Universidade Federal do Tocantins
Campus Universitário de Gurupi
Programa de Pós-Graduação em Biotecnologia

GRECIA ESTHEFANY BARRIGA MONTALVO

DISCOVERY OF NOVEL BIOACTIVE PEPTIDES FROM *Spirulina (Arthrospira) maxima.*

GURUPI - TO
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Dissertação apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade Federal do Tocantins como parte dos requisitos para a obtenção do título de Mestre em Biotecnologia.

Orientador: Prof. Dr. Carlos Ricardo Soccol

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Discovery of novel bioactive peptides from Spirulina maxima.

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RESUMO

Nos últimos anos o interesse pelos peptídeos bioativos de *Spirulina* sp. (*Arthrospira*) tem aumentado enormemente devido a seu estado Geralmente reconhecido como seguro (GRAS) e seus potenciais benefícios para a saúde. Essa microalga pode ser utilizada em diferentes alimentos funcionais, para fins médicos, cosméticos e nutracêuticos devido às suas propriedades biológicas como anti-hipertensivas, antioxidantes, anti-hialuronidase e outras. No presente trabalho de investigação, as fracções de peptídeos de *Spirulina maxima* foram produzidas através de três processos diferentes de hidrólises, utilizando duas proteases diferentes. Em seguida os extratos foram submetidos a um processo de purificação pelo método de filtração tangencial (membranas de 2 µM e 10 kDa), e os fragmentos obtidos no permeado de 10 kDa foram parcialmente caracterizados e avaliadas suas capacidades antioxidantes (sequestro de radicais, atividade quelante de ferro, antimicrobiana, anti-inflamatória e anticolagenase. Fração de peptídeos obtido do primeiro hidrolisado (PHA) apresentou atividade antioxidante capturando aos radicais DPPH com um valor de IC_{50} 21,25 µg/ml e ABTS com IC_{50} 9,5 µg/ml e TEAC 465,7 Trolox µM/µg de amostra, e atividade quelante mostrou uma inibição de 97,3% e um IC_{50} 6,99 µg/ml. Fração de peptídeos obtido do segundo hidrolisado (PHP) apresentou atividade antimicrobiana com concentração inibitória media (IC_{50}) e concentração bactericida mínima (CBM) de 0,34 e 0,63 mg/ml (*Bacillus subtilis*), IC_{50} e CBM 0,62 e 0,63 mg/ml (*Staphylococcus aureus*), IC_{50} e CBM 0,99 e 1,25 mg/ml (*Salmonella typhi*), IC_{50} e CBM 0,94 e 1,25 mg/ml (*Escherichia coli*). Fração de peptídeos obtido das duas enzimas (PHS) apresentou atividade antioxidante capturando aos radicais DPPH com um valor de IC_{50} 17,93 µg/ml e ABTS com IC_{50} 8,6 µg/ml e TEAC 540,7 Trolox µM/µg de amostra, anti-inflamatória com inibição da enzima hialuronidase 39% e IC_{50} 0,92 mg/ml, e anticolagenase com uma inibição 92,5% e IC_{50} 32.49 µg/ml. Os resultados permitem concluir que isolado proteico de *Spirulina* pode ser uma fonte para obtenção de extratos peptídicos (PHA, PHP e PHS) com atividade antioxidante, quelante, antimicrobiana, anti-inflamatória, anticolagenase.

Palavras-chave: *Spirulina* máxima, peptídeo, atividade antioxidante, quelante, antimicrobiana, anti-inflamatória, anticolagenase.

ABSTRACT

In the last years, the interest by the bioactive peptides *Spirulina* sp. (*Arthrospira*) is increasing, because of its Generally Regarded as Safe (GRAS) status, and their potential health benefits. These peptides can be used in different functional foods, for medical purposes, cosmetic and nutraceuticals, due to biological properties such as antihypertensive, antioxidative, anti-hyaluronidase and others. In the present investigation, *Spirulina maxima* were produced through of three hydrolyses process. After, the peptides fractions were purified through tangential filtration (membrane of 2 µM and 10kDa), and the fragments obtained below 10 kDa were partially characterized and determinate their antioxidant (radical scavenging, iron-chelating), antimicrobial, anti-inflammatory, and anti- collagenase. The peptide fraction obtained from the first hydrolyzed (PHA) presented antioxidant activity by capturing the DPPH radicals with a value of IC_{50} 21.25 µg/ml and ABTS with IC_{50} 9.5 µg/ml and TEAC 465.7 Trolox µM/µg sample, iron-chelating showed inhibition of 97.3% and IC_{50} 6.99 µg/ml. Meanwhile, the peptide fraction obtained from the second hydrolyzed (PHP) presented antimicrobial activity with the half maximal inhibitory concentration (IC_{50}) and the minimum bactericidal concentration (MBC) 0.34 mg/ml and 0.63 mg/ml (*Bacillus subtilis*), IC_{50} and MBC 0.62 mg/ml and 0.63 mg/ml (*Staphylococcus aureus*), IC_{50} and MBC 0.99 mg/ml and 1.25 mg/ml (*Salmonella typhi*), IC_{50} and MBC 0.94 mg/ml 1.25 mg/ml (*Escherichia coli*). The peptide fraction obtained from the two enzymes (PHS) showed antioxidant activity by capturing the DPPH radicals with a value of IC_{50} 17.93 µg/ml and ABTS with IC_{50} 8.6 µg/ml and TEAC 540.7 Trolox µM/µg sample, anti-inflammatory with inhibition of 39% and IC_{50} 0.92 mg/ml, and anti-collagenase with inhibition 92.5% and IC_{50} 32.49 µg/ml. The results indicated that protein isolate from *Spirulina maxima* can be a source for obtaining of peptides fractions (PHA, PHP and PHS) with activity antioxidants, iron-chelating, antimicrobial, anti-inflammatories and anti-collagenase.

Keywords: *Spirulina maxima*, peptide, activity antioxidants, iron-chelating, antimicrobial, anti-inflammatories and anti-collagenase.

SUMMARY

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LIST OF ABBREVIATIONS

ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

AMPs: Antimicrobial peptides

BPs: Bioactive peptides

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DMAB: 4-dimethylaminobenzaldehyde

DH: Degree of hydrolysis

ECM: Extracellular matrix

E/S ratio: Enzyme/Substrate ratio

EDTA-Na_2: Disodium ethylenediaminetetraacetate dihydrate

FALGPA: N-[3-(2- furyl)acryloyl]-Leu-Gly-Pro-Ala

FDA: Food and Drug Administration

$FeCl_2$: Iron dichloride

GRAS: Generally Regarded as Safe

GI: Gastrointestinal

HPLC: High performance liquid chromatography

H_2O_2: Hydrogen peroxide

IC_{50}: Measure of a compound's inhibition (50% inhibition)

MBC: Minimum bactericidal concentration

MIC: Minimal inhibitory concentration

MHB: Mueller hinton broth

O^{2-}: Superoxide anion

$^1O_2$: Singlet oxygen

OH^-: Hydroxyl

PHA: Peptide fraction obtained from the first hydrolyzed

PHP: Peptide fraction obtained from the second hydrolyzed

PHS: Peptide fraction obtained from the two enzymes

ROS: Reactive oxygen species

RP- HPLC: Stationary phases for reversed phase high performance liquid chromatography

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TEAC: Trolox equivalent antioxidant capacity

TCA: Trichloroacetic acid

LIST OF AMINO ACIDS AND THEIR SYMBOLS

Alanine	Ala
Cysteine	Cys
Aspartic acid	Asp
Glutamic acid	Glu
Phenylanine	Phe
Glycine	Gly
Histidine	His
Isoleucine	Ile
Lysine	Lys
Leucine	Leu
Methionine	Met
Asparagine	Asn
Proline	Pro
Glutamine	Gln
Arginine	Arg
Serine	Ser
Threonine	Thr
Valine	Val
Tryptophan	Trp
Tyrosine	Tyr

INTRODUCTION

In the actuality, numerous diseases and physiological and morphological disorders are increasing and affecting the quality of life. Many of these disorders are associated with abuse of synthetic drugs and additives. For this reason, increasing the search for new drugs and functional food products able to fight degenerative illnesses has increased. Natural sources are especially appealing in this context, en especially proteins and their derivatives, including peptides (SEGURA C. et al., 2011; SULTAN et al., 2016).

Bioactive peptides (BPs) are specific protein fragments, formed by amino acids joined by peptide bonds, that are produced by cleavage from parent proteins, usually composed by one chain of 2 - 20 amino acids (HARNEDY; FITZGERALD, 2012; LAFARGA; ÁLVAREZ; HAYES, 2017; SULTAN et al., 2016). The BPs present a positive impact on body functions and may influence in the health, as they show high biological activities (e.g. opioid, hypersensitive, antithrombotic, and antimicrobial) associated with low toxicity and high specificity (SHARMA; SINGH; RANA, 2011; SINGH; VIJ; HATI, 2014). The activity of these peptides is dependent on the structure, hydrophobicity, charge, and other factors, and a single peptide may have more than one activity (MEISEL; FITZGERALD, 2003; WIJESEKARA; KIM, 2010).

Bioactive peptides are produced by means of the following mechanisms: a) during the fermentation of food using proteolytic starter cultures, b) as a result of the degradation of dietary proteins by digestive enzymes in vivo, c) as a result of the enzymatic action of digestive enzymes in vitro (CICERO; FOGACCI; COLLETTI, 2017; MUKHOPADHYA; SWEENEY, 2016; OVANDO et al., 2016). The most common method is enzymatic hydrolysis, which does not use organic solvents or toxic chemicals, and is more specific and controlled (CLARE; SWAISGOOD, 2000; ZINOVIADOU; GALANAKIS, 2017).

Marine cyanobacteria are significant sources of several bioactive compounds and, therefore, they may be used in several biological applications related to health benefits, and nutraceuticals (DE JESUS RAPOSO; DE MORAIS; DE MORAIS, 2013; MI et al., 2017). *Spirulina* (*Arthrospira*) has been used by humans as food since ancient times due to its high content of proteins (43-70%), essential amino acids, and vitamins (BILLS; KUNG, 2014; KHAN; BHADOURIA; BISEN, 2005) Furthermore, the

pharmaceutical industry has shown great interest in *Spirulina* for its biotechnological and nutritional properties, as well as its GRAS (Generally Regarded as Safe) status by Food and Drug Administration (FDA) (OLIVEIRA et al., 2013).

