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Chemical characterization and bioactivities of sericin extracted from silkworm cocoons from two regions of Portugal

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Preface

This dissertation reflects the work developed as a student in the Master's degree in Biotechnological Engineering, under the scope of the project "Sericin: a waste product from the silk industry with biomedical potential (PTDC/BTA-BTA/0696/2020)."

This dissertation is organized based on the publications produced as a result of the work carried out. In the introduction is presented the review manuscript "Silk Sericin: A Promising Sustainable Biomaterial for Biomedical and Pharmaceutical Applications." published in the journal Polymers, in 2022. This article outlines the influence of the extraction method on sericin biosynthesis and physicochemical profile. Moreover, this work covers the prospective uses of sericin for biomedical and pharmaceutical applications, focusing on the development of smart drug delivery systems and sericin-based products for tissue engineering. This work allows to establish the best conditions of extraction process and biochemical characterization to evaluate the bioactivities of sericin obtained from different sources.

Afterwards, are presented the goals of this work. Next, in the third section are described the materials and methodology used in the characterization of sericin samples obtained from Bragança, Castelo Branco, Sigma-Aldrich and FUJIFILM Wako Chemicals. After the results and discussion section, the conclusions and the future are presented.

Author's declaration

Under the terms of the "Decreto-lei nº 216/92, de 13 de Outubro", is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.

Under the terms of the "Decreto-lei nº 216/92, de 13 de Outubro", is hereby declared that the following original articles/communications were prepared in the scope of this dissertation.

Scientific publications

Andreia S. Silva, Elisabete C. Costa, Sara Reis, Carina Spencer, Ricardo C. Calhelha, Sónia P. Miguel, Maximiano P. Ribeiro, Lillian Barros, Josiana A. Vaz and Paula Coutinho (2022). Silk Sericin: A Promising Sustainable Biomaterial for Biomedical and Pharmaceutical Applications. Polymers, 14(22), 4931. https://doi.org/10.3390/polym14224931

Oral communication

Sara Reis, Paula Coutinho, Josiana A. Vaz. Caracterização química e das bioatividades de sericina extraída de casulos de bicho-da-seda de duas regiões de Portugal. VII Encontro de Jovens Investigadores, 24 novembro 2022, Bragança.

Abstract

Along the years, sericin has been undervalued and discarded as waste from the textile silk industry. However, recent studies have shown that sericin has great potential for biomedical applications. The potential for medicinal applications depends on its physicochemical properties and molecular heterogeneity. In addition, the characteristics of sericin are influenced by its extraction method, its origin and the variety of cocoon. This work aimed to characterize the biochemical and bioactivities of sericin from Bragança and Castelo Branco. Sericin was extracted using the autoclave method, and its physicochemical properties were then characterized by analyzing its amino acid content by HPLC. In addition, its potential for antioxidant by TBARS and CAA assays, anti-inflammatory by NO inhibition, antimicrobial by microdilution method, anti-proliferative by SBR assay, and anticoagulant activities by APTT method was evaluated. In its pure state, sericin did not have a high content of free amino acids, with tyrosine being identified as the most abundant. On the other hand, when hydrolyzed sericin showed a higher content of amino acids and serine was the most abundant. In terms of bioactivities, the sericin tested did not show antioxidant or anti-inflammatory potential in the tests carried out. Despite this, it showed anti-proliferative activity in contact with human tumor cell lines and in non-tumor cell lines at a minimum concentration of 0.52 and 0.32 mg /mL, respectively. As far as antimicrobial activity is concerned, sericin proved capable of inhibiting the growth of the bacteria and fungi tested at concentrations between 5 and 10 mg/mL. Finally, sericin was also shown to be able to prolong the coagulation time in adult mice plasma, presenting anticoagulant potential. The sericin from Bragança and Castelo Branco, collected in 2019, did not differ greatly, differences in amino acid composition were identified. In addition, the sericin collected in Bragança in 2021 and 2022, S3 and S4, respectively, showed differences compared to the other samples and showed the best antiproliferative, antibacterial and anticoagulant potential. Additionally, there are also differences between extracted and commercial samples.

Keywords: silk protein; amino acids; bioactive compounds; natural products; silk waste.

Resumo

Ao longo do tempo, a sericina foi desvalorizada e descartada como resíduo da indústria têxtil da seda, no entanto, estudos recentes comprovam o grande potencial da sericina em aplicações biomédicas. O potencial para aplicações medicinais depende das suas propriedades físico-químicas e heterogeneidade molecular. Além disso, as características da sericina são influenciadas pelo seu método de extração, a sua origem e a variedade do casulo. Este trabalho teve como objetivo a caracterização bioquímica e bioactiva da sericina com origem em Bragança e Castelo Branco. A sericina foi extraída através do método da autoclave, e, posteriormente as propriedades físico-químicas foram caracterizadas através da análise do teor de aminoácidos por HPLC. Além disso, foi avaliado o seu potencial para as atividades antioxidante pelos ensaios TBARS e CAA, anti-inflamatória pela inibição de NO, antimicrobiana pelo método da microdiluição, anti proliferativa pelo método SRB, e, anticoagulante pelo método APTT. No seu estado puro, a sericina não apresentou um grande teor de aminoácidos livres, tendo sido identificado como mais abundante a tirosina. Por outro lado, quando hidrolisada, a sericina apresentou um teor mais elevado, sendo a serina a mais abundante. Relativamente às bioatividades, a sericina testada não apresentou potencial antioxidante nem anti-inflamatória nos ensaios realizados. Apesar disso, apresentou atividade anti proliferativa em contacto com linhas celulares tumorais humanas e em linhas celulares não tumorais a uma concentração mínima de 0.52 e 0.32 mg /mL, respectivamente. Relativamente à atividade antimicrobiana, a sericina mostrou ser capaz de inibir o crescimento das bactérias e fungos testados para concentrações compreendidas entre 5 e 10 mg/ mL. A sericina também mostrou ser capaz de prolongar o tempo de coagulação em plasma de ratos adultos, apresentando potencial anticoagulante. Além disso, as sericinas de Bragança e Castelo Branco, de 2019, não diferiram muito, contudo foram identificadas diferenças na sua composição de aminoácidos. Por outro lado, a sericina recolhida em Bragança em 2021 e 2022, S3 e S4, respetivamente, apresentou diferenças em relação às outras amostras e apresentou o melhor potencial antiproliferativo, antibacteriano e anticoagulante. Adicionalmente, existem também diferenças entre as amostras extraídas e as comerciais.

