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PRODUÇÃO, CARACTERIZAÇÃO E AVALIAÇÃO DAS PROPRIEDADES BIOLÓGICAS DE OLIGOSSACARÍDEOS DE QUITOSANA OBTIDOS A PARTIR DE RESÍDUOS DO PROCESSAMENTO DO CAMARÃO MARINHO *Litopenaeus vannamei*

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2019

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de doutora em Ciencias Biológicas.

Área de concentração: Biotecnologia

Orientador: Prof. Dr. Ranilson de Souza Bezerra
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RESUMO

A quitosana é um polissacarídeo pseudonatural, biodegradável, não tóxico, com a natureza catiônica, que possui diversas aplicações biotecnológicas. No entanto, em consequência a sua elevada massa molecular, este polímero apresenta baixa solubilidade em pH neutro e alta viscosidade, limitando assim o seu uso em alimentos, cosméticos, produtos farmacêuticos e indústrias agrícolas. A produção de oligossacarídeos de quitosana, também chamados de quitoligossacarídeos (QOS), é uma forma eficaz de melhorar a solubilidade da quitosana e diminuir a sua viscosidade, aumentando assim as suas possíveis aplicações. Dessa forma, a presente tese reportará a produção, caracterização da estrutura química, propriedades e funções biológicas dos QOS obtidos a partir dos subprodutos do processamento do camarão marinho *Litopenaeus vannamei*. O capítulo um, traz a produção dos QOS a partir de hidrólise enzimática inespecífica utilizando a pepsina, sua caracterização estrutural e a avaliação de sua atividade antimicrobiana e antioxidante frente a modelos de radicais livres. No capítulo dois, os QOS produzidos são avaliados quanto a sua atividade antitumoral (*in vitro* e *in vivo*), toxicidade aguda, atividade analgésica e anti-inflamatória. Por fim, no capítulo três foi estudado o efeito hipocolesterolêmico dos QOS em animais submetidos a dieta hiperlipídica base de cérebro bovino, manteiga comercial e ração de camundongo. Foram realizadas análises bioquímicas (glicose, triglicerídeos, HDL, não-HDL, AST, ALT, uréia e creatinina) no início, durante e ao término da experimentação. Como resultados preliminares apresentados na presente tese, foi observado que, os QOS foram produzidos com sucesso por hidrólise enzimática não específica utilizando pepsina e apresentaram possuir sua estrutura química semelhante ao da quitosana, contudo, apresentaram baixo peso molecular e, consequentemente, solúveis em pH neutro com baixa viscosidade. Os resultados preliminares mostram a eficácia destes oligômeros, em atuar como agentes antioxidantes, atuarem como antibacteriano, serem atóxicos, além de conferirem ação analgésica, antitumoral e hipocolesterolêmica. Estudos futuros são importantes para determinar claramente as atividades biológicas destes compostos.

Palavras-chave: **s**ubprodutos da indústria pesqueira; quitina; quitoligossacarídeos; atividades biológicas.

ABSTRACT

Chitosan is a biodegradable, non-toxic pseudonatural polysaccharide with the cationic nature, which has several biotechnological applications. However, as a consequence of its high molecular weight, this polymer has low solubility at neutral pH and high viscosity, thus limiting its use in foods, cosmetics, pharmaceuticals and agricultural industries. The production of chitosan oligosaccharides (COS), also called Chitooligosaccharides (QOS), is an effective way of improving the solubility of chitosan and decreasing its viscosity, thus increasing its possible applications. Therefore, the present thesis will report the production, chemical structure, properties and biological functions of the QOS obtained from the byproducts of Litopenaeus vannamei sea shrimp processing. Chapter one, brings the production of QOS from non-specific enzymatic hydrolysis using pepsin, its structural characterization and the evaluation of its antimicrobian and antioxidant activity against free radical models. In chapter two, the produced QOS are evaluated for their antitumor activity (in vitro and in vivo), acute toxicity, analgesic and anti-inflammatory activity. Finally, in chapter three we will study the hypocholesterolemic effect of the QOS in animals submitted to a hyperlipidic diet. As preliminary results presented in this thesis, it was observed that, QOS were successfully produced by non-specific enzymatic hydrolysis using pepsin and presented to possess its chemical structure similar to the one of chitosan, however, presented low molecular weight and consequently soluble at neutral pH with low viscosity. Preliminary results show the effectiveness of these oligomers in acting as antioxidants, act as antibacterial, are nontoxic, and confer analgesic, antitumoral and hypocholesterolemic action. Future studies are important to clearly determine the biological activities of these compounds.

Keywords: by-products; fishing industry; chitin; quitoligosaccharides; biological activities.

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Table 4 - Markers of liver and kidney injuries in mice under high fat diet treated with chitooligosaccharid. *$p<0.001$ vs Control, MANOVA followed Bonferroni's Test.

LISTA DE ABREVIATURAS E SIGLAS

5-FU	Quimioterápico 5-fluorouracil
ABTS	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
CH	Quitosana
COS	Quitoligossacarídeos/oligossacarídeos de quitosana
DD(%) ou GD(%)	Deacetylation degree / Grau de desacetilação
DMEM	Meio de cultura (Meio Eagle Modificado por Dulbe
DMSO	Dimetil sulfóxido
EDTA	Ácido Etilenodiaminotetracético
FAO	Organização da Nações Unidas para Agricultura e Alimentos
FT-IR	Espectroscopia de Infra Vermelho por Transformad Fourier
GlcN	Glucosamina (2-desoxi-2-amino glucopiranosídeo)
GlcNAc	N-acetil glucosamina (2-desoxi-2-acetamido glucopiranosídeo)
HEp-2	Carcinoma laringeo humano
HT-29	Cancêr colo humano
LMWC	Quitosana de baixo peso molecular
LABENZ	Laboratório de Enzimologia
MPA	Ministério da Pesca e Aquicultura
MALDI-TOF	Matrix-assisted laser desorption/ionization
MCF-7	Carcinoma mamário humao

MTT	(3-[4,5-dimetiltiazol-2-il]-2,5-difenilterazzólio
NC1-H292	Carcinoma mucoepidermoide pulmonar humano
QTS	Quitoligossacarídeos/oligossacarídeos de quitosana
RAEC	Células endoteliais de aorta de coelho
SSA	Aspirina
RMN/NMR	Resonancia Magética nuclear
TBE	Tris/Borato/EDTA
TROLOX	6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid
TWI	Inibição tumoral

SUMÁRIO

1 – INTRODUÇÃO

O pescado e os produtos pesqueiros estão entre os alimentos mais comercializados no mundo. Estes são oriundos tanto da pesca extrativa, quanto da aquicultura. Embora a captura de organismos aquáticos apresente maior percentual do total de pescado fornecido, é a aquicultura que é responsável por um crescimento significativo, contribuindo dessa forma para o incremento na oferta global de produtos da pesca (FAO, 2018).

No entanto, com a expansão do mercado de pescados em todo mundo, a indústria pesqueira tem gerado consequentemente grande quantidade de resíduos e subprodutos. No ano de 2014, resíduos sólidos gerados na cadeia produtiva da pesca e da aquicultura corresponderam a 20% do volume de 167,2 milhões de toneladas que foram produzidos no referido ano (FAO, 2016).

Dessa forma, a geração de resíduos é um desafio para o setor pesqueiro, visto que cerca de 50% do volume processado diariamente nas indústrias é resíduo sólido que é descartado em lixões, córregos, rios e mares (ARRUDA, 2004; PINTO, 2017). Estes subprodutos, embora sejam biodegradáveis, seu acúmulo excessivo no ambiente causa problemas ambientais e sociais, se transformado em perigo para a saúde pública (CIRA. et al., 2002; MARTONE et al., 2005; ROCHA, et al., 2004).

Os subprodutos gerados pela Carcinicultura são formados principalmente por cabeças, exoesqueleto e cauda. Tais produtos têm sido alvo de pesquisas que demostram que esse material é uma importante fonte de biomoléculas, tais como: proteínas, quitina, minerais, glicosaminoglicanos sulfatados e carotenoides. Esses materiais representam uma importante fonte de diversas moléculas bioativas, com amplo potencial de aplicabilidade biotecnológica (CAHÚ et al., 2012).

Dentre estas biomoléculas, a quitina tem recebido considerável atenção. Sua principal destinação encontra-se na produção de quitosana, um composto obtido a partir da desacetilação alcalina da quitina (DALLAN, 2005). A quitosana, devido a sua composição química, apresenta propriedades funcionais como baixa toxicidade, biodegradabilidade, biocompatibilidade, capacidade antimicrobiana e antioxidante, podendo ser usada na área farmacêutica, médica, cosmética e alimentícia (KIM & RAJAPAKSE, 2005). Porém, pelo fato da quitosana apresentar alta massa molecular e ser insolúvel em pH neutro, suas aplicações biológicas são limitadas, o que restringe o seu uso (KUMAR e THARANATHAN, 2004; LI et al., 2005).

Como alternativa a este problema, diversos estudos surgiram propondo metodologias de obtenção e otimização da síntese de derivados hidrossolúveis de quitosana, garantindo assim um aumento significativo das aplicações deste polímero (RONCAL et al., 2007; KANATT et al., 2008; ZHANG et al., 2010). Umas das alternativas para a utilização eficiente da quitosana é através da sua despolimerização, obtendo assim produtos com baixa massa molecular. Esse procedimento origina moléculas que são então classificadas como oligossacarídeos de quitosana (STOYACHENKO & VARLAMOV, 1994).

Os oligossacarídeos de quitosana, por apresentarem tamanho molecular menor que a quitosana, são passíveis de solubilização em água, tornando possível a obtenção de soluções desses oligossacarídeos com baixa viscosidade e em pH neutro. Diversas pesquisas relacionadas com a avaliação das atividades biológicas dos oligossacarídeos de quitosana vêm sendo desenvolvidas nos últimos anos, estando evidenciada atividade hipocolesterolêmica efeitos antitumorais e imunológicos, e aplicações como em carreadores de drogas, aceleradores na absorção de ferro e cálcio, entre outras (KIM & RAJAPAKSE, 2005).

O presente trabalho teve como objetivo estudar os oligossacarídeos de quitosana produzidos a partir de resíduos da indústria pesqueira e avaliar suas propriedades através de sua caracterização e estudo de suas atividades biológicas. Para isso, após sua produção através de hidrólise enzimática inespecífica, os oligômeros foram submetidos a análise estrutural por RMN, FT-IR e MALDI-TOF. Para a avaliação de suas atividades, foi analisado seu potencial antioxidante frente aos testes de ABTS, quelante de ferro e teste de proteção ao DNA. Foi avaliada a toxicidade celular, e aguda desses compostos como também sua ação analgésica. Posteriormente, os oligossacarídeos foram avaliados frente a modelos tumorais *in vitro e in vivo e* sua atuação como composto hipocolesterolêmico.

Os resultados sugerem que os produtos obtidos, além de apresentarem importância econômica e ambiental, também apresentam importância biotecnológica, pois os oligossacarídeos de quitosana apresentam interessantes propriedades que os tornam potenciais alvos para novos estudos com sua provável aplicação.

2 – OBJETIVOS

2.1 - Objetivo Geral

Produzir, caracterizar e avaliar as atividades biológicas dos oligossacarídeos de quitosana produzidos a partir de resíduos do camarão marinho Litopenaeus vannamei.

2.2 - Objetivos Específicos

- Produzir, a partir de quitosana obtida de resíduos do processamento do camarão marinho Litopenaeus vannamei, quitooligossacarídeos por hidrólise enzimática.
- Caracterizar quimicamente as amostras por viscosimetria, espectroscopia de infravermelho (FT-IR), ressonância magnética nuclear (13C e 1H) e espectrometria de massas (MALDI-TOF);
- Avaliar a atividade antioxidante frente a modelos de radicais livres;
- Analisar a atividade antimicrobiana da quitosana e seus derivados;
- Analisar a citotoxicidade, toxicidade aguda e antineoplásica sobre tumores experimentais em camundongos;
- Avaliar a atividade analgésica e anti-inflamatória dos compostos obtidos;
- Analisar a ação hipocolesterolêmica dos oligossacarídeos de quitosana em animais submetidos a dieta hiperlipidica.

3 - REVISÃO BIBLIOGRÁFICA

3.1 – Panorama da indústria pesqueira

O pescado e os derivados pesqueiros encontram-se entre os produtos alimentícios mais comercializados no mundo, representando 1% do comércio mundial de mercadorias em termos de valor. Estes produtos são oriundos tanto da pesca extrativa, que envolve a captura de organismos aquáticos, quanto da aquicultura que envolve o cultivo desses animais (FAO, 2018).

No ano de 2016, a produção mundial de pescado atingiu 170,9 milhões de toneladas, das quais 90,9 milhões, cerca de 53,18%, foram oriundos da pesca e 80 milhões, cerca de 46,8% da aquicultura (Tabela 1). Mais uma vez, o grupo mais produzido foi o de peixes com 54,1 milhões de toneladas, seguido de algas, com 30,1 milhões de toneladas, moluscos com 17,1 milhões de toneladas e crustáceos com 7,9 milhões de toneladas produzidas (FAO, 2018).

Tabela 1 - Produção mundial de pescado a partir da pesca extrativa e aquicultura.

Category	2011	2012	2013	2014	2015	2016
Production						
Capture						
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total capture	**92.2**	**89.5**	**90.6**	**91.2**	**92.7**	**90.9**
Aquaculture						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total aquaculture	**61.8**	**66.4**	**70.2**	**73.7**	**76.1**	**80.0**
Total world fisheries and aquaculture	**154.0**	**156.0**	**160.7**	**164.9**	**168.7**	**170.9**
Utilization[b]						
Human consumption	130.0	136.4	140.1	144.8	148.4	151.2
Non-food uses	24.0	19.6	20.6	20.0	20.3	19.7
Population (*billions*)[c]	7.0	7.1	7.2	7.3	7.3	7.4
Per capita apparent consumption (*kg*)	18.5	19.2	19.5	19.9	20.2	20.3

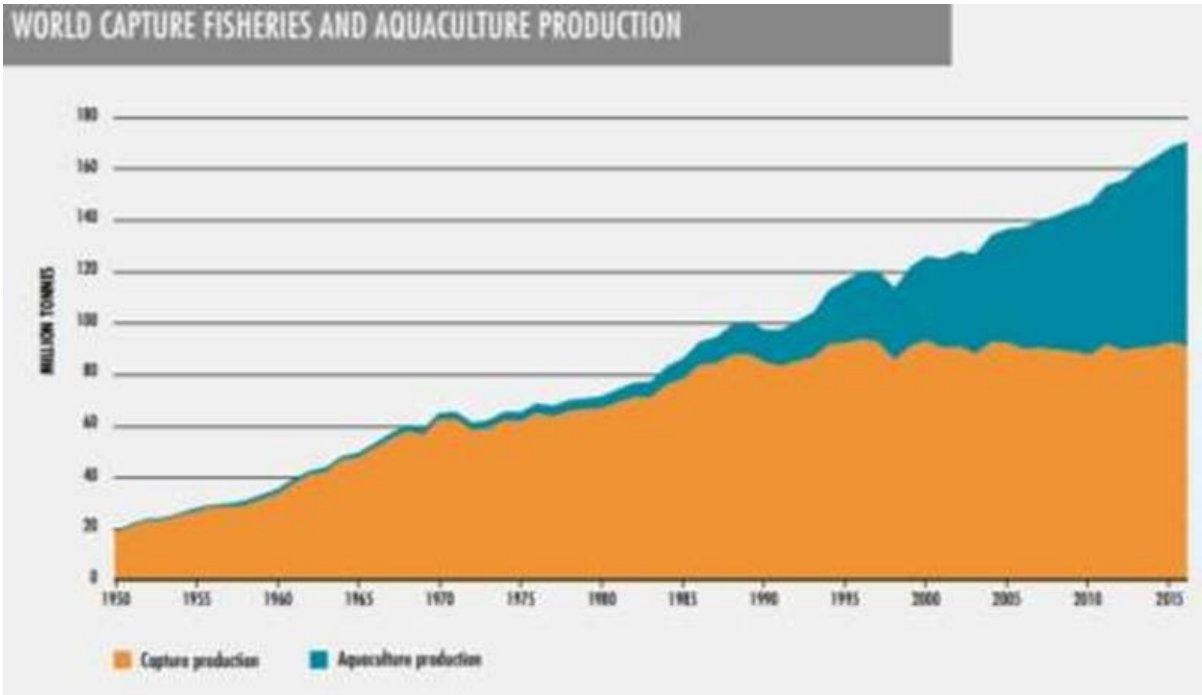
[a] Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants.
[b] Utilization data for 2014–2016 are provisional estimates.
[c] Source of population figures: UN, 2015e.

Fonte: FAO, 2018.

Embora a captura de organismos aquáticos, apresente maior percentual do total de pescado fornecido, a produção mundial de pesccado vem apresentando estabilidade de produção desde a década de 80 (Figura 1). Por outro lado, a aquicultura nas últimas décadas vem apresentando um crescimento significativo, alcançando uma taxa média de

crescimento anual de 8,8% (FAO, 2016). O rápido crescimento da aquicultura nos últimos anos demonstra o bom desempenho desta atividade no cumprimento de seu papel em satisfazer a crescente demanda de consumidores por pescados, através de um incremento na oferta global de produtos da pesca (FAO, 2018).

Figura 1 - Produção mundial da pesca e aquicultura.



Fonte: FAO, 2018.

A aquicultura é um dos sistemas de produção de alimentos com maior taxa de crescimento no mundo, o que coloca esta atividade em foco pela grande oportunidade de produção de alimentos, geração de postos de trabalho e desenvolvimento de negócios (FAO, 2018). Esse sistema de produção abrange as seguintes especialidades: piscicultura, malococultura, ostreicultura, miltucultura, algicultura, ranicultura, criação de jacarés e carcinicutura.

A carcinicultura, por sua vez, é a modalidade da aquicultura que envolve a criação de camarão em viveiros, ou ainda de caranguejo e siri. Entre os crustáceos, o mais cultivado é o *Litopenaeus vannamei*, com 4,1 milhões de toneladas (51,89%) (FAO, 2018).

O *L. vannamei*, também conhecido como camarão branco do pacífico (Figura 2), é considerada a espécie mais importante para a carcinicultura no Hemisfério Ocidental. Esta espécie é oriunda da Costa Sul Americana do Oceano Pacífico, predominante na

faixa costeira do Equador. É uma espécie que vem sendo cultivada em todos os países produtores de camarão do mundo ocidental. Considerada de porte médio, apresenta um elevado desempenho reprodutivo em laboratório, com ótimo desempenho zootécnico, possuindo taxas de crescimento uniformes e fácil adaptabilidade a diferentes condições ambientais (FAO, 2012b; FREITAS et al., 2009).

Figura 2 - Camarão marinho *Litopenaeus vannamei*.

Fonte: https://www.google.com.br/search?q=litopenaeus+vannamei&biw; acesso em 16/06/2019.

3.2 – Subprodutos da indústria pesqueira

Com a expansão do mercado de pescados em todo mundo e, consequentemente, do volume de pescado processado, a indústria pesqueira tem gerado grande quantidade de resíduo e subprodutos. Estima-se que cerca de 50% do pescado produzido é descartado na forma de resíduo (ARRUDA, 2004; SUCASAS, 2011). Considerando que em 2016 foram produzidas 170,9 milhões de toneladas de pescado no mundo (FAO, 2018), isso equivaleria a 85,45 milhões de toneladas de resíduos. Estes subprodutos, embora sejam biodegradáveis, seu acúmulo excessivo no ambiente, associado à sua natureza altamente perecível, gera um grande problema de ordem social. Consequentemente se transforma em perigo para a saúde pública, por tornar-se rapidamente colonizado por organismos de deterioração e atraírem insetos e roedores. Outro agravante é que estes resíduos são, em geral, clandestinamente enterrados ou jogados nos rios e mares, causando problemas ambientais (CIRA et al., 2002; MARTONE et al., 2005; ROCHA, et al., 2004).

Com o intuito de agregar valor ao resíduo de processamento do pescado, estudos vêm sendo realizados visando o reaproveitamento desses biomateriais, tornando assim a atividade aquícola mais sustentável e viável ecologicamente (BEZERRA, et al. 2001). No caso dos subprodutos gerados pela carcinicultura (que são formados principalmente

por cabeças, exoesqueleto e cauda), pesquisas têm demostrado que esse material é uma importante fonte de biomoléculas tais como, proteínas, quitina, minerais, glicosaminoglicanos sulfatados, carotenoides e compostos aromáticos. Desta forma, constitui um material riquíssimo por possuir uma variedade de moléculas bioativas, com amplo potencial de aplicabilidade biotecnológica (COWARD-KELLY et al., 2006; ARVANITOYANNIS et al., 2008;CAHÚ et al., 2012; BOUGATEF, 2013).

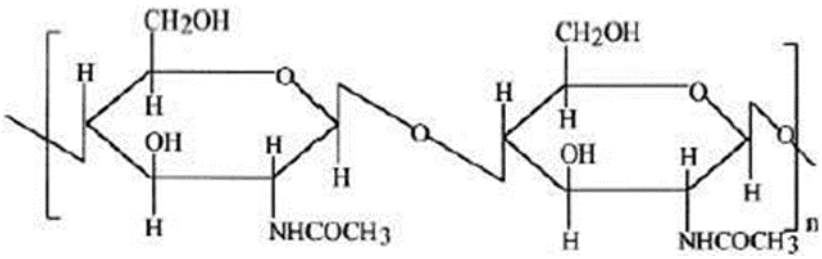
As proteínas e os carotenoides podem ser extraídos para uso na indústria farmacêutica e alimentícia (HE et al., 2013; MAZZOMO et al., 2011; SANTOS et al., 2012). O carbonato de cálcio pode ser utilizado como matéria-prima em construção de edifícios e na produção de cal (óxido de cálcio) (YANG et al., 2005). Os glicosaminoglicanos sulfatados podem ser amplamente utilizados em aplicações biomédicas (CHEN et al., 2011) e a quitina tem uma variedade de utilizações nas mais diferentes áreas, principalmente quando é convertida em quitosana (SALAH et al 2013; SANSONE et al., 2014; ISLAM et al., 2014).