Studies have shown that *Spirulina* proteins hydrolyzed with commercial proteases result in bioactive peptides with possible health promoting properties such as antioxidant effect (SAFITRI et al., 2017), Anti-allergic effect (VO et al., 2014), anticancer effect, antimicrobial activity (JANG; PARK, 2016), angiotensin I-converting enzyme (ACE) inhibiting activity (HEO et al., 2015). In addition, a large variety of peptides of marine origin have been discovered, but few *Spirulina* peptides have been identified. Thus, the objective of this study is the generation of bioactive peptides of *Spirulina maxima* through enzymatic hydrolysis for determination of the *in-vitro* bioactivity of the hydrolysates.

OBJETIVE

The aim of this study was the production of bioactive peptides of *Spirulina maxima* through enzymatic hydrolysis for determination of biological activity.

Secondary objectives

- Evaluate the proximal composition of biomass of *Spirulina maxima*.
- Obtain the protein isolate of biomass from *Spirulina maxima*.
- Hydrolyze the protein isolate by two methods (single-step and sequential-step).
- Evaluate the degree of hydrolysis and the concentration of peptides of hydrolyzes.
- Purify and identify the peptides fractions of *Spirulina maxima.*
- Evaluate the antioxidant, iron-chelating, antimicrobial, anti-inflammatory and anti-collagenase activity *in-vitro.*

CHAPTER 1: THEORETICAL FRAMEWORK

1. Bioactive Peptide

Bioactive peptides (BPs) are specific protein fragments, and in addition to act as amino acids and nitrogen sources, they have a positive impact on body functions and may influence in the health (HARNEDY; FITZGERALD, 2012; SHARMA; SINGH; RANA, 2011; SINGH; VIJ; HATI, 2014). BPs can be obtained from diverse raw materials, such as: plants, animals, macroalgae, microalgae, seafood, and fungi (HAYES, 2013; KITTS; WEILER, 2003; SHARMA; SINGH; RANA, 2011).

The bioactive peptides are naturally occurring biomolecules, and are released by processes such as (a) microbial fermentation of proteins by proteolytic microbes, (b) proteolysis by enzymes from plants or microorganisms, and (c) proteolysis by gastrointestinal enzymes (AGYEI et al., 2016; SAMARAKOON; JEON, 2012). Nevertheless, especially in food and pharmaceutical industries the enzymatic hydrolysis method is preferred for production of BPs, because of the lack residual organic compounds and toxic chemicals in the end product (KADAM et al., 2015; LAFARGA; ÁLVAREZ; HAYES, 2017). Thus, two factors can determine the generated bioactive peptide: the protein substrate and the specificity of the enzyme(s) which is used to generate the peptide (HARNEDY; FITZGERALD, 2012). The size of BPs usually contains 3–20 amino acid residues in length (WALTHER; SIEBER, 2011). The bioactive peptide can be absorbed by the intestine and be transported out intact in the circulatory system, where they exert physiological effects or local effects on the gastrointestinal system (MARCONE; BELTON; FITZGERALD, 2017; SEGURA C. et al., 2011). The bioavailability of peptides molecules depends on their ability to cross the intestinal mucosa and by the resistance to peptidase degradation of both the intestinal tract (RENUKUNTLA et al., 2013; VERMEIRSSEN et al., 2004). Furthermore, depending on the amino acid sequence, structure, molecular weight, hydrophobicity, charge, and other factors, they may be induced several biological functions such as antioxidant, anticancer, antihypertensive, antimicrobial, anti-obesity, immunomodulatory , metal-chelating (SULTAN et al., 2016). Also, some peptides may exhibit several properties, where specific peptide sequences may possess two or more different biological activities (Table 3) (HARNEDY; FITZGERALD, 2012; KIM; WIJESEKARA, 2010).

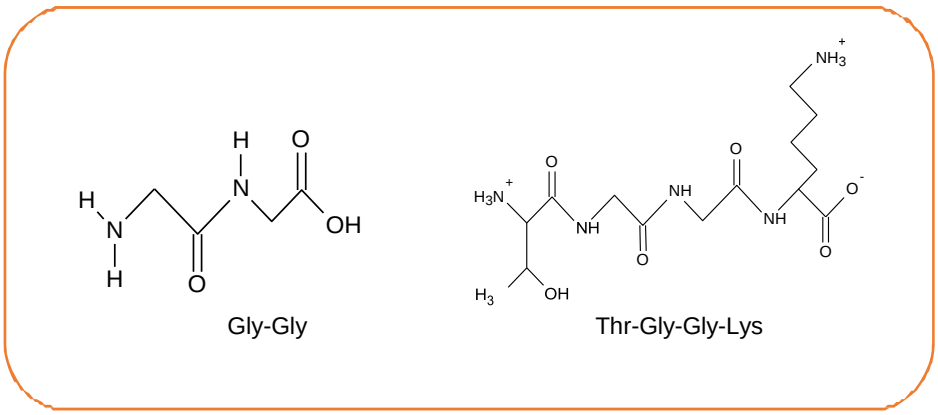

Figure 1-Example of possible structures of bioactive peptides.

2. Enzymatic hydrolysis for peptide production

In the actuality, the methodology most used for protein breakdown and to generate functional peptide is enzymatic hydrolysis, where the enzymes applied could be non-gastrointestinal (non-GI) proteases (e.g., papain, alcalase and thermolysin) from bacterial, fungal or plant sources or gastrointestinal (GI) proteases (e.g., pepsin, trypsin and chymotrypsin) of animal origin (KORHONEN; PIHLANTO, 2003; SULTAN et al., 2016). For production of peptides with potential impact on health or food quality is important to select the appropriate proteolytic enzyme and in addition perform the process at the optimal physicochemical conditions (pH, temperature, incubation time, flow rate), in order to maximize the yield (hydrolysis degree), the activity of proteolytic enzyme, due to considerably influence the molecular weight distribution and fractions of peptides; affecting the target bioactivity (LIU et al., 2016; SARMADI; ISMAIL, 2010). Furthermore, in this process of hydrolysis, if is necessary, can be used simultaneously two or more enzyme or sequentially for production of novel BPs (ALUKO, 2012; OVANDO et al., 2016). For this reason, is fundamental the search of suitable enzymes for targeted bioactivity and optimize the hydrolysis degree using the bioactivity as response factor also.

Marine bioactive peptides possess a variety of beneficial biological functionalities and have many physiological effects in the human body (HAYES, 2013; KIM; WIJESEKARA, 2010). For example, several studies have shown that *Spirulina* protein hydrolyzed with commercial GI proteases and non-GI proteases generates peptides

with therapeutical effects in the health (KIM; WIJESEKARA, 2010; OVANDO et al., 2016) (Table 1). For the food and pharmaceutical industries, the use of enzymatic hydrolysis is preferred, in the production of bioactive peptides, because the process gives better yields and purities than organic solvent extractions (ZAMBROWICZ et al., 2013).

Table 1- Enzymes and optimum conditions used in hydrolysis of marine-derived.

Enzymes and ratio E:S	**Buffer/Solvent**	**pH**	**T**	**Time**	**Bioactivity**	**Reference**
Proteomax 580L (1:25)	Sodium carbonate bicarbonate buffer	9.5	60 °C	3.5h	Antioxidant	(LISBOA et al., 2016)
Papain (1:50)		7	70 °C	9 h	Anti-hypertensive	(PAN et al., 2015)
Alcalase (1:500)	Sodium phosphate 10 mM	8	55 °C	1 h	Iron-chelating	(KIM et al., 2014)
Flavourzyme (1:50)		7	50 °C	8 h		
Pepsin (1:50)	Water	2	50 °C	15 h	Anticancer	(SHEIH et al., 2010)
Pepsin (1:250)		2	37 °C	2 h	Ace-inhibitory and antioxidant	(NAKAJIMA; YOSHIE-STARK; OGUSHI, 2009)
Pancreatin (1:100)		7	37 °C	3 h		
Termolysin (50:1)		7	37 °C	5 h		

FONT: The author.

3. Production and identification of bioactive peptides

For the discovery and production of bioactive peptides involves a serial of steps, first identifying a suitable protein source, second releasing peptide fragments with bioactivity through hydrolysis of peptide bonds, usually by the proteolytic action of enzymes sourced exogenously (e.g. chymotrypsin, pancreatin, trypsin, and pepsin) (LI-CHAN, 2015). In order to identify bioactive peptides following hydrolysis, the crude hydrolysates are assayed for various bioactivities and size fractionated. Finally identification of BPs and synthesis (Figure 2).

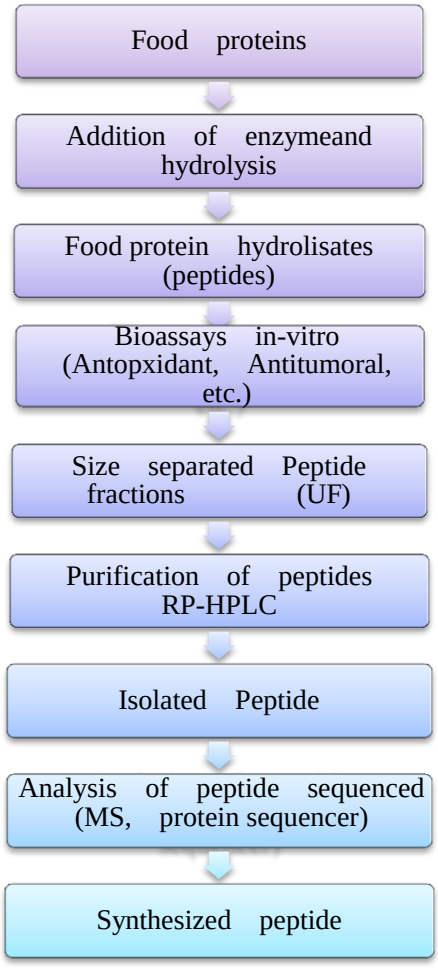

Figure 2-Flowchart showing the various steps involved in the production of bioactive peptides from food proteins. Adapted from CHALAMAIAH; YU; WU, (2018).

For the adequate detection of bioactive peptides, it is necessary to remove impurities in order to increase the product selectivity using ultrafiltration membrane system (10, 5, 3 KDa), allowing for improved studies of structure, physicochemical properties, and evaluation of the bioactive properties (AGYEI et al., 2016; SHAHIDI; ZHONG, 2008).

The most common technique for the purification of bioactive peptides is RP- HPLC (Stationary phases for reversed phase high performance liquid chromatography), is used for separating peptides based on the interaction between their side chains and the stationary phase and the mobile phase (AGUILAR, 2004; HARA et al., 2015; HUGHES ANDREW, 2010;

LEMES et al., 2016). Furthermore, by to identify individual peptide fractions a combination of HPLC and mass spectrometry (LC-MS/MS) and protein sequencing are useful tools (ARIHARA, 2006).