Palavras-chave: proteína de seda; aminoácidos; compostos bioativos; produtos naturais; resíduos de seda.

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List of abbreviations

$ABTS^{\cdot+}$ – 2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid)

AGS – epithelial cells from the stomach tissue with gastric adenocarcinoma

Ala – alanine

APTT – activated partial thromboplastin clotting time

Arg – arginine

Asn – asparagine

Asp – aspartic acid

CaCo-2– epithelial cells isolated from colon tissue derived from a male with colorectal adenocarcinoma

CFU – colony forming units

Cys – cysteine

DCFH – 2',7'-dichlorohydrofluorescein

DMSO – dimethyl sulfoxide

DPPH – 2,2-difenil-1-picrylhydrazyl

ECACC – European collection of authenticated cell cultures

FBS – fetal bovine serum

FHC – normal colonic mucosal cells

FRAP – ferric-reducing antioxidant power

GI_{50} – inhibit cell growth in 50%

Gln – glutamine

Glu – glutamic acid

Gly – glycine

HeLa – epithelial cell from the cervix with adenocarcinoma

His – histidine

HPLC-FLD – high performance liquid chromatography-FLD

Hyp – hydroxyproline

IC_{50} – concentration responsible for 50% inhibition of activity

Ile – isoleucine

INT – *p*-iodonitrotetrazolium chloride

Leu – leucine

LOD – limits of detection

LOQ – limits of quantification

LPS – liposaccharide solution

Lys – lysine

MBC – minimum bactericidal concentration

MCF-7 – epithelial cells from the breasts with ductal carcinoma

MDA – malondialdehyde

MEB – malt extract broth

Met – methionine

MIC – minimum inhibitory concentration

NCI-H460 – epithelial cells from the pleural fluid with large cell lung cancer

NO – nitric oxide

NPs – nanoparticles

Phe – phenylalanine

Pro – proline

RAW 264.7 – macrophage cell line, established from a tumor in a male mouse induced with the Abelson murine leukemia virus

ROS – reactive oxygen species

RT– retention times

Ser – serine

SFB – fetal bovine serum

SRB – sulforrodamine B

SW480 – human colorectal cancer cells

Tau – taurine

TBA – thiobarbituric acid

TBARS – thiobarbituric acid reactive substances

TCA – trichloroacetic acid

Thr – threonine

Tris – tris(hydroxymethyl)aminomethane

Tris-HCl – tris-(hidroximetil)-aminometano)-HCl

Trp – tryptophan

TSB – tryptic Soy Broth

Tyr – tyrosine

Val – valine

Vero – epithelial cells from kidney tissue derived from a normal, adult African green monkey

1. Introduction

polymers

MDPI

Review

Silk Sericin: A Promising Sustainable Biomaterial for Biomedical and Pharmaceutical Applications

Andreia S. Silva [1,2], Elisabete C. Costa [3], Sara Reis [1,2], Carina Spencer [3], Ricardo C. Calhelha [1,2,*], Sónia P. Miguel [3,4], Maximiano P. Ribeiro [3,4], Lillian Barros [1,2], Josiana A. Vaz [1,2] and Paula Coutinho [3,4,*]

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Abstract: Silk is a natural composite fiber composed mainly of hydrophobic fibroin and hydrophilic sericin, produced by the silkworm *Bombyx mori*. In the textile industry, the cocoons of *B. mori* are processed into silk fabric, where the sericin is substantially removed and usually discarded in wastewater. This wastewater pollutes the environment and water sources. However, sericin has been recognized as a potential biomaterial due to its biocompatibility, immunocompatibility, biodegradability, anti-inflammatory, antibacterial, antioxidant and photoprotective properties. Moreover, sericin can produce hydrogels, films, sponges, foams, dressings, particles, fibers, etc., for various biomedical and pharmaceutical applications (e.g., tissue engineering, wound healing, drug delivery, cosmetics). Given the severe environmental pollution caused by the disposal of sericin and its beneficial properties, there has been growing interest in upcycling this biomaterial, which could have a strong and positive economic, social and environmental impact.

Keywords: *Bombyx mori*; silk sericin; biomaterials; biomedical and pharmaceutical applications; tissue engineering; drug delivery systems

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1. Introduction

Silk is a natural composite fiber produced by the silkworm *Bombyx mori* (*B. mori*) to assemble the cocoon that provides the ideal conditions for the larvae to metamorphose into adults. In 1865, the chemist Emil Cramer reported that the two main components of silk are the hydrophobic fibroin and the hydrophilic sericin [1]. The sericin binds and coats the two fibroin filaments in the raw silk. Although it is known that the textile industry has been using silk for more than 5000 years [2], there is evidence that humans were using silk as early as 8500 years ago [3]. In the textile industry, sericin is removed from the raw silk (degumming process), rendering a much finer silk fiber with a better luster and texture, which is used to make yarns and fabrics [4]. Global silk production in 2018–2019 was 192,692 metric tons [5], with China and India being the main producers [6]. Since sericin is the second largest component of raw silk (after fibroin), it is estimated that out of 400,000 tons of dry cocoons produced worldwide, 50,000 tons of sericin are (usually) discarded in the effluent [7], causing environmental problems [8,9]. The deposited organic load in aqueous effluents with chemical oxygen demand can deplete oxygen in water systems, leading to eutrophication and thus threatening aquatic life [8–10].

Sericin, removed during the degumming of silk, is considered waste or by-product despite its biological properties such as biocompatibility, immunocompatibility, biodegradability, anti-inflammatory, antibacterial, antioxidant and photoprotective, among others

(as previously reviewed [7,11–17] and represented in Figure 1). This protein has been recognized as a potential sustainable biomaterial for various biomedical and pharmaceutical applications [7,11–23].