3.3 – Quitina

A Quitina foi descrita pela primeira vez em 1811 pelo francês Henri Branconnot, professor de história natural, que durante suas pesquisas com *Agaricus volvaccus* e outros fungos, com solução alcalina, obteve uma substância identificada em plantas, a qual denominou de “fungine” ou “fungina” (ANJOS, 2005; SKAUGRUD; SARGENTE, 1990). Em 1823, Odier isolou uma substância contida nas carapaças de insetos semelhante à encontrada em plantas, a chamou de quitina, que em grego “Khitón” significa túnica, envelope ou cobertura. Ledderhose em 1878 identificou a quitina como sendo um composto de glucosamina e Gilson, em 1894, confirmou a presença de glucosamina na quitina (STAMFORD, 2007).

A quitina é um polissacarídeo natural, insolúvel em água, linear composto por unidades β- (1→4)-N-acetil-D-glucosamina (Figura 3)(CANELA E GARCIA, 2001).

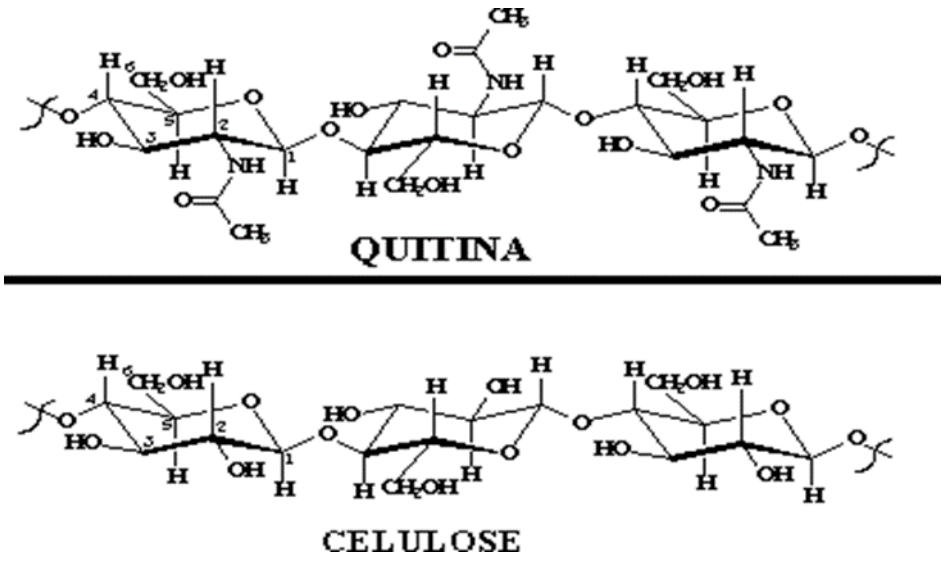
Figura 3 - Estrutura química da quitina.



Fonte: Adaptada de KUMAR (2000).

Esse biopolímero é sintetizado por diversos organismos vivos, e é o polissacarídeo mais abundante na natureza, depois da celulose (KUMAR, 2000; KURITA 2001). A estrutura da quitina é similar à da celulose, exceto pelo fato de que o grupo hidroxila (-OH) do carbono na posição 2 do anel glicopiranosídico é substituído pelo grupo acetamida (Figura 4). Esta semelhança estrutural é refletida nas funções análogas desses dois polissacarídeos na natureza, pois ambos atuam como material estrutural e protetor (SIGNINI, 2002; CAMPOS-TAKAKI, 2005).

Figura 4 – Estrutura química da quitina e da celulose.



Fonte: Signini, 2002.

A quitina é encontrada na natureza sob forma de microfibrilas cristalinas, formando o componente estrutural dos exoesqueletos de artrópodes e das paredes celulares de fungos e leveduras. Além de ser produzida por estes organismos, também

pode ser sintetizada por uma série de outros organismos dos reinos animal e vegetal, surgindo quando reforço e resistência são necessários (RINAUDO, 2006).

Embora encontrada em animais e fungos, as principais fontes comerciais de quitina são as carapaças de camarão e de caranguejos, que são descartadas pelas indústrias pesqueiras (ZOHURIAAN-MEHR, 2005). Caso não seja aproveitada, a quitina pode se tornar um grande problema de poluição (KUMAR, 2000).

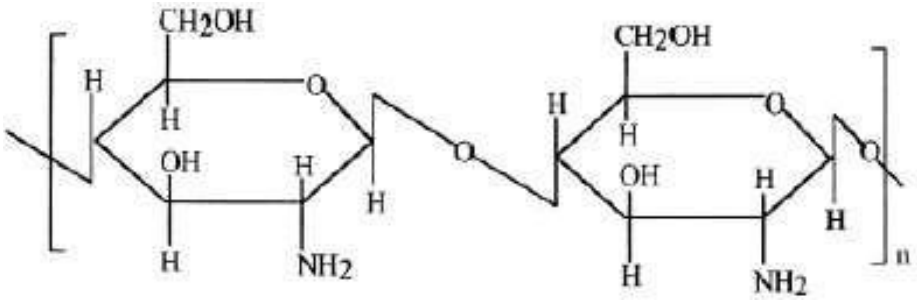
As cascas secas dos crustáceos possuem cerca de 20 a 30% de quitina, esta que está firmemente associada aos demais constituintes do exoesqueleto como proteínas (30- 40%), carbonato e fosfato de cálcio (30-50%), além de pigmentos como os carotenoides astaxantina, cantaxantina, luteína e β-caroteno (CANPANA-FILHO et al , 2007; CAHU et al., 2012; ARANCIBIA et al., 2014; CANELLA e GARCIA, 2001). Em consequência da associação da quitina com os demais componentes do exoesqueleto das cascas dos crustáceos é necessário um tratamento ácido para dissolver o carbonato, seguido de uma extração alcalina para solubilizar as proteínas e despigmentação para remoção dos pigmentos residuais. A quitina resultante deve ser classificada em termos de pureza e cor, dado que resíduos de proteínas podem inviabilizar seu uso especialmente para aplicações biomédicas (RINAUDO, 2006).

O uso da quitina é restrito, pois é insolúvel na maioria dos solventes usuais. Sua principal aplicação encontra-se na produção de quitosana, um composto produzido a partir da sua desacetilação alcalina, com propriedades que permitem que a quitosana seja utilizada em diversas áreas (DALLAN, 2005)

3.4 – Quitosana

A quitosana é um polissacarídeo que foi descoberto em 1859 por Rouget, quando a quitina entrou em contato com uma solução de hidróxido de potássio em ebulição (DALLAN, 2005). Ela é um polissacarídeo linear composto por unidades repetidas de 2- amino-2-deoxi-D-glucosamina (GlcN) (Figura 5) que estão unidos por ligação glicosídica β (1→4) (KURUTA, 2001; PETER, 2005).

Figura 5 - Estrutura da quitosana.

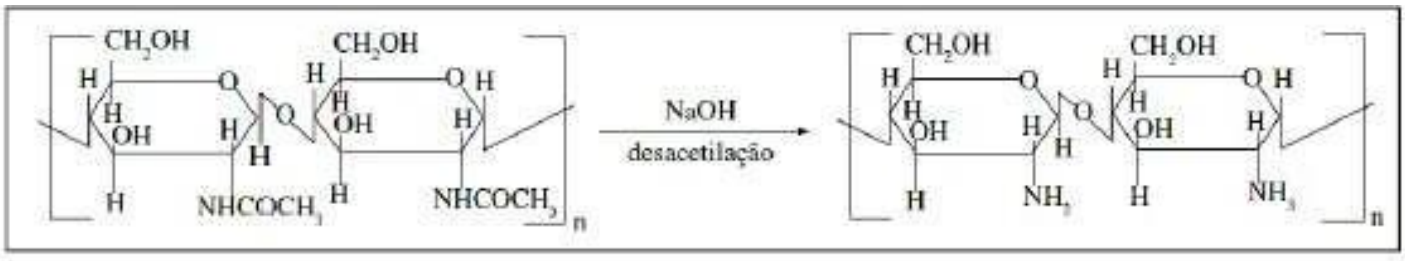


Fonte: Adaptada de KUMAR (2000).

A quitosana é uma molécula obtida a partir da desacetilação alcalina da quitina. Durante este processo de desacetilação, as ligações N-acetil são hidrolisadas, formando-se a D-glicosamina, que contém um grupo amino livre (DALLAN, 2005). Este processo também pode ocorrer utilizando enzimas específicas, como a quitina desacetilase ou pela ação de microrganismos que sintetizem tal tipo de enzima (MONTEIRO JÚNIOR1999; KIM E RAJAPAKSE, 2005).

A figura 6 ilustra a reação de desacetilação alcalina em que a quitina é submetida para obtenção da quitosana. Nesse processo os grupamentos acetoamido (-$NHCOCH_3$) são transformados em grupos amino (-NH_2), dando origem a quitosana. A extensão de desacetilação da quitina é classificada como grau de desacetilação. Quando a quantidade de D-glicosamina, ou seja, o grau de desacetilação se torna maior que 50%, a quitina passa a ser solúvel no meio aquoso, em pH ácido, e o polímero é denominado quitosana (DALLAN, 2005). A completa desacetilação da quitina é dificilmente realizada, pois à medida que este grau aumenta, a possibilidade de degradação do polímero também aumenta (ABRAM, 2004).

Figura 6 - Produção da quitosana a partir da desacetilação alcalina da quitina, onde n é o grau de polimerização.



Fonte: SPIN-NETO e colaboradores (2008).

A presença dos grupamentos de amino livres (NH2) no carbono 2 de cada resíduo monomérico da quitosana, confere a este biopolímero o comportamento polieletrólito catiônico (pKa = 6,3). Em consequência disto, a quitosana é insolúvel em água, soluções básicas e solventes orgânicos, porém é solúvel na maioria das soluções orgânicas ácidas quando o pH desta solução seja menor que 6,3. Em meio aquoso ácido, esta tem seus grupos amínicos protonados (NH_3^+) apresentado uma elevada densidade de carga positiva. (RODRÍGUEZ-PEDROSO et al., 2009).

3.4.1 – Propriedades da quitosana

A quitosana é um dos poucos polissacarídeos pseudonatural com caráter catiônico, e esta característica é responsável pela maioria de suas propriedades. Essa particularidade possibilita a sua interação com cargas negativas, geralmente presentes na superfície de biomoléculas como proteínas, polissacarídeos aniônicos, ácidos nucléicos e ácidos graxos, justificando seus efeitos sobre elas (SANTOS et al., 2003). Este fato contribui com a atividade antimicrobiana contra diversas bactérias e fungos, pois os grupamentos amino positivos das unidades glicosamina possivelmente interagem com os componentes negativos das paredes celulares das bactérias, suprimindo a biossíntese (SHI et al., 2006). Além disso, a quitosana interrompe o transporte de nutrientes através da parede celular e causa o vazamento de organelas celulares, acelerando a morte da bactéria. Outro mecanismo proposto envolve a penetração de quitosana de baixa massa molar na célula a qual se liga ao DNA inibindo a síntese de RNA e proteínas (VINSOVA e VAVARIKOVA, 2008).

Estudos sugerem que os efeitos da quitosana sobre as bactérias gram-positivas e gram-negativas sejam distintos. No caso das gram-positivas, a hipótese é que a quitosana de alta massa molar forme películas ao redor da célula e gera a inibição da absorção de nutrientes. Por outro lado, a quitosana de baixa massa molar penetra mais facilmente em bactérias gram-negativas, causando distúrbios no metabolismo desses organismos (COSTA SILVA et al., 2006).

Alguns investigadores têm reportado que quitina e quitosana induzem a analgesia. (SILVA, et al., Allan et al., 1984). Allan et al.(1984) verificaram que a quitosana forneceu uma sensação refrescante, agradável e calmante tópico quando aplicada em feridas abertas Okamoto et al.(1995) e Shigemasa & Minami (1996) observaram em animais que, quitina e quitosana aceleram a cicatrização de feridas,

reduzem a frequência de tratamento, diminuem a dor e protegem a superfície da ferida. Os animais não sentiram dor quando suas feridas foram cobertas com quitina e quitosana.

Tanto a quitina, como a quitosana reduzem o tempo de coagulação sanguínea de forma dose-dependente, sendo a quitosana mais eficaz. Este fato se deve à capacidade da quitosana em agregar tanto as plaquetas, como hemácias pela interação entre as cargas positivas dos grupos amínicos livres com as cargas negativas dos receptores. A ação da quitosana sobre as plaquetas produz mais um efeito benéfico, que é a liberação de fator de crescimento derivado de plaquetas AB e fator de transformação do crescimento β1, que desempenham papel importante a cicatrização (COSTA SILVA et al., 2006).

A quitosana também possui propriedade imunomoduladora, que é devida à sua capacidade de ativar quase que exclusivamente os macrófagos. Isto explica não só o papel da quitosana na aceleração da cicatrização de lesões, mas também a degradação desse polímero no organismo. Os macrófagos, ativados pelos oligômeros de quitosana liberam interleucina 1, que estimula a proliferação de fibroblastos e influencia a estrutura do colágeno. Liberam também, N-acetilglicosaminidase, que hidrolisa a quitosana a monômeros de N-acetilglicosamina e glicosamina, unidades de açúcares necessárias á biossíntese de ácido hialurônico e outros glicosaminoglicanos da matriz extracelular dos fibroblastos. Eles promovem a migração de neutrófilos, facilitando a resolução da resposta inflamatória. As atividades bactericidas e bacteriostáticas sugerem que este polímero pode prevenir infecções, quando aplicado diretamente no local da lesão (SUZUKI et al., 1986; TOKORO et al., 1988; XIA, 2003).

Outra importante propriedade da quitosana é de agir como quelante, o que confere a quitosana a capacidade de se ligar seletivamente a moléculas de colesterol, gorduras, proteínas, células tumorais e íons metálicos. Fatores que afetam a capacidade quelante deste biopolímero são bastante complexos, sendo sugerido que o mecanismo envolve a ligação com o grupo hidroxila do carbono 6 e, principalmente, com o grupo amino situado no carbono 2 (GOY et al., 2004; YEN et al., 2008). Esse grupamento foi apontado como responsável pela capacidade antioxidante da quitosana contra diferentes espécies de radicais instáveis (ARANAZ et al., 2009; ARANCIBIA et al., 2014).

A quitosana tem sido amplamente estudada e é alvo de grande interesse das indústrias médica, farmacêutica, cosmética e alimentícia, pois possui propriedades interessantes como atividade biológica (antioxidante, antitumoral, hipocolesterolêmica,

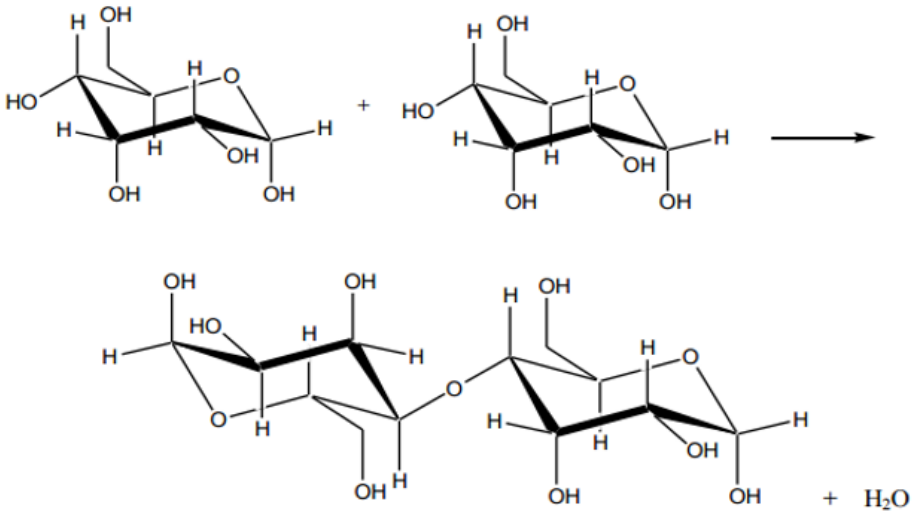
entre outras), excelente biocompatibilidade, biodegradabilidade e baixa toxicidade. Desta forma, são muitas as possíveis aplicações desse polissacarídeo, devido a sua versatilidade (SILVA et al., 2006; PRASHANTH & THARANATHAN,2007). Porém existem relatos que demonstram que a quitosana apresenta, *in vivo,* atividade comprometida devido à sua baixa absorção pelos organismos. A maioria dos intestinos de animais, principalmente o trato gastrointestinal humano, não produzem enzimas capazes de hidrolisar a quitosana. Além disso, a alta massa molecular e viscosidade da quitosana restringem o uso particularmente na medicina e na indústria de alimentos (KUMAR e THARANATHAN, 2004; LI et al., 2005).

Evidências têm mostrado que quitosanas de baixa massa molecular possuem significantes atividades biológicas (KUMAR e THARANATHAN, 2004; LI et al., 2005). Para uma utilização eficiente da quitosana *in vivo,* faz-se necessário a despolimerização para a obtenção de produtos com baixo peso molecular, esse procedimento origina oligossacarídeos de baixa massa molar, sendo então classificados como quitooligossacarídeos (QOS) (STOYACHENKO e VARLAMOV, 1994).

3.5 – Oligossacarídeos de quitosana

Os oligossacarídeos são polímeros que possuem de 2 a 10 unidades de monossacarídeos unidos por ligações glicosídicas. Na polimerização de *n* moléculas de monossacarídeos, ocorre a liberação de *n-1* moléculas de água, obtidas a partir da condensação do grupo hidroxila anomérico de um monossacarídeo com as hidroxilas da unidade adjacente (FIGURA 7). É essa hidroxila anomérica que confere propriedades redutoras ao monossacarídeo e reduz, principalmente, íons metálicos como cobre e prata e se oxida a ácido carboxílico. Esses carboidratos são denominados redutores devido essa propriedade (RIBEIRO E SERAVALLI, 2007).

Figura 7. Formação de um dissacarídeo através de uma ligação glicosídica.



Fonte: Nelson & Cox (2010).

Os oligossacarídeos pertencem a um importante grupo de carboidratos poliméricos que podem ser encontrados em todos os organismos vivos, seja na forma a livre ou combinado a outros compostos como a glicoproteínas. Vários tipos de oligossacarídeos podem ser encontrados em diversos alimentos, incluindo frutas, vegetais, leite e mel. Quando estão presentes em alimentos, melhoram a qualidade do produto, promovendo efeitos benéficos no organismo, sendo por isso, classificado como alimento nutracêutico (NAKAKUKI, 2002).

Os oligossacarídeos de quitosana, portanto, resultam da despolimerização deste polissacarídeo. Por terem tamanho molecular menor que a quitosana, não apresentam dificuldades quanto à solubilização em água, tornando possível a obtenção de soluções desses oligossacarídeos com baixa viscosidade e em pH neutro. Além dessas propriedades, os oligossacarídeos de quitosana apresentam também atividade antitumoral (SUZUKI et al., 1986; MAEDA & KIMURA 2004; PRASHANTH & THARANATHAN, 2005),

atividade antimicrobiana (JEON, PARK, KIM, 2001; ZHENG & ZHU, 2003) e atividade prebiótica (LEE et al.,2002). Tais propriedades têm atraído o interesse de muitos pesquisadores em utilizar os oligossacarídeos derivados da quitosana, principalmente nas áreas médicas e nutricional, em que se observa a possibilidade destes em melhorar a qualidade dos alimentos e saúde humana (NAKAKUKI, 2002; KIM & RAJAPAKSE, 2005).

3.5.1 – Produção dos oligassacarídeos por hidrólise da quitosana

O processo de hidrólise da quitosana é semelhante ao que acorre com outros polissacarídeos, onde a presença de determinados agentes rompe as ligações glicosídicas. A degradação dessas ligações pode ser obtida por diferentes metodologias, nas quais os produtos gerados, nesse caso, oligossacarídeos de quitosana, variam tanto o grau de polimerização quanto o número e sequência das unidades de GlcN (2-amino-2-deoxi-D-glicose) e GlcNAc (2-aceto-amino-2-deoxi-D-glicose) no oligômero gerado. Dentre os métodos já descritos encontram-se o da hidrólise ácida, hidrólise enzimática (utilizando de enzimas específicas ou não específicas), degradação oxidativa com peróxido de hidrogênio, degradação ultrassônica, químico-enzimático (KIM & RAJAPAKSE, 2005) e radiação (HAIet al., 2003). Dentre estes métodos os mais empregados são o da hidrólise ácida e enzimática.

3.5.1.1 – Hidrólise ácida da quitosana

Para a produção em grande escala de oligômeros de quitosana, a hidrolise ácida pode ser utilizada para romper as ligações glicosídicas da quitosana, sendo empregada para essa metodologia o HCl (DOMARD & CAARTIER, 1989) e HNO2 (TOMMERAAS et al., 2001). Essa metodologia é de fácil execução, embora esse mecanismo resulte em um baixo rendimento de oligômeros e grande quantidade de monômeros (D-glucosamina), além de, pela possível presença de contaminação por compostos químicos tóxicos, não poderem ser utilizados como material bioativo (CABRERA & CUTSEM, 2005). Outro inconveniente desse método é a necessidade em utilizar altas temperaturas e grandes concentrações de reagentes, podendo gerar possíveis problemas ambientais (RONCAL et al., 2007).

3.5.1.2 – Hidrólise enzimática da quitosana

Ao contrário do que ocorre no processo de hidrólise ácida da quitosana, a hidrólise enzimática é realizada em condições brandas. Neste processo, as enzimas realizam a hidrólise de forma mais específica que o ácido, e permitem o controle da reação ao decorrer do processo e, consequentemente, do grau de polimerização dos oligômeros gerados (KIM & RAJAPAKSE, 2005; MING et al., 2006; RONCAL et al., 2007; KUO, CHEN & CHIANG, 2004).