4. *Spirulina* as source of novel bioactive peptides

Marine microalgae and cyanobacteria are very rich in several bioactive compounds and, therefore, they may be used in several biological applications related to health benefits, and nutraceuticals (DE JESUS RAPOSO; DE MORAIS; DE MORAIS, 2013). Among several cyanobacteria is considered that an excellent source of bioactive substances in especially of bioactive peptides is *Spirulina* (BELAY, 2002).

Spirulina (*Arthrospira*) is a microscopic and filamentous cyanobacterium belonging to the family Oscillatoriaceae, and their filaments have a form spiral or helical nature (KITTS; WEILER, 2003). The *Spirulina* has been used by humans as a food supplement since ancient times, due to having a complete nutritional composition of minerals, polysaccharides, essential fatty acids and vitamins. Besides, of present a high content of proteins (60-70%), containing all amino acid essential (BABADZHANOV et al., 2004; YU et al., 2016).

Spirulina is well recognized as a therapeutic source. Many research studies show that *Spirulina* its effectiveness in the treatment of anti-inflammatory, antioxidant, antiviral, anti-bacterial, hypertensive, immunomodulatory, anticancer, anti-virus and others activities (JANG; PARK, 2016; WU et al., 2016). Since 1981 is considered Generally Regarded as Safe (GRAS) status by Food and Drug Administration (FDA) (OLIVEIRA et al., 2013). Also, is recognized by World Health Organization (WHO) for therapeutics and nutritional properties (ABD EL-BAKY; EL-BAROTY, 2012).

These attributes combined with great interest of cosmetic, nutraceutical and pharmaceutical industry for its biotechnological and nutritional properties, make a *Spirulina* an attractive source for exploration and production of bioactive peptides. Table 2 shows a list of the recent studies of specific peptides derived from *Spirulina* biomass with different bioactivities.

Table 2- *Spirulina* bioactive peptides. Adapted from OVANDO (2016).

Bioactivity	**Amino acid sequence**	**Enzyme**	**Source**	**Reference**
	Ile-Ala-Glu Phe-Ala-Leu Ala-Glu-Leu Ile-Ala-Pro-Gly Val-Ala-Phe	Pepsin	*Spirulina platensis*	(SUETSUNA; CHEN, 2001)
Anti-Hypertensive	Thr-Met-Glu-Pro-Gly-Lys-Pro	Pepsin, Trypsin, α-chymotrypsin	*Spirulina* sp.	(HEO et al., 2015)
	Ile-Gln-Pro	Alcalase	*Spirulina platensis*	(LU et al., 2010; PAN et al., 2015)
	Val-Glu-Pro	Papain	*Spirulina platensis*	(LU et al., 2011)
Anti-allergic	Leu-Asp-Ala-Val-Asn-Arg Met-Met-Leu-Asp-Phe	Pepsin, Trypsin, α-chymotrypsin	*Spirulina maxima*	(VO et al., 2014)
	Ala-Gly-Gly-Ala-Ser-Ley-Leu-Leu-Leu-Arg Leu-Ala-Gly-His-Val-Gly-Val-Arg	Alcalase, Papain	*Spirulina platensis*	(WANG; ZHANG, 2016a)
Antitumor	Lys-Phe-Leu-Val-Cys-Leu-Arg			
	His-Val-Ser-Arg-Ala-Pro-Arg	Pepsin, Trypsin, α-chymotrypsin	*Spirulina platensis*	(WANG; ZHANG, 2016b)
	Tyr-Gly-Phe-Met-Pro-Arg-Ser-Gly-Leu-Trp-Phe-Arg	Papain	*Spirulina platensis*	(WANG; ZHANG, 2016c)
Anti-inflammatory	Leu-Asp-Ala-Val-Asn-Arg Met-Met-Leu-Asp-Phe	Pepsin, Trypsin, α-chymotrypsin	*Spirulina maxima*	(VO; RYU; KIM, 2013)
Antibacterial	Lys-Leu-Val-Asp-Ala-Ser-His-Arg-Leu-Ala-Thr-Gly-Asp-Val-Ala-Val-Arg-Ala	Papain	*Spirulina platensis*	(SUN et al., 2016a)
Antiviral	Ser-Met	Pepsin, Trypsin, α-chymotrypsin	*Spirulina maxima*	(JANG; PARK, 2016)
Anti-atherosclerotic	Leu-Asp-Ala-Val-Asn-Arg Met-Met-Leu-Asp-Phe	Pepsin, Trypsin, α-chymotrypsin	*Spirulina maxima*	(VO; KIM, 2013)
Iron-chelating	Thr-Asp-Pro-Ile(Leu)-Ala-Ala-	Alcalase,	*Spirulina*	(KIM et al.,

	Cys-Ile(Leu)	Flavourzyme	sp.	2014)
Antioxidant	Phe-Ser-Glu-Ser-Ser-Ala-Pro-Glu-Gln-His-Tyr	Thermolysin, pepsin, trypsin, α-chymotrypsin	*Spirulina platensis*	(SAFITRI et al., 2017)
	Pro-Asn-Asn		*Spirulina platensis*	(YU et al., 2016)

5. Bioactivities investigated in the present thesis

5.1. Antioxidant activity

Food quality can suffer a deterioration of their attributes such as flavor, aroma, texture and color on account of oxidative reactions, and can provoke serious diseases. The antioxidant mechanisms possess multiple pathways including inactivation reactive oxygen species (ROS), metal-ion chelation, free-radical scavenging, and reduction of hydroperoxides (ELIAS; KELLERBY; DECKER, 2008; SU; SHYU; CHIEN, 2008).

Antioxidants protect the body, during metabolism and respiration, reactive oxygen species (ROS) are constantly and inevitably produce, such as superoxide anion (O^{2-}) and hydroxyl (OH^{-}) radicals, and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which can exert oxidative damage to proteins, lipids and DNA by subtracting electrons, thus starting chain reactions (JENSEN et al., 2013; NURDIANI et al., 2016; YU et al., 2016). Also, when ROS are overproduced, redox-active transition metal ions such as iron (II) or copper (II) can cause severe oxidative stress and thus damage tissues and the cellular DNA (KAUR et al., 2006).

The oxidative damage cause by ROS can lead to different diseases diabetes, anemia, aging process, allergies, cancer, cardiovascular and neurodegenerative (GALLEGOS T. et al., 2011; PIÑERO E.; BERMEJO B.; VILLAR DEL FRESNO, 2001). Meanwhile, the antioxidant system can under normal conditions remove reactive species through enzymatic (e.g. superoxide dismutase SOD) and non-enzymatic antioxidants (e.g. antioxidant vitamins and trace elements), but if this

endogenous defense system present a problem or is aging can not to protect the body and produce a major quantity of ROS (LAFARGA; ÁLVAREZ; HAYES, 2017). One way to suppress oxidative stress is intake of dietary antioxidative peptides and that can act as radical scavengers (HAYES, 2013; SARMADI; ISMAIL, 2010).

5.1.1. Antioxidant Peptides

The antioxidant peptide acts by preventing binding of oxygen to another molecule, and by the inhibition of free-radicals action (KANG et al., 2011). These peptides are important, because of their protective effect in lessening the severity of diseases; considering that in our body oxidative stress can cause serious damage to cells or tissues (NURDIANI et al., 2016; RAHAL et al., 2014). Additionally, the antioxidant peptides can present structures that contain nucleophilic sulfur-contain side chains cysteine (Cys) and methionine (Met) or aromatic side chains with amino acids histidine (His), tyrosine (Tyr) and methionine (Met) which can easily donate hydrogen atoms. Also, hydrophobicity and position of amino acids in the peptide are believed to play an essential role regarding antioxidant activity of a peptide (HAYES, 2013). The Antioxidant peptides have been found in differents sources such as plants, marine-derived, animal-derived and other (Table 3). In the resent years, a lot of research has focused on antioxidant peptides derived from *Spirulina*.

5.1.2. Iron-chelating peptides

The iron-chelating peptides have a capacity the increase iron absorption and bioavaility, due to these peptides combined with non-heme iron facilitating direct absorption in the intestine (NGO, 2013; WU et al., 2012). The Iron-chelating activity can determine by measuring the formation of the Fe^{2+} -ferrozine complex. The iron-chelating peptides may present structure that contains methionine (Met), glutamine (Gln), lysine (Lys) and arginine (Arg) (DE CASTRO; SATO, 2015) (Table 3).

5.2 Antimicrobial activity

Currently, all living organisms are constantly exposed several pathogens, which

can cause damage on the health (ANDERSSON; HUGHES; KUBICEK S., 2016). The survival of such pathogens in the host organisms depends, of an innate mechanism of immune system of the host and by acquired immune responses (molecules endogenous). The endogenous compounds can proportion responses faster and effective means of defense against the pathogen (REDDY; YEDERY; ARANHA, 2004). These compounds called antimicrobial peptide (AMPs) that have primitive immune defense mechanism (BECHINGER, 2004). The antimicrobial action of AMPs is divided in two mechanism classes: membrane-disruptive and non-membrane-disruptive (NAWROT et al., 2013; NICOLAS, 2009). These mechanisms depend on the peptide physicochemical properties and the membrane composition of the pathogen (bacterial Gram negative or Gram positive) (SHAI, 1999; SMITH; DESBOIS; DYRYNDA, 2010).

The membrane-disruption mechanism has been explained through barrel-stave, micellar-aggregate, and carpet models (MALMSTEN, 2014). The barrel-stave model describes how amphipathic peptides re-orient and perpendicularly align to the membrane in a manner in which the hydrophobic side chain of AMP drives into the lipid environment while the polar side chains align to form a transmembrane pore (POWERS; HANCOCK, 2003), this pore act as a channel that allows the escape of cellular components. In the carpet model, peptides self-associate onto the acidic phospholipid-rich regions of lipid bilayers so peptides are absorbed and accumulated in the membrane surface. Exceeding the monomers threshold causes permeation and disintegration of the membrane (PELEGRINI et al., 2011; SMITH; DESBOIS; DYRYNDA, 2010). Equally important, the micelle-aggregate model describes that the peptides reorient and associate with other membrane-spanning micellar; apart that indicate that collapse this aggregates could explain translocation into the cytoplasm and provoke the membrane disruption (CHEUNG; NG; WONG, 2015).