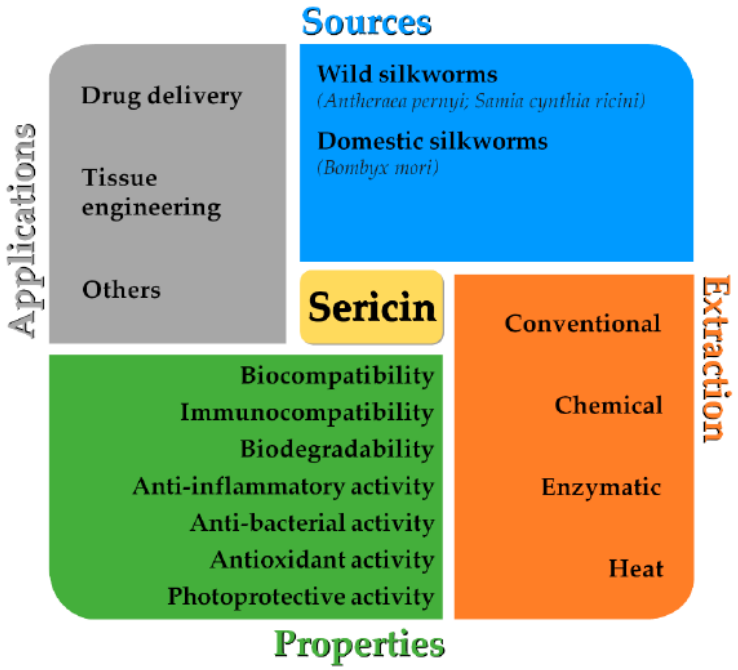

Figure 1. Schematic diagram of sericin sources, extraction methods, properties, and biomedical and pharmaceutical applications.

Indeed, sericin is a highly hydrophilic molecule consisting of polar hydroxyl, carboxyl, and several amino groups [12]. The organic composition, solubility and structural organization of these polar chemical groups are responsible for the biological properties but also allow the formation of blends with other polymers through crosslinking, copolymerization or blending, improving the mechanical resistance of sericin-based biomaterials [24]. When sericin is crosslinked or combined with other polymers, it can be incorporated into hydrogels, films, sponges, particles and fibers with particular properties relevant to biomedical applications [25–27], such as tissue engineering [14,28], wound dressings [29] and drug delivery systems [28,30], and to pharmaceutical applications [31].

Given the severe environmental pollution caused by the disposal of sericin and because of its beneficial properties, interest in the recovery of sericin has increased. Thus, the valorization of sericin for biomedical and pharmaceutical applications could have a substantial economic, social, and environmental impact. This article reviews the properties of sericin and recent advances in the use of this protein for biomedical and pharmaceutical applications, focusing on the development of smart drug delivery systems and reported clinical trials using sericin-based products for tissue engineering.

2. Biosynthesis and Genetics of Sericin and Its Physical and Chemical Properties

Along with cotton, wool, linen and hemp, silk is one of the most abundant naturally derived fiber. Silk is produced by various animals, such as spiders (*Nephila clavipes* and *Araneus diadematus*), domestic silkworms (*B. mori*) and wild silkworms (*Antheraea pernyi* and *Samia cynthia ricini*) [32]—Figure 1. As the availability of spiders is limited, mainly domestic

silkworms are used to obtain silk for the production of textile products—sericulture. For this purpose, silkworm eggs of *B. mori*, a holometabolous insect belonging to the Lepidoptera order and *Bombycidae* family, are laid and incubated before the larvae hatch [32]. Afterwards, the larvae are fed with mulberry leaves for six weeks. Then, until the end of the fifth larval instar stage, the larvae form the silk cocoons that protect them during metamorphosis and create the necessary conditions for larval metamorphosis into adults [4,12]. Before the silkworms turn into pupae, they are sacrificed, and the cocoons are recovered for silk extraction. The killing of the silkworm is necessary to preserve the quality and length of the fiber, as it digests the cocoon as a way out. The finished *B. mori* silk consists of various types of chemical components, mostly fibroin (70–80%) and sericin (20–30%), as well as others (carbohydrates (1.2–1.6%), inorganic matter (0.6–0.7%), wax matter (0.4–0.8%) and pigments (0.2–0.3%)) [15,33]. Figure 2 illustrates the chemical structure of silk polymer and the intermolecular hydrogen bonds between fibroin and sericin [34]. Fibroin acts as the inner core and gives the fiber mechanical strength, while sericin is the outer glue-like coating. Each silk fiber contains two fibroin filaments coated with sericin. Both fibroin and sericin are constituted by a repeated amino acid sequence capable of forming the β-sheet structure. Fibroin has the sequence [GAGAGS]n, and part of the repeat sequence in the sericin has the sequence GSVSSTGSSSNTDSST, where G, A, S, T, V, N and D denote glycine, alanine, serine, threonine, valine, asparagine and aspartic acid, respectively [34].

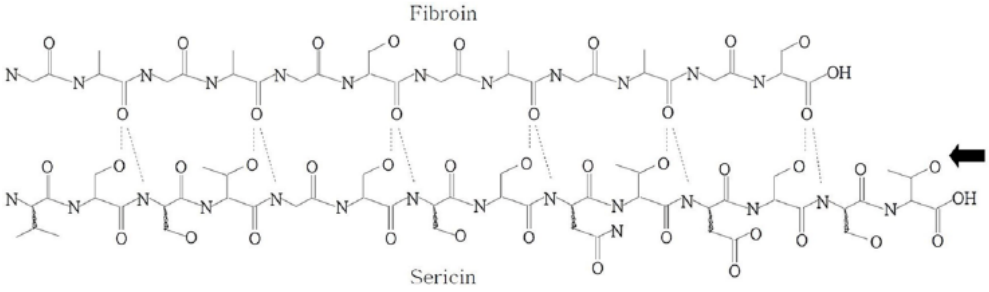

Figure 2. Chemical structure of silk sericin showing the intermolecular hydrogen bonds between the fibroin and sericin. Reprinted with permission from Lee [34]. Copyright © 2022 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Silk sericin is produced in the labial glands of *B. mori*, commonly referred to as silk glands [32,35]. The silk glands are long and paired structures originating from the labial region. They are anatomically and physiologically divided into three major compartments: the anterior silk gland (which forms the excretory duct), the middle silk gland (which secretes three types of sericin) and the posterior silk gland (which secretes fibroin)—Figure 3 [35–37].

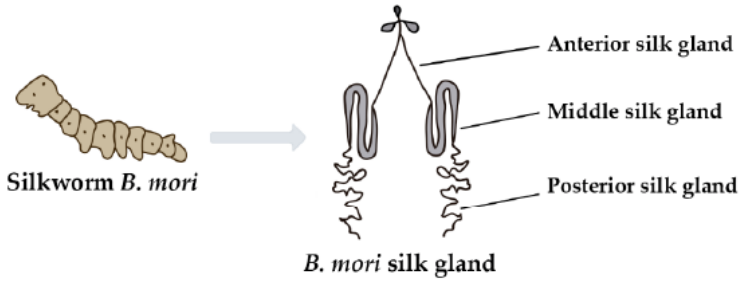

Figure 3. *B. mori* silk gland: anterior silk gland, middle silk gland and posterior silk gland.

Sericin is produced by alternative splicing of sericin genes [38,39]. The expression of the genes is temporally regulated depending on larval development, resulting in a certain homogeneity between exons, and it is responsible for the large protein diversity. There are at least three genes responsible for sericin synthesis, *Ser1*, *Ser2*, and *Ser3*. Sericin is a globular protein composed of random coil and β-sheets with a molecular weight ranging from 20 to 400 kDa, which is mainly influenced by the extraction method (described in detail in the next section). Sericin comprises 18 amino acids with 45.8% hydroxy amino acids (serine and threonine), 42.3% polar amino acids and 12.2% non-polar amino acids [40–42].

The water solubility of sericin is influenced by its position in the silk fiber, i.e., sericin located in the outer layer of the fiber is most soluble in warm water (α-sericin); while sericin located in the inner layer of the fiber (next to fibroin) is insoluble in hot water (β-sericin) [37].

The gelation process of sericin is possible due to the conversion of the random coil into a β-structure, i.e., the sericin random coil is soluble in hot water. However, at low temperature (10 °C, pH about 6–7), this structure converts to a β-sheet structure which facilitates the formation of a three-dimensional network and promotes the formation of the sericin gel [43–45]. This phenomenon is reversible when the sample is heated (50–60 °C) [43–45]. On the other hand, gelation of sericin can also be achieved by chemical crosslinking (e.g., with glutaraldehyde), leading to the formation of a stable β-sheet structure.

3. Extraction Methods of Sericin from Silk Cocoons

The degumming of silk is the process that leads to the cleavage of the peptide bonds (Figure 2) by the hydrolysis of sericin and the subsequent detachment of sericin from fibroin. The extraction of sericin from silk is possible because fibroin is hydrophobic and insoluble in water, while sericin is hydrophilic and can be dissolved in water.

The conventional method used by the silk industry to degum silk uses detergents/soaps (e.g., Marseille) and a solution of sodium bicarbonate (Na_2CO_3)—Table 1. This method successfully removes sericin from cocoons, allowing the recovery of clean and isolated fibroin that the textile industry can use. However, the recovered sericin is highly degraded, reducing its molecular weight and losing some functional properties [46]. In addition, the separation of soap and sericin is very complex. Consequently, traces of soap may remain in the sericin, limiting its use for biomedical and pharmaceutical purposes [47].

Therefore, other degumming methods have been developed to favor the recovery of sericin instead of fibroin, including heat, chemical and enzymatic methods [28]—Table 1 and Figure 1. All these methods can be adapted in terms of time, temperature, chemical additives and others [12,28,48–50]. Sometimes, these methods are combined to obtain sericin with desired yield and properties.

In the method that uses heat, the cocoons are usually heated/boiled in water (in combination with/without high pressure by autoclaving). The high temperature and pressure cause instability of the hydrogen bonds between the hydroxyl groups, allowing water to interact with the hydroxyl groups of polar amino acids and further detachment of sericin and fibroin [51].

Acids (citric, tartaric, succinic, etc.) or bases (sodium carbonate, sodium phosphate, sodium silicate, sodium hydrosulfite, etc.) are used to extract the sericin from silk because these chemicals hydrolyze the sericin by breaking the peptide bonds of the amino acid into small molecules, releasing the sericin into the alkaline or acidic solution, in which sericin is highly soluble [52,53]. For example, sodium carbonate converts the -COOH in the sericin molecules to -$COONa^+$, which increases its solubility due to the strong hydration of Na^+ [54].

Proteolytic enzymes (e.g., alcalase, savinase, degummase, papain, trypsin) have also been used to extract sericin [50]. These enzymes cause the hydrolysis of the peptide bonds of the amino acids between the carboxyl group of lysine/arginine and the amino group (NH_2) of the neighboring amino acids.

Table 1. Overview of the main methods used to extract sericin from *B. mori* cocoons.

Extraction Method	Approach	Advantages	Limitations	Ref. (s)
Conventional	Detergents/soaps (e.g., Marseille) and sodium bicarbonate	Effective	Sericin is highly degraded Sericin recovery is difficult It is not environment-friendly/effluent problems	[49]
Chemical	Alkaline solutions (e.g., sodium carbonate, sodium phosphate, sodium silicate, and sodium hydrosulfite)	Quick Low-cost Efficient	Sericin is degraded Sericin recovery is difficult Purification steps are needed It is not environment-friendly/effluent problems	[28,49]
	Acidic solutions (e.g., citric, tartaric, succinic acid).	Sericin is less degraded than when using alkaline solutions	Sericin is degraded Not efficient Purification steps are needed It is not environment-friendly/effluent problems	[28,49]
	Urea (with or without mercaptoethanol)	Effective Time-consuming Sericin is poorly degraded	Purification steps are needed to remove the chemical impurities Toxic to cells	[28,49]
Enzymatic	Proteolytic enzymes (e.g., bromelain, pancreatin, alcalase, savinase, degummase, papain, trypsin, etc.)	Effective Environment-friendly/no effluent problems	Expensive Sericin is degraded Time-consuming	[28,49,50]
Heat	Boiled in water (associated or not with high pressure by autoclaving)	Simple Low-cost Time-consuming No purification steps needed Environment-friendly/no effluent problems	Sericin is degraded (when used at high temperatures) Damages fibroin Removes only the outer layer of sericin	[28,49]

All these methods have advantages and disadvantages, as outlined in Table 1. The use of acids and bases to extract sericin can significantly degrade the protein [52]. Moreover, the sericin extracted using acids and bases must be purified in additional steps to remove the chemical impurities. On the other hand, urea-degradation extraction (with 2-mercaptoethanol) has a lower degradative effect on sericin. With this method, about 95% of the total sericin present in the fiber can be extracted without damage. However, this method is expensive and time-consuming [55], and sericin extracted using urea is highly toxic to cells [56]. Considering these limitations, heat is the most commonly used method for sericin extraction. Although this method also causes some degradation of sericin, especially when high temperatures are used or the solutions are applied during long periods [57], sericin retains its remarkable properties. Moreover, the silk is heated in hot distilled water to which no other chemicals are added so that the sericin is obtained without impurities.

In recent years, new technologies have been developed to extract sericin from silk in a greener, more effective and sustainable manner, such as those that use infrared heat, carbon dioxide supercritical fluid and ultrasounds [28,49]. However, these techniques require the use of extra equipment.

Influence of the Extraction Method on Sericin Yield and Characteristics

It is important to note that the extraction method of *B. mori* silk sericin affects not only its yield but also its physical, chemical and mechanical properties, which in turn will influence the biological properties of sericin.

The extraction of sericin by urea and by autoclave had higher yields (18.60–23.10% and 17.00–21.27%, respectively) compared to extraction using citric acid and sodium carbonate solutions (8.33–15.19% and 5.93–12.69%, respectively) [58].

Sericin with higher molecular weights (10 to >225 kDa) was obtained when extracted with urea. In contrast, the molecular weights of sericin obtained by acid, alkali and heat degradation were 50–150 kDa, 15–75 kDa, and 25–150 kDa, respectively [56]. Furthermore, extraction with the urea solution was the only method that showed clearer protein bands when analyzed by polyacrylamide gel electrophoresis (SDS-PAGE). This demonstrates that the urea-based extraction method has a lower impact on sericin degradation and thus allows sericin recovery with more defined molecular weights.

The conformational changes in the secondary structure of sericin were also observed—Table 2. Sericin extracted by the conventional method and alkali-degradation showed the presence of α-helix, random coil and turns. In contrast, autoclaving (heat)-extracted sericin lacked α-helical structures [16].

Table 2. Percentage (%) of secondary structures (α-helix, β-sheet, turns and random coil) of silk sericin from *B. mori* extracted through different methods. Adapted from [16].

Extraction Method	Secondary Structure (%)			
	α-Helix	β-Sheet	Turns	Random Coils
Conventional	28.8	0.0	35.1	36.1
Heat (boiling in water)	0.0	56.2	2.5	41.3
Urea-degradation	2.8	54.5	4.0	38.7
Alkali-degradation	28.5	0.0	33.8	37.8
Acid-degradation	14.9	34.8	17.0	33.3

Measurements demonstrated that sericin zeta potential is negative independently of the extraction method used. The zeta potential of sericin from urea extraction yielded the highest negative charge (−68.36 mV), followed by acid-degraded sericin (−32.12 mV), heat-degraded sericin (−20.69 mV) and finally, alkali-degraded sericin (−15.87 mV) [56].

Table 3 summarizes the most significant changes in the amino acid composition of sericin under different extraction methods. As shown by Aramwit et al. [56], regardless of the extraction method, serine was the most abundant amino acid in sericin, followed by aspartic acid and glycine. The amount of methionine found in sericin extracted by heat was significantly higher than in sericin extracted by other methods. In comparison, the amount of tyrosine found in urea-extracted sericin was substantially lower than in sericin extracted by other methods.

Significantly, the content of secondary metabolites (phenols and flavonoids) associated with sericin also differed depending on the extraction methods [16]—Table 4. The total phenol content was higher when sericin was extracted by heat (boiling water) and lower when sericin was extracted with urea solutions. Acid-degraded sericin showed the highest total flavonoid content, while alkali degradation resulted in the lowest flavonoid levels.

It is important to point out that the extraction method, and therefore the properties of sericin, affect the use of this protein. Sericin amino acids assembly (aggregation stands, β-sheets, β-turns formation, etc.) influences cell behavior. In fact, avoiding the chemical degradation of sericin promotes cell growth and attachment due to the arrangement of methionine and cysteine amino acids [59]. The amino acid content also affects sericin's biological properties (discussed in more detail in the following section) and, thus its performance as a biomaterial. For instance, sericin with higher content of serine and

threonine amino acids have higher antioxidant and photoprotective activity. It has also been observed that sericin extracted by different methods affects cell viability and collagen production differently [56]. On the other hand, the molecular weight of sericin affects its application. While low molecular weight (<20 kDa) sericin is generally used in hair care, cosmetics and medications, >20 kDa sericin is preferred for manufacturing products (e.g., drug delivery systems, membranes, hydrogels, fibers) for tissue engineering and other purposes [59].

Table 3. Composition of the amino acid (in mole%) of silk sericin from *B. mori* extracted using various methods. Adapted from [56].

Amino Acid	Extraction Method			
	Heat	Urea-Degradation	Acid-Degradation	Alkali-Degradation
Serine	33.63	31.27	31.86	30.01
Aspartic acid	15.64	18.31	15.93	19.88
Glutamic acid	4.61	5.27	5.75	5.93
Glycine	15.03	11.23	10.49	11.01
Histidine	1.06	3.26	2.47	1.72
Arginine	2.87	5.41	4.92	4.92
Threonine	8.16	8.36	8.51	6.49
Valine	2.88	2.96	2.95	2.94
Methionine	3.39	0.12	0.06	0.15
Lysine	2.35	3.14	3.48	2.89
Isoleucine	0.56	0.96	0.87	0.75
Leucine	1.00	1.58	1.43	1.56
Phenylalanine	0.28	0.60	0.71	0.81

Table 4. Total phenol and flavonoid content of silk sericin from *B. mori* extracted through different methods. Adapted from [16].

Extraction Method	Total Phenol Content (mg GAE/10 g)	Total Flavonoid Content (mg CE/10 g)
Conventional	253.40 ± 9.10	328.79 ± 47.81
Heat (boiling in water)	319.40 ± 5.70	381.00 ± 47.45
Urea-degradation	200.23 ± 13.50	539.93 ± 46.8
Alkali-degradation	257.73 ± 12.00	210.01 ± 30.09
Acid-degradation	256.07 ± 12.37	708.80 ± 54.49

4. Sericin Properties Favorable for Biomedical and Pharmaceutical Applications

The most relevant and promising silk sericin biological properties, such as biodegradability, biocompatibility, immunocompatibility, anti-inflammatory, antibacterial, antioxidant and photoprotective activities, are summarized in Figure 1.

4.1. Biocompatibility and Immunological Response

Biocompatibility is the main requirement of any biomedical material. When in contact with the human body, a biomaterial should not induce any adverse effects (e.g., immune response) [60].

Regarding sericin, its biocompatibility has been demonstrated in different works since this protein is an immunologically inert material. The addition of sericin to the culture media of several cell lines has shown that it does not promote cytotoxicity, indicating

sericin's safety to cells [61,62]. Moreover, sericin does not induce immunological responses. In work carried out by Gil et al. [63], it was demonstrated that sericin does not induce macrophage activation. On the other hand, Chlapanidas et al. [64] studied the activity of sericin, obtained from different silkworm strains, in peripheral blood mononuclear cells. The results revealed that sericin (in some strains) promoted a decrease in the in vitro secretion of interferon-gamma (IFNγ). At the same time, no effect was observed on the release of tumor necrosis factor-alpha (TNFα) and interleukin 10 (IL10).

On the other hand, Aramwit et al. [65] evaluated the inflammatory response of a sericin-based cream when applied to induced wounds in rats. The quantification of interleukin 1 beta (IL1β) and TNFα was performed after 7 days of application of the cream. The results demonstrated a significant decrease in the release of these cytokines. This effect was also verified in the works carried out by Dash et al. [18] and Mandal et al. [66], in which sericin decreases the levels of cytokines produced by macrophages and monocytes. Thus, it is possible to confirm that sericin is a biocompatible material which does not provoke severe immune responses.

4.2. Biodegradability

A biodegradable biomaterial can be broken down into other substances by the organism through different biological processes. The use of biodegradable biomaterials to produce biomedical products is preferred in most cases since these biomaterials are only maintained in the human body until they serve their purpose, being eliminated gradually and naturally after their degradation. A clear advantage of using biodegradable biomaterials is visible when they are used to treat wounds. Applying a biodegradable wound dressing avoids the need to replace/remove it from the wound site, reducing the pain and discomfort for the patient and the damage to the newly formed tissue [67].

Sericin is a biodegradable polymer, and its degradation is mediated both in vitro and in vivo by proteolytic enzymes (e.g., protease XIV, α-chymotrypsin, proteinase K, papain, matrix metalloproteinases, and collagenase) which act on the amorphous hydrophilic segments of the heavy and light chains of silk [67,68]. The products resulting from the degradation of sericin are amino acids, which are absorbed by the body, not inducing any immune response. In the literature, some works report that materials produced based on sericin fibers are reabsorbed after 6 weeks of implantation in vivo [68].

Concerning in vitro assays, researchers used protease XI as a model enzyme to assess the degradation profile of sericin [67]. However, it is essential to consider that the rate of enzymatic degradation depends on several factors, namely the structural and morphological characteristics of the structures composed of sericin (e.g., fibers, films, sponges), processing conditions, characteristics of the biological environment at the implantation site and the presence of mechanical and chemical stresses [68].

4.3. Anti-Inflammatory Activity

Inflammation is one of the phases of the healing process, in which phagocytosis of necrotic tissues and possible contaminants present at the wound site occurs [69]. In addition, at this stage, inflammatory cells secrete cytokines and growth factors that recruit the cells responsible for forming new tissue. However, this phase must be controlled since the uncontrolled, exuberant expression of inflammatory cytokines promotes the expression of metalloproteinases, which are responsible for the degradation of the extracellular matrix. In this sense, biomaterials developed for wound treatment must be able to control the inflammatory process [70].

In general, assays to determine anti-inflammatory activity are often based on the evaluation of the expression/release of inflammatory cytokines (interleukin 1 (IL-1) and tumor necrosis factor-alpha (TNF-α)). As described in the literature, IL-1 and TNF-α are the most important inflammatory mediators. They also induce the expression of adhesion molecules essential for the proliferative phase [70]. Thus, in vitro and in vivo assays have

already demonstrated that sericin controls the release of the inflammatory cytokines IL-1β and TNF-α [12].

4.4. Antibacterial Activity

A biomaterial has antibacterial properties if it destroys bacteria or suppresses their growth or ability for multiplication [71]. In recent years, infectious disease management has become an increasing challenge for healthcare systems. For instance, the occurrence of infections during the wound-healing process is considered one of the most severe problems in wound care. The presence of microorganisms at the wound site prevents the healing process from normally occurring, causing other more severe complications at the local and systemic level [72]. Therefore, using biomaterials with antibacterial properties is a promising tool for producing biomedical products.

According to what has been reported in the literature, the antimicrobial activity of sericin may be related to the presence of cysteine in its composition, an uncharged polar amino acid, due to its sulfhydryl groups [73]. In turn, these sulfhydryl groups can form weak hydrogen bonds with oxygen or nitrogen, producing an extremely reactive compound that affects various enzymatic reactions and metabolic functions of microorganisms [74].

The antimicrobial activity of sericin has been demonstrated against Gram-positive and Gram-negative bacteria. Studies carried out by Ahamad and KumarVootla [75] revealed that sericin has antimicrobial effects against *Escherichia coli*, *Staphylococcus aureus* and fungi such as *Candida albicans* and *Aspergillus flavus*. Similarly, Jassim and Al-Saree [76] found that when sericin concentration was increased (10–20 mg/mL), the inhibitory effect on the growth of *Streptococcus pneumoniae*, *Pseudomonas aeruginosa* and *E. coli* was also potentiated. Additionally, the authors also found that the number of colonies was reduced from 146 to 29 after treatment with a sericin solution (2%) for 7 days [76].

In addition to antibacterial activity, silk sericin has been reported as an anti-biofilm agent. Recently, Aramwit and other authors developed an in vitro study to investigate the sericin's potential to inhibit biofilm formation (prevention) and disrupt already formed biofilm (treatment) [77]. They concluded that sericin extracted by urea, heat or acid degradation was able to inhibit and/or reduce biofilm formation, urea-extracted sericin having the highest antibiofilm activity (against *Streptococcus mutans*). This suggests that sericin can be used as an anti-biofilm agent.

4.5. Antioxidant and Photoprotective Activity

Reactive oxygen species (ROS) are formed during normal cellular metabolism but become toxic when present in high concentrations. Free radicals and ROS are unstable and react readily with other groups or substances in the body, leading to cell or tissue injury, and are responsible for many diseases (cancer, cirrhosis, ischemic reperfusion, etc.) [78]. Sericin is well known for its potent antioxidant activity. The antioxidant properties of sericin result from its ROS scavenging activity, as well as inhibition of lipid peroxidation, and anti-tyrosinase and anti-elastase activities, as demonstrated by Kato et al. [79]. Moreover, Li et al. [80] showed that sericin might enhance the activity of antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase.

The antioxidant activities of sericin are correlated with its high serine and threonine content, whose hydroxyl groups act as chelating trace elements such as copper and iron [26,28]. On the other hand, the pigment molecules (e.g., flavonoids and carotenoids) accumulated in sericin layers may be one of the causes that endow sericin with antioxidant properties and anti-tyrosinase activity. Aramwit et al. demonstrated that sericin obtained from cocoons submitted to the pigment extraction had anti-tyrosinase activity, which was higher than the sericin obtained from cocoons with pigments [58].

Sericin is also reported to have photoprotective activity since it can effectively absorb ultraviolet (UV) radiation and prevent oxidative damage by maintaining redox balance. It was previously reported that the topical delivery of *B. mori* sericin protected female hairless mouse from UVB radiation-induced sunburn and tumor initiation [81]. Sericin possesses

various amino-based groups rich in hydrogen, oxygen and nitrogen which facilitate strong absorption of UV light wavelengths under 200 nm [82].

5. Sericin Biomedical and Pharmaceutical Applications

Various biomaterials have been used for biomedical and pharmaceutical applications. Generally, naturally derived polymers such as agarose, alginate, cellulose, chitosan, collagen, keratin, sericin, etc. (Table 5) are preferred as they have several advantages over the synthetic polymers (poly (anhydride), poly (caprolactone) (PCL), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (lactic-co-glycolic acid) (PLGA), etc.). The biocompatibility, non-toxicity and biodegradability are the most relevant bioactivities of these polymers, similar to native extracellular matrix (ECM), as extensively reviewed in previous works [83–85].

Table 5. Naturally occurring polymers: origin and major advantages/disadvantages for biomedical and pharmaceutical applications.

Polymer	Origin	Advantages	Disadvantages	Ref.(s)
Agarose	Purified from agar that is obtained from red seaweed (e.g., *Ahnfeltia plicata*, *Gelidium amansii*, *Eucheuma*)	Biocompatible Non-immunogenic Thermo-reversible behavior Easy gelling Inexpensive	Soluble only at high temperatures Low cell adhesion Poorly-degradable in humans	[83,86]
Alginate	Brown seaweed (e.g., *Laminaria*, *Macrocystis*, *Ascophyllum*)	Biocompatible Easy chemical modification Easy gelling Inexpensive	Poorly-degradable in humans Sterilization causes degradation Low cell adhesion Weak mechanical strength.	[83,86,87]
Cellulose	Plants and bacteria	Biocompatible Good mechanical strength Porous stable matrix Inexpensive	Poorly-degradable in humans	[83,88,89]
Chitosan	Deacetylation of chitin that is obtained from crustacean exoskeletons, insects and fungal cell walls	Biocompatible Biodegradable Non-toxic Non-antigenic Non-allergenic Bioactive Inexpensive	Immunogenic Non-soluble at physiological pH Low long-term stability Weak mechanical strength	[87–90]
Collagen	Connective tissue (e.g., cartilage, bones, tendons, ligaments and skin)	Biocompatible Biodegradable Non-toxic Non-antigenic Non-immunogenic Bioactive Native ECM protein	Viral contamination Low stability Sterilization causes degradation Weak mechanical strength Expensive	[86,88,89]
Fibrin	Converted from fibrinogen that is obtained from blood serum	Biocompatible Biodegradable Non-immunogenic Native ECM protein Bioactive	Viral contamination Rapid degradation Weak mechanical strength	[83,91]

Table 5. *Cont.*

Polymer	Origin	Advantages	Disadvantages	Ref.(s)
Fibroin	Silk of different animals (e.g., spiders (*Nephila clavipes* and *Araneus diadematus*), silkworms (*B. mori*, *Antheraea pernyi* and *Samia cynthia ricini*))	Biocompatible Biodegradable Bioactive Good mechanical strength Thermostable	Non-soluble in water Expensive	[37,83,91–93]
Gelatin	Degraded collagen that is obtained from connective tissue	Biocompatible Biodegradable Non-immunogenic Poorly-antigenic Bioactive Thermal crosslinking Easy gelling	Rapid degradation Weak mechanical strength	[83,87,91]
Hyaluronic acid	ECM of the connective tissue (e.g., cartilage, bones, tendons, ligaments, and skin), synovial fluid and other tissues	Biocompatible Biodegradable Non-immunogenic Native ECM protein Bioactive	Viral contamination Rapid degradation Weak mechanical strength Expensive	[83,86,87]
Keratin	Hair, nails, horn, hoofs, wool and feathers	Biocompatible Biodegradable Bioactive	Weak mechanical strength	[83,93,94]
Sericin	Silk of different animals (e.g., spiders (*Nephila clavipes* and *Araneus diadematus*), silkworms (*B. mori*, *Antheraea pernyi* and *Samia cynthia ricini*))	Biocompatible Biodegradable Bioactive Thermo-reversible behavior/easy gelling	Weak mechanical strength	[12,37,83]

ECM—extracellular matrix

Because of its biological properties, but also considering the environmental problems caused by sericin as a discharged waste of the textile industry, this protein has been studied for different applications in different industries [26,27]—Table 6 and Figure 1. Hereafter, this review will be focused on the major biomedical and pharmaceutical applications of sericin, namely tissue engineering, drug delivery and other applications such as metabolic disorders and cosmetic formulations.

Table 6. Silk sericin applications in different industries. Adapted from [26,27].

Industry	Applications
Biomedical and pharmaceutical	Supplement in culture media Antitumor activity Metabolic effects (in the gastrointestinal tract, in the circulatory and immune systems, on lipid metabolism and obesity) Tissue engineering Wound healing Drug delivery Contact lenses Matrix for implants Vehicle for cell amplification Stabilizer in vaccines Skincare: skin elasticity, anti-wrinkle and anti-aging influences, UV protection impact Nailcare: prevents cracks and brittleness, and raises the inherent brightness Haircare: conditioner; prevents hair damage Gel: moisturizing property

Table 6. *Cont.*

Industry	Applications
Food	To enhance the taste and touch of porridge Prevents browning reactions in a variety of ingredients Antioxidants Mineral absorption is accelerated Additive as a nutrient
Textile	In fabrics to absorb moisture Cleaning fabrics Improved antibacterial activity Fabricated nanofiber UV protection of textiles Medical textiles Nanofibers
Others	Treating industrial wastewater with adsorptive pollutants Air filter products Anti-frosting agent for roads and roofs Artificial leather products Roads and roofs Art pigments

5.1. Drug Delivery

Scaffolds, films, hydrogels, fibers, foams, spheres, capsules and microneedles, among others, can be used for local and systemic drug delivery. Since sericin has an amphiphilic character (polar side chains and hydrophobic domains), it can be used as a vehicle because it easily binds charged therapeutic molecules or hydrophobic and hydrophilic drugs [95]. In addition, sericin has a long half-life in vivo and high moisture absorption and desorption abilities, which are also favorable properties for its application for drug delivery purposes [28].

Sericin-based structures, mostly hydrogels, prepared by crosslinking, ethanol precipitation, or blending with other polymers, can be used for drug delivery.

Yan et al. [96] developed a hydrogel using sericin and poly (ethylene glycol) diacrylate (PEGDA) solution in a 1:1 volume ratio. The 20% sericin-containing hydrogel displayed a greater specific surface area and suitable mechanical properties. Additionally, the behavior of the hydrogel in terms of swelling, drug release, and in vitro cytotoxicity demonstrated its appropriateness for drug delivery when loaded with berberine. Further, antibacterial studies against *S. aureus* and *E. coli.* confirmed that the released berberine maintained the antibacterial activity of this natural compound.

In another study, a crosslinked sericin/dextran injectable hydrogel was synthesized to display efficient drug loading and controlled release of both macromolecular (protein enzyme (horseradish peroxidase, HRP)) and small molecular (antitumor drug doxorubicin (DOX)) drugs [97]. Furthermore, the hydrogel could be used as a photoluminescence-trackable drug delivery system since the sericin's photoluminescence from this hydrogel was directly and stably correlated with its degradation, enabling long-term in vivo imaging and real-time monitoring of the remaining drug.

Further, sericin can be used to produce drug carriers such as nanoparticles and microparticles due to its chemical reactivity that allows the easy binding of molecules [28]. In this context, in 2019, Yalcin et al. [98] developed albumin–sericin nanoparticles modified with poly-L-lysine (PLL) and decorated with hyaluronic acid (HA) as a novel small interfering RNA (siRNA) delivery system for laryngeal cancer treatment. In vitro studies carried out with Hep-2 cells demonstrated that the nanoparticles (with albumin and sericin at a ratio of 2:1 (*w*/*w*)) promoted gene silencing, resulting in significant inhibition of cell growth and inducing the cells' apoptosis.

In turn, stable micelles were developed by Deng et al. [99] by conjugating hydrophilic sericin with hydrophobic cholesterol, folic acid (tumor-targeting agent) and a near-infrared dye (IR780 iodide). The results showed that the micelles could be absorbed by folic acid-positive gastric cancer cells (BGC-823) through folic acid receptors. Moreover, the nanoparticles showed remarkable effectiveness in photodynamic and photothermal therapy for cancer cells.

Recently, in 2022, Xu et al. [100] reported an effective strategy to fabricate a silk sericin nanospheres systems for the delivery of recombinant human lactoferrin (a promising protein to treat ulcerative colitis (UC)). To this end, authors optimized transgenic silkworms to generate genetically engineered silk fibers with significant quantities of recombinant human lactoferrin. The nanoparticle uptake by cells in the inflamed colon of mice was more efficient than that of free lactoferrin in solution. Moreover, a low dose of nanoparticles significantly relieved symptoms of UC in mice and achieved a comparable therapeutic effect to the high dose of free lactoferrin in solution.

Sericin is sensitive to pH due to its strongly polar side groups (hydroxyl, carboxyl, and amino). It has been extensively investigated to produce smart delivery systems, i.e., pH-responsive systems. The use of pH-responsive delivery systems is quite advantageous because they can control the release of the therapeutic compound in an external acidic/alkaline environment, improving its specificity and thus enhancing its efficiency and reducing its side effects [101,102].

Wang et al. [103] produced an injectable hydrogel with sericin (2% *w*/*v*). The hydrogel had excellent cell adhesion capability and effectively promoted cell attachment, proliferation and long-term survival of various cell types (e.g., mouse myoblasts (C2C12) and human keratinocytes (HaCaT)). Compared with alginate hydrogels (2% and 4% *w*/*v*), the sericin hydrogel exhibited higher compressive strength and lower compressive modulus, indicating that the sericin hydrogel has good mechanical properties that allow easy handling during implantation. More importantly, these authors observed that the sericin hydrogel had a pH-responsive character since the maximum degree of swelling was reduced in the acidic environment (pH 3). The swelling behavior of sericin depends on its net charge. Since the isoelectric point of sericin is near pH 4 [104], the net charge of sericin at pH 4 is nearly zero. Consequently, there are equal numbers of negatively and positively charged amino acids. Therefore, the attractive force between negative and positive charges prevents the swelling of sericin molecules. This pH-responsive swelling behavior of the sericin hydrogel makes it useful for delivering therapeutic agents in environments with acidic-neutral pH, such as the microenvironment of tumors.

In a study performed by Huang et al. [105], folate-conjugated sericin nanoparticles were used for tumor targeting and pH-responsive subcellular delivery of doxorubicin (DOX) to treat cancer. The nanoparticles targeted the folate-receptor-rich human oral epithelium carcinoma cell line (KB). Further, the acid environment of the lysosomes that contained the endocytosed nanoparticles prompted the rapid release of DOX to nuclei.

Hu et al. [106] developed pH-triggered nanoparticles based on silk sericin for enhanced cellular uptake and delivery of DOX. In this work, nanoparticles were prepared by physically reacting the negatively charged sericin with positively charged chitosan. Under mild acidic conditions (e.g., in the tumor microenvironment), the surface charge of the nanoparticles changes from negative to positive charge (due to the increased amino/carboxyl ratio), which improves their cellular uptake due to their high affinity for the negatively charged cell membranes.

Oh et al. [107] also produced a pH-responsive sericin drug delivery system for oral administration. The authors observed that the release of diclofenac (nonsteroidal anti-inflammatory drug) was slightly more efficient at higher pH values (7.4 and 9.2 versus 2.2 and 4). In addition, only 10% of the sericin beads were dissolved at pH 2.2 in the presence of pepsin (proteolytic enzyme in the stomach), whereas 45% of the beads were dissolved at pH 7.4 in the presence of trypsin (proteolytic enzyme in the intestine). There-

fore, the beads can be used for oral administration of drugs that cause side effects when released into the stomach.

5.2. Tissue Engineering

As already mentioned, sericin-based scaffolds can be used as drug delivery systems and as hydrogels, films, sponges and others for tissue engineering purposes [108,109]. In fact, up to now, sericin is a biomaterial that has been mostly investigated for the regeneration of skin and bone, but also cartilage and adipose tissues, among others [28].

Sericin alone can be used for bone regeneration, which was already evaluated by Noosak et al. [110], where it was verified that sericin could increase the proliferation of osteoblast cells (MC3T3-E1) up to 135%, compared with the untreated control. Nevertheless, for the regeneration of bone tissue, the conjugation of sericin with hydroxyapatite or other calcium phosphate-based materials is the most common blend of biomaterials used to produce scaffolds (reviewed in [111,112]) since these biomaterials enable the mimicking of the organic and inorganic matrixes of the bone, respectively. Furthermore, since sericin has poor mechanical properties, its mixture with other biomaterials is crucial to obtain scaffolds with suitable mechanical properties for bone regeneration.

Recently, Ming et al. [113] developed a membrane of sericin and hydroxyapatite with osteogenