melon (*Cucumis melo* var. acidulus) and its nanoencapsulation for therapeutic use. **Materials and Methods:** For the analysis of anticoagulant activity, thrombin time, prothrombin time and activated partial thromboplastin time were performed. The nanoparticles were produced by a double emulsion using poly(lactic-co-glycolic acid) and poly (vinyl alcohol) and, subsequently, were analyzed morphologically by scanning electron microscopy and characterized in the Zetasizer Nano ZS90. **Results:** The pectin tests showed promising anticoagulant activity. In order to increase the biological activity of this substance, nanoparticles of sulfated pectin were obtained, attaining particle size 301.03 nm, polydispersity index of 0.18, zeta potential of -16.63 mV and Acid pH, the results were considered satisfactory by the researchers. The encapsulation efficiency of the sulfated pectin was 76.31% with controlled release. The scanning electron microscopy (SEM) showed uniform and spherical nanoparticles. **Conclusion:** The results indicated that the sulfated pectin nanoparticles can be seen as an innovative alternative in anticoagulant therapy.

Keywords: Anticoagulant, Heparin, Nanoparticles, Sulfated polysaccharides.

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DOI: 10.5530/jyp.2020.12.27

INTRODUCTION

Unfractionated heparin (UFH) and its derived Low Molecular Weight Heparin (LMWH) are the chosen drugs for the treatment of thromboembolic disturbances. However, reports of adverse effects related to these drugs are frequent, such as hemorrhage, thrombocytopenia and osteoporosis, as well as problems such as structural diversity and contamination by animal pathogens, which justifies the growth by the search for new compounds with similar anticoagulant activities, fewer adverse effects and different of those which come from animal sources.[1,2]

Along the compounds that can be used as anticoagulant agents, the sulfated polysaccharides stand out, because the literature states that its property is related to the presence of sulfate groups and their distribution along the chain.[2,3] Factors such as molecular weight, chemical composition, sulfate content and position and uronic acid content may interfere with the polysaccharide anticoagulant activity.[4] Algae and invertebrates that have naturally sulfated polysaccharides in their constitution are already confirmed to possess anticoagulant and antithrombotic activity.[1] However, as these species are limited, chemically polysaccharides sulfates may represent a good alternative.[5]

Aiming at the fruit valorization, the increase of the commercialization profits and the sustainable reuse, this work analyzed the anticoagulant action of the pectin in Caipira melon (*Cucumis melo* var. acidulus), which is widely produced in the Northeast of Brazil and possess irrelevant commercial value. It is a high molecular weight natural polysaccharide, biocompatible, non-toxic, anionic, already isolated and chemically sulfated in a previous study.[1,6] Therefore, it was found that the viability of this sulfated pectin represents a new pharmaceutical alternative in the treatment of coagulation disorders.

Studies assert that the drug can be nanoencapsulated to improve its pharmacokinetic profile, improve treatment efficacy, minimize side effects and increase its chemical and conformational stability, confirming the efficiency of the nanoencapsulation of anticoagulant drugs.[7,8] Therefore, the purpose of this paper was to evaluate the anticoagulant activity of pectin isolated and later chemically sulfated and, from these results, produce and characterize a nanocarrier system to expand its therapeutic potential.

MATERIALS AND METHODS

Materials

Samples of sulfated and non-sulfated polysaccharides were provided by the Department of Chemistry, at the Federal University of Ceara. Unfractionated heparin (Hemofol 5000UI / ml - Lote18030036) was purchased commercially. The Kits and the commercial kits used in the coagulation tests (APTT, PT and TT) were purchased commercially (Bios Diagnóstica®). Poly (lactic-co-glycolic acid) (PLGA) with a 50:50 lactide co-glycolide ratio (MW 40-75 kDa) and poly (vinyl alcohol) (PVA) (MW 13-23 kDa, 87- 89% hydrolyzed) were purchased from Sigma-Aldrich.

Samples Blood: the samples for plasma separation used in *in vitro* tests were obtained from donors from the Hemocenter of Ceara (HEMOCE), obeying the following criteria: adult donors who do not take any type of medicine; healthy and donors of both sexes. Samples from hemolyzed patients were excluded. The project was approved by the Research Ethics Committee of the Federal University of Ceara, with the protocol number 1.572.008.

Assay of anticoagulant activity

The Activated Partial Thromboplastin Time (APTT) clotting assay was carried out according to the method of Castro *et al.*,[9] using normal human plasma. In the assay, plasma samples (90 µL) were mixed with different amounts of polysaccharide in 0.9% NaCl (10 µL) and incubated at 37°C for 60 seconds and prewarmed at 100 µL. APTT assay reagent was added and allowed to incubate at 37°C for 2 minutes. Thenceforth, the substance was prewarmed and 0.25 M calcium chloride (100 µL) was then added. The APTT recorded as the time for clot formation in a coagulometer. For thrombin time (TT) clotting assay, citrated normal human plasma (90 µL) was mixed with 10 µL of a solution of polysaccharide and incubated at 37°C for 60 seconds. Then, 200 µL of TT assay reagent preheated to 37°C was added and the clotting time was recorded one more time. Prothrombin time (PT) clotting assay was performed as follows: citrated normal human plasma (90 µL) was mixed with 10 µL of a solution of polysaccharide and incubated at 37°C for 1 min. Then, 200 µL of PT assay reagent pre-incubated at 37°C for 10 mins was added and clotting time was recorded. The bioassay results were expressed as means ± standard deviation (SD). The experimental data were subjected to an analysis of variance for a completely random design and three samples were prepared for assays of every attribute.

Preparation of nanoparticles

The NSP was prepared according to the method described by Chereddy *et al.*[10] considering some modifications. Briefly, 100µl sulfated pectin (1mg/ml) was then discarded in the 100mg 50:50 solution of PLGA (poly (lactic-co-glycolic acid)), in 5ml dichloromethane (DCM) and sonicated for 15 seconds (50% Amplitude, 5 seconds with pulse on and 2 seconds with pulse off), using an ultrasonic processor (Sonifier® model W-450D Branson). Once prepared, this first emulsion was then poured into 100mg PVA, dissolved in 10ml deionized water and sonicated again for 15 seconds (Amplitude 50%, pulse 5 seconds "on" and 2 seconds "off") using the processor again (Sonifier® Model W -450D Branson). The formed water/oil/water double emulsion (w/o/w) was then stirred using a magnetic stirrer, to remove excess DCM for 6 hours. The nanoparticles were separated by centrifugation (5810R Centrifuge, Eppendorf, USA) at 3.000rpm for 15 minutes, using a separation filter (15ml Amicon Ultra 100 KDa) and finally, the sample was lyophilized (Labconco, USA). Empty nanoparticles were prepared with the same procedure, considering the addition of sulfated pectin during formulation preparation.[11,12]

Characterization of Nanoparticles

The mean particle size (nm), the polydispersity index (PDI) and the Zeta potential (ZP) of NSP were determined using a Zetasizer Nano ZS90 instrument (Malvern Instruments, Malvern, UK) at 25ºC, by dynamic light scattering (DLS). All measurements were carried out in triplicate (n=3).

Scanning Electron Microscopy (SEM)

The particles were characterized by Scanning Electron Microscopy (SEM) (Quanta 450 FEG, FEI Company, USA) at a voltage of 30kV, after prior coating with the silver under vacuum by sputtering using a QT150 ES (Quorum Technologies, USA) apparatus.

Differential Scanning Calorimetry (DSC)

The physical state of sulfated pectin inside the nanoparticles was characterized by the analysis of the DSC curves. The curves were obtained in DSC cell (Shimadzu, Kyoto, Japan) using aluminum crucibles with approximately 5mg of samples, under dynamic air atmosphere (40 mL/min) and a heating rate of 10°C/min at a temperature range from 25°C to 600°C.[13]

Thermogravimetric Analysis (TG) and derived thermogravimetry (DTG)

The thermal stability of the samples was verified by simultaneous TG and DTG, using a Q 50 TGA (TA INSTRUMENTS). TG-DTG curves were obtained in the temperature range from 30°C to 800°C, using aluminum crucibles with approximately 2mg of samples, under dynamic air atmosphere (40 mL/min) and heating rate of 10°C /min.[14]

Determination of Entrapment Efficiency (EE)

The calculation of the quantity of nanoencapsulated substance was indirect; first the SP present in the supernatant was quantified using a method based on the linearity of the APTT results of increasing concentrations of this pectin in the plasma of healthy patients. The dose-dependent linearity of APTT in the analyzed concentrations made it viable to establish a dose-response curve that, then, made it possible to quantify the free polysaccharide. Later, the formula below permitted the calculation of these nanoencapsulated substances.[14,15]

$$EE(\%) = \frac{\text{Total initial drug} - \text{Drug in supernatant}}{\text{Total initial drug}} \times 100$$

Stabilities studies

The samples were stored in room temperature and sheltered from light and heat for 30 days. To evaluate the stability of the obtained nanoparticles, the following parameters were monitored: Zeta potential (mV), polydispersity index, particle size (nm) on a Zetasizer Software Malvern Version 7.12, by dynamic mirroring of light at times 0 and 30 days. The pH of the samples was also analyzed in both times using pH meter Quimis, Q400AS.

Nanoparticles release study

Initially, 30mg NSP of lyophilized sulfated pectin was dispersed in 3 ml of phosphate-buffered saline (PBS). The NP were incubated at room temperature while stirring for 6 days. After 1 and 2 hours and after 1, 2 and 5 days, 1ml of the supernatant was collected from the environment and filtered on an ultracentrifugation tube and the same PBS was reposted on the releasing environment. The amount of sulfated pectin released on determined times was calculated through the dose-response curve already described on the Encapsulation Efficiency tests.[10]

Rheology study of SP and UFH (Unfractionated Heparin) solutions

The study of the rheological behavior of UFH solutions using the AR 2000 controlled tension rheometer (TA INSTRUMENTS), with a conical plate geometry that possesses a diameter of 40cm and angle 1 0 '47', maintaining a range of 27 µm. The solutions of SP, nanoparticles of SP and UFH (0.25 mg/ml) were placed in the container and the rate of shear stress applied by gradual rotation of the rod measured the torque required to calculate the shear stress from 1 to 1000 s^{-1}. The samples were shaken for approximately 2 minutes before the beginning of the experiment. A comparison was made between the rheological properties of the analyzed samples and all experiments were done in triplicate.

Statistical Analysis

All obtained results were analyzed by the Shapiro-Wilk test to measure the normality distribution of data in which showed parametric and non-parametric distributions. Therefore, the results were presented as the meanor median ± measures of dispersion. The statistical differences between the groups were calculated using the *t* test, Analysis of Variance (ANOVA) test followed by the Bonferroni posttest or their correspondent non-parametric tests, considering a critical alpha of 0.05 significance level. The program used for was GraphPad Prism, version 5.01.

RESULTS

In vitro coagulation tests

According to Figure 1, sulfation has interfered with the anticoagulant activity and the isolated pectin shows no anticoagulant Oliveiraeffect. The APTT result of the SP was statistically similar to UFH, used as a positive control. ($p< 0.001$).

Anticoagulant activity of sulfated pectin

Observing the results of Table 1. It was verified the alterations on the TT, as expected, since the APTT results were extended dose-dependently.

The prothrombin time (PT) shows no significant change.

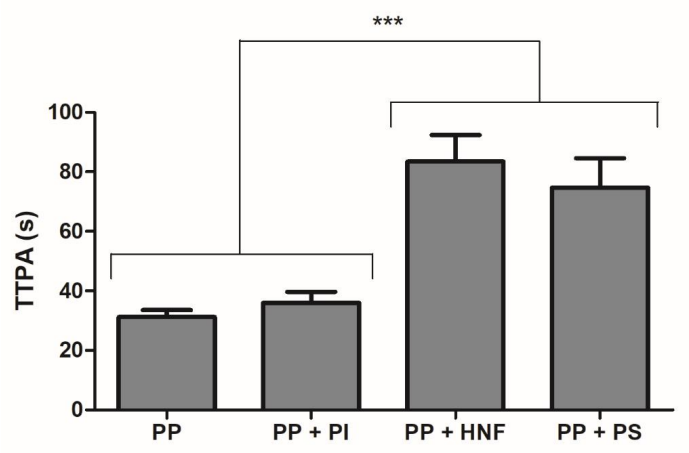

Figure 1: The activated partial thromboplastin time (APTT) in seconds of pure plasma (PP) in vitro solution combined with isolated pectin 0.5 ml/mL (P+IP), unfractionated heparin (P+UFH) and sulfated pectin 0.5 mg/mL (P+SP). The data are presented as mean ± SD of six independent replicates and statistically compared using the ANOVA followed by Bonferroni posttest. Significant differences: $p<0.001$***.

The E50 of the SP was calculated from the statistical analysis of the data in Table 1. The confidence interval of the active dose of SP able to achieve 50% of the maximum anticoagulant effect is between 0.25 and 0.40 mg/ml.

Development and characterization of the nanoparticles

The double emulsion method with the evaporation of the solvent used in this study allows to achieve homogeneous nanoparticles. All samples have shown average diameters of particles (particle size) of 301.00 ± 7.19 nm and the polydispersity index of the samples was <0.2.

Both nanoparticles of SP have shown negative superficial electrical density (zeta potential).

Morphology analysis

In Figure 2 it is possible to observe spherical, regular nanoparticles in the range of 300nm average diameter.

Entrapment efficiency (EE)

In this study, the EE achieved for the NPS was 76.4%.

Release studies

In Figure 3, it is possible to observe the maximum sulfated pectin (SP) release, reaching approximately 49% in 5 days.

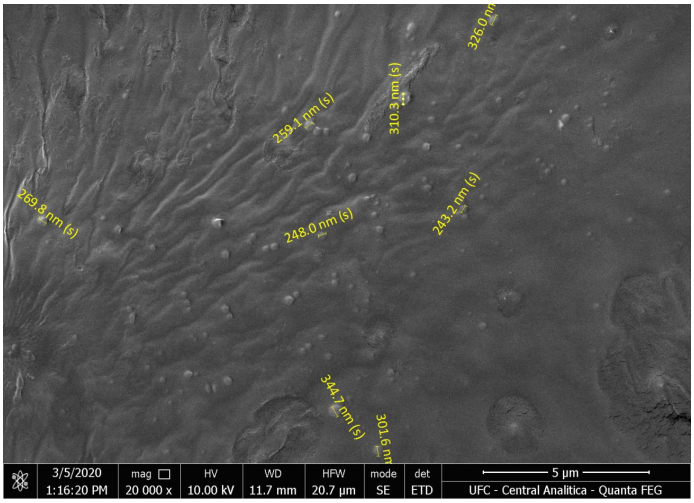

Figure 2: Scanning electron microscope images of sulfated pectin nanoparticles showed nano sized spherical particles.

Table 1: Prothrombin time (PT), activated partial thromboplastin time (APTT) and thrombin time (TT) of pure plasma solutions alone or in association with isolated pectin (PP + PI) and pure plasma with sulfated pectin (PP + PS) in six concentrations studied.

(mg/ml)	APTT	PT	TT			
	seconds	p-value	seconds	p-value	seconds	p-value
Plasma puro (PP)	31.21±0.7		14.36±0.4		18.2±0.7	
PP + PI 1,0	35.2±0.5	$p<0.01$	14.34±0.9	NS	20.1±0.6	NS
PP + PS 1,0	114.76±1.3	$p<0.001$	14.06±0.8	NS	87.2±0.9	p<0.001
PP + PS 0,75	92.13±1.7	$p<0.001$	14.03±0.9	NS	66.7±3.3	p<0.001
PP + PS 0,5	74.43±2.1	$p<0.001$	14.56±0.6	NS	56.6±2.0	p<0.001
PP + PS 0,25	58.4±1.6	$p<0.001$	14.03±1.0	NS	24.3±1.8	p<0.001
PP + PS 0,125	46.06±0.9	$p<0.001$	13.76±1.5	NS	19.9±2.7	NS
PP + PS 0,0625	37.3±1.0	NS	13.65±0.4	NS	19.1±1.6	NS

Values are represented as means ± SD (n = 3). Significant differences compared to plasma pure alone using ANOVA followed by Bonferroni posttest. NS: not significant.

Thermogravimetric analysis of NPS

Figure 4 shows the presence of three endothermic peaks: a first event in the temperature range of 69.11°C followed by two consecutive events at 193.7°C and 278.1°C. No crystallinity event was detected in relation to the encapsulated drug. The TG-DTG curve has confirmed the data achieved on DSC. The nanoparticles show thermal stability on the temperature range of 190°C when compared to the pure polymer. Weight loss occurs in two stages: 314°C – 58.38 % and 432 – 32.38%, associated with exothermic events between the temperatures of 206°C and 499°C.

Stability tests

According to the analysis made, the nanoparticles of sulfated pectin (NSP) remained stable for 30 days at room temperature and sheltered from light and heat, as it is shown in Table 2. The formulations developed show no significant difference compared to the parameters analyzed in the evaluated times.

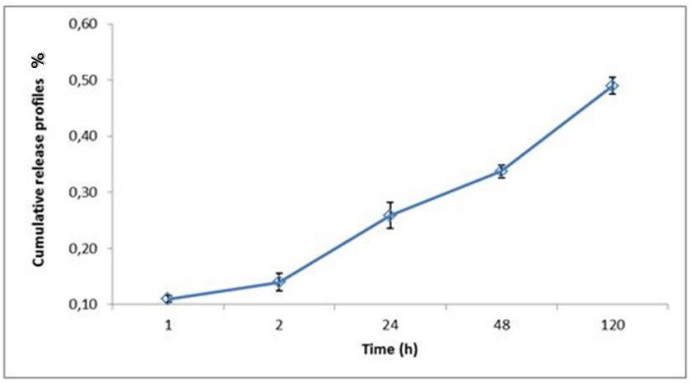

Figure 3: Cumulative release profiles of sulfated pectin nanoparticles in phosphate-buffered saline (pH=7.4) at 37°C.

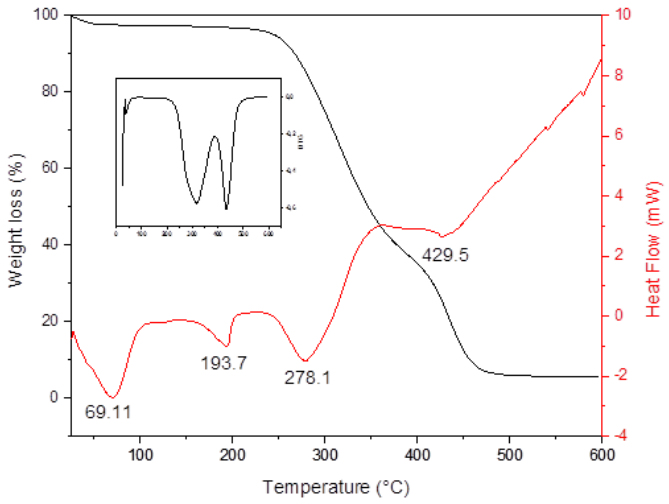

Figure 4: Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of NSP. (The derivative graph is embedded).

Rheological behavior studies

It was possible to observe the similarity of rheological behavior of sulfated pectin (SP), nanoparticles of sulfated pectin (NSP) and unfractionated heparin (UFH) aqueous solutions in a concentration of 0.25 mg/ml.

DISCUSSION

According to the results, it was possible to verify that the chemical sulfation of the sulfated pectin promoted its anticoagulant activity. Studies[1,2,16] assert that the introduction of sulfate groups has a direct influence on antithrombotic and anticoagulant activities. Similar results were obtained by Hu[5] *et al.* and Chaouch *et al.*[16] that chemically sulfated other pectins and promoted an extension in the APTT. Along with these studies, the non-sulfated pectin showed no anticoagulant activity as well. For these results, APTT, TT and PT *in vitro* stitching tests were performed.

It is known that the APTT assesses intrinsic pathway coagulation factors. TP is used to characterize extrinsic coagulation factors, while an increase in TT suggests inhibition of thrombin or fibrin production.[17,18] Looking at the results in Table 1. It was found the changes in TT and APTT, with APTT altering in a dose-dependent manner, as described by Cui *et al.*[19] and Wang *et al.*[20] in similar works.

As the TP displayed no significant change, it was concluded that the samples involved in these tests acted only in the intrinsic coagulation pathway. This result corroborates with the research by Adrien *et al.*[4] and Da Silva *et al.*,[21] where sulfated polysaccharides were also subjected to these tests and only significant variations in the APTT were verified. These authors state that the intrinsic pathway is the main target of sulfated polysaccharides and the extrinsic pathway is not affected by these substances.[9]

For the nanoparticles obtained, the particle size is one of the most important parameters, as it determines the biocompatibilities and bioactivities of the nanoparticles, in addition to possessing direct relevance to the stability of the formulation.[22] Similar results have been reported in other studies that reached 195nm to 251nm nanoparticles.[10] In a similar test, medium-sized nanoparticles 512.8nm and 269nm were produced for nanoencapsulated low molecular weight heparin, using PVA and PLGA as well.[12,23]

Regarding low PDI, the literature confirms that low values reveal good homogeneity of the samples.[21] Already analyzing the zeta potential, this parameter is probably related to the free carboxylic groups on the surface.[24] According to Sun *et al.*[22] in the case of charged particles, the greater the Zeta potential, the greater the repulsive interaction, leading to the formation of more stable particles with a more uniform size distribution.

According to MEV and EE, the results were similar to others reported in the literature regarding the production of sulfated polysaccharide nanoparticles using PVA and PLGA.[25,26]

According to the authors, the efficiency of encapsulation is related to the concentration of polymers and surfactants. As the concentration of

Table 2: Stability of the nanoparticles of sulfated pectin (NSP) in terms of zeta potential (mV), polydispersity index, particle size (nm) and pH, for 30 days.

Day	Zeta potential (mV)	Polydispersity index	Particle size (nm)	pH	p-value
1	-16.63± 1.96	0.18± 0.04	301± 7.19	4.20± 0.01	p>0.05
30	-14.22± 0.51	0.14± 0.02	304± 3.59	4.40± 0.08	

Values are represented as means ± SD (n = 3). Significant differences between day 1 and day 30 using ANOVA followed by Bonferroni posttest. Not significant: p>0.05.

surfactants increases, the size of the particles and the surface area decrease, which leads to an increase in the release of drugs by the nanoparticles.[11] Analyzing this release, it can be found that in the first two hours it was faster. However, over time, the speed decreased. Therefore, with a longer release, the drug remained in the body for a longer time, because one of the main disadvantages of heparins is its low bioavailability and the need for many daily doses.[8]

The DSC curves of the NPS show that the first event may be related to the release of acetyl groups that have been transformed into acetic acid and the successive catalytic degradation of the main chain at higher temperatures. The TG-DTG curve revealed no events related to the melting point of the drug. This fact reinforces the idea that it is encapsulated in amorphous nanoparticles, as confirmed by DSC.[1,25]

As for stability, it is relevant to emphasize that monitoring pH over time is important. Decreasing pH values may indicate the beginning of polymer degradation.[27] According to the results obtained, all formulations pointed acid pH (4.0-4.5) for 30 days due to the negative characteristics of the sulfated polysaccharides and, therefore, remained stable.[12]

The results of the rheological behavior clarified as well that the NSP in the studied concentration has a Newtonian character, since the relationship between shear stress and local velocity gradient is linear, with the proportionality being constant to the viscosity of the fluid. Similar behavior is demonstrated by UFH and SP, suggesting that the solution with SP nanoparticles can be administered intravenously.

CONCLUSION

In vitro and *in vivo* studies of the applications of sulfated polysaccharides as anticoagulants are increasing and expanding among the scientific community. They are receiving significant attention from the pharmaceutical market. Due to the problems shown by the use of heparins, the chemically modified SP extracted from Japanese melon can be a new alternative to non-toxic, efficient and accessible anticoagulant therapy.

Coagulation tests results were promising. The nanoencapsulation methodology was successful with satisfactory encapsulation efficiency. The nanoparticles produced are stable, homogeneous, possess controlled release and rheological behavior similar to UFH.

The small number of articles found about the species of sulfated pectin used as anticoagulants shown the need for a continuous effort of researchers and pharmaceutical industry to increase the studies of these species and, eventually, lead them to the development of an innovative, safe and efficient drug.

ACKNOWLEDGMENTS

The Coordination of Improvement of Higher Education Personnel (CAPES)/Ministry of Education (MEC). The authors would like to thank the Central Analítica-UFC/CT-INFRA/MCTI-SISANO/CAPES for the support.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

APTT: Activated Partial Thromboplastin Time; **DCM:** Dichloromethane; **DLS:** Dynamic Light Scattering; **DSC:** Differential Scanning Calorimetry; **EE:** Entrapment efficiency; **HEMOCE:** Hemocenter of Ceará; **IP:** Isolated pectin; **LMWH:** Low Molecular Weight Heparin; **MW:** Molecular weight; **NSP:** Nanoparticles of sulfated pectin; **PBS:** Phosphate-buffered saline; **PDI:** Polydispersity index; **PLGA:** Poly (lactic-co-glycolic acid); **PP:** Pure plasm; **PT:** Prothrombin time; **PVA:** poly (vinyl alcohol); **SD:** Standard deviation; **SEM:** Scanning Electron Microscopy; **SP:** Sulfated Pectin; **TG:** Thermogravimetric Analysis; **TT:** Thrombin time; **UFH:** Unfractionated Heparin; **ZP:** Zeta potential.

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Article History: Submission Date : 10-01-2020; Revised Date : 18-03-2020; Acceptance Date : 10-06-2020.
Cite this article: Oliveira RCR, Almeida RR, Uchoa AFJ, Santos AP, Ricardo NMPS, Lemes RPG and Araújo TG. Production and Characterization of Melon Sulfated Pectin (*Cucumis melo* var. acidulus) Nanoparticles with Anticoagulant Potential. J Young Pharm. 2020;12(2):135-40.