The non-membrane-disruptive mechanism some peptides have the capacity to induce transcriptional changes in the bacteria cytoplasm (ZENG et al., 2013). This mechanism is used by antimicrobial peptides in order to affect bacterial growth. Likewise, antibiotic molecules have the necessity of interacting

with biological membranes which induces changes in the structure of the lipid bilayer. There are five factors involved in this interaction: hydrophobicity, steric-effect, conformation and self-association, net charge, and bilayer insertion (ZENG et al., 2013).

The AMPs are of enormous interest because they are powerful stimulators of the immune system. They also demonstrate beneficial impacts in health due to their inhibitor effect towards microorganisms (MAHLAPUU et al., 2016; SUN et al., 2014; ZHANG; GALLO, 2016). Several studies demonstrate that antimicrobial peptides play other important roles in processes such as angiogenesis, attraction of leukocytes, inflammation, and cell proliferation (PHOENIX; DENNISON; HARRIS, 2012).

The AMPs present common characteristics: high proportion of hydrophobic residues such as Leucine (Leu), Isoleucine (Ile), Valine (Val), Phenylalanine (Phe), and Tryptophan (Trp) (HANEY; HANCOCK, 2014). AMPs can also be classified based on their secondary structure and amino acid sequences as linear α-helical peptides, cyclic peptides, looped peptides and linear peptides. The AMPs has been found in differents sources such as plants, marine-derived, animal-derived and other (FALANGA et al., 2016; GAGNON et al., 2016; SPERSTAD et al., 2011)(Table 3).

5.3 Anti-inflammatory activity

The inflammation performed a physiological role in wound healing and infection tissues (MARCONE; BELTON; FITZGERALD, 2017). An excessive inflammation may cause an uncontrolled production of pro-inflammatory cytokines, eicosanoids derived from arachidonic acid and also oxygen reactive species can be produced (VERNAZA et al., 2012). The inflammation and remodeling of tissue occur in the synthesis and degradation of extracellular matrix, in this process is involving the activation and inhibition of hyaluronidase enzyme (PRADO et al., 2016). The hyaluronidase is an enzyme that has a capacity of hydrolyzes hyaluronic acid; this acid is a viscous polymer and whose function is to ensure that cells remain adhered to one other. By the action of hyaluronidase, the polymer is

transformed into small fragments, significantly reducing its viscosity and facilitating the proliferation of these cells from the tissues, leading to a degradation of the extracellular matrix (ECM) that promotes inflammation. The excessive degradation of the ECM may development several diseases (e.g. arthritis, rheumatism). Due to importance of hyaluronic acid is necessary procured natural inhibitor such as peptides, phenolic compounds and flavonoids for hyaluronidase enzyme (GIRISH et al., 2009; MARCHESAN et al., 2006).

The anti-inflammatory peptides have received much attention due to its potential in the therapeutic treatment for several diseases (cancer, tumor progression, allergy, asthma, autoimmune diseases, and coeliac disease) (VO; RYU; KIM, 2013) (Table 3), with respect their structures and characteristics not yet elucidated.

5.4 Anti-collagenase activity

The aging is a natural process that all people undergo with the time pass. The process of skin aging, like other organs are divided into two categories: (a) Intrinsic skin aging, those occur due to passage of time, genetic influence and the intrinsic factors (telomere shortening), the imbalance between free radicals and hormonal changes. (b) Extrinsic skin aging is cause for exposure to solar radiation and provokes leathery appearance, dark/light pigmentation (CHATTUWATTHANA; OKELLO, 2015; THRING; HILI; NAUGHTON, 2009). These structural alterations occur when the extracellular matrix (ECM) is degraded and cause increase in activity of certain enzymes (e.g. elastase, collagenase), proteolytic breakdown, and breakup of dermal fibers (e. g. collagen, elastin) (NDLOVU et al., 2013). The ECM is the non-cellular component present within all tissues and organs and is composed by proteoglycans (PGs) and fibrous proteins (collagens, elastins, fibronectins and laminins) (FRANTZ; STEWART; VALERIE M. WEAVER, 2010).

The collagenase (metalloproteinase) posse a capacity of cleaving the X-Gly bond of collagen causing the skin aging, due to collagen is responsible for the elasticity and strength of the skin. On the other hand, for detaining the action of this enzyme

is using inhibitors synthetics, that can be caused several secondary effects (eg. colitis, esophagitis), for this reason, is necessary procured natural inhibitors (e.g. peptides, phenolic) (NORZAGARAY V. et al., 2017).

Actuality, only know that anti-collagenase peptides posse a capacity of a block the activity of the enzyme and can present a sequence which most closely resembles that around the cleavage site in native collagen, their structure can compose of hydrophobic residues such as Leucine (Leu), Isoleucine (Ile), Valine (Val), and Phenylalanine (Phe) (AURELI et al., 2008; THRING; HILI; NAUGHTON, 2009) (Table 3).

Table 3- Bioactive peptides and possible bioactivities from protein hydrolysates of the marine-derived.

Source	Amino acid	Bioactivity	Reference
Shrimp	Ile-Phe-Val-Pro-Ala-Phe		(HAI-LUN et al., 2006)
Chlorella vulgaris	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Gln-Phe		(SHEIH; FANG; WU, 2009)
Undaria pinnatifida	Val-Tyr Ile-Tyr Ala-Trp Phe-Tyr Val-Trp Ile-Trp Leu-Trp	Anti-Hypertensive	(SATO et al., 2002)
Chlorella vulgaris	Val-Glu-Cys-Tyr-Gly-Pro-Ans-Arg-Pro-Gln-Phe		(SHEIH; WU; FANG, 2009)
Conger eel	Leu-Gly-Leu-Asn-Gly-Asp-Asp-Val-Asn	Antioxidant	(RANATHUNGA; RAJAPAKSE; KIM, 2006)
Platycephalus fuscus	Met-Gly-Pro-Pro-Gly-Leu-Ala- Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg		(NURDIANI et al., 2016)
American lobster	Ala-Ala-Ala-Leu Ala-Gly-Gly-Val Ala-Ala-Val-Lys-Met.	Antimicrobial	(SILA et al., 2014)
Oyster	CgPep33		(LIU et al., 2008)
Green sea	Cys		(LI et al., 2008)

urchin			
Platycephalus fuscus	Met-Gly-Pro-Pro-Gly-Leu-Ala-Gly-Ala-Pro-Gly-Glu-Ala-Gly-Arg	Anticancer	(NURDIANI et al., 2016)
Tuna dark muscle	Leu-Pro-His-Val-Leu-Thr-Pro-Glu-Ala-Gly-Ala-Thr Pro-Thr-Ala-Glu-Gly-Gly-Val-Tyr-Met-Val-Thr		(HSU; LI-CHAN; JAO, 2011)
Sea hare	Dolastatin		(MADDEN et al., 2000)
Salmon pectoral	Pro-Try-Leu	Anti-inflammatory	(AHN; CHO; JE, 2015)
Salmon	SPHF1		(AHN; JE; CHO, 2012)
Engraulis japonicus	Ser-(Gly)7-Leu-Gly-Ser-(Gly)2-Ser-Ile-Arg Ile-(Glu)2-Leu-(Glu)3-Ile-Glu-Ala-Glu-Arg.	Metal-chelating	(WU et al., 2012)
Hoki frame	Val-Leu-Ser-Gly-Gly-Thr-Thr-Mrt-Tyr-Ala-Ser-Leu-Tyr-Ala-Glu		(JUNG; KIM, 2007)
Echiuroid worm	Gly-Glu-Leu-Tyr-Pro-Glu-Ser-Gly-Pro-Asp-Leu-Phe-Val-His-Phe-Asp-Gly-Pro-Ser-Tyr-Ser-Leu Try-Ala-Asp-Ala-Val-Pro-Arg	Anticoagulant	(JO; JUNG; KIM, 2008)
Nori	Asn-met-Glu-Lys-Gly-Ser-Ser-Ser-Val-Val-Ser-Ser-Arg-Met		(INDUMATHI; MEHTA, 2016)
Hippocampus, (*Syngnathidae*)	SHP-1	Anti-collagenase	(RYU; QIAN; KIM, 2010)

FONT: The author.

CHAPTER 2:

PROMISING BIOLOGICAL ACTIVITIES FROM NEW PEPTIDES OF *Spirulina maxima*.

ABSTRACT

The interest in biological peptides from *Spirulina* (*Arthrospira*) is increasing in the last time at reason of great potential to produce new products for the food, cosmetic and pharmaceutical industry. The aim of this study was the production of bioactive peptides of *Spirulina maxima* through enzymatic hydrolysis. In this work, *Spirulina maxima* proteins were hydrolyzed using single and sequential digestion. These hydrolysates were purified by ultrafiltration (<10kDa) to evaluate peptide concentration and determinate their biological activities. Three peptide fractions were analyzed; the best antioxidant activity was obtained by first hydrolysis (PHA) displayed an for the capture of DPPH radicals with an IC_{50} value of 21.25 µg/ml, against ABTS with an IC_{50} 9.5 µg/ml, a TEAC activity of 465.7 Trolox µM/µg sample, together with a 97.3% inhibition of iron-chelation and IC_{50} 6.99 µg/ml. For the antimicrobial activity maximal inhibitory concentration (IC_{50}) and the minimum bactericidal concentration (MBC) the best was second hydrolysis (PHP) that presented 0.34 mg/ml and 0.63 mg/ml for *Bacillus subtilis;* 0.62 mg/ml and 0.63 mg/ml for *Staphylococcus aureus;* 0.99 mg/ml and 1.25 mg/ml for *Salmonella typhi* and 0.94 mg/ml and 1.25 mg/ml for *Escherichia coli*. While the peptide fraction obtained from the two enzymes (PHS) showed multi biological activity as antioxidant against DPHH with a IC_{50} value of 17.93 µg/ml and against ABTS with IC_{50} 8.6 µg/ml, TEAC of 540.7 Trolox µM/µg sample, anti-inflammatory with inhibition of hyaluronidase of 39% and IC_{50} 0.99 µg/ml, and anti-collagenase with 92.5% inhibition and IC_{50} 32.49 µg/ml. The results indicated that the three peptides possessed diverse activities and could be potential candidates for used in the pharmaceutical, cosmetic and food industry.

Keywords: *Spirulina maxima*, peptide, antioxidant, iron-chelating, antimicrobial, anti-inflammatory, anti-collagenase.

1. Introduction

The importance of novel bioactive compounds significantly increased in the last years. Bioactive peptides (BPs) are specific protein fragments, they have a positive impact on body functions and may influence health (HARNEDY; FITZGERALD, 2012; SHARMA; SINGH; RANA, 2011; SINGH; VIJ; HATI, 2014). BPs can be obtained from diverse raw materials, such as plants, macroalgae, microalgae, seafood, and fungi (HAYES, 2013; KITTS; WEILER, 2003; SHARMA; SINGH; RANA, 2011).

Recently, there has been great interest in the use and evaluation of peptides that show biological activities. The antioxidant peptides are important, because of their protective effect in lessening the severity of diseases; considering that in our body oxidative stress can cause serious damage to proteins, lipids, and DNA by subtracting electrons (NURDIANI et al., 2016; RAHAL et al., 2014). These peptides act by preventing binding of other molecules to oxygen, and by the inhibition of free-radicals action (KANG et al., 2011). On the other hand, the antioxidant peptides can present structures that contain nucleophilic sulfur-contain side chains (Cys and Met) or aromatic side chains with amino acids histidine (His), tyrosine (Tyr) and methionine (Met) which can easily donate hydrogen atoms (HAYES, 2013). Also, the iron-chelating peptides, due to act in the metabolical pathways of autoxidation mechanisms and have the capacity the increase non-heme iron absorption and bioavaility in the body (HELI; MIRTORABI; KARIMIAN, 2011; NGO, 2013; WU et al., 2012). These peptides may present structure that contains methionine (Met), glutamine (Gln), lysine (Lys) and arginine (Arg) (DE CASTRO; SATO, 2015).

The antimicrobial peptides (AMPs) are of enormous interest because of their inhibitory activity against several pathogens and their ability as stimulators of the human immune system. AMPs are known as host defense peptides due to their innate presence in the immune system in animals, insect, plants, and humans with the role of defending against the diversity of bacterial, fungal, viral, and other pathogenic infection (MAHLAPUU et al., 2016; WANG, 2014; ZHANG; GALLO, 2016). Furthermore, it is known that AMPs have the capacity to play other important roles in such processes as angiogenesis, an attraction of leukocytes, inflammation, and inhibition cell proliferation (PHOENIX; DENNISON; HARRIS, 2013). The AMPs present common characteristics: high proportion of hydrophobic residues such as Leucine (Leu), Isoleucine (Ile),

Valine (Val), Phenylalanine (Phe), and Tryptophan (Trp) (HANEY; HANCOCK, 2014).

The same way, the anti-inflammatory peptides have received much attention due to its potential in the therapeutic treatment for several diseases (cancer, aging, allergy, asthma, autoimmune diseases, and coeliac disease)(VO; RYU; KIM, 2013). These peptides act block hyaluronidase enzyme and prevent hydrolysis of hyaluronic acid, helping to regeneration, proliferation, and reparation of tissues. As well, can increase elasticity and decrease the loss of moisture on the skin (PRADO et al., 2016; SULERIA et al., 2016). On the other hand, the anti-collagenase peptides prevent degradation of the extracellular matrix (ECM) by blocking the action of the collagenase (CHATTUWATTHANA; OKELLO, 2015; NDLOVU et al., 2013; THRING; HILI; NAUGHTON, 2009). These peptides can present a sequence which most closely resembles that around the cleavage site in native collagen, their structure can compose of hydrophobic residues such as Leucine (Leu), Isoleucine (Ile), Valine (Val), and Phenylalanine (Phe) (AURELI et al., 2008; THRING; HILI; NAUGHTON, 2009).

The cyanobacteria *Spirulina* has been used by humans as food since ancient times due to its high protein content (43-70%), which can be hydrolyzed into BPs (BILLS; KUNG, 2014; YU et al., 2016). It has been experimentally proven to be effective for the treatment of certain conditions, such as anti-inflammatory, antioxidant, antiviral, anti-bacterial, hypertensive, immunomodulatory, anticancer (JANG; PARK, 2016; OVANDO et al., 2016; SHIH et al., 2009). Furthermore, the pharmaceutical industry has shown a great interest in *Spirulina* for its nutritional and biotechnological properties, as well as its Generally Regarded as Safe (GRAS) status by the Food and Drug Administration (FDA) (OLIVEIRA et al., 2013). Thus, the objective of this study is the generation of bioactive peptides from *Spirulina maxima* through an enzymatic hydrolysis in order to determine the *in vitro* bioactivity of the hydrolysates.

2. Material and methods

A general schema is of the peptides fraction production and a biological activities test is present in Figure 7.

2.1. Material

Spirulina maxima biomass was provided by the Ouro Fino Agribusiness, Ribeirão Preto,São

Paulo, Brazil. All solvents used were of analytical grade. 1,1-diphenyl-2- picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate (Ferrozine), Iron(II) chloride, Disodium ethylenediaminetetraacetate dihydrate (EDTA-Na_2), hyaluronidase from bovine testes (EC 3.2.1.35) and a Collagenase Activity Colorimetric Assay Kit were all purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Proximal composition

Analyses of *S. maxima* biomass were performed in order to determine the proximal composition. Analyses of total protein, ashes, carbohydrate and lipids composition were carried out according to the methods described by the Association of Official Analytical Chemists (HORWITZ, WILLIAM; LATIMER, 2005).

2.3. Protein Extraction from *Spirulina maxima*

Soluble protein extraction was performed as described previously (Wang and Zhang, 2016), with modifications. *S. maxima* powder (100 g) was dissolved in 1 L sodium phosphate buffer (PBS) (0.1 M). The solution was frozen at −20 °C for 4 h and thawed at 37 °C, with 4 freeze–thaw cycles in total. After homogenization (2800 x *g* 30 s, 11000 x *g* 1 min, 2800 x *g* 30 s), the mixture was ultrasonicated under 160 W power for 25 min (every 10s with 13s interval) in an ice bath. Afterwards the lysate solution was centrifuged at 10000 x *g* and 4 ^{o}C for 15 min. The protein content of the supernatant was determined by the Bradford Protein Assay.

2.4. Enzymatic hydrolysates

The protein fraction of *S. maxima* was initially diluted to 3% in citrate phosphate buffer in different pH (0.1 M pH 7 and pH 3) and hydrolyzed with two types of endopeptidases under specific conditions. The conditions under which these two enzymes were worked on in the enzyme process were based on previous studies for peptides production (KIM, 2013; LISBOA et al., 2016; LU et al., 2010; WANG; ZHANG, 2016). The first hydrolysate was prepared using protease 1 in the following conditions, enzyme/substrate (E/S) ratio of 2% w/w, 60 ^{o}C, pH 6.5 and 6 h reaction time. The second hydrolysate was prepared with protease 2, E/S of 4% w/w, 37

^{o}C, pH 4 and 4 h of reaction time. The last hydrolysate was prepared using both enzyme systems, sequentially. The solution was first treated using protease 1 under the above conditions and with a 4 h reaction time; after inactivation at 85 ^{o}C, the solution was then hydrolyzed by protease 2 under the conditions described above and with a 3 h reaction time. The reactions were stopped by heating the solution in a boiling water bath for 10 min. The obtained hydrolysates were centrifuged at 6000 x *g* for 10 min (Figure 8).

2.5. Determination of degree of hydrolysis

The method used to determine the degree of hydrolysis (DH) was performed as described previously (Hoyle and Merritt, 1994). Three hydrolysis systems were evaluated: 1 ml aliquots were inactivated by the addition of 9 ml of 6.25% (w/v) trichloroacetic acid (TCA) solution and left to settle for 10 min. The solution was then centrifuged for 5 min at 3000 x *g* and the precipitate removed. The soluble proteins content was determinate using the Bradford (1976) method. DH was calculated as shown in Eq. 1:

$$\mathrm{DH}(\%) = \frac{(\mathrm{PSti\text{-}PSto})}{\mathrm{Ptotal}} \mathrm{X}100 \qquad \text{(Eq. 1)}$$

Where: PSto, corresponds to the amount of soluble protein in TCA 6.25% w/v before the addition of enzyme; PSti, is the protein soluble after the addition of enzyme and P total is the amount of total protein in the sample.

2.6. Purification of proteins hydrolyses

Peptides obtained from enzymatic hydrolysis were purified by ultrafiltration through a Vivaflow 200 Sartorius (tangential filtration) system. First the hydrolysate was microfiltrated using a 2 µM membrane, after it was ultrafiltrated using a membrane of 10 kDa molecular weight cut off (MWCO). The permeate fraction containing molecules below 10 kDa was collected and stored at – 80 ^{o}C, before lyophilization.

2.7. Peptide's quantification

The lyophilized peptide extract (<10 kDa) of *S. maxima* was solubilized in ultrapure water for obtained at concentration of 1 mg/ml. The content of peptides present in the solution was

determined using the Micro BCA Protein Assay Kit (Thermo fisher).

2.8. SDS-PAGE gel electrophoresis

The lyophilized *S. maxima* peptide isolates of were resolved on a 17% polyacrylamide gel and stained with silver nitrate. The molecular weight marker was Protein MW marker, low range K-880 (3.5-31.0 kDa) (AMRESCO, Fountain Parkway Solon, OH, USA). The GelAnalyzer 2010 software (Lazar et al., 2010) was used to calculate the molecular weights .

2.9. DPPH Radical Scavenging -*in vitro* assay

The capacity of the peptides for sequestering the free radical 2,2-diphenyl-1-picryl-hidrazol (DPPH) was performed as described previously (Yu et al., 2016). For the preparation of the DPPH reagent, 4 mg DPPH (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100 ml 95% methanol. For each peptide extract, concentrations of 2, 5, 10, 25, 50, 100 µg/ml was used for this assay of each peptides extracts. Vitamin C (0.1 mg/ml) was used as a positive control. A 96-well microplate was used to determine the scavenging activity, where 100 µl of the samples or standard were mixed with 100 µl of DPPH reagent, and incubated for 30 min in the dark at room temperature. After this time the absorbance was measured by using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, USA) at 517 nm. The percentage of DPPH radical scavenging was calculated as shown in Eq. 2:

$$\text{DPPH radical scavenging (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] * 100 \qquad \text{(Eq. 2)}$$

Where A_0 was the absorbance control, A_1 was the absorbance of the sample.

2.10. ABTS Radical Scavenging- *in vitro* assay

The 2,2'-azinobis-3-etilbenzothiazoline-6-sulfonic acid (ABTS) Radical Scavenging assay was performed as described previously (Lee et al., 2015). The ABTS reagent was prepared by mixing 5 ml of 7 mM ABTS (Sigma-Aldrich, St. Louis, MO, USA) with 88 µl of 140 mM potassium persulfate, and reacting for 16 h at room temperature in the dark. After this time, the ABTS reagent was diluted to 1:45 with ethanol (99%) until reaching an absorbance of 0.700, which was measured in the spectrophotometer at 734 nm. Trolox (6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, St. Louis, MO, USA) was prepared from a stock solution (1 mM) over the concentration range (200, 100, 50, 25, 10, 5, 2 µM). In the case of peptide extracts and with the positive control (vitamin C), was used with the concentrations (100, 75, 50, 25, 10, 5, 2 µg/ml). For determining the scavenging activity a 96-well microplate was used where 100 µl of the samples or standard were mixed with 100 µl of ABTS reagent, in the dark at room temperature. The absorbance was measured by using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, USA) at 734 nm. The percentage of ABTS radical scavenging was calculated as shown in Eq. 3:

$$\text{ABTS radical scavenging (\%)} = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \qquad \text{(Eq. 3)}$$

Where A_0 was the absorbance control, A_1 was the absorbance of the sample.

2.11. Ferrous ion-chelating activity - *in vitro* assay

The ferrous ion-chelating activity was performed as described previously (Wang et al., 2009). Peptide extract (100 µl) was added in the following concentrations (1.25, 2.5,5,10, 25 µg/ml) and mixed with 135 µl of distilled water and 5 µl of 2 mM $FeCl_2$ in the microplate. The reaction was initiated by the addition of 10 µl of 5 mM ferrozine, and mixed for 10 min at room temperature. After incubation, the absorbance was measured at 562 nm with a PowerWave XS Microplate Spectrophotometer. Distilled water (100 µl) instead of sample was used as the control. For the blank distilled water (10 µl) instead of ferrozine was used. EDTA-Na_2 was used as reference standards. All measurements were performed in triplicate. The ferrous ion-chelating activity was calculated as shown in Eq. 4:

$$\text{Ferrous ion-chelating activity (\%)} = \frac{[(A_0 - (A_1 - A_2)]}{A_0} \times 100 \qquad \text{(Eq. 4)}$$

Where A_0 was the absorbance control, A_1 was the absorbance of the sample or standard and A_2 was the absorbance of the blank.

2.12 Antimicrobial activity - *in vitro* assay

Broth microdilution is a method used for determining the minimal inhibitory concentration

(MIC) of a substance. It is considered as the best methodology for examination of susceptibility or resistance of bacteria to antimicrobials (ELSHIKH et al., 2016).

2.12.1 Minimum Inhibitory Concentration (MIC) Determination

This assay determines the antimicrobial potential of the peptide extract. A 96-well microplate assay was used to determine the MIC: 80 µl of Mueller Hinton Broth (MHB) was put in wells, mixed with 100 µl of peptides extracts (0.13, 0.63, 1.25, 6.25 mg/ml) and inoculated with 20 µl of bacterial suspension (1.0x 10^7 UFC/ml) (*Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), *Salmonella typhi* (ATCC 14028), *Escherichia coli* (ATCC 35218)). Chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) was used as the positive control, and only culture medium plus peptide extract for the negative control. The microplate was sealed tape (Corning) and incubated at 37 ^{o}C for 24 h. After incubation, the absorbance was measured at 600 nm. The percentage of growth inhibition was calculated as shown in Eq. 5:

$$\text{Grow inhibition (\%)} = \left[1 - \left(\frac{\text{Ac}}{\text{Ao}}\right)\right] * 100 \qquad \text{(Eq. 5)}$$

Where Ac was the absorbance of the sample, Ao was the absorbance of the control. Finally, the microplate was colored with 30 µl of resazurin indicator solution (0.1%), and incubated for 2 h.

2.12.2. Minimum Bactericidal Concentration (MBC) Determination

After MIC determination of the peptide extracts, an aliquot of 5 µl from all microplate assay wells was seeded in Mueller Hinton Agar (MHA) plates. The plates were then further incubated at 37 °C for 24h. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the MHA plates.

2.13 Anti-inflammatory activity - *in vitro* assay

The anti-inflammatory activity was evaluated by the inhibition of the enzyme hyaluronidase (Type IV), as described previously (Prado et al., 2016), with slight modification. Briefly, the three lyophilized peptide isolates (<10 kDa) of *S. maxima* were used in different concentrations (3.3, 10, 33,100, 333 µg/ml). The propolis commercial extract (Bitmel, São José do Rio Preto –

SP) was used as positive control.

To begin the analysis, 100 µl of peptide extract or the positive control was added to 500 µl of the potassium salt of hyaluronic acid (Sigma-Aldrich, St. Louis, MO, USA), and incubated for 5 min at 37 ^{o}C. Then, 350 units of the enzyme hyaluronidase type IV-S were added (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 ^{o}C for 40 min. The reaction was inactivated by adding 10 ml of sodium hydroxide solution (4 N) and 100 µl of potassium tetraborate at 0.8 M, and incubated for a further 3 min at 100 ^{o}C. Afterwards, 3 ml of 4-dimethylaminobenzaldehyde (DMAB) was added to the tubes, mixed and transferred to a water bath for 20 min at 37 ^{o}C. Finally, the absorbance was measured in the spectrophotometer at 585 nm. The percentage of inhibition was calculated as shown in Eq. 6:

$$\text{Hyaluronidase inhibition activity (\%)} = \frac{\text{Am}*100}{\text{Ac}} \qquad \text{(Eq. 6)}$$

Where: Am is the absorbance of sample after interaction with the enzyme hyaluronidase, Ac corresponds to the absorbance of the control.

2.14. Collagenase inhibition - *in vitro* assay

This assay was performed according to the descriptive instructions supplied by the Collagenase Activity Colorimetric Assay Kit. The principles of this assay are based on the enzyme-substrate interaction between collagenase from *Clostridium histolyticum* and the synthetic N-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA). For this assay each peptide extract (10 µl) was added in the following concentrations (10, 25, 50, 75 µg/ml). Negative controls were performed with water and positive control was performed 10-Phenanthroline. Absorbance at 345 nm was measured. All measurements were performed in triplicate. The collagenase inhibition activity was calculated as shown in Eq. 7:

$$\text{Collagenase inhibition activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100 \qquad \text{(Eq. 7)}$$

Where A_0 was the absorbance control, A_1 was the absorbance of the sample.

2.15. Statistical analysis

Statistical analysis was performed using one-way and two-way ANOVA test on Graphpad Prism6 305 software.

3. Results

3.1. Characterization of peptide extracts

The composition of *S. maxima* was as follows: 57.04±0.031% (w/w) proteins, 11.2±0.36% (w/w) lipids, 10.67±0.12% (w/w) carbohydrates and 5.65±0.276% (w/w) ash. On the other hand, the protein isolate had a concentration of 2983±0.06 mg/ml, a recovery of approximately 80% of the total proteins by using water extraction, freeze–thawing, homogenization and ultrasonication. Single-step and two-step hydrolysis of the extracted proteins with proteases were performed under controlled conditions. The characterization of peptides extracts was shown Table 4. Peptide profiles in PHS, PHA and PHP were realized using electrophoretic analysis and presented a large number of bands with very different molecular masses, are shown in Figure 3.

Table 4- Characterization of protein hydrolysis from *Spirulina maxima*.

Sample	**Degree hydrolysis (%)**	**concentration (µg/ml)**	**Molecular mass (kDa)**
PHA	49.5	2395.6	>3.5
PHP	43	2831.5	2.6-10.6
PHS	43.3	2651.1	3.6-10.9

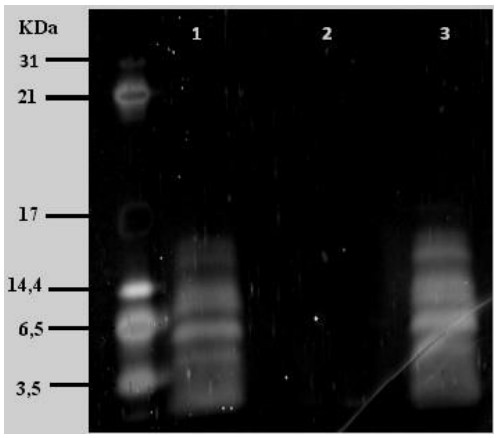

Figure 3-Electrophoretic analysis of peptides. The sample of peptides extracts with concentration of 40 µg/ml of PHS (1), PHA (2) and PHP (3) is submitted (SDS-PAGE) 17%.

3.2. Free-radical scavenging activity-*in vitro*

The samples PHA, PHP and PHS were evaluated for their capacity to free-radicals scavenging and known antioxidant capacity in the DPPH and $ABTS^{+}$ assays. For each peptide, six concentrations from 100 to 2.5 µg/ml were used. All samples exhibited antioxidant activity (Figure 4)**.** At a concentration of 100 µg peptide/ml extract, PHA had an antioxidant activity in the DPPH assay of 78±0.44%, PHS exhibited a value of 78±0.21% and PHP an activity of 77 ±0.71%. Meanwhile in the $ABTS^{+}$ assay, with the same concentration of peptide, PHA exhibited an activity of 98.4±0.71%, PHP exhibited 97.3±0.71% and PHS displayed an activity of 96.1 ±0.9% The IC_{50} and TEAC of these assays are shown in Table 5.

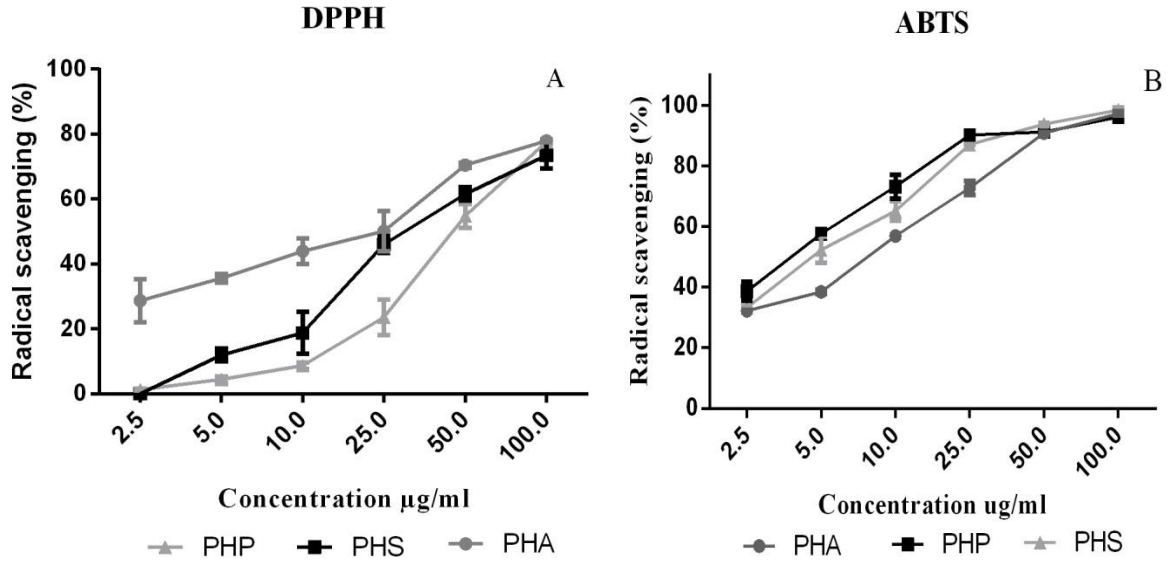

Figure 4-(A) Percentage of radical scavenging using DPPH assay. (B) Percentage of radical scavenging using ABTS assay. The PHP, PHA and PHS have shown six concentrations (2.5, 5, 10, 25, 50, 100 µg/ml).

3.3 Iron-chelating activity-*in vitro*

The peptide extracts (PHP, PHS, and PHA) were assayed for their Fe^{2+}chelating activity at different concentrations and this activity was compared with the chelating activity of the synthetic metal chelator EDTA. PHA at 25 µg/ml showed a percentage of chelating activity of 97.3 ± 0.4%. While PHP and PHS at the same concentration showed values of less than 30%. EDTA-NA_2 presented a chelating activity 61±1.3% at 25 µg/ml. Furthermore, with respected to their IC_{50}, PHA showed a lower value compared with the others samples and the commercial chelator EDTA-NA_2 (see Table 5).

Table 5-Antioxidant and iron-chelating activities of extracts peptides from *Spirulina maxima.*

	Scavenging of DPPH radical scavenging	**Scavenging of $ABTS^+$ radical scavenging**		**Fe^{2*} Chelating activity [c]**
Samples	IC_{50} (µg/ml)	IC_{50} (µg/ml)	TEAC (Trolox µM/µg sample)	IC_{50} (µg/ml)
PHA	21.25 [a]	9.5 [b]	465.7 [b]	6.98 [c]
PHP	34.63 [a]	15.63 [b]	282.2 [b]	724.7 [c]

PHS	17.93 [a]	8.6 [b]	540.7 [b]	492.2 [c]
Vitamin C	11.97	6.1	Nd	nd
Trolox	nd*	44.11	Nd	nd
EDTA-NA_2	Nd	nd	Nd	14.31

[a] Analysis of variance showed p=0.006 (PHA *vs* PHP; PHA *vs* PHS; PHP *vs* PHS).

[b] Analysis of variance showed p=0.0113 (PHA *vs* PHP; PHA *vs* PHS; PHP *vs* PHS).

[c] Analysis of variance showed p=<0.0001 (PHA *vs* PHP; PHA *vs* PHS; PHP *vs* PHS)

*nd=not determinate

3.4 Antimicrobial activity-*in vitro*

The three protein hydrolyzes were evaluated MIC, MBC and percentage of growth inhibition in presence of human pathogenic bacteria (*E. coli*, *S. typhi*, *B. subtilis* and *S. aureus*). For each peptides extract five concentrations were used, from 6.25 to 0.13 mg/ml. The PHA, PHP and PHS values of MIC, MBC and IC_{50} are shown in Table 6.

Table 6- Antimicrobial activity of peptides from *Spirulina maxima* determined by MIC, MBC and IC_{50}.

Sample	**PHA**			**PHP**			**PHS**		
Bacteria	IC_{50} (µg/ml)	MIC (mg/ml)	MBC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	IC_{50} (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
B. subtilis	2.17	6.25	>6.25	0.34	0.63	0.63	1.25	1.25	6.25
S. aureus	1.62	6.25	>6.25	0.62	0.63	0.63	0.88	1.25	>6.25
S. typhi	7.15	>6.25	>6.25	0.99	1.25	1.25	1.22	1.25	6.25
E. coli	11.89	>6.25	>6.25	0.94	1.25	1.25	0.79	1.25	6.25

Analysis of variance showed p = <0.0001 (significant).

MIC= minimum inhibitory concentration

MBC= minimum bactericidal concentration

3.5 Anti-inflammatory activity-*in vitro*

For anti-inflammatory activity, the inhibition by PHP, PHA and PHS of hyaluronidase Type IV was evaluated. Six concentrations from 3.3 to 333 µg/ml of each peptide extract were evaluated and the results are shown in Figure 5. PHP showed 30.4± 0.15% at 333 µg/ml, PHA 32.3±1.1% and PHS 38.8±1.1% at the same concentration. The three peptide isolates showed anti-inflammatory activity. The PHS showed IC_{50} 0.92 mg/ml, PHA presented IC_{50} 1.63 mg/ml,

and PHP showed IC_{50} 1.66 mg/ml and control + showed IC_{50} 23.61 mg/ml.

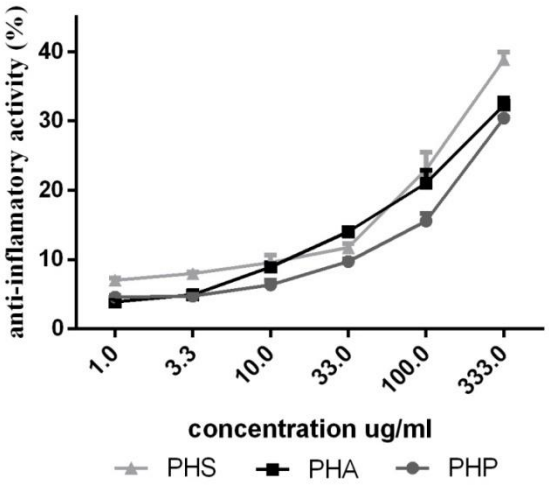

Figure 5-Percentage of inhibition of hyaluronidase enzyme. The PHP, PHA, and PHS have shown six concentrations (1.0, 3.3, 10, 33, 100, 333 µg/ml) (p=0.009).

3.6 Anti-collagenase activity-*in vitro*

Collagen, the major component of the skin, is degraded by the enzyme collagenase. The peptide extracts (PHP, PHS, PHA) at different concentrations were assayed for their ability to inhibit collagenase and this activity was compared with the synthetic inhibitor 10-Phenanthroline. PHS at 75 µg/ml showed an inhibition activity of 92.5 ± 0.5% and an IC_{50} 32.5 µg/ml, while PHP and PHA showed values of <70% at the same concentration and IC_{50} values of 43.9 and 96.7 µg/ml. The 10-Phenanthroline presented an inhibition activity of 57.13±1.9% at 75 µg/ml. Results are shown in Figure 6.

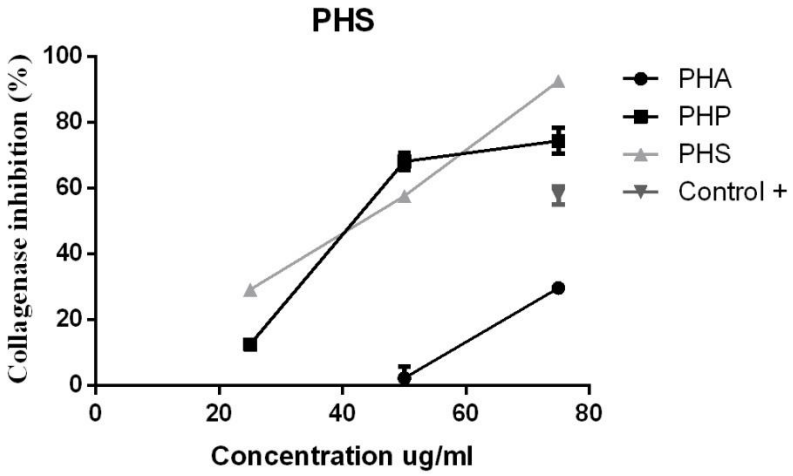

Figure 6-Percentage of inhibition of collagenase enzyme. The PHP, PHA, and PHS have shown four concentrations (10, 20, 50, 75 µg/ml) (p=<0.0001).

4. Discussion

Currently, bioactive peptides have received plenty of attention due to their ability to play a significant role in regulation and metabolic modulation, which suggest potential use as nutraceutical and functional food for human health promotion and reducing the risk of disease (HARNEDY; FITZGERALD, 2012; MOHANTY et al., 2016; SHARMA; SINGH; RANA, 2011). BPs have previously been obtained from various raw materials that contain high protein concentration through enzymatic hydrolyzes. In this current study, for the generation of *Spirulina* BPs, the proteins were subjected to an individual hydrolysis process or sequential. The biomass of the *S. maxima* used in this work contained 57.04±0.031% (w/w) proteins. This is within the range (43-70% w/w) of proteins previously described for *Spirulina* (BILLS; KUNG, 2014; EPPINK; BARBOSA; WIJFFELS, 2012; SARAVANAMUTHU, 2010). The single or combined hydrolysis process here employed resulted in three extracts with values in the range of 43-50% degree of hydrolysis, thus generating a large population of peptides. According to previous research, these enzymes have been used for the production of peptides with antioxidant, anticancer, anti-microbial and ACE-inhibitory, among other activities (KANG et al., 2011; QU et al., 2010; SHEIH et al., 2010; THÉOLIER et al., 2013). The PHA showed only one band, the PHP resulted in the formation of four bands and the PHS showed four bands (Table 4). According to the literature peptides of less than 4 kDa are generally generated, peptides as large

as 12 kDa could also be identified (MAIER et al., 2013; SILVA et al., 2003; SLOMIANY; SLOMIANY, 1993). The total antioxidant potential of the three peptides extracts (PHA, PHP, and PHS) was studied using two commonly used methods. The first one was to measure their ability to scavenge free radicals, where the ABTS and DPPH assay was used. The PHA showed the highest percentage of ABTS radical scavenging with value of 98.4±0.71% at 0.1 mg/ml, while PHS showed value of scavenging of 96.1±0.9% at 0.1 mg/ml and lowest values of IC_{50} and a TEAC of 5407*10$^{\wedge 2}$ Trolox mM/g sample. The *S. platensis* hydrolysate showed antioxidant activity 85.21 ± 1.59% at 10 mg/ml (YU et al., 2016). Lisboa et al. (2016) reported an antioxidant activity of 73.25±0.34% at 2.5 mg/ml of and a TEAC of 0.248 Trolox mM /g sample, using peptides of *Spirulina.* Valenzuela et al. (2017), reported the hydrolysis of three species of microalgae, *D. tertiolecta, T. suecica* and *Nannochloropsis* sp. which gave a TEAC value of 437.01±1.34 Trolox µM /g protein hydrolysate, 696.99±1.82 Trolox µM /g protein hydrolysate, 519.44±4.46 Trolox µM /g protein hydrolysate, respectively. The second method here used was iron-chelating, where the only extract that exhibited good activity was PHA with an inhibition rate of 97.3% at 0.075 mg/ml and an IC_{50} 0.007 mg/ml. On the other hand, comparing with a known chelating agent (EDTA-NA_2), PHA showed a higher percentage of iron chelation. Kim et al. (2014), reported that the peptide Thr-Asp-Pro-Ile(Leu)-Ala-Ala-Cys-Ile(Leu) from *Spirulina* sp. gave a value of 80% iron-chelating activity. Wu et al. (2012), reported that hydrolysis of Anchovy resulted in values of IC_{50} 0.048 and 0.086 mg/ml. Comparing our sample (PHS and PHA) with those previously reported in the literature, we observe that our process obtained a better radical scavenging and chelating activity, suggesting that the peptides contain cysteine (Cys) and methionine (Met), glutamine (Glu), lysine (Lys) and arginine (Arg) or aromatic side chains with amino acids histidine (His) and tyrosine (Tyr). Furthermore, based on these results, it can be said that PHS and PHA have the ability to scavenging of free radicals, and prevent oxidative damage to proteins, lipids, and DNA (NURDIANI et al., 2016). In addition, the chelating action of the PHA extract could be used as an agent to preserve foods with high lipid content and as a catalysts for metal ions to reduce cell damage (Castilla et al., 2012). But, these iron-chelating peptides not only act in the metabolical pathways of autoxidation mechanisms, but also have the capacity to promote the absorption and bioavailability of non-heme iron (WU et al., 2012).

The antimicrobial activity against four pathogenic bacteria (*E. coli, S. typhi, B. subtilis* and *S.*

aureus) was evaluated. This activity is important because these pathogens mutating and present resistance to existing drug, for this reason is necessary to search for alternatives (ALLEN et al., 2014). In our study, we have demonstrated that PHP was the most effective growth inhibitor, and displayed better MIC and IC_{50} values against *B. subtilis, S. aureus and S. typhi*. Additionally, this peptide extract is unique in that it showed bactericidal action against all four pathogenic bacteria used in this assay. PHS was most efficient against *E. coli*. The PHP showed activity against Gram positive and Gram negative Bacterial, confirmed this sample have antibacterial activity and can be used as antibiotic. The AMPs can kill Gram-positive and Gram-negative bacteria but also have the capacity to simultaneously neutralize released pathogenic factors (PINI et al., 2010). *Spirulina* is renowned by have antibacterial activity through the production of phycocyanins and carotenoids, while the existence of antibacterial peptides is rarely reported (OZDEMIR et al., 2004). Sun et al. (SUN et al., 2016b), showed an antimicrobial activity against *E. coli* and *S. aureus* with a peptide from *S. platensis*, with MIC values of 8 and 16 mg/ml, respectively. Comparing, our PHP extract to that reported in the literature PHP exhibits a better antimicrobial activity against *E coli* and *S. aureus*.

Anti-inflammatory activity was evaluated as a percentage of inhibition of hyaluronidase due to that this enzyme is involved in degradation of the extracellular skin matrix and the vascular system. Therefore, it may be expected that hyaluronidase is responsible for the processes causing inflammatory, aging and allergic reactions (GIRISH et al., 2009; KOLAYLI et al., 2016; PRADO et al., 2016). PHS showed an IC_{50} of 0.92 mg/ml while the positive control showed an IC_{50} of 23.61 mg/ml. These results suggest that the peptides generated by the sequential action of both proteases has a higher anti-inflammatory activity at lower doses than for example propolis ethanol extract, in addition to increasing tissue regeneration, proliferation, and repair by inhibiting hyaluronic acid hydrolysis. Valenzuela et al. (2017) reported that *D. tertiolecta* exhibited IC_{50} 5.542 mg/ml and *T. suecica* showedIC_{50} 5.907 mg/ml. Here we show that PHS is a potent anti-inflammatory agent when compared with similar compounds reported in the literature. Furthermore, the capacity of these peptide extracts to inhibit the enzyme collagenase, which causes alterations in the extracellular matrix, was also evaluated. PHS also displayed the best inhibition value (92%) of collagenase, especially compared to the positive control (10-Phenanthroline) that resulted only in a 57% inhibition, and would indicate that PHS possesses the capacity to prevent the cutting of collagen, delaying the process of pre-collagen fibers

formation and the subsequent aging process. It must also be considered that hyaluronidase and collagenase enzyme can act together in the aging process by damaging the extracellular matrix, causing loss of strength, flexibility, elasticity and moisture loss in the skin (CHATTUWATTHANA; OKELLO, 2015; NDLOVU et al., 2013; THRING; HILI; NAUGHTON, 2009). Making such BPs through the combined hydrolysis with both proteases provides a potential alternative for anti-aging treatment. To our knowledge no existing reports exist of peptides from *Spirulina* displaying anti-aging activity.

5. Conclusion

The present study demonstrates that the three peptides fractions (PHA, PHP, and PHS) obtained by single or sequential hydrolysis from *Spirulina maxima* proteins, exhibited noticeable biological activities in different in vitro test models. According to the results, PHA could be used as a potential antioxidant and chelating agent. PHP could be used as an antimicrobial agent, above all, against Staphylococcus and Salmonella. PHS showed antioxidant, anti-hyaluronidase and anti-collagenase activity, by presenting these activities possesses a high potential for aging treatment and as an anti-inflammatory agent. The peptides fractions obtained from *S. maxima* showed great potential to be used in a large variety of products of pharmaceutical, cosmetic and food industries.

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CONCLUSION AND PERSPECTIVE

In the present study, we demonstrate that the peptides extracts obtained by singly or in sequence hydrolysis from proteins of *Spirulina maxima* exhibited several biological activities in different test models in vitro. The high hydrolysate yields, peptide recoveries, and the gel electrophoretic profiles of the hydrolysates indicated that the enzymatic processes employed were efficient in hydrolyzing proteins of *S. maxima* into low molecular weight peptides.

The PHA showed iron-chelating, and antioxidant. Meanwhile, the PHP showed antimicrobial activity. Furthermore, the PHA presented better antioxidant activity, anti-inflammatory, and anti-aging potential. In summary, the peptides extracts could be potential candidates for use as bioactive ingredients in functional foods, nutraceuticals, cosmetics and pharmaceuticals industry.

Further studies should be conducted such as identify the peptide with better biological activity, chemical synthesis of peptides, Analysis of the stability of peptide and test the peptides in-vivo models.

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SUPPLEMENTARY MATERIAL

Figure 7- General schema of peptides fractions production and their biological activities tests.

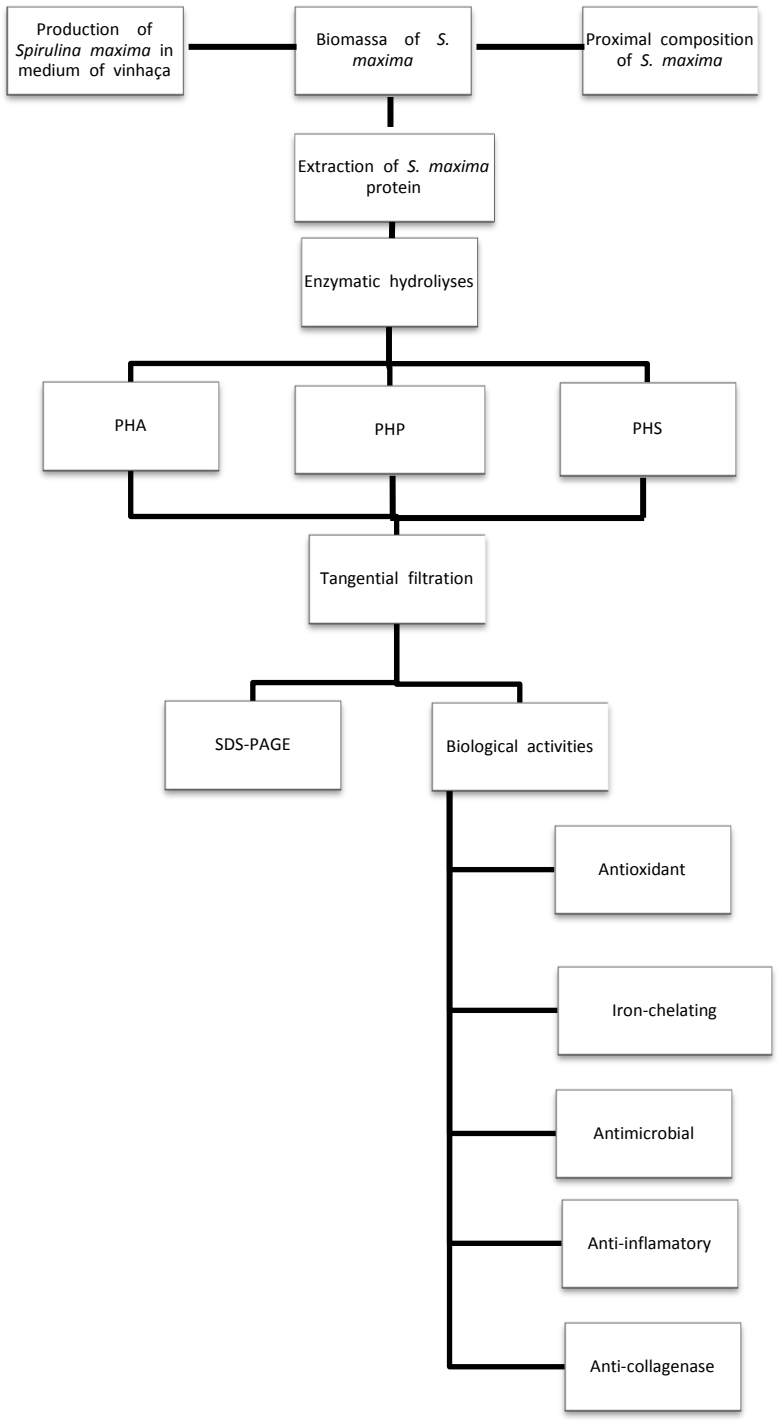

Figure 8-Schema of enzymatic hydrolysis process.