Como citado anteriormente, a hidrólise enzimática da quitosana pode ocorrer utilizando enzimas específicas ou enzimas não específicas. Como enzima específica para este processo tem-se a quitosanase (B-D-2-deoxi-2-amino 1→4 glucosidade). Esta enzima pode ser encontrada em uma grande variedade de microrganismos, incluindo bactérias, actinomicetos e fungos e, em pequena quantidade, em plantas (CHEN; XIA; YU, 2005). A produção de oligossacarídeos de quitosana por via enzimática, em que se utilize a quitosanase, é limitada devido a ausência de uma produção constante e eficiente de quitosanases que garantam um processo de baixo custo. Como alternativa, estudos foram realizados utilizando enzimas inespecíficas que diminuíssem o custo de produção e alcançasse êxito na produção desses oligômeros (CABRERA & CUTSEM, 2005; RONCAL et al., 2007).

A hidrólise enzimática realizada por enzimas inespecíficas tem sido descrita na literatura como uma alternativa efetiva na obtenção de oligossacarídeos e de polímeros com menor massa molar. É atrativa pelo menor custo relativo em comparação com enzimas específicas. Em consequência disso, alguns pesquisadores têm estudado enzimas comerciais não específicas (PANTALEONE, YALPANI & SCOLLAR, 1992; VISHU KUMAR et al., 2004; VISHU KUMAR AND THARANATHAN, 2004; CHENG, CHANG, WU, & LI, 2006; RONCAL et al., 2007; LIN et al.,2009; XIE et al. 2009; pois estas têm sido usadas em indústrias alimentícias por muitos anos e são relativamente seguras e com menor custo.

Roncal e colaboradores (2007) descrevem um trabalho em que se investigou a capacidade de enzimas inespecíficas, como a celulase, pepsina e lipase, em hidrolisar a quitosana em comparação com quitosanase. Como resultado foi observado que em 1 hora de reação a viscosidade diminuiu em 80% e em 20 horas, 89% quando utilizado a pepsina. A quitosanase em 1 hora reduziu a viscosidade em 65% e em 20 horas a 96%. Também foi observada a formação dos terminais redutores ao longo da hidrólise, onde a lipase A e a quitosanase foram mais eficientes em gerar tais terminais (Tabela 2).

Tabela 2. Hidrólise de quitosana catalisada por diferentes enzimas comerciais.

Enzyme	Viscosity decrease (%)		Reducing ends (mM)	
	$t = 1$ h	$t = 20$ h	$t = 1$ h	$t = 20$ h
Bromelain	61	86	—	0.51
Lysozyme	28	41	—	0.20
Cellulase	69	85	0.34	3.11
Pepsin	80	89	0.43	3.32
Lipase A[b]	82	97	0.26	4.69
Papain	42	70	—	0.28
Protease type XIV	37	82	—	0.42
Chitosanase	65	96	0.41	5.46

Fonte: Roncal e colaboradores, 2007.

A atividade enzimática foi mensurada a partir da diminuição da viscosidade e pela formação dos terminais redutores. b A lipase A foi submetida a ao pH de 3,0, diferente das demais que foi 4,5.

Pôde-se observar, através de estudos cinéticos, que as enzimas testadas possuem ação de endo-enzimas, pois a hidrólise da solução de quitosana provocou intensa redução da viscosidade do meio devido à degradação do polímero em produtos de cadeia menor (RONCAL, et al.; 2007). O mecanismo de degradação da quitosana por essas enzimas não está muito claro, entretanto, foi demonstrada a atividade hidrolítica sobre a quitosana para as enzimas papaína (de origem vegetal), lipase, celulase, pectinase, glucanase e protease (de origem microbiana) (MUZZARELLI et al.; 1995; IZUME et al.; 1992; RONCAL et al.; 2007).

Porém, apesar do baixo custo na utilização de enzimas inespecíficas na produção de oligossacarídeos, estudos realizados por Mengíbar (2013), demostraram que os oligossacarídeos de quitosana, produzidos por quitosanase, apresentam melhor atividade biológica em relação aos oligômeros produzidos por hidrólise inespecífica realizada com lisozima. Dessa forma é importante analisar se a hidrólise da quitosana por pepsina, oferece realmente um melhor custo benefício em relação a hidrólise por enzimas específicas, já que os oligômeros produzidos por quitosanase apresentariam melhores efeitos biológicos.

Diversas pesquisas relacionadas com a avaliação das atividades biológicas dos oligossacarídeos de quitosana, sendo a quitosana de origem comercial, vêm sendo desenvolvidas nos últimos anos, estando evidenciada atividade antioxidante, antitumoral, anti-inflamatória, antimicrobiana, atividade hipocolesterolêmica, agentes

aceleradores na absorção de ferro e cálcio, agentes imunomoduladores e pré-bióticos (KIM & RAJAPAKSE, 2005).

2.5.2 – Aplicações dos oligassacarídeos de quitosana

Os oligossacarídeos obtidos a partir da hidrólise da quitosana têm despertado muito interesse na área farmacêutica, alimentícia e médica, devido às suas propriedades biológicas.

Diversas pesquisas relacionadas com a avaliação dessas atividades biológicas vêm sendo desenvolvidas nos últimos anos, sendo evidenciada atividade antimicrobiana (ALLAN & HADWIGER, 1979; KENDRA & ADWIGER, 1984; NO, ARK, LEE, & MEYERS, 2002; SEKIGUCHI et al., 1994; SUDARSHAN, HOOVER, & KNORR, 1992; UCHIDA, LZUME, & OHTAKARA, 1989; WEI & XIA, 2003; ZHAO & XIA, 2006),hipocolesterolêmica (KIM & RAJAPAKSE, 2005; MAEZAKI et al., 1996; SUGANO et al., 1980; SUGANO, WATANABE, KISHI, IZUME, & OHTAKARA, 1988; ZHOU, XIA, ZHANG, & YU, 2006), efeitos antitumorais e imunológicos (SUZUKI et al., 1986; TOKORO et al., 1988; XIA, 2003), carreadores de drogas, (BRAVO- OSUNA, MILLOTTI, VAUTHIER, & PONCHEL, 2007; LIAO et al., 2007; PARK, SARAVANAKUMAR, KIM, & KWON, 2010; SINSWAT & TENGAMNUAY, 2003; THANOU, VERHOEF, & JUNGINGER, 2001), aceleradores na absorção de ferro e cálcio (BRAVO-OSUNA et al., 2007; DEUCHI, KANAUCHI, SHIZUKUISH, & KOBAYASHI, 1995; JEON, SHAHIDI, & KIM, 2000; JUNG, MOON, & KIM, 2006; LIAO et al., 2007;SINSWAT & TENGAMNUAY, 2003; XIA, 2003), além de antioxidante, e outras.

2.5.2.1 – Propriedade antioxidante dos oligossacarídeos de quitosana

O estresse oxidativo tem sido identificado como um ponto comum para várias doenças crônicas como diabetes, artrite, doenças neurodegenerativas, cardiovasculares e tumores. Essas doenças estão diretamente relacionadas com a oxidação de biomoléculas por espécies reativas de oxigênio geradas extensivamente pelos tecidos (CALABRESE et al.,2005). Em algumas doenças, tratamentos envolvendo antioxidantes têm mostrado ser efetivo em reduzir marcadores do estresse oxidativo levando ao crescente interesse na progressão do desenvolvimento mundial para explorar efetivos antioxidantes de moléculas sequestradoras de radicais livres, especialmente as de origem natural (MENDIS et al., 2007). Os sequestradores de radicais livres são antioxidantes

preventivos e a presença destes compostos pode inibir eventos e as sequências oxidativas em diferentes níveis (KIM & RAJAPAKSE, 2005).

As propriedades antioxidantes dos oligossacarídeos têm atraído atenção, principalmente devido a sua habilidade de doar prótons, pois o radical livre pode reagir com o grupo NH_2 formando o grupo NH_3^+ em consequência da absorção do íon hidrogênio da solução. Além destes grupos, as hidroxilas dos carbonos 2, 3 e 6 do anel piranosídico pode reagir com radicais livres instáveis para formar moléculas estabilizadas (KIM & RAJAPAKSE, 2005). Essa propriedade antioxidante depende do grau de desacetilação e da massa molecular desses oligossacarídeos pois essa atividade está diretamente relacionada com a protonação dos grupos amina (RAJAPAKSE et al., 2007; XIE, W., XU, P., LIU, Q., 2011). Baseados em resultados obtidos em estudos utilizando a técnica de aprisionamento de elétron (Electron Spin Trapping), os oligossacarídeos de quitosana com um peso molecular entre 1-3 KDa têm sido identificados por seu alto potencial em sequestrar radicais livres (PARK, JE & Kim, 2003a).

A geração de espécies reativas de oxigênio por células endoteliais está envolvida em várias condições clínicas associadas à aterosclerose, hipercolesterêmica e coagulação intravascular disseminada (PANDIAN et al., 2005; FASANARO et al., 2006). A utilização de oligossacarídeos de quitosana em cultura de células endoteliais mostrou a melhora na injúria celular em associação com o estresse oxidativo. Além disso, influencia na apoptose e na progressão do ciclo celular por atenuar o estresse oxidativo exógeno (LIU & ZENG, 2009).

2.5.2.2 – Efeitos antitumorais dos oligossacarídeos de quitosana

O câncer é um conjunto de doenças caracterizadas pelo desenvolvimento de massa tecidual resultante de uma divisão celular exacerbada. No desenvolvimento dessas doenças, o crescimento do tecido neoplásico supera o número de células do tecido sadio (KIRCHNER, 2014). Entre as linhas para a terapêutica do câncer, tem-se o uso de fármacos antineoplásicos, que apesar de ser o principal meio de tratamento, apresenta alguns obstáculos devido ao seu baixo índice terapêutico, fazendo com que a dose terapêutica seja similar a dose tóxica, o que provoca muitos efeitos colaterais ao paciente (FUKUMASU et al., 2008). Entre as principais drogas utilizadas na terapêutica, tem-se o 5-fluoruracil. Este quando administrado é metabolizado em monofosfato de fluorodeoxiurina (FdUMP), um metabólito ativo que inibe a ação da

enzima timidilado sintase, bloqueando a biossíntese de DNA, promovendo a apoptose (MALET-MARTINO et al,. 2002). O 5-fluoruracil apresenta variabilidade na resposta terapêutica e na toxicidade (BARATTE et al., 2010).

O crescimento do número de novos casos de câncer tem impulsionado pesquisas que visam encontrar alternativas de tratamento para esse conjunto de doenças (HURYN, 2013; REITZ, 2013). Estudos com novos compostos com atividade antitumoral, a cultura de células tem sido uma ferramenta fundamental.

Atividade biológica dos oligossacarídeos de quitosana em inibir o crescimento de tumores foi descrito na literatura. Essa atividade é dependente da característica estrutural destes compostos, como o grau de desacetilação e o peso molecular dos oligômeros (KIM & RAJAPAKSE, 2005) (Tabela 3).

Tabela 3. Efeito dos oligossacarídeos de quitosana (COS) no crescimento de diferentes tumores em camundongos.

COS			Tumor type	Inhibition (%)[a]	Ref.
MW (kDa)	DD (%)	Dose (mg/(kg/day))			
~1	100	10	Meth-A solid tumor	41	Tokoro et al. (1998)
~1	100	300	Sarcoma 180 solid tumor	93	Suzuki et al. (1986)
~1	100	500	MM 46 solid tumor	55	Suzuki et al. (1986)
6.5–12	90	10	Sarcoma 180 solid tumor	61.7	Jeon and Kim (2002)
1.5–5.5	90	10	Sarcoma 180 solid tumor	66.7	Jeon and Kim (2002)
1.5–5.5	90	50	Uterine cervix tumor	73.6	Jeon and Kim (2002)
1.4	85	50	Sarcoma 180 solid tumor	50.4	Qin et al. (2002)
3–10	80	200	Sarcoma 180 solid tumor	56.9	Qin et al. (2002)

Fonte: KIM & RAJAPAKSE, 2005.

A inibição do crescimento de tumores calculado como porcentagem, a partir da comparação dos pesos dos tumores tratados com COS e o peso dos tumores do grupo controle.

Estudos sugerem que a atividade antitumoral dos oligossacarídeos de quitosana atuaria não apenas matando as células tumorais, mas também partir de seus efeitos imunológicos aumentando a produção de linfócitos, gerando uma resposta antitumoral de linfócitos-T citotóxicos (CD8) (TOKORO, TATEWAKI, SUZUKI, MIKAMI, SUZUKI AND SUZUKI, 1998). Os oligossacarídeos também induzem apoptose celular, foi o que Xu et al. (2008) observaram ao tratar células de hepatocarcioma (SMMC-7721) com oligossacarídeos. O possível mecanismo é que esses compostos regulam a expressão da proteína pró-apoptótica BA e a ativação de caspases (serino proteases), acionando o programa de apoptose da célula.

Foi descrito ainda, que os oligossacarídeos de quitosana manifestam um efeito inibitório de crescimento e antimetastático em carcinoma de pulmão de ratos com administração intramuscular (TSUKADA et al., 1990).

Os oligossacarídeos podem ser fracionados e testados as atividades biológicas dessas frações. Em estudos realizados sobre o efeito antitumoral foi evidenciado que estes compostos possuem forte atividade inibitória para o tumor ascítico em camundongos BALB e que os quitoligossacarídeos do tipo N-acetilquitohexose e quitohexose apresentaram efeitos inibitórios muito fortes para o Sarcoma-180 e crescimento do tumor sólido MM 156 em ratos, como foi descrito por TOKORO et al . (1988).

Os oligossacarídeos de quitosana possuem potenciais propriedades que os permitem ser aplicados em diversas áreas. Além de suas interessantes qualidades, a obtenção destes a partir de resíduos da indústria pesqueira faz com que sua produção tenha melhor relação custo/benefício, quando comparados a outros polímeros atualmente utilizados no mercado podendo ser cada vez mais promissor o seu uso.

4 – RESULTADOS

Os resultados dessa tese estão apresentados na forma de artigos científicos.

4.1 – Artigo 1

ENZYMATIC HYDROLYSIS OF SHRIMP CHITOSAN FOR PRODUCTION OF WATER-SOLUBLE OLIGOSACCHARIDES WITH ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY

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ABSTRACT

Chitosan polysaccharides have been widely studied and applied due to their unique properties among other natural and pseudo-natural sugars. In order to obtain more suitable acid-free water-soluble chitosan, different extents of backbone depolymerization is used to broaden the utilization while maintain the attractive chitosan chraracteristics. This work aimed to obtain chitosan polymers and oligomers from shrimp processing by-products and evaluate their chemical features, antioxidant and antimicrobial potential. Chitosan was produced from purified shrimp chitin by enzymatic hydrolysis for 20 hours at 40 ° C using pepsin, resulting in chitoligosaccharides and low molecular weight chitosan. The obtained oligo- and polysaccharides were evaluated with infrared spectroscopy (FT-IR), ^{13}C nuclear magnetic resonance (^{13}C NMR) and mass spectrometry (MALDI-TOF). To determine the antioxidant potential, experiments using ABTS radical, reducing power and chelating potential of Fe^{2+}, total antioxidant capacity by phosphomolybdenum (CAT) were evaluated, and protection of DNA molecule submitted to Fenton reaction. Antibacterial assay was performed for potential of growth inhibition of different chitosan in liquid media for *Escherichia coli* and *Staphylococcus aureus*. Chitoligosaccharides (COS) showed complete solubility and low viscosity in neutral aqueous media. The structural profile were similar to that of chitosan. MALDI-TOF analysis showed that the COS molecular weight is between 0.8 and 2.6 kDa. The maximum antioxidant activity for ABTS, DPPH and chelating abilities on ferrous ion was 37.94%, 30.7% and 22.38% (at 1mg/ml) respectively. COS exhibited full DNA protection at 400 μg/mL. Chitosan and its derivatives showed antibacterial activity against *E. coli* and *S. aureus*. COS are easy to produce by pepsin degradation with a narrow DP range and no presence of monomers. These results show the efficiency of these oligomers as chitosan analogues, allowing advanced utilization while maintaining biological properties. COS utilization represents an improvement of chitosan application in biomedical research.

Keywords: By-products; Chitosan hydrolysis; Oligosaccharides; Pepsin.

Introduction

Chitin is an aminosugar component of the external carapace of crustaceans and other arthropods and the second most abundant polysaccharide in nature. It is commercially obtained from shrimp and crab processing by-products, which represent most of the residual products in the shellfish industry (Lodhi et al., 2014; Li et al., 2017). The conversion of chitin by deacetylation is performed to obtain chitosan, which is a more soluble and charged biopolymer, naturally being found in the cell walls of certain fungi and algae (Benhabiles et al., 2013; Mohammed et al., 2013; Xin et al., 2012). The presence of amino group and two hydroxyl groups in each pyranoside ring facilitates the reaction with negatively charged polymers (Yousef, et al., 2015; Yousef, et al., 2018). Thus, chitosan is known for its wide variety of bioactive properties, such as biocompatibility, biodegradability, antimicrobial, antitumor and antioxidant activities (Shukla et al., 2013; Babu et al., 2001; Chien et al., 2016; Moreno-Vásquez et al., 2017).

The low solubility of chitosan in pH above 6.3 and high viscosity of its acidic solutions, a consequence of its molecular weight, is undesirable for many biological and technological applications. The production of low molecular weight chitosan (LMWC) and chitooligosaccharides (COS) is an effective way to improve solubility and decrease solution viscosity, improving their potential as functional materials for biotechnological applications (Mengíbar et al., 2013).

COS can be produced using acid hydrolysis, physical methods and enzymatic depolymerization (Aam et al., 2010). Among the methods already described for producing COS, enzymatic hydrolysis is known to be performed under mild conditions and high specificity (Wu et al., 2011). In addition, COS can also be produced by some non-specific enzymes including lysozyme, pepsin, cellulase, lipase, and papain (Li et al., 2005; Roncal et al., 2007; Kim et al., 2005; Ming et al., 2006).

Enzymatic hydrolysis carried out by nonspecific enzymes has been described in the literature as an effective alternative to obtain oligosaccharides and polymers with lower molecular weight. It is attractive for its feasibility compared to specific enzymes, with the advantage of yielding well defined oligomer lengths and low monosaccharide generation (Pan et al., 2016). Consequently, some researchers have studied nonspecific commercial enzyme preparations, as these have been used in the food industry for many years and are relatively safer and less costly (Kumar et al., 2004; Liu et al., 2016; Xia et al., 2008; Suwan et al., 2009). This work aimed to obtain chitosan from shrimp chitin

extracted from industrial wastes of *L. vannamei*. The non-specific enzymatic hydrolysis was performed using pepsin, generating LMWC and COS which were analyzed by their chemical, antioxidant and antibacterial properties.

Materials and methods

Materials

Shrimp processing wastes (cephalothorax), of *Litopenaeus vannamei* adults (total body commercial weight 11±2g) were provided by a local fishery processing plant (Noronha Pescados Ltd., Recife PE, Brazil). Fresh heads were immediately stored on ice and transported to the laboratory where they were packed in plastic bags (1 kg per bag) and stored at −20°C until use. Only analytical-grade reagents were used: 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p′*-disulfonic acid monosodium salt hydrate (ferrozine), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and pepsin from porcine gastric mucosa were all purchased from Sigma-Aldrich (St Louis, USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

Chitosan production

Shrimp heads were mixed with distilled water at a ratio of 1 kg of wet raw-material to 1 L of distilled water and blended in an industrial food processor (Engefrio, Recife, Brazil). Then, the mixture was hydrolyzed, according to the method of Cahú et al. (2012) for *L. vannamei*, without the addition of any commercial proteases, in a vessel placed in water bath at 40°C for 2 h with constant stirring (700 rpm). The preparation was then boiled for 10 min and filtered (1 mm^2 mesh) to retain the carapace (solid phase), which was used for chitin extraction and chitosan (CH) production. The solid phase (chitinous residue) was dried at 70 ◦C and treated with 1 M HCl and 1 M NaOH to remove minerals and proteins, respectively. The depigmentation was performed with 0.5% (w/v) $KMnO_4$ and 1% (w/v) $Na_2S_2O_4$. The chitin flakes (25 g) were washed with abundant distilled water and dried at 70°C. Chitin was added to 500 mL of 50% (w/v) NaOH and incubated in a water bath at 65 ◦C for 24 h with constant stirring. N-deacetylation was performed a second time under the same conditions. The chitosan was washed with distilled water until a neutral pH was reached, then dried at 70 ◦C and pulverized in an electric mill (IKA® A11 Basic, IKA®-Works Inc., China) to reduce

the particle size to below 250 m. Finally, the chitosan (1%, w/v) was solubilized in 3% (v/v) acetic acid, filtered through paper filter (14 m), precipitated with 1 M NaOH until a pH of 11.0 was reached, neutralized to pH 7.0 and centrifuged (10,000 × g for 15 min at 25 ◦C). This purified chitosan was lyophilized and collected as a fine powder.

Degree of acetylation and viscometric molecular weight of chitosan

Fourier transform infrared spectroscopy (FT-IR) was employed, wherein FT-IR spectra were measured in KBr pellets in transmission mode within a range of 4000–500 cm^{-1} using an FT-IR Bomem MB100 spectrophotometer. The degree of deacetylation (DD) was calculated from the infrared spectra, according to the formula: DD(%)=100–((A1320/A1420-0.3822)/0.03133) (Brugnerotto et al., 2001). Intrinsic viscosities in sodium acetate buffer (0.25M, pH 4.5) were measured using an Ostwald n°100 capillary viscometer, with a diameter of 0.9 mm and solution concentrations lower than 1% (w/v) at 25±0.1°C. The following Mark-Houwink-Sakurada (MHS) equation for chitosan was used to calculate molecular weight:

$$[\eta] = K M_w^{\alpha}$$

Where [η] is the intrinsic viscosity calculated from viscosity data and Mw is the molecular weight. The viscometric constants, *K* and α were $1.49x10^{-4}$ dL/g and 0.79, respectively, for polydisperse chitosan samples in sodium acetate buffer (0.25M) at 25°C, with the degree of acetylation (DA) between 20 and 26%, based on an empirically modified MHS equation at 25 °C (Kasaai et al., 2000).

Production of COS

COS were obtained by enzymatic hydrolysis with pepsin from porcine gastric mucosa, according to Roncal et al. (2007) with slight modifications. Briefly, chitosan (1%) in 0.2 M sodium acetate buffer pH 4.5 and pepsin (1:100) were incubated in a water bath at 40°C for 20h. After this, the pH was adjusted to 7.0 with 1 M NaOH and the reaction was boiled for 10 min to inactivate the enzyme and centrifuged. The supernatant was freeze-dried and resuspended with methanol, and then precipitated and washed with 2 volumes of acetone, and finally dried under vacuum.

Nuclear magnetic resonance and MALDI

Chitosan and COS were analyzed by nuclear magnetic resonance (NMR) and matrix-assisted laser desorption/ionization (MALDI-TOF). Decoupled ^{13}C NMR spectra were obtained using a Bruker Avance DRX-400 spectrometer with a 5-mm inverse probe. ^{13}C NMR acquisitions were performed using the WALTZ-16 pulse sequence with the following parameters: FIDRES: 0.8466 Hz; AQ: 0.5906 s; DW: 15.75 s; DE: 5.5 μs; D1: 110 ms; D2: 3.4 ms; PL12: 17 dB (decoupler 1H). The molecular weight of COS obtained from the enzymatic hydrolysis of chitosan was determined by MALDI-TOF MS analysis (Voyager-DE TM STR Biospectrometry Workstation, Applied Biosystems Inc., NCIRF, Korea)

Antioxidant activity

ABTS

To determine antioxidant activity, the radical reduction method $ABTS^{\bullet+}$ was used (Re et al., 1999). Initially formed $ABTS^{\bullet+}$ radical, from reacting 5 ml of 7 mM ABTS • + 88μL of a 2.45 mM potassium persulfate ($K_2S_2O_8$) solution, was incubated with COS at room temperature and in the absence of light for 16 hours. Subsequently, the $ABTS^{\bullet+}$ solution was diluted in ethanol to obtain a solution with an absorbance of 0.70 (± 0.05) at 734 nm. Prepared in triplicate, the final concentrations of COS and standard were 15, 10, 8, 6, 4, 2.1, 0.5 and 0.05 mg/mL. In the absence of light and using 20 μL of each concentration of COS and standards in test tubes with 2000 μL of the ABTS radical $^{\bullet+}$, the absorbance reading was performed for six minutes in a spectrophotometer. The results were expressed as an average of three replicates, as TEAC (Trolox Equivalent Antioxidant Capacity) and the percentage inhibition of oxidation was calculated, using the following equation:

$$\%Inhibition = \frac{ABScontrol - ABSsample}{ABScontrol} \times 100.$$

The determination of IC_{50}, which is the concentration of samples necessary to scavenge 50% of radicals was calculated by linear plot.

Ferrous ion (Fe^{2+}) chelating activity

The Fe^{2+} ion chelating activity was determined by Decker and Welch (1990) with modifications (Decker et al., 1990). A 100 µL sample at different concentrations (0.05; 0.5; 1.0; 2.0; 4.0; 6.0; 8.0; 10.0 mM) was mixed with 100 µL of distilled water. Then 25 µL of 0.5 mmol/L $FeCl_2$ solution and 25 µL of 2.5 mmol/L ferrozine solution were added. This mixture was kept at room temperature for 20 minutes and the absorbance was read at 550 nm. Distilled water was used as the control. The chitosan chelating activity was expressed by the following equation:

$$HCA\ (\%) = \left[1 - \left(\frac{ABSsample}{ABScontrol}\right)\right] \times 100$$

DNA nicking assay

The DNA nicking assay was performed using the pBR 322 plasmid. The reaction mixture contained 0.3 µL of plasmid DNA, 10 µL of Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid, and 80 mM $FeCl_3$) followed by the addition of COS at different concentrations (100, 200, 400 µM). The final volume of the mixture was brought up to 200 µL using distilled water. The mixture was then incubated for 30 min at 37°C and the DNA was analyzed by electrophoresis in 1% agarose gel (Kaur et al., 2008). The measurement of DNA damage protection was analyzed by TotalLab Quant.

DPPH scavenging potential

DPPH scavenging activity was performed according to Blois et al. (1958). Briefly, 100 µL of samples, at different concentrations (0.08-15mg/ml), were mixed with 100 µL of 150 µM DPPH in a 99.5% methanol solution. This mixture was kept in the dark at room temperature for 30 minutes and the absorbances were measured at 517 nm using a Bio-rad xMark™ spectrophotometer. The radical scavenging activity (RSA) was measured using the following equation: DPPH inhibition = $[1-(A_{sample} - A_{blank})/A_{control}]\times100$. Where the blank was the sample mixed with methanol and the control was the DPPH solution without

sample. The determination of $IC_{50,}$ which is the concentration of samples necessary to scavenge 50% of radicals was calculated by linear plot.

Total antioxidant capacity by phosphomolybdenum (TAC)

The total antioxidant capacity of the compounds was evaluated by the phosphomolybdenum method, which consisted in the ability of the composition to reduce molybdenum and form the phosphate-molybdate complex (Pietro et al., 1999). The formation of this complex results in a change in color from yellow to purple-blue. Briefly, the composition was added to a solution of 40 mM ammonium molybdate, 60 mM sulfuric acid, 280 mM sodium phosphate solution and distilled water. The reaction occurred in a water bath at 90 ° C for 90 minutes. The absorbances were read in a spectrophotometer at 695 nm. The sample was replaced with methanol and ascorbic acid at the concentration of 1 mg/ml in the positive control. The absorbance of the sample was subtracted by the absorbance of the blank. The result of this subtraction was divided by the absorbance of ascorbic acid minus the absorbance of positive control. The value obtained in the division was multiplied by 100 and the result expressed as percentage of total antioxidant activity in relation to ascorbic acid (% AAT). The determination of $IC_{50,}$ which is the concentration of samples necessary to scavenge 50% of radicals was calculated by linear plot.

Antibacterial assay

Escherichia coli (UFPEDA224) and *Staphylococcus aureus* (UFPEDA02) bacterial strains were provided by the Department of Antibiotics, Federal University of Pernambuco (Brazil), in Difco Nutrient Agar and stored at 4°C. Inhibitory assays on bacterial growth were performed in sterile microplates, with a modified method (Cahú et al, 2017; Gaidamashvli et al., 2002), using a Smart 3000 Spectrophotometer. To each well (n=6) 200 μL of bacteria cultures (in Difco Nutrient Broth) were added in the exponential growth phase plus 50 μL of chitosan solution (1 mg/mL in 0.025 mol/L acetate buffer, pH 6.0) or COS (1 mg/mL in ultrapure water), and the absorbance was determined at 600 nm every hour for 10 h and then in intervals of 8 hours at 37°C. A positive control was carried out using bacterial suspensions in Nutrient Broth (Costa et al., 2011).

Statistical analysis

Graphs and one-way analysis of variance (ANOVA) with Tukey's post-test were performed using GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego, CA USA, www.graphpad.com).

Results and Discussion

Production of COS

Chitosan oligosaccharides were obtained by non-specific enzymatic hydrolysis using pepsin from porcine gastric mucosa (Sigma-Aldrich), as proposed by Roncal et al. (2007) and the hydrolysis efficiency obtained was comparable to chitosanase. Among the enzymes studied, pepsin presented good performance in its action, reducing the viscosity of chitosan solution by 60% in just 10 minutes, combined with formation of reducing terminals which resulted from the hydrolysis of polysaccharides, at 0.43 to 3.32 mM in 20 hours of reaction with no monomer production. When compared with the performance of the specific enzyme, i.e. chitosanase, the viscosity of the solution was reduced from 65 to 96% in 20 hours, and a reducing end of 0.41 to 5.46 mM was generated (Roncal et al., 2007).

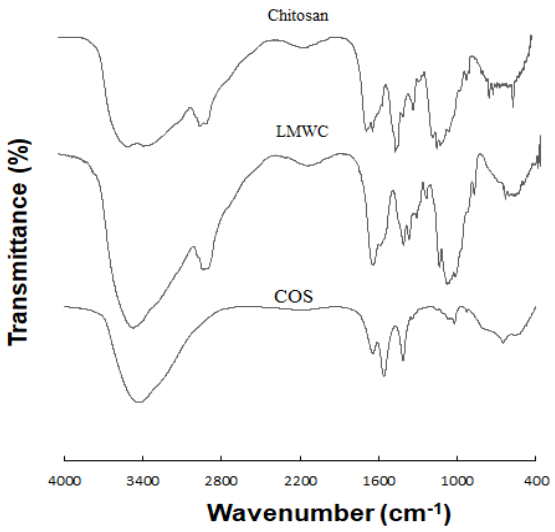
Corroborating these results, a study performed by Kumar and Tharanathan (2004) analyzed the depolymerization reaction of chitosan using some non-specific enzymes. As a result, kinetic parameters obeyed Michaelis-Menten kinetics and *Km* and *Vmax* values indicated high affinity of pepsin to chitosan (Kumar et al., 2004). Although chitosanase is found in a variety of microorganisms, its utilization is still limited due to the lack of steady and efficient production, which has encouraged the search for a more feasible process. The mechanism is not entirely clear of how pepsin hydrolyzes chitosan, but studies have shown that chitosan solutions show a remarkable decrease in viscosity when submitted to incubation with pepsin, thus suggesting that pepsin acts on this polysaccharide as an endoglycosidase, reducing its molecular mass (Roncal et al., 2007). From the enzymatic hydrolysis we obtained two types of molecules: low molecular weight chitosan (LMWC) and chitosan oligosaccharides (COS).

Characterization of COS

The characterization of chitosan and enzymatic hydrolysis products (LMWC and COS) was performed by infrared spectroscopy (FT-IR), ^{13}C nuclear magnetic resonance (^{13}C NMR) and mass spectrometry (MALDI-TOF).

Spectroscopy in the infrared region allows one to observe and classify some bands related to the characteristic vibrations of functional groups present in the structure of chitosan, LMWC and COS. The FT-IR graphics obtained for chitosan and the products of enzymatic hydrolysis (Figure 1) showed a broad peak near 3400 cm^{-1} in the region corresponding to the -OH stretch, which appears as a superimposed axial deformation of the amine group band (-NH) (Nunthanid et al., 2001). The peak near 2888 cm^{-1} represents the –CH group stretch and peak around 1661 and 1671 cm^{-1} are assigned to the vibrations of the amide I group and correspond to C=O stretching, indicating that acetylated units remained (Paulino et al., 2006). The peak around 1370 cm^{-1} indicates the C-O stretch of the primary alcohol group (-CH_2OH). The axial deformation of amide CN appears around 1428 cm^{-1} peak while the presence of a 1555 cm^{-1} amine band indicates the deacetylated form. The intense band between 800 and 1200 cm^{-1} is related to the presence of pyranoside rings (Shigemaza et al., 1996). This last band was not intense in the spectrum for COS, which shows less pyranoside rings relative to chitosan and LMWC.

Figure 1 - FT-IR spectrums of Chitosan and products from non-specific enzymatic hydrolysis



Source: Prepared by the author (2016).

From the FT-IR, using the relation between the absorbance in the wavelengths 1655 and 3450 cm^{-1}, it was possible to estimate the degree of deacetylation of these biomolecules. This analysis is considered one of the main parameters to characterize chitosan. The degree of deacetylation is defined as the number of amine groups in relation to the

number of amide groups in the polymer chain. As a result, the CH showed 81.02%, LWMC 80.68% and COS 81.58% degrees of deacetylation (Table 1).

Table 1 - Average molecular weight (Mw) by MALDI-TF MS and deacetylation degree (DD) values (%) for chitosan (CH), low molecular weight chitosan (LWMCH) and COS by FT-IR.

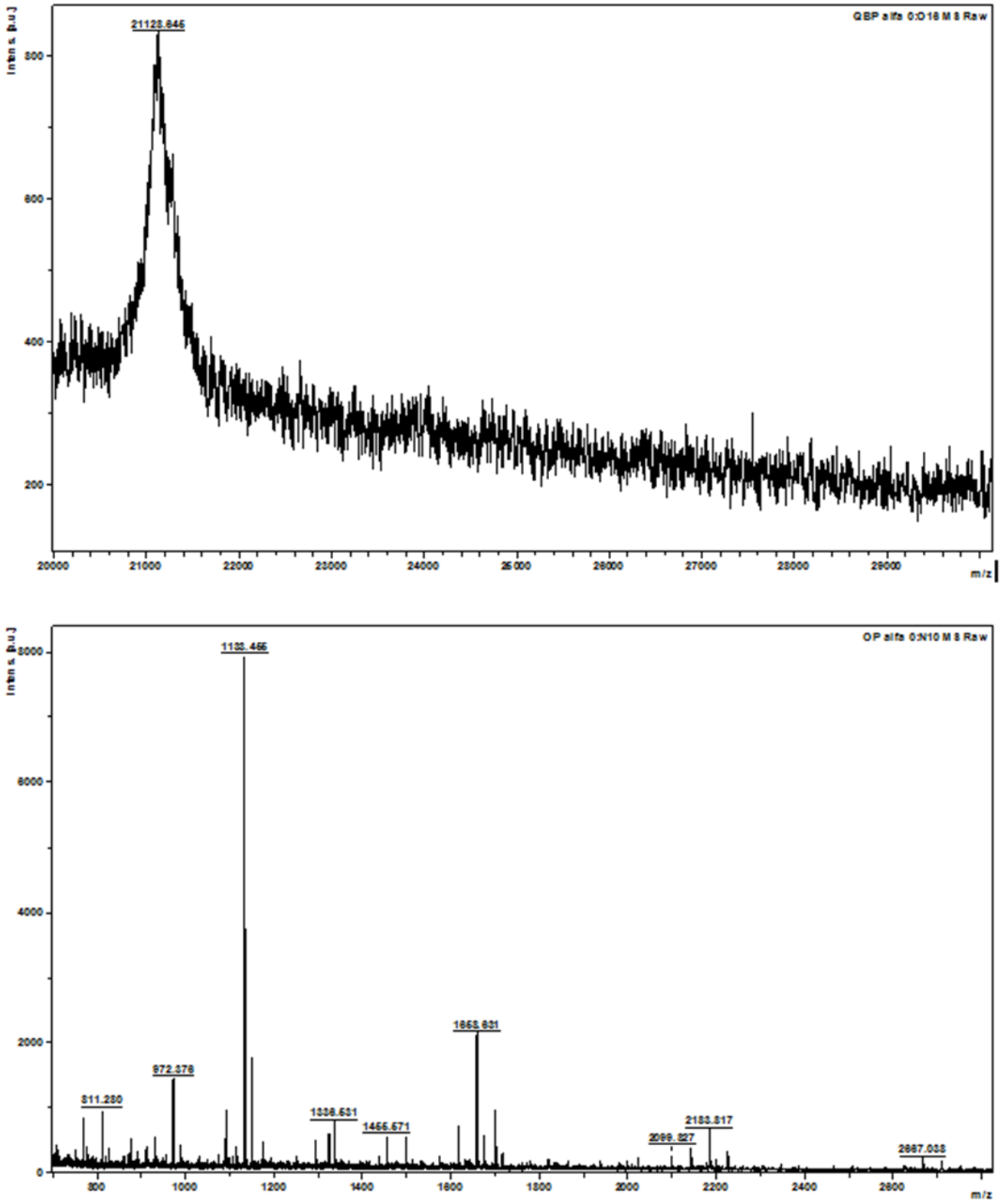
Samples	***DD (%)***	***M_w (KDa)***
CH	88.02	1200†
LWMCH	80.68	21.1‡
COS	81.58	0.811-2.667‡

†Calculated from viscometric measurements with Mark-Houwink-Sakurada (MHS) equation.
‡Data from MALDI-TOF analysis.
Source: Prepared by the author (2016).

The ^{13}C spectra of COS showed chemical shifts corresponding to the chitosan structure. The peak at 178.8 corresponds to C=O, at 104 (C1), 58.8 and 57.2 (C2). C3 and C5, which appear as a single signal at 72.8, 74.6. 64 (C4) and 63.2 (C6). This profile was similar to that described by Cahú et al (2012) [23], found for marine shrimp (*Litopenaeus vannamei*) chitosan.

Chitosan, LMWC and COS were analyzed by MALDI-TOF-MS. In the case of chitosan, no spectra were detected because of high molecular weight. Thus, chitosan was analyzed by capillary viscometry which identified an average molecular weight of 1200 KDa calculated from intrinsic viscosity and the MHS equation. Low molecular weight chitosan, when analyzed by MALDI-TOF-MS F, showed an average mass of approximately 21 kDa (Figure 2).

Figure 2 - MALDI-TOF MS spectrums of low molecular weight Chitosan (A) and COS (B).



Source: Prepared by the author (2016).

MALDI-TOF for COS shows 9 peaks corresponding to the glucosamine oligomers with the degrees of polymerization (DP) 5 to 16 monitored in the spectrum.

The mass/charge (m/z) of the main peaks were 811.280, 972.376, 1133.455, 1336.531, 1455.571, 1658.631, 2099.827, 2183.817 and 2667.038. Nine peaks were all [M+Na+] ion-peaks with approximately 203 Da mass larger than the next peak, which was the molecular mass of a GlcN and GlcNAc residue respectively (Li et al., 2012). To summarize, this analysis displayed that the distribution of COS ranged from 5 to 16 DP and consisted of a mixture of 0.811-2.66 kDa saccharides, showing a major peak of 1.13 kDa, which suggests that the sample is composed of heptamers (DP 7) (Table 2).

Table 2 - Prediction of the molecular weight of chitosan oligosaccharides (COS) by MALDI-TOF MS analysis.

Chitosan oligosaccharides	***DP***	***m/z***
GlcN	1	180
GlcNAc	1	220
(GlcN)5	5	811
(GlcN)6	6	972
(GlcN)7	7	1133
(GlcN)5 + GlcNAc + (GlcN)2 or (GlcN)7 + GlcNAc	8	1336
(GlcN)9	9	1455
(GlcN)5 + GlcNAc + (GlcN)3 or (GlcN)9 + (GlcNAc)1	10	1658
(GlcN)13; (GlcN)11 + (GlcNAc)2	13	2099; 2183
(GlcN)14 + (GlcNAc)2	16	2667

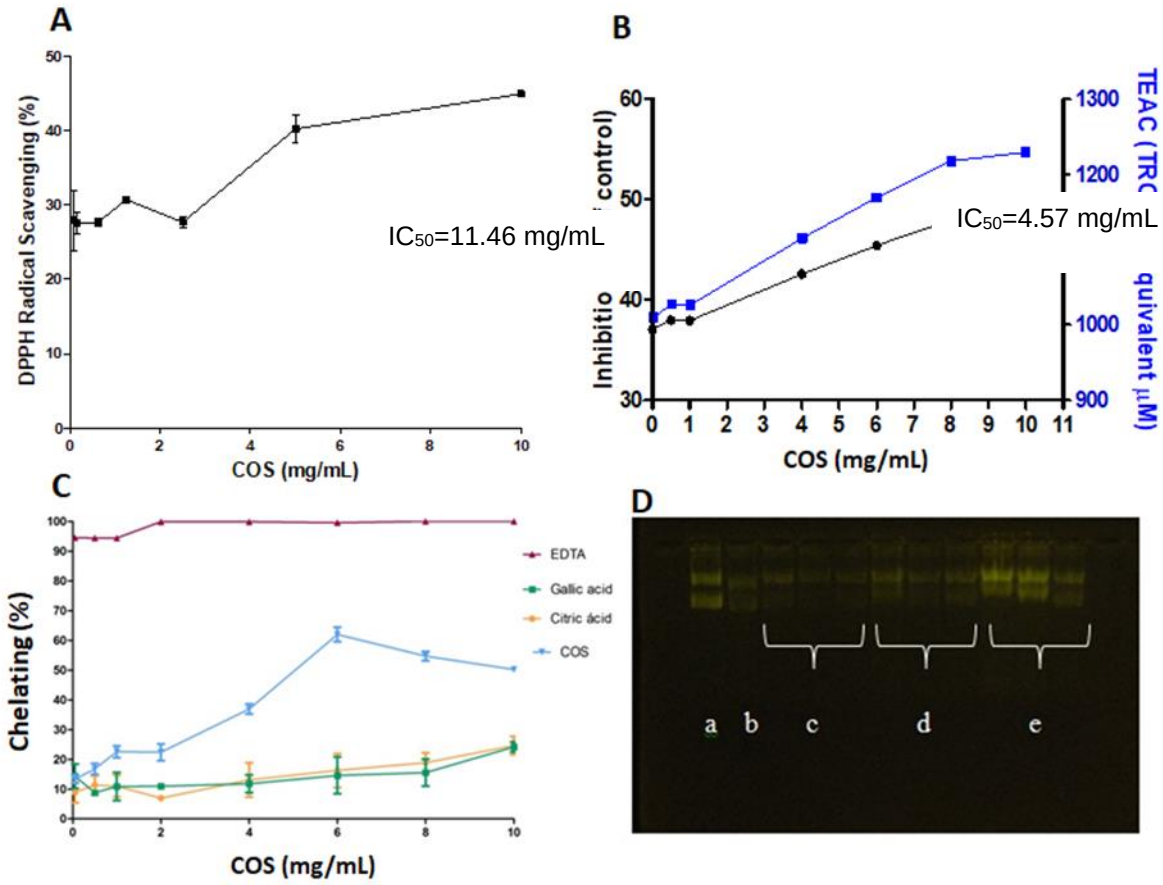
Source: Prepared by the author (2016).

Oligosaccharides obtained from the hydrolysis of chitosan have aroused much interest not only because COS are water soluble and have low molecular weight, but also because they have many functional properties (Huang et al., 2006; Chen et al., 2003; Je et al., 2004).

In vitro antioxidant activity

Two of the most commonly used methods to measure antioxidant activity is by capturing the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis radical (3-ethylbenzthiazoline-6- sulfonic acid) (ABTS. +), which is generated by a chemical reaction. COS proved to be a promising source of antioxidant compounds as shown in Figure 3. The scavenging potential for COS towards DPPH radical was 44.93% at 10 mg/mL. The activity towards DPPH radical may be explained due to the organic reaction medium, and limited solubility of COS in methanol solution. The calculated IC_{50} for COS in DPPH scavenging assay was 11.46 mg/mL.

Figure 3 - Antioxidant activity of COS (A) DPPH (B) ABTS, (C) Chelating activity, (D) Protective effects of COS in DNA nicking assay. (a) negative control (distilled water + DNA), (b) control (DNA + Fenton's reagent), (C) COS 100µg/mL+ Fenton's reagent, (d) COS 200 µg/mL) + Fenton's reagent, (e) COS 400 µg/mL) + Fenton's reagent.



Source: Prepared by the author (2016).

The antioxidant activity by radical reduction method of COS as a function of ABTS concentration is shown in Fig 3 B, with oxidative inhibition of 48.56% at 10mg/ml, in 6 min, equivalent to TEAC of 1229.17 μM Trolox. The COS showed significant antioxidant activity as a function of concentration, reaching 37.06% inhibition (TEAC of 1010 μM Trolox) at a concentration of 50 μg/ml in 6 min of reaction. Inhibition gradually increased at higher concentration of COS. The calculated IC_{50} value for COS in ABTS assay was 4.57 mg/mL.

Total antioxidant capacity (TAC) for chitosan, LMWC and COS calculated IC_{50} was, respectively, 8.54, 7.75 and 14.3 mg/mL. Reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) are generated by normal metabolic processes in all aerobic organisms. However, the overproduction of these species may hamper cellular antioxidant defenses and lead to oxidative stress. Oxidative stress has been implicated in the pathogenesis of several diseases by various mechanisms, which may involve DNA mutations, protein oxidation and lipid peroxidation (Park et al., 2003; Finkel & Holbrook, 2007; Valko et al., 2007). In some diseases, treatments involving antioxidants have been shown to be effective in reducing oxidative stress markers, leading to growing interest to explore effective antioxidants, especially those of natural origin (Mendis et al., 2007). Free radical scavengers are preventive antioxidants and the presence of these compounds can inhibit oxidative sequences at different levels (Kim et al., 2005). Thus, they have helped pave the way for the interest in discovering new compounds that are potential antioxidants that may help prevent or even be used in the treatment of certain diseases (Anraku et al.,2018).

Yin et al. (2013) report that chitosan oligosaccharide and its derivatives showed a higher scavenging effect than chitosan and its derivatives. This study suggests that chitosan and its derivatives exhibited a stronger H bonding between their polysaccharide chains. The strong effect of intramolecular hydrogen bonds weakens the activities of hydroxyl and amino groups. The amount and the activity of hydroxyl and amino groups are important factors associated with antioxidant activities of chitosan derivatives while chitosan oligosaccharide elicited the highest scavenging effect because no active groups were substituted (Xie et al., 2001).

The ferrous ion-chelating effect of COS is shown in Figure 3C. The COS showed moderate abilities, with concentration-dependent chelating ability. The COS in 10 mg/ml reached 50% ability to chelate iron ions. COS, when compared to antioxidant pattern, with the exception of EDTA, achieved better ability to chelate iron ion than

citric acid and gallic acid, which achieved 24.65 and 24.65% respectively for ability to chelate iron ions. It has been reported that transition metal ions promote lipid peroxidation, starting a chain reaction that can lead to the deterioration of food and be correlated with the pathogenesis of various human diseases. Chitosan and its derivatives possess the ability to effectively inhibit pro-oxidant activity of iron ions by converting the ferric ion to ferrous ion, thus retarding the oxidation of lipids (Magalhães et al., 2008). According to the results by Mengíbar (2013), low molecular weight COS undergo reactions thought hydroxyl and amine groups with different radicals and therefore there is a markedly capacity to donate electrons and reduce Fe^{3+}.

This increased antioxidant activity of compounds with lower molecular weight has been reported previously (Tomida et al., 2009; Huang et al., 2012; Mengíbar et al., 2013). Mengíbar (2013) analyzed the iron chelating activity of COS produced by enzymatic hydrolysis with chitosanase and lysozyme. This study showed that COS produced by chitosanase were better compared to lysozyme. The different methodologies to produce COS could produce fractions with different acetylation versus deacetylation rates.

It has been reported that chitosan with higher molecular weights could present strong intermolecular interactions due to hydrogen bonds, which would reduce the ability of the amine and hydroxyl groups to react with different radicals. In the case of COS, which have lower molecular weights, this intramolecular force would be lower compared to the high molecular weight CH, and therefore have better activity in chelating ions (Feng et al., 2008; Sayas-Barbera et al., 2011; Sun et al., 2007; Yang et al., 2006; Yen et al., 2008; Zhong et al., 2007).

The DNA nicking assay was performed to verify the ability of COS to protect supercoiled pBR322 plasmid DNA against oxidation. In this assay antioxidant activity was measured by the degree of protection of DNA from devastating effects of hydroxyl radicals generated by Fenton's reagent. DNA damage is related to the conversion of the supercoiled form of this plasmid DNA to the open circular and further linear forms (Jung et al., 2001). Fig 3D shows the antioxidant and DNA protection abilities of COS. Due to exposure to Fenton's reagent, the supercoiled form decreased and was converted into the open-circular and linear forms. In Fig. 3Da, the reaction took place without the presence of Fenton's reagent, thus the intensity in the gel demonstrates the presence of intact supercoiled DNA. At concentrations of 100 µM and 200 µM, COS does not appear to have provided protection against DNA damage. Meanwhile, at a concentration

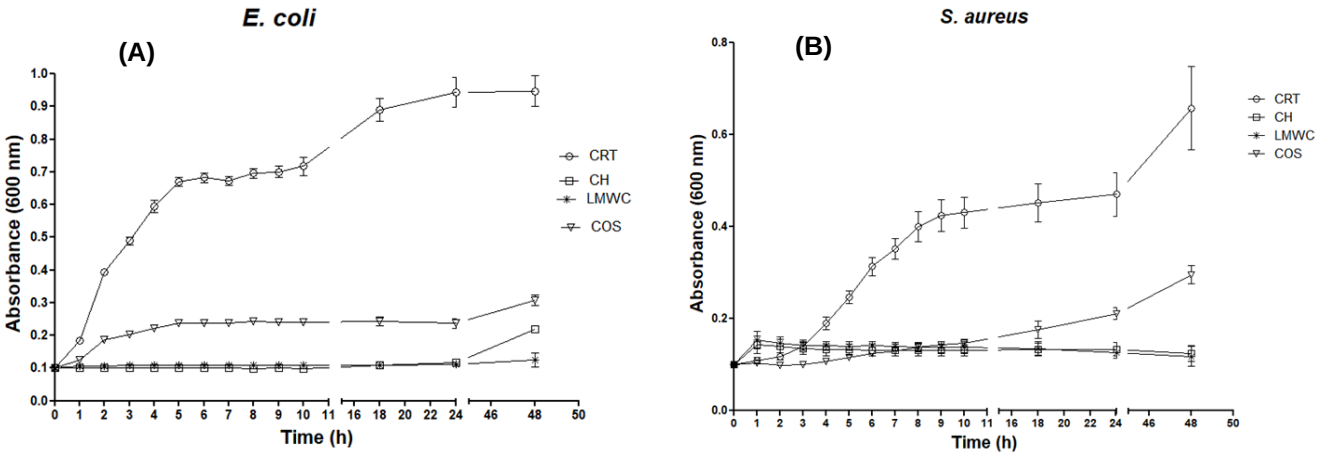
of 400 µM, the intensity in the gel demonstrates the presence of super-coiled DNA, indicating DNA protection. Hydroxyl and superoxide radicals are highly reactive and have been shown to be highly damaging to health, being able to damage almost all cellular molecules. This radical has the ability to join nucleotides in DNA strand breaks causing it to contribute to carcinogenesis, mutagenesis and cytotoxicity (Hochestein et al., 1988; Babu et al., 2001). As there is no specific enzyme or antioxidant system for protection against these free radicals in the body, the discovery of products with antioxidant properties has become increasingly important.

The antioxidant properties of oligosaccharides have attracted attention, mainly due to their ability to receive and donate protons, since the free radical can react with the NH_2 group forming the NH_3^+. In these groups, the hydroxyl carbons 2, 3 and 6 of the pyranose ring may react with unstable free radicals to form a stabilized molecule (Kim et al., 2005).

Antibacterial activity

All derivative products from chitosan inhibited the growth of *S. aureus* and *E. coli* in nutrient broth when compared to the control without chitosan (Figure 4).

Figure 4 - Antibacterial effect of chitosan and derivate samples on growth of (A) *Escherichia coli* and (B) *Staphylococcus aureus* in nutrient broth.



Source: Prepared by the author (2016).

Similar results were obtained by Cahú et al. (2017). Chitosan is one of the few pseudo-natural polysaccharides with a cationic character, and this characteristic is responsible for most of its properties. This peculiarity makes possible its interaction

with negatively charged molecules, which are usually present on the surface of biomolecules in cell membranes and walls such as proteins, anionic polysaccharides and fatty acids, explaining its effects on them (Liu et al., 2004). This fact contributes to its antimicrobial activity against several bacteria and fungi, because the amino-positive groups of the glucosamine units possibly interact with the negative components of the cell walls of the bacteria, suppressing biosynthesis (Shi et al., 2006). In addition, chitosan disrupts the transport of nutrients through the cell wall and causes the leakage of cellular organelles, accelerating the death of the bacteria. Another proposed mechanism involves the penetration of low molecular weight into the cell which binds to the DNA, inhibiting the synthesis of RNA and proteins (Visnova et al., 2008). Studies have suggested that the effects of chitosan on gram-positive and gram-negative bacteria are distinct. In the case of gram-positive bacteria, the hypothesis is that high molecular weight forms films around the cell and generates inhibition of the absorption of nutrients. On the other hand, chitosan of low molar mass penetrates more easily into gram-negative bacteria, causing disturbances in the metabolism of these organisms (Costa et al., 2006). The amino groups in chitosan units also provide unique functional properties, such as mucoadhesivity (Dash et al., 2011; Sinha et al., 2004) owing to the attractive molecular forces from electrostatic interaction between positively charged chitosan and negatively charged mucosal surfaces (He et al., 1998; Lehr et al., 1992). A commonly reported property of chitosan is antimicrobial activity, which may be the result of positively charged amino groups that interact with the negatively charged cell membranes of microbes. This interaction can cause leakage of intracellular constituents of the microbe and lead to cell death (Kong et al., 2010; Kumirska et al., 2011). These results advocate the use of COS as chitosan analogues with enhanced solubility while maintaining bioactive properties.

Conclusions

COS were obtained by non-specific enzymatic hydrolysis using pepsin and exhibited moderate antioxidant activity, as well as the ability to inhibit the growth of *S. aureus* and *E. coli* in nutrient broth. Thus, COS, besides being obtained from low added-value raw material, can be considered potential biomolecules with extensive application in the medical, pharmaceutical or food industries.

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4.2 – Artigo 2

EVALUATION OF ANTITUMOR ACTIVITIES OF CHITOLIGOSACCHARIDES OBTAINED FROM CHITOSAN OF MARINE SHRIMP LITOPENAEUS VANNAMEI PROCESSING WASTE

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ABSTRACT

The production of chitoligosaccharides is an effective way to improve the chitosan solubility and decrease its viscosity, thereby enhancing its potential application. This work aimed to produce and characterize chitosan oligosaccharides obtained from processing residues of the marine shrimp *Litopenaeus vannamei* and evaluate its cytotoxic, acute toxicity, analgesic and antitumor activity Chitosan was produced from shrimp heads by enzymatic autolysis and alkaline deacetylaton method, followed by nonspecific enzymatic hydrolysis using pepsin to obtain chitoligosaccharides and low molecular weight chitosan. Infrared spectroscopy (FT-IR), nuclear magnetic resonance (^{13}C NMR) and mass spectrometry (MALDI-TOF) was performed for sample characterization. The cytotoxicity was evaluated using endothelial normal cells from rabbit aorta (RAEC) and NCI-H292, HT-29, MCF-7, Hep-2 human tumor cell lines. Acute toxicity of these compounds was tested in mice, as well as the analgesic and antitumor activity *in vivo*. Oligosaccharides showed complete solubility and low viscosity in neutral aqueous media. The FT-IR and NMR spectra profile was similar to that of chitosan. MALDI-TOF analysis showed that oligosaccharides molecular weight is between 0.8 and 2.6 kDa. COS did not show cytotoxicity towards RAEC and presented low inhibition against tumor cell lines at a concentration of 25 μM. The compounds showed analgesic activity dose dependent and interesting ability to tumor inhibition *in vivo*. For Ehrlich carcinoma tumors COS inhibited 61.8% while for sarcoma 180, COS inhibited 63.1%, of the growth of tumor in mice These results show the efficiency of these oligomers obtained from shrimp waste heads, which have similar structures to those of chitosan, allowing the improvement of its properties. Future studies are important to determine clearly the biological activities of these compounds.

Keywords: By-products of the fishing industry; chitosan; oligosaccharides; antioxidant activity; cytotoxicity; acute toxicity; analgesic activity; antitumor activity

1. INTRODUCTION

Due of the growth of world fishery production, the fishing industry has generated large amounts of waste and by-products. It is estimated that approximately 50% of the produced fish to be discarded as residue (ARRUDA, 2004). The generation of such materials encourages the search for alternative ways of utilization, in order to avoid environmental pollution and loss of interesting bioactive molecules.

Chitin, the second most abundant polysaccharide in nature, is obtained primarily from shrimp and crab by-products (shells). From deacetylation of chitin is obtained chitosan, which is a more soluble, polycationic and non-toxic biopolymer, being found in the cell walls of fungi and yeast (SHAHIDI AND ABUZAYTOUN, 2005). Chitosan is known to exhibit a wide variety of physiological activities such as antitumor (QIN et al., 2002), antimicrobial (YANG et al., 2005) and antimutagenic activity (KOGAN et al., 2004).

However, the low solubility of chitosan and the high viscosity of its solutions due to its high molecular weight constitute disadvantages for many biological and technological applications. The production of chitooligosaccharides (COS) is an effective way to improve the solubility of chitosan and decrease the viscosity of its solution enhancing, the potential as functional materials for biotechnological applications (MENGÍBAR et al., 2013).

Among the methods already described for producing COS, enzymatic hydrolysis is known to be performed under mild conditions, and unlike of acid hydrolysis, for example, presents as a more specific process that allows control of the reaction during the process and thus of the degree polymerization of generated oligomers (KIM & RAJAPAKSE, 2005; MING et al , 2006;. RONCAL et al, 2007;. KUO, CHEN & CHIANG, 2004).

However, COS production by enzymatic means, in which use specific enzymes is limited due to the absence of a steady and efficient production chitosanases to ensure a low-cost process. Alternatively, studies were conducted using non-specific enzymes which diminish the production cost for successful production of these oligomers (CABRERA & CUTSEM, 2005; RONCAL et al., 2007). The enzymatic hydrolysis carried out by nonspecific enzymes have been described in the literature as an effective alternative to obtain oligosaccharides and polymers with lower molecular weight. It is attractive for the feasibility compared to specific enzymes. As a consequence, some researchers have studied nonspecific commercial enzyme (PANTALEONE, YALPANI

& SCOLLAR, 1992), as these have been used in the food industry for many years and are relatively safer and less costly.

Oligosaccharides obtained from the hydrolysis of chitosan have aroused much interest. not only because COS and low molecular weight water soluble, but they also have a lot of functional properties including anti-tumor (HARISH & PRASHANTH THARANATHAN., 2005; HUANG et al , 2006), antioxidant (CHEN et al , 2003), elimination of free radical (JE et al , 2004; PARK et al 2003), the anti-hepatotoxic (CHEN et al., 2005a), and anti-angiogenesis activities (HARISH PRASHANTH AND THARANATHAN, 2005).

This work aimed to study the COS produced from shrimp processing waste and evaluate its properties, characterization, its antioxidant potential, its acute toxicity, its analgesic action and its antitumor activity.

2. MATERIALS AND METHODS

2.1. Materials

Processing waste (cephalothorax and carapace), from *L. vannamei* juveniles heads (total body weight between 10 g and 12 g) were provided by a local fishery processing plant (EMPAF Ltd.). Fresh heads were immediately stored on ice (0 ◦C) and transported to the laboratory where they were packed in plastic bags (1 kg per bag) and stored at −20 ◦C until use. All other chemicals and reagents used in this study were of analytical grade and commercially available.

2.2. Chitosan from marine shrimp processing wast

Shrimp heads were mixed with distilled water at a ratio of 1 kg of wet raw-material to 1L of distilled water and grinded in an industrial food processor (Engefrio, Recife, Brazil). Then, the mixture was hydrolyzed, according to the method of Leal et al . (2010). for *L.vannamei*, without the addition of any commercial proteases, in a vessel placed in a water bath at 40 ◦C for 2 h with constant stirring (700 rpm). The preparation was then heated for 10 min at 100 °C and filtered (1 mm^2 mesh) to retain the head carapace (solid phase) used for chitin extraction and chitosan (CH) production, using the previously described method by Cahú et al . (2012).

2.3. Production of Chitoligosaccharides

Chitoligosaccharides (COS) were obtained by enzymatic hydrolysis with pepsin from

gastric porcine, according to Roncal et al., (2007) with slightly modifications. Briefly, chitosan (1%) in 0.2 M sodium acetate buffer pH 4.5 and pepsin were incubed (1:100) in a water bath at 40°C for 20h. After this, pH was adjusted to 7.0 with 1 M NaOH and the reaction was boiled for 10 min to inactivate the enzyme and centrifuged. The supernatant was freeze dried and resuspended with methanol, and then precipitated and washed with 2 vol of acetone, and finally dried under vacuum.

2.3 Characterization

Chitoligosaccharides were analyzed by Fourier transform infrared spectroscopy (FT-IR), Nuclear magnetic resonance (NMR), Viscosimetric molecular and Matrix-assisted laser desorption/ionization (MALDI-TOF). Decoupled 13C NMR spectra were obtained using a Bruker Avance DRX-400 spectrometer with a 5-mm inverse probe. ^{13}C NMR acquisitions were performed using the WALTZ-16 pulse sequence with the following parameters: FIDRES: 0.8466 Hz; AQ: 0.5906 s; DW: 15.75 s; DE: 5.5 μs; D1: 110 ms; D2: 3.4 ms; PL12: 17 dB (decoupler 1H). FT-IR spectra were measured in KBr pellets in transmission mode within a range of 4000–500 cm^{-1} using an FT-IR Bomem MB100 spectrophotometer. Intrinsic viscosities in sodium acetate buffer (0.25M, pH 4.5) were measured using an Ostwald n°100 capillary viscometer, with a diameter of 0.9 mm and solution concentrations lower than 1% (w/v) at 25±0.1°C. The following Mark-Houwink- Sakurada (MHS) equation for chitosan was used to calculate the molecular weight:

$$[\eta]=KM\_w\text{^}\alpha$$

Where [η] is the intrinsic viscosity calculated from the equation and Mw is the molecular weight. The viscometric constants, K and α, for 1.49x10-4 dL/g and 0,79, respectively, for polydisperse chitosan samples in sodium acetate buffer (0.25M) at 25°C, with DA between 20 and 26% were an empirically modified MHS equation at 25 °C[24]. The molecular weight of Chitoligosaccharides obtained from the enzymatic hydrolysis of chitosan was determined by MALDI-TOF MS analysis (Voyager-DE TM STR Biospectrometry Workstation, Applied Biosystems Inc., NCIRF, Korea) The degree of deacetylation (DD) were calculated by infrared spectra, according $100–((A_{1320}/A_{1420}-0,3822)/0,03133))$ by Brugnerotto et al . (2001).

2.4 Antitumor activity in vitro/Cytotoxitcity assay

The cytotoxic activity was performed using the MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) method (Sigma-Aldrich, St Louis, MO, USA) (ALLEY et al., 1988; MOSMANN, 1983). Human tumor cell lines NCI-H292 (human pulmonary mucoepidermoid carcinoma), HEp-2 (human laryngeal carcinoma), HT-29 (human colon cancer) and human mammary carcinoma cells (MCF-7), maintained in DMEM medium culture. The media was supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin and streptomycin). The cells were maintained in an incubator at 37 ° C in a humid atmosphere enriched with 5% CO_2.

The NCI-H292 cells, HT-29, and Hep-2 (10^5 cells/ml) were plated in 96 well plates and incubated for 24h. Then the samples dissolved in DMSO (0.5%) were added to the wells at final concentration of 25 μM. The drug doxorubicin (5 mg/mL) was used as standard. After 72 h reincubation was added 25 μL of MTT (5 mg/ml) and after 3 h incubation, the culture medium with MTT was aspirated and 100 μL of DMSO was added to each well. The absorbance was measured in a microplate reader at a wavelength of 560 nm.

Endothelial cells (RAEC) derived from rabbit aorta used in this study (Colburn & Buinassisi, 1982) were grown in F-12 medium (Gibco BRL, Grand Island, USA) supplemented with 10% fetal calf serum (FCS) (Cultilab, Campinas, SP, Brazil) in the presence of penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown at 37°C in a humidified, 2.5% CO_2 atmosphere and sub-cultured every week with Pancreatin (Sigma-Aldrich, St. Louis, MO, USA). Cells (8×10^3) were subcultured in 96-well plates until confluence and then exposed to different concentrations of chitosan oligosaccharides diluted in F-12 medium for 24 h. After that, the medium was aspired and 0.2 mL of F-12 medium containing 1 mg/mL MTT was added the reaction was allowed for 2 h in culture conditions. Afterwards, the medium was removed and 0.1 mL of dimethylsulfoxide was added to lyse cells and dissolve formazan crystals. The solution was homogenized in a plate shaker and the absorbance was read in 540 nm. The experiments were performed in sixtuplicate and the percent inhibition was calculated with the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

2.5 Assessment of acute toxicity

This study has approval from the Ethics in Animal Use Committee of the Universidade Federal de Pernambuco (nº 23076.029665/2015-10). Increasing doses (500, 1000,

2000 mg/kg) were administered orally in 5 mouse per group (US Environmental Protection Agency, 1992). On the first day, the animals were observed every 10 minutes for 4 hours, followed by two daily observations after administration of 24 hours, 48 hours and 72 hours to observe possible changes in spontaneous motor activity, reflects, motion, respiration, contortions and piloerection plus mortality.

2.6 Analgesic Activity: Abdominal constriction response caused by intraperitoneal injection of diluted acetic acid

Abdominal constrictions were induced by intraperitoneal injection (0.8% acetic acid), according to the procedures described for Schneider, et al.,(1968) with minor modifications. Animals were pre-treated with COS (100, 200, 400 mg/kg) or standard drugs (ASS) , intraperitoneally, 1 hour prior to acetic acid injection. The control animals received a similar volume of 0.9% NaCl (10 mL/kg, i.p.). After the challenge, each mouse was placed in a separate glass funnel and the number of contractions of the abdominal muscles, together with stretching, was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of abdominal contractions, comparing the control animals with the mice pretreated with COS.

2.7 Antitumor activity in vivo

2.7.1 Animals

For the evaluation of antitumor activity, were used Swiss albino mice (*Mus muscullus*) males, aged 35-60 days and average weight of 35 g, obtained from the animal house of the Department of Biochemistry-UFPE. The animals were kept at room temperature (28°C) and natural day/night cycle (12h light and 12h dark) with free access to food and water.

2.7.2 Treatment

Sarcoma 180 and Ehrlich carcinoma were used as models of experimental tumors, from the Academic Center of Vitória (CAV-UFPE). Tumors were transferred and maintained in the ascitic form in mice every seven days. Tumor cells were implanted subcutaneously in the right axillary region of the mice at a concentration of 2.5×10^8 ml^{-1} thus developing tumors in solid form. After 24 hours implantation of tumors was initiated the treatment which lasted seven days. Twenty four animals were used for each

type of tumor; each experimental group consisted of eight animals. The animals received the solutions containing the COS concentration of 400 mg/kg day body weight by intraperitoneal injections. COS were prepared in saline 1%. The negative control animals received only saline and the positive control received 5-fluorouracil chemotherapy in equal conditions.

2.7.3 Tumor weight

Twenty-four hours after the last drug administration, the mice were anesthetized with urethane (1.25g/kg). Tumors were removed, dissected and weighed. Inhibition was determined from the average weight of animals treated groups compared to the untreated control group. The assessment of tumor inhibition (TWI%) was performed by calculating: TWI = ((TC) / C) x100, where C: average tumor weight of negative control group, T: average weight of the tumors treated.

3. RESULTS AND DISCUSSION

3.1 Prodution of Chitoligosaccharides

Chitosan oligosaccharides were obtained from by non-specific enzymatic hydrolysis using pepsin, as proposed by Roncal et al. (2007) who obtained hydrolysis efficiency comparable to chitosanase. Among the enzymes studied, pepsin was the one with better performance in its action, reducing the viscosity of chitosan solution by 60% in just 10 minutes, combined with formation of reducing terminals which are resulting from the hydrolysis of polysaccharides in question, in 3:32 to 0.43 mM in 20 hours of reaction. When compared with the performance of the specific enzyme, that is chitosanase, this reduced the viscosity of the solution 65 to 96% in 20 hours, and generating reducing end of 0.41 to 5.46 mM, while no monomer was produced.

Corroborating these results, Kumar and Tharanathan (2004), analyzed the depolymerization reaction of chitosan using some non-specific enzymes. As a result, kinetic parameters obeyed Michaelis-Menten kinetics and *Km* and *Vmax* values indicated high affinity of pepsin to chitosan. Although chitosanase is found in a variety of microorganisms, its utilization is still limited due to the lack of a steady and efficient production which ensures that the process is more feasible. It is not entirely clear the mechanism which pepsin hydrolyzes chitosan, but studies have shown that chitosan solutions show markable decrease in viscosity when hydrolysed by pepsin, thus suggesting that pepsin operates in this polysaccharide as an endoglucosidase reducing

its molecular mass. From the enzymatic hydrolysis were obtained two types of molecules: Low molecular weight chitosan and chitosan oligosaccharides.

3.2 Characterization of Chitoligosaccharides

The characterization of chitosan and enzymatic hydrolysis products (low molecular weight chitosan and chitooligosaccharide) was performed by infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR) of ^{13}C and mass spectrometry (MALDI-TOF).

Spectroscopy in the infrared region allows to observe and classify some bands related to the characteristic vibrations of functional groups present in the structure of chitosan, low molecular weight chitosan (LMWC) and chitooligosaccharide (COS). The FT-IR graphics obtained for chitosan and the products of enzymatic hydrolysis of chitosan (LMWC and COS) (Fig 1) showed a broad peak near 3400 cm^{-1} in the region corresponding to the OH stretch, which appears superimposed axial deformation of the amine group band (-NH) (NUNTHANID et al., 2001). The peak near 2888 cm^{-1} represents –CH group stretch and peak around 1661 and 1671 cm^{-1} are assigned to the vibrations of the amide I group and corresponds to C=O stretching, indicating that remained acetylated units (FIGUEIREDO, 2002). The peak around 1370 cm^{-1} indicates stretch C-O of the primary alcohol group (- CH_2OH). The axial deformation of amide CN appears around 1428 cm^{-1} peak. The intense band between 800 and 1200 cm^{-1} is related to the presence of pyranoside rings (SHIGEMASA et al., 1996). This last band was not intense in the spectrum for COS, which shows less pyranoside rings relative to chitosan and chitosan of low molecular weight.

From the FT-IR, using the relation between the absorbance in the wavelengths 1655 and 3450 cm^{-1}, it was possible to estimate the degree of deacetylation of these biomolecules. This analysis is considered one of the main parameters to characterize chitosan. The degree of deacetylation is defined as the number of amine groups in relation to the number of amide groups in the polymer chain. As a result, the CH showed 81.02%, LWMC 80.68% and COS 81.58% degree of deacetylation (Table 1).

The ^{13} C spectra of chitoligosaccharides are displayed in Fig. 2 The peak at 178.8 correspond to C=O, at 104 (C1), 58.8 and 57.2 (C2). C3 and C5, which appear as a single signal at 72.8 and 74.6. 64 (C4) and 63.2 (C6). This profile was similar to that described by Cahú et al (2012), found for marine shrimp chitosan of *Litopenaeus vannamei*.

The chitosan, chitosan low weight molecular and chitoligosaccharides was analyzed by

MALDI-TOF-MS. In the case of chitosan no spectra were detected because of hight molecular weight and difficult to ionize. The low molecular weight chitosan when analyzed showed a molecular weight of approximately 21 kDa (Fig 3). MALDI-TOF for COS clearly shows 9 peaks corresponding to the glucosamine oligomers with degree of polymerization (DP) 5 to 16 were monitored in the spectrum. The mass/charge (m/z) of the main peaks were 811.280, 972.376, 1133,455, 1336,531, 1455.571, 1658.631, 2099.827, 2183.817 and 2667.038, respectively. Nine peaks were all [M+Na+] ion-peaks with approximately 161 or 203 Da mass larger than the peak ahead, which was the molecular mass of a GlcN and GlcNAc residue respectively as it was reported by Li et al .(2012). To summarize, this analysis displayed that the distribution of COS ranged from 5 to 16-mers consisted of a mixture of saccharides of 0,811-2,66 KDa, showing a marjor weight of 1.13 KDa, which suggests that the sample in composed by heptamers (DP 7)(Tale 2).

3.3 Antitumor activity in vitro/Cytotoxitcity assay

The cytotoxicity of COS is shown in Table 3 with their percentage inhibition. COS were tested at a concentration of 25 μM in NCI-H292 (human pulmonary mucoepidermoid carcinoma), HEp-2 (human laryngeal carcinoma) and HT-29 (human colon cancer) cell cultures. Results showed that COS were able to inhibit the growth lineages of NCI-H292 in 29.8% HEP-2 in 20.1% and HT-29 27.1%. The COS showed no inhibition percentage for the cell line MCF-7.The doxorubicin compound, a drug widely used in chemotherapy for cancer, was used as a positive control showing in a concentration of 25 μM inhibitions of 94.15% for NCI-H292, 79.39% for HEP-2, 64.1 % for HT-29 and 74.7% for MCF-7. The effects of COS on viability of rabbit aortic endothelial cells (RAEC) are shown in table which presented low or negligible inhibition of viability, even in higher concentration at 200 μM. This data suggest that chitosan and derivates are not toxic to RAEC. According to the methodology here applied, antiploriferative substances exhibit inhibition values above 75% this *cut-off* qualifies substance to be included in screening compounds having antitumor activity according to the protocol of the National Cancer Institute (NCI). According to this protocol compounds with antiproliferative action between 20 and 50% have little antitumor activity *in vitro*, what was observed in compounds tested, showing no cytotoxicity in this *in vitro* methodology.

De Assis (2012) examined the cytotoxic effect COS produced from commercial

chitosan on some cell lines cells such as the fibroblast 3T3, hepatocarcinome HepG2 cells and HeLa cervical adenocarcinome cells. The HepG2 cells in this study, when treated with COS at the concentration of 0.2 mg/ml, cell viability was of 20% and at higher concentrations, it remained practically unaltered. These results corroborate literature data, in which chitooligosaccharides did not show any activity against Hep_3B cells. In this same study, however, COS decreased cell viability was dose dependent against HeLa cells. Cell viability decreased by 60% at a concentration of 0.5 mg/mL. Similar results were found by Jeon and Kim (2002), where chitooligosaccharides inhibited uterine tumors in rats by 73.6%. Kim and Rajapakse (2005) observed that a mixture of chitosan oligomers from tetramer to pentamer could inhibit tumor cell growth in rats. It was observed that HeLa cells were more sensitive than the other ones showing that biological functions of chitooligosaccharides depend not only on the degree of polymerization, but also on its molecular weight (KIM AND RAJAPAKSE, 2005; SHEN et al., 2009).

This leads to the hypothesis that COS have antitumor activity related to mechanisms that cannot be assessed by *in vitro* assays such as angiogenesis inhibition, a mechanism of fundamental importance for tumor growth, on the tumor microenvironment, or on the epithelium-mesenchymal transition, a process in which epithelial cells undergo change in its polarization and consequently morphological and biochemical changes, making it migratory and invasive capacity, and resistance to apoptosis (ONUCHIC, 2010; HEINTEL, 2013).

3.4 Assessment of acute toxicity

The toxicity evaluation is performed in order to determine the potential of new substances and products harmful to human health. Tests assessing acute systemic toxicity are used to classify and appropriately label substances according to their potential lethality or toxicity as laid down by legislation (COECKE et al., 2005).

In this study, none of the doses tested (500, 1000, 2000 mg/Kg) showed toxicity in the animals, there was no death thereof. It was not identified behavioral changes such as in motor behavior, gait, breathing, piloerection or contortions.

Similar results were found by Fernandes et al., (2010), which examined the acute toxicity of a mixture of COS with distinct average molecular weights—1.2 and 5.3 kDa and possessing a degree of deacetylation in the 80–85% range. In this study, treatment was administered between 50 and 1000 mg / kg, and no significant change in behavior

was observed.

3.5 Analgesic Activity

In Fig 4 is represented the number of abdominal writhing in mice by administration of test compounds and acetic acid. Animals that were treated with saline, showed an average of 35 writhings; SSA (aspirin) 6.2; COS (100 mg/kg) 5.2; COS (200 mg / kg) 0.6 and COS 400 mg/Kg showed no abdominal writhing. Thus it is suggested that the COS have analgesic activity, proportional to its concentration.

Concern for the clinical use of new substances with analgesic activity, mainly used for the treatment of various types of pain (both neurogenic or inflammatory origin) is increasing significantly. Various nociception models in laboratory animals were developed to verify the analgesic activity compounds. The test of abdominal writhing induced by acetic acid in mice is widely used as a screening analgesic substances (COLHIER et al; 1968). The reaction of pain to exogenous compouds results in the release of various endogenous substances, called algogenic that activate nociceptors involved in this process (FALEIROS et al; 1997) This test can be considered a preclinical test importance to evaluate the antinociceptive effect because it allows a correlation between the appropriate value of the effective dose in animals obtained, and doses analgesic in human (COLLIER et al., 1968). Okamoto et al.(2002) claim that chitosan also has topical analgesic effect resulting from the capture of acidic hydrogens released at the site of inflammation by ionization of the amine group (NH_2) to form NH^+. As chitosan, the COS possible exert its analgesic effect through absorption of proton ions released in the inflammatory site.

3.6 Antitumor activity in vivo

Fig 5 shows the inhibition of Ehrlich carcinoma and sarcoma 180 tumor from the treatment of COS (400 mg/kg/day) and 5-FU drug. The dose chosen for this analysis was 400 mg/kg/day because it was from that concentration was total inhibition of pain as reported previously. COS presented themselves as potential inhibitors of Ehrlich carcinoma and sarcoma 180, promoting regression of the tumor mass. With the administration of COS, tumor weights that were 2.38 g and 1.8 g (saline control) became 0.9 and 0.66 for Ehrlich carcinoma and sarcoma 180, respectively while 5-FU decreases tumor weight to 0.6 for both tumor types. (Fig 6 and 7) From this data it was calculated the percentage of inhibition that the studied compounds provided. For Ehrlich

carcinoma tumors COS inhibited 61.8% while the 5-FU 76.6%. For sarcoma 180, COS inhibited 63.1%, whereas 5-FU inhibited 76.8% of the growth of tumor in mice (Fig 5).

Cancer is a group of diseases characterized by the development of tissue mass resulting in an enhanced cell division. In developing these diseases, the growth of tumor tissue exceeds the number of healthy tissue cells (KIRCHNER, 2014). The growing number of new cancer cases has driven research aimed at finding alternative treatment for this group of diseases (HURYN, 2013; REITZ, 2013).

Biological activity of chitosan oligosaccharides to inhibit growth of tumors has been described in the literature. This activity is dependent on the structural feature of these compounds, as the degree of deacetylation and the molecular weight of the oligomers.

Quin et al (2002) studied the *in vivo* antitumor activity of COS with molecular weight between 3-10 kDa, and degree of polymerization of 80%, it has shown that these compounds at a dosage of 200 mg / kg, inhibited 56.9% the Sarcoma 180 tumor growth. Suzuki et al (1986), demonstrated tumor inhibition in 93% of COS with a molecular weight around 1 kDa and degree of polymerization of 100%.

Studies suggest that the antitumor activity of COS act not only killing tumor cells, but also from their immunological effects by increasing the lymphocyte production, generating an antitumor response of cytotoxic T-lymphocytes. (TOKORO, TATEWAKI, SUZUKI, MIKAMI, SUZUKI AND SUZUKI, 1998).

Oligosaccharides also induce apoptosis, according to Xu et al. (2008), observed when treating cells hepatocarcioma (SMMC-7721) with oligosaccharides. The possible mechanism is that the COS regulate the expression of pro-apoptotic protein BA and caspase activation (serine proteases), triggering the cell apoptosis program. It was also described that the COS manifested an inhibitory effect of growth and anti-metastatic on lung carcinoma in mice with intramuscular administration (TSUKADA et al., 1990).

4. CONCLUSIONS

This work reported the production of chitosan oligosaccharides from marine shrimp *Litopenaeus vannamei* processing waste. The products were obtained by non-specific enzymatic hydrolysis using pepsin. The COS produced, showed no toxicity, analgesic activity and interesting ability to tumor inhibition *in vivo.* Thus, the production of COS, besides being obtained from low added value raw-material, they can be considered potential biomolecules with extensive application in medical field, pharmaceutical or food industries.

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Figures:

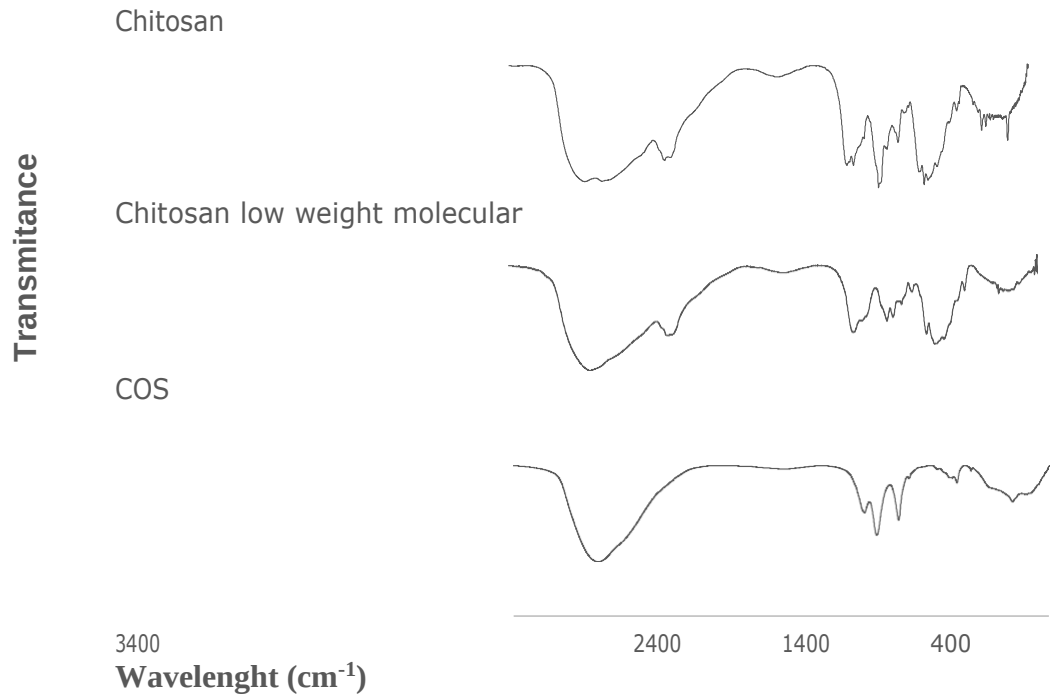

Fig 1. FT-IR spectruns of Chitosan and products from no-speicific enzymatic hydrolysis

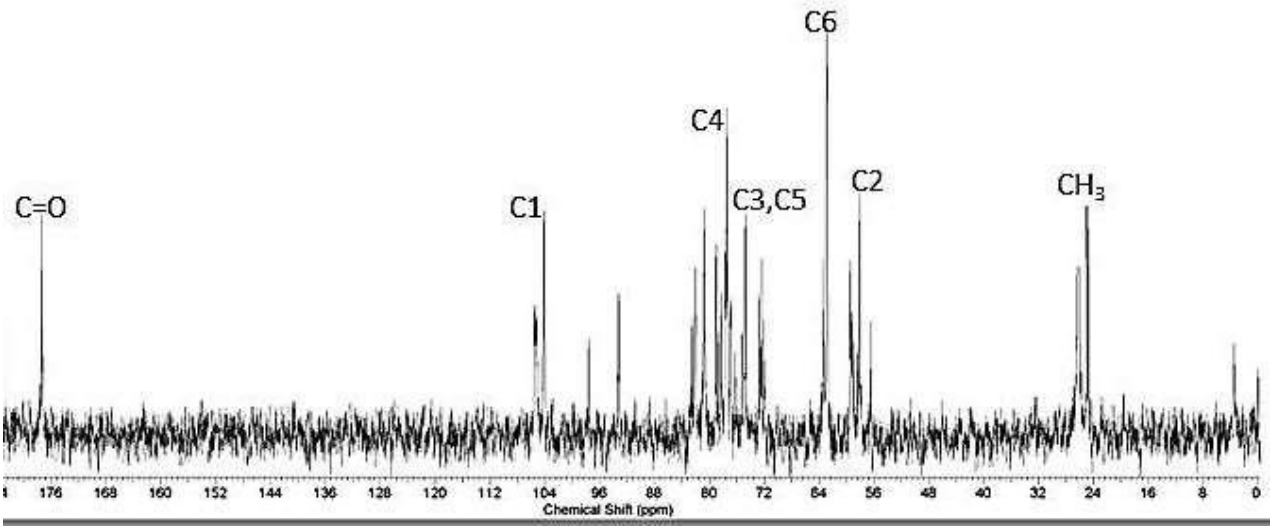

Fig 2 ^{13}C NMR spectra of COS.

A

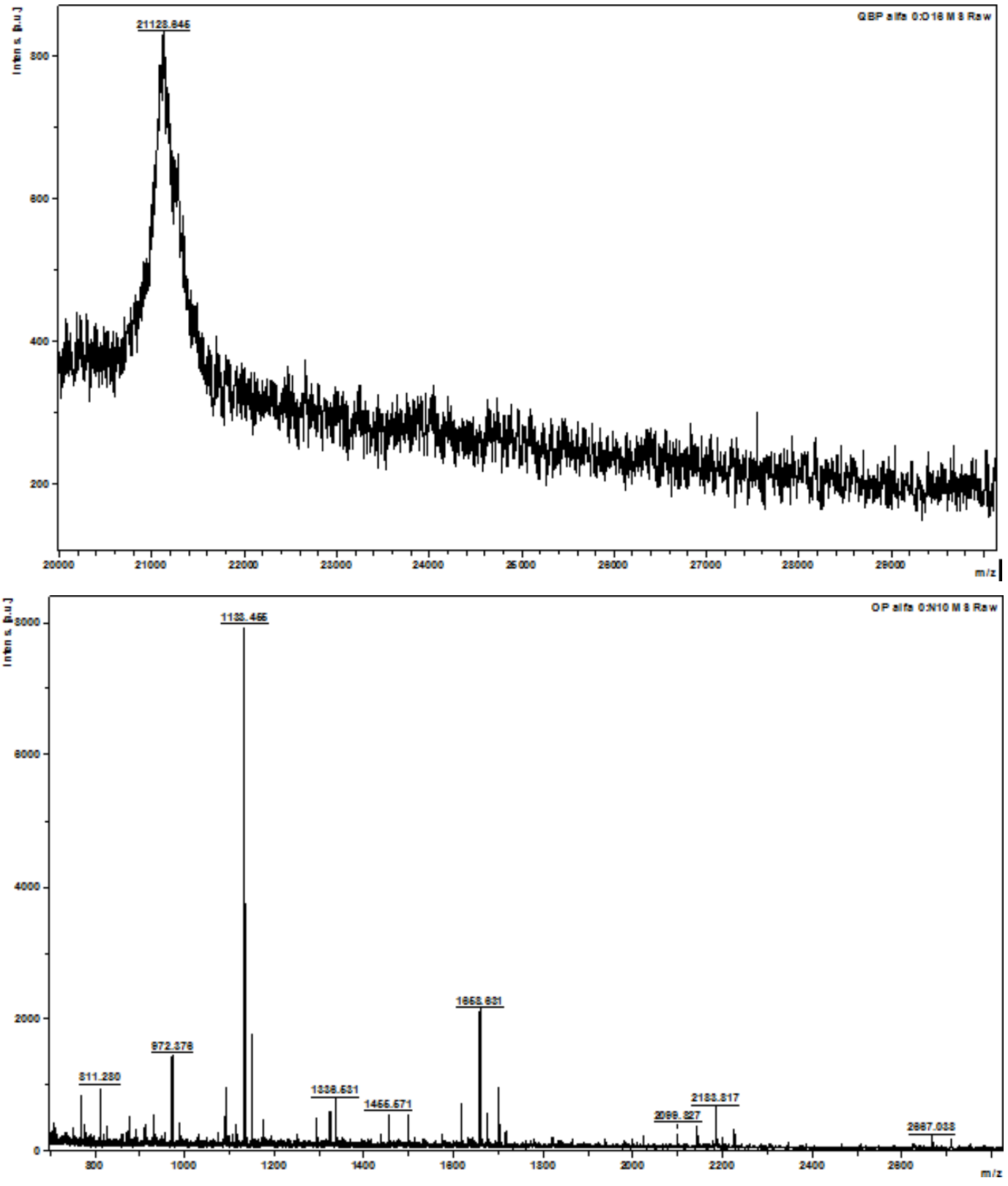

Fig. 3 MALDI-TOFMS spectrums of Chitosan low weight molecular (A) and Chitoligosaccharides (B).

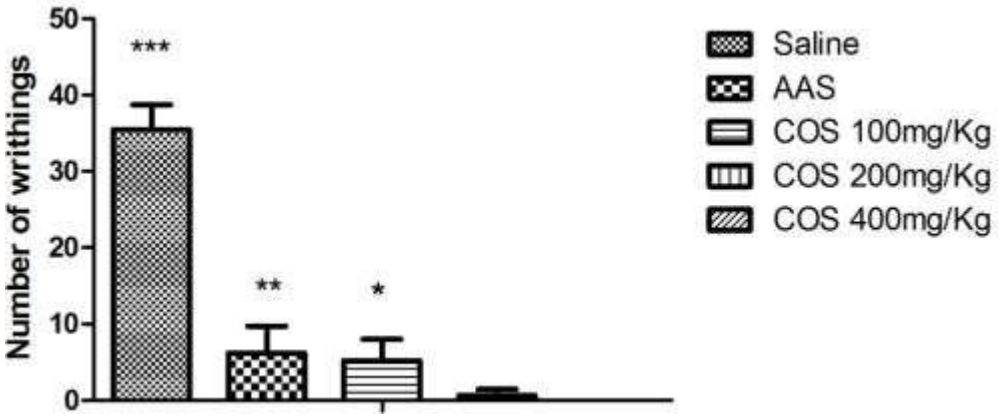

Fig 4.Analgesic Activity: Abdominal constriction response caused by intraperitoneal injection of diluted acetic acid. Animals that were treated with saline; AAS (aspirin); COS (100 mg/kg); COS (200 mg/kg) and COS to 400 mg/Kg. Statistical differences were determined by one-way ANOVA followed by Tukey test ($P < 0.05$)

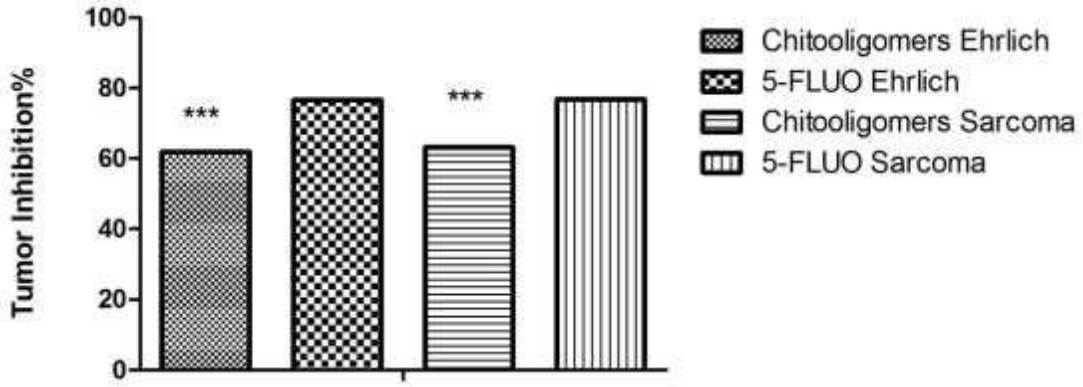

Fig 5. Inhibition of tumor Ehrlich carcinoma and Sarcoma 180 from the treatment of COS (400 mg/kg) and 5-FU drug. The data represent the mean the standard deviation ± statistical differences were determined by one-way ANOVA followed by Tukey test.

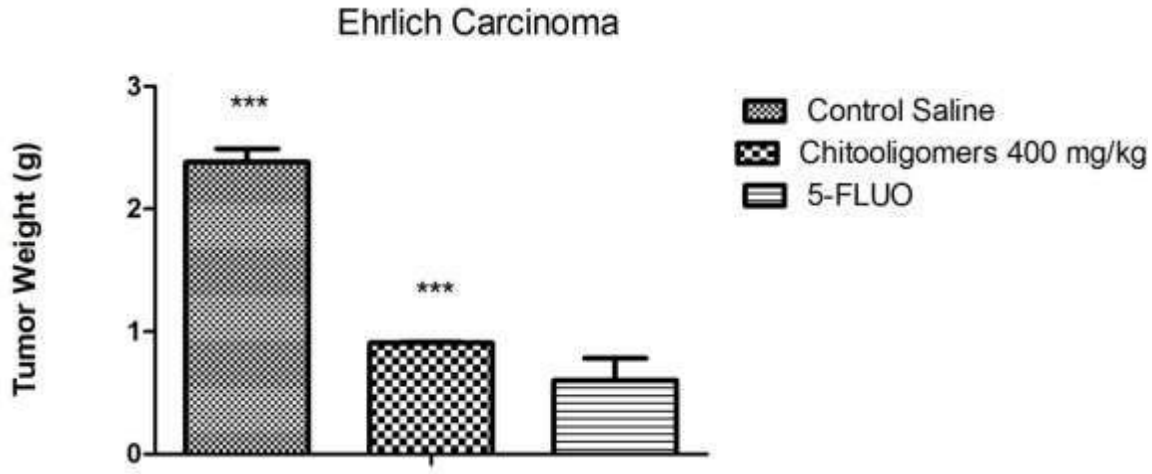

Fig 6. Weight of solid tumors Ehrlich carcinoma of the groups treated with saline, COS (400 mg / kg) and 5- FU. The data represent the mean standard deviation. The control of the statistical differences were determined by one way ANOVA followed by Tukey's test.

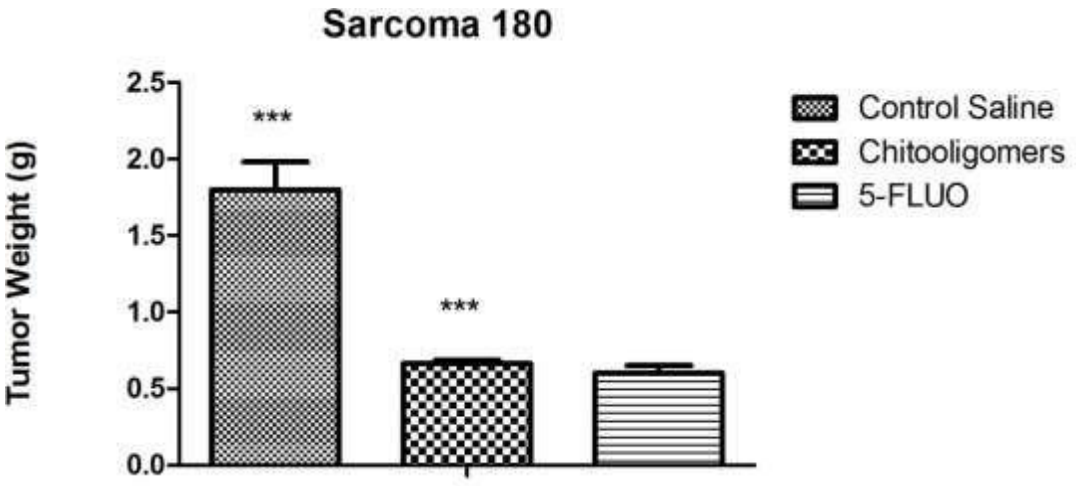

Fig 7. Weight of solid tumors Sarcoma 180 of the groups treated with saline, COS (400 mg / kg) and 5-FU. The data represent the mean standard deviation. The control of the statistical differences were determined by one way ANOVA followed by Tukey's test.

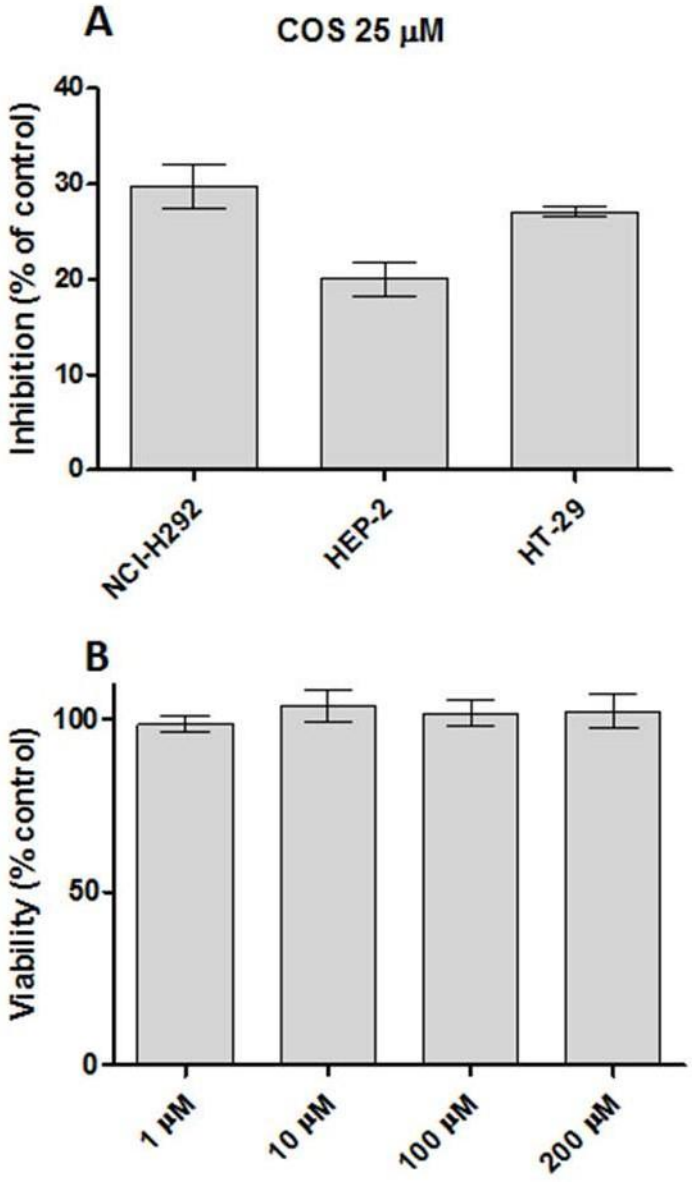

Fig 8. **A**: The inhibition effect of COS in human tumor cell lines (NCI-H292,HEP-2, HT-29,) concentration of 25 μg/mL; **B**: Effect o COS concentrations to RAEC cells.

TABLES

Table 1. Average molecular weigth (Mw) by MALDI-TF MS and desacetylation degree (DD) values (%) for chitosan (CH), low molecular weight chitosan (LWMCH) and chitoligosaccharides (COS) by FT-IR.

Samples	***DD (%)***	***Mw (KDa)***
CH	88.02	-
LWMCH	80.68	21
COS	81.58	0.811-2.667

Table 2. Prediction of the molecular weight of chitosan oligosaccharides (CTS-OS) by MALDI-TOF MS analysis.

Chitosan oligosaccharides (COS)	***DP***	***m/z***
GlcN	1	161
GlcNAc	1	203
$(GlcN)_5$	5	811
$(GlcN)_6$	6	972
$(GlcN)_7$	**7**	**1133**
$(GlcN)_5$ + GlcNAc + $(GlcN)_2$ Or $(GlcN)_7$ + GlcNAc	8	1336
$(GlcN)_9$	9	1455
$(GlcN)_5$ + GlcNAc + $(GlcN)_3$ Or $(GlcN)_9$ + $(GlcNAc)_1$	10	1658
$(GlcN)_{13;}$ $(GlcN)_{11}$ + $(GlcNAc)_2$	13	2099; 2183
$(GlcN)_{14}$ + $(GlcNAc)_2$	16	2667.038

Table 3. A: The inhibition effect of COS in human tumor cell lines (NCI-H292,HEP-2, HT-29,) concentration of 25 μg/mL; B: Effect o COS concentrations to RAEC cells.

Test products	**% Inhibition**							
	NCI-H292	DP	**HEP-2**	DP	**HT-29**	DP	**MCF-7**	DP
COS	29.8	2.3	20.1	1.8	27.1	0.6	0.0	0.0
Doxorubicin	94.15	1.99	79.39	2.65	64.1	1.1	74.7	2.1

4.3 – Artigo 3

BIOORGANIC & MEDICINAL CHEMISTRY
(F.I. 2,802 ;QUALIS CB I: B1

Antihyperlipidemic activity of chitoligosaccharides in mice submitted to a model of dyslipidemia

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Abstract

Chitosan polymers are biocompatible with potential for biomedical uses such as therapeutic drugs and novel biopolymer-based devices. Production of chitooligosaccharides (COS) is an effective way to improve chitosan solubility and broaden its applications. This study proposed to evaluate the effects of COS on Swiss albino mice receiving a high fat diet (HFD). COS were produced from purified shrimp chitin by enzymatic hydrolysis using pepsin. The obtained oligosaccharides were characterized, and to determine their potential, animals were submitted to HFD based on bovine brain, commercial butter and mouse chow for 28 days. During the experiment, animals were also treated with the COS at concentrations of 50 mg/kg and 100 mg/kg. Biochemical analysis of glucose, triglycerides, high density lipoprotein cholesterol (HDL-c), non-HDL, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine were performed to evaluate the effect of COS on the metabolism of animals with HFD. The lipid profile of the animals treated with COS was decreased significantly. The total cholesterol, the triglyceride, and the non-HDL cholesterol fraction levels were reduced, whereas the HDL-c (45.8±2.1 mg/dL at 100 mg/Kg) was increased when compared to untreated animals with HFD (24.6±4.6 mg/dL).Hepatic markers, AST and ALT, did not have significant changes in the groups that received COS when compared to control group. However, COS groups showed a significant decrease in ALT concentration (51.4±3.5mg/dL at 100mg/Kg) when compared to HFD group (80.8±2.0 mg/dL), demonstrating that these oligomers are effective in protecting the liver against acute lesions. Quantification of urea and creatinine were not altered at the end of the experiment, indicating that COS did not cause renal damage to treated animals. Glycemia of groups that received COS (91.2 mg/dL at 100mg/Kg) were significantly reduced when compared to the HFD group (160.2 mg/dL), demonstrating the efficacy of the compounds in decreasing insulin resistance linked to a hyperlipidemic diet. Results demonstrate that COS are promising compounds in the treatment of obesity and its associated complications, such as insulin resistance and lipid control.

Keywords: Chitosan; Oligosaccharides; Hyperlipidemia; Insulin resistance.

1. Introduction

Obesity and dyslipidemia is considered as one of the contributing risk factors in the prevalence of coronary heart diseases. It is considered as the main cause of morbidity and mortality nationwide as well as worldwide [1]. The elevated serum level of triglycerides, cholesterol and LDL are major risk factors for the premature development of cardiovascular diseases like arthrosclerosis, hypertension, coronary heart disease etc. Increased plasma lipid levels mainly total cholesterol; triglycerides and LDL along with decrease in HDL are known to cause hyperlipidemia which is the reason for initiation and progression of atherosclerosis process[2].

Among the drugs most used to combat dyslipidemias may have numerous adverse reactions, such as gastrointestinal complications, nausea, insomnia, headache, decreased leukocytes, and liver damage such as elevation of hepatic transaminases and creatinine kinases [3]. In this way, research has been carried out aiming at alternatives that can minimize the damages related to obesity. The research for new bioactive compounds that combat several prevalent diseases, such as cardiovascular diseases. and ways to minimize side effects, has been the target of several studies worldwide [4].

Growing evidences indicate that chitosan can reduce serum and liver triglyceride (TG) and total cholesterol (TC) levels proposed feasible options for COS development as anti-obesity and hypocholesterolemic agente [5-8].

Chitosan is a natural biodegradable and biocompatible polysaccharide derived by deacetylation of chitin (polysaccharide found in the exoskeleton of crustaceans and insects [9]. It is known for its wide variety of physiological properties such as antimicrobial, antitumor and antioxidant activities [10-12]. The low solubility of chitosan in pH above 6.3 and high viscosity of its acidic solutions, a consequence of its high molecular weight, is undesirable for many biological and technological applications [13]. The production of low molecular weight chitosan (LMWC) and its oligomers (COS) is an effective way to improve solubility and decrease solution viscosity, improving their potential as functional materials for biotechnological applications [13]. Unlike high-molecular weight chitosan, COSs are easily absorbed through the intestine, quickly enter the blood flow, and have systemic biological effects in organisms [14]. Much previous research work has been done to study the hypolipidemic mechanism of chitosan and its derivates, which can generally be divided into two different approaches. After oral administration, majority of chitosan is directly

excreted out, which binds large amounts of lipids in feces [15,16]. For another approach, a fraction of chitosan can be absorbed by the body through intestinal epithelial cells, and then regulates the lipid metabolisms [17,18]. The absorption of chitosan can be increased by decreasing its molecular weight [19], which might enhance the second approach.

This work aimed to obtain chitosan from shrimp chitin extracted from industrial wastes of *Litopenaeus. vannamei*. The non-specific enzymatic hydrolysis was performed using pepsin, generating LMWC and COS which were analyzed by their chemical structure, hypocolesterolemical properties.

2. Methodology

2.1. Chitosan production

Shrimp heads were mixed with distilled water at a ratio of 1 kg of wet raw material to 1 L of distilled water and blended in an industrial food processor (Engefrio, Recife, Brazil). Then, the mixture was hydrolyzed, according to the method of Cahú et al. (2012) [20] for *L. vannamei,* without the addition of any commercial proteases, in a vessel placed in water bath at 40°C for 2 h with constant stirring (700 rpm). The preparation was then boiled for 10 min and filtered (1 mm^2 mesh) to retain the carapace (solid phase), which was used for chitin extraction and chitosan (CH) production. The solid phase (chitinous residue) was dried at 70 ◦C and treated with 1 M HCl and 1 M NaOH to remove minerals and proteins, respectively. The depigmentation was performed with 0.5% (w/v) $KMnO_4$ and 1% (w/v) $Na_2S_2O_4$. The chitin flakes (25 g) were washed with abundant distilled water and dried at 70°C. Chitin was added to 500 mL of 50% (w/v) NaOH and incubated in a water bath at 65 ◦C for 24 h with constant stirring. N-deacetylation was performed a second time under the same conditions. The chitosan was washed with distilled water until a neutral pH was reached, then dried at 70 ◦C and pulverized in an electric mill (IKA® A11 Basic, IKA®-Works Inc., China) to reduce the particle size to below 250 m. Finally, the chitosan (1%, w/v) was solubilized in 3% (v/v) acetic acid, filtered through paper filter (14 m), precipitated with 1 M NaOH until a pH of 11.0 was reached, neutralized to pH 7.0 and centrifuged (10,000 × g for 15 min at 25 ◦C). This purified chitosan was lyophilized and collected as a fine powder.

2.2 Degree of acetylation and viscometric molecular weight of chitosan

Fourier transform infrared spectroscopy (FT-IR) was employed, wherein FT-IR spectra were measured in KBr pellets in transmission mode within a range of 4000–500 cm^{-1}

using an FT-IR Bomem MB100 spectrophotometer. The degree of deacetylation (DD) was calculated from the infrared spectra, according to the formula: DD(%)=100–((A$_{1320}$/A$_{1420}$-0.3822)/0.03133) [21]. Intrinsic viscosities in sodium acetate buffer (0.25M, pH 4.5) were measured using an Ostwald n°100 capillary viscometer, with a diameter of 0.9 mm and solution concentrations lower than 1% (w/v) at 25±0.1°C. The following Mark-Houwink-Sakurada (MHS) equation for chitosan was used to calculate molecular weight:

$$[\eta] = K M_w^{\alpha}$$

Where [η] is the intrinsic viscosity calculated from viscosity data and Mw is the molecular weight. The viscometric constants, *K* and α were 1.49x10^{-4} dL/g and 0.79, respectively, for polydisperse chitosan samples in sodium acetate buffer (0.25M) at 25°C, with the degree of acetylation (DA) between 20 and 26%, based on an empirically modified MHS equation at 25 °C (Kasaai et al., 2000).

2.3. Production of COS

COS were obtained by enzymatic hydrolysis with pepsin from porcine gastric mucosa, according to Roncal et al. (2007) [22] with slight modifications. Briefly, chitosan (1%) in 0.2 M sodium acetate buffer pH 4.5 and pepsin (1:100) were incubated in a water bath at 40°C for 20h. After this, the pH was adjusted to 7.0 with 1 M NaOH and the reaction was boiled for 10 min to inactivate the enzyme and centrifuged. The supernatant was freeze-dried and resuspended with methanol, and then precipitated and washed with 2 volumes of acetone, and finally dried under vacuum.

2.4 Nuclear magnetic resonance and MALDI

Chitosan and COS were analyzed by nuclear magnetic resonance (NMR) and matrix-assisted laser desorption/ionization (MALDI-TOF). Decoupled ^{13}C NMR spectra were obtained using a Bruker Avance DRX-400 spectrometer with a 5-mm inverse probe. ^{13}C NMR acquisitions were performed using the WALTZ-16 pulse sequence with the following parameters: FIDRES: 0.8466 Hz; AQ: 0.5906 s; DW: 15.75 s; DE: 5.5 μs; D1: 110 ms; D2: 3.4 ms; PL12: 17 dB (decoupler 1H). The molecular weight of COS obtained from the enzymatic hydrolysis of chitosan was determined by MALDI-TOF MS analysis (Voyager-DE TM STR Biospectrometry Workstation, Applied Biosystems Inc., NCIRF, Korea)

2.5 Animals, diet and protocol.

This study has approval from the Ethics in Animal Use Committee of the Universidade Federal de Pernambuco (nº 23076.029665/2015-10) Animals from the Laboratory of Imunopathology Keiso Azami (LIKA-UFPE) were housed two weeks in the Department of Biochemistry of the Universidade Federal de Pernambuco prior to experiments for accommodations. Albino Swiss mice, male, with three months of age, were placed in 4 different groups during 28 days (n=5). Animals receiving regular diet (Control), animals receiving high fat diet (HFD), this diet has as main composition of fat (Table 1), animals receiving high fat diet and being treated with 50 mg/kg of COS daily (HFD + COS 50 mg/kg), and, animals receiving high fat diet and being treated with 100 mg/kg of COS daily (HFD + COS 100 mg/kg). HFD was based on the study of Araújo et al. (2011) [23], in which 43 % of powdered chow pellet diet were added to 40 % of dried bovine brain, 14.6 % of butter fat, 2 % of sodium cholate, and 0.4 % of choline citrate. Diet was made continuously to assure freshness.

Energy (kJ/g)	Commercial	HFD
Total	13.40	20.48
Fat	8.00 %	51.65 %
Carbohydrates	54.00 %	20.95 %
Protein	26.00 %	27.40 %

Table 1. Energy value of high fat diet in contrast with commercial chow

2.6 Body, muscle and fat weight.

During the protocol, body weight was measured with digital scale; and, on the 28th day, mice were anesthetized and had epididymal fat and gastrocnemius muscle removed for weight analysis in digital scale.

2.7 Swim-forced test.

Mice had 5% of body weight attached to the proximal third of their tail, then were placed in a cylindrical chamber with water (20 cm depth) as described by Ikeuchi et al. (2006) [24]. Mice were observed, and failure was considered when mice stopped returning to the surface after 8 seconds. Then, mice were taken off the chamber, placed in a warm and dry towel, and time was recorded.

2.8 Biochemical analysis.

On Days 1 and 28 of protocol, mice were anesthetized, and blood was collected from retro-orbital plexus. Blood was centrifuged, and plasma was used for the quantification of glucose, triglycerides, total cholesterol, high-density-cholesterol (HDL-cholesterol), aspartate aminotransferase (AST),alanine aminotransferase (ALT), urea and creatinine as indicated by LabTest kits (LabTest, Brazil). Non-HDL-cholesterol was measured from the subtraction of HDL-cholesterol quantity form the total cholesterol quantity.

2.9 Statistical analysis.

Data was expressed as mean ± standard deviation, and analyzed in Prism 5.0 (GraphPad, USA). For data analyzed only on day 28, one-way ANOVA followed by Tuckey's Test investigated significant difference. Two-way ANOVA followed by Bonferroni's Test was used to analyze data of days 1 and 28, or more. For statistical difference, it was considered $p<0.001$.

3 Results and discussios

A chemical and structural characterization was discussed in Chapter 1.

3.1 Analysis of body weight

The body weight of the animals under study was evaluated every 7 days. The animals in the control group (received commercial chow), had their body weights higher in comparison to the other groups that received the hyperlipid diet. This happened because the animals that received the HFD diet had an adaptation time until they became accustomed to the new food provided. This result was already expected, as described by Araujo et al (2011) [23] in which the HFD diet causes significant weight gain from the second month of diet. As this study lasted 28 days these changes were not observed. Throughout the experiment the animals that were treated with COS (HFD + COS 50 mg/kg; HFD + COS 100 mg/kg) showed a decrease in weight when compared to the HFD group, demonstrating their capacity in reducing body weight. Although it did not change the body weight of the COS-treated group in relation to the control group, when the body composition of the animals was evaluated, it was observed that the weight of the gastrocnemius muscle was higher in the treated groups than in the HFD group, suggesting that the COS were able to maintain muscle mass. When the weight of the

epididimal fat was evaluated, the groups that received the treatment with the COS, were shown to be significantly lower, evidencing the effectiveness of the oligomers in reducing body fat.

Body Weight (g)	Control	HFD	HFD + COS 50 mg/kg	HFD + COS 100 mg/kg
Day 1	48.2±2.9	54.4±7.8	55.2±7.0	60.2±5.1
Day 7	51.0±4.1	45.0±3.5*	44.0±4.0*	46.0±6.5*
Day 14	52.0±4.3	44.4±4.1*	43.9±5.5*	43.0±5.6*
Day 21	52.9±4.0	44.8±4.5*	46.0±9.6*	43.3±4.9*
Day 28	51.8±3.5	46.1±3.1*	42.5±6.0*	40.9±5.0*
Gastrocnemius Muscle Mass (g)				
	0.181±0.04	0.141±0.04	0.144±0.05	0.172±0.01
Epididymal Fat (g)				
	0.323±0.08	0.411±0.10	0.202±0.05**	0.201±0.09**

Table 2. Body, gastrocnemius muscle mass and epididymal fat weight of mice with high fat diet treated with chitooligosaccharides. *p<0.001 vs Control, MANOVA followed by Bonferroni's Test. **p<0.001 vs Control, ANOVA followed by Tuckey's Test.

Similar results were reported by Huang et al (2015) [24] who showed that COS have shown anti-obese effects in high-fat diet (HFD)-induced obese rat models improved dyslipidemia and prevented body weight gains by inhibiting the adipocyte differentiation in obese rats induced by a high-fat diet. Furthermore, the expression levels of PPAR-γ (receivers activated by peroxisome necessary and sufficient to differentiate adipocytes). were explicitly reduced. Other mechanisms that are described is that COS reduces food intake by increasing serum leptin and lowering serum ghrelin. COS also inhibits pancreatic lipase activity and bile acid function, resulting in reduced fat absorption and increased fecal fat excretion [25].

3.2 *Swim-forced test.*

The swim-forced test is used to evaluate the ability of animals to strenuous activities. As results (figure 1), it was found that the control group had a mean swimming time of 33.6 seconds. The animals in the HFD group, meanwhile, had a mean swimming time of 8.2 seconds.

However, the groups that received the hyperlipid diet but were treated with COS at 50mg / kg and 100mg / kg, presented an average time of 33.6 seconds and 30.6 seconds, respectively. This result shows the ability of the COS to maintain the animals' physical condition in the opposite way to what happened to the HFD diet animals.

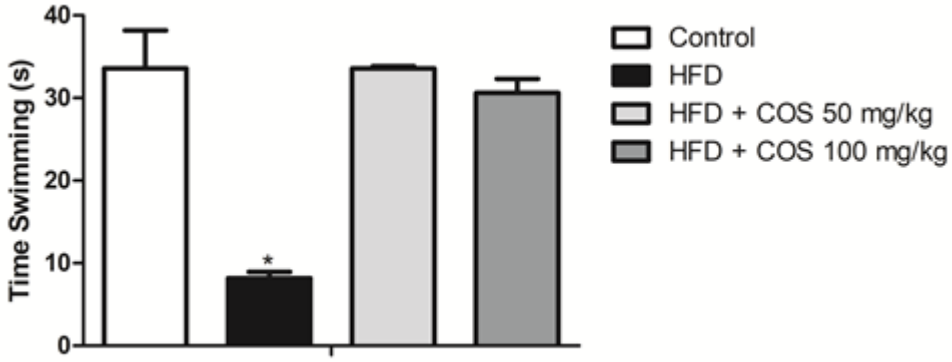

Figure 1. Swimming performance of mice with high fat diet treated with chitooligosaccharides. *p<0.0001 vs Control, ANOVA followed by Tuckey's Test.

In addition to being demonstrated in this work, COS are effective in decreasing the body weight of treated animals, when the forced swim test it is evidenced that the animals that received the treatment with the COS had a greater physical performance in comparison to the group that only received the diet HFD.

One of the foundations that can explain the action of the COS in mater the physical capacity of the animals, is that these oligomers can avoid insulin resistance, which is due to obesity. As insulin is in its normal ability to act, the glucose is internalized in the cells initiating the process of cellular respiration, producing energy to perform the required functions of the organism [26].

3.3 Biochemical Analysis

COS had their effects on the biochemistry profile of the test animals evaluated at the beginning and at the end of the treatment (Figure 2, Table 3 and 4).

When the glucose concentration was evaluated at the beginning of the experiment, no significant differences were observed between the groups (Figure 2). The glucose concentration was between 65 and 79 mg/dL. At the end of the experiment, on the 28th, this profile had an important change.

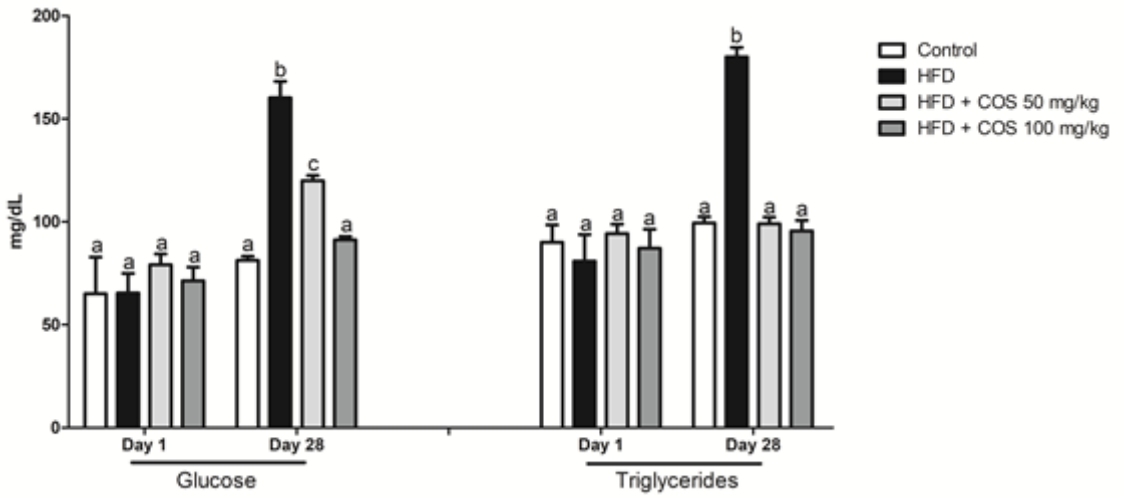

Figure 2. Glucose and triglycerides plasma levels of mice with high fat diet treated with chitooligosaccharides. Different letters mean statistical difference, $p<0.001$, ANOVA followed by Tuckey's Test.

The control group and the group treated with COS at 100 mg/kg did not present significant statistical differences between them, evidencing that the COS, dose-dependent, have the ability to maintain glucose levels close to normal dietary patterns. The opposite occurred with the HFD group which had a significant increase in plasma glucose from 65.3 mg/dL to 160.2 mg/dL. This can be explained by studies by Murata et al (2012) [27] and Harish et al (2000) [28] who observed that Polymeric chitosan was effective in reducing blood glucose and LMW chitosan was effective in preventing the development of hyperglycemia in a slowly progressive model of Diabetes mellitus. Other studies performed by Liu et al (2015) [26] have shown that COS activates AMPK in muscle cells and other adipocytes, resulting in increased glucose transporter 4 (GLUT4) membrane insertion hence successively increases glucose uptake.

When the effects of COS on triglycerides were evaluated (Figure2), it was observed that on the first day of the experiment the groups showed no significant difference in the concentration of these compounds, as expected. The triglycerides concentration was between 80.8 and 94.3mg/dL.

On the last day of the experiment, the groups of animals that received hyperlipidic diet and were treated with COS at 50mg/kg and 100mg/kg, a triglyceride concentration was 99mg/dL and 95.5mg/dl, respectively, against 180.1 mg/dL of the HFD group and 99.4 mg/dL of the control group.

These results corroborate with the results found when the weight of the animals was evaluated during this experiment. In the evaluation of the cholesterol and lipoproteins (Table 3) it was observed that the COS positively altered the metabolism of these molecular aggregates. On the first day of the experiment, the values between the groups did not present significant statistical differences. At the end of the experiment, on the twenty-eighth day, the values of cholesterol between the control group, HFD + COS 50 mg/kg and HFD + COS 100 mg/kg (119 mg/dL, 144.5 mg/dL and 129.2 mg/dL, respectively) remained without significant changes contrary to the concentration of cholesterol of the HFD group (195.5 mg/dL). High cholesterol is a risk factor for cardiovascular disease. That way it is important to have your levels controlled. These results evidenced the ability of COS to act by inhibiting the elevation of this macromolecule.

Total Cholesterol (mg/dL)	Control	HFD	HFD + COS 50 mg/kg	HFD + COS 100 mg/kg
Day 1	112.1±27.3^{a}	119.5±13.8^{a}	105.7±19.0^{a}	100.5±12.5^{a}
Day 28	119.8±16.8^{a}	195.5±7.3^{b}	144.5±17.2^{c}	129.2±7.5^{a}
HDL-cholesterol				
Day 1	52.2±9.1^{a}	53.5±5.0^{a}	49.9±10.4^{a}	44.5±3.0^{a}
Day 28	55.3±8.8^{a}	24.6±4.6^{b}	48.7±6.5^{a}	45.8±2.1^{a}
Non-HDL-cholesterol				
Day 1	59.9±29.9^{a}	62.8±18.2^{a}	55.7±17.6^{a}	55.9±13.4^{a}
Day 28	64.4±22.8^{a}	170.8±8.7^{b}	93.7±24.7^{a}	83.4±6.8^{a}

Table 3. Levels of total cholesterol and its fractions in mice under high fat diet treated with chitooligosaccharides. Different letters mean statistical difference, $p<0.001$, MANOVA followed by Bonferroni's Test.

Other important analyzes, are those of lipoproteins (Table 3), because their values are extremely important to evaluate the cardiovascular effect that the COS generated.

When we observed HDL, which is a high density lipoprotein and has the function of transporting cholesterol from vessels and tissues to the liver, it was observed that on day 1 of the experiment the groups did not present significantly different values (Control: 52.2 mg/dL; HFD: 53.5 mg/dL; HFD + COS 50 mg/kg: 49.9 mg/dL; HFD + COS 100 mg/Kg: 44.5 mg/dL) as expected.

At the end of the experiment, on the twenty-eighth day, HDL was dosed: Control: 55.3 mg/dL; HFD: 24.6 mg/dL; HFD + COS 50 mg/kg: 48.7 mg/dL; HFD + COS 100 mg/kg: 45.8 mg/dL. The HFD group showed a significant reduction of the HDL fraction while the groups treated with the COS had a value close to the control group.

Corroborating with these results, the non-HDL-cholesterol dosage (table 3), that is, fractions comprising LDL, VLDL and other lipoproteins, which are directly associated with cardiovascular diseases, had as results on the 28th day of the experiment: Control: 64.4 mg/dL; HFD: 170.8 mg/dL; HFD + COS 50 mg/kg: 93.7 mg/dL; HFD + COS 100 mg/kg: 83.4 mg/dL.

Some studies have shown that low-molecular weight chitosan is a more effective anti-obese agent in high-fat diet induced obesity animal models. [31]. Studies have shown that COS significantly decreased lipid accumulation, a marker of adipogenesis, in a dose dependent manner [32]. The low molecular mass COS (1–3 kDa) were more effective in inhibiting adipocyte differentiation in 3T3-L1 cells [33,34]. COS treatment notably decreased the expression of peroxisome proliferator-activated receptor γ (PPARγ), a key adipogenic transcription factor.

Hepatic markers, AST and ALT, and renal markers, urea and creatinine, were also measured trying to understand the effects of COS on these organs (Table 4).

AST	Control	HFD	HFD + COS 50 mg/kg	HFD + COS 100
Day 1	76.0±3.0	78+8±2.1	79.3±2.4	79.6±2.5
Day 28	80.8±0.9	80.9±2.3	80.0±0.3	79.8±0.6
ALT				
Day 1	50.7±2.1	49.2±1.3	48.5±1.2	50.2±1.2
Day 28	49.5±0.7	80.8±2.0*	63.9±3.5	51.4±3.5
Urea				
	48.7±9.8	47.9±2.5	45.8±2.6	46.0±2.9
Creatinine				
	0.15±0.03	0.19±0.05	0.17±0.02	0.12±0.02

Table 4. Markers of liver and kidney injuries in mice under high fat diet treated with chitooligosaccharides. *$p<0.001$ vs Control, MANOVA followed by Bonferroni's Test.

As with the results, no significant changes in AST concentration were observed at the end of the experiment. This hepatic marker is found in hepatocyte mitochondria and is associated with chronic lesion. When evaluated the ALT enzyme, which is found in the cytoplasm of hepatocytes, and was associated with acute lesions, it was observed that

only the HFD group had a high concentration (80.8 mg / dL), the control group (49.5 mg / dL) and the groups that received COS at 50 mg / kg and 100 mg / dL (63.9 mg / dL and 51.4 mg / dL, respectively).

Renal markers were evaluated on the last day of the experiment (Table 4). As a result of urea and creatinine concentrations, no significant changes were observed in either group, meaning that no animal had kidney damage as a result of diet and COS used in treatment. Studies report that through the Urine analysis of MW of intra-peritoneal administered COS / chitosan (300 kDa) fascinatingly ranged from less than 10 kDa to 40 kDa as excreted [35, 36]. Thus, these data essentially specify that COS / chitosan or their derivatives are primarily excreted from the body via urine after biometabolism in hepatocytes and kidney tissues, not generating any renal injury

4 Conclusion

With the results observed above, we conclude that the COS are efficient compounds in the reduction of the levels of lipids and lipoproteins, they maintained the physical strength of treated animalsbesides promoting a decrease of glycemia of the animals in hyperlipidica diet. In addition, these compounds were able to prevent acute hepatic lesions and did not cause renal damage. The data presented demonstrate that the compounds present therapeutic safety and may become postulants in the treatment of obesity and its complications.

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5 – CONSIDERAÇÕES FINAIS

- A partir da quitosana obtida de resíduos do processamento do camarão *Litopenaeus vannamei*, os oligossacarídeos foram produzidos com sucesso por hidrólise enzimática não específica utilizando pepsina;
- Os oligossacarídeos, bem como a quitosana de baixo peso molecular foram caracterizados quimicamente apresentando perfil semelhante ao da quitosana; estes apresentaram baixo peso molecular;
- Quando avaliados frente a modelos de radicais livres, mostraram capacidade em sequestrar radicais instáveis, quelar íons e proteger o DNA contra danos oxidativos;
- A quitosana, quitosana de baixo peso molecular e os oligossacarídeos de quitosana obtidos neste trabalho possuiu a capacidade de inibir o crescimento de *S. aureus* e *E. coli* em caldo nutriente.
- Na avaliação da citotoxicidade, demostraram ter atividade inibitória contra células tumorais e baixa toxicidade frente a células normais;
- Não apresentaram toxicidade em modelos experimentais *in vivo* e demostraram sua capacidade analgésica;
- Quando avaliada sua atividade antitumoral *in vivo*, estes demostraram interessante capacidade em inibir o crescimento tumoral;
- Os oligossacarídeos de quitosana foram eficientes na redução dos níveis de lipídeos e lipoproteínas, mantiveram a força física dos animais tratados além de promover a diminuição da glicemia dos animais que receberam a dieta hiperlipídica.
- Além disso, esses compostos foram capazes de prevenir lesões hepáticas agudas e não causaram danos renais.
- Além de suas interessantes propriedades, a obtenção destes produtos a partir de resíduos da indústria pesqueira, faz com que sua produção tenha melhor relação custo/ benefício, quando comparados a outros polímeros atualmente utilizados no mercado se configurando como potenciais produtos para serem aplicados nas indústrias biomédicas, farmacêutica e alimentícia, sendo necessário futuros estudos.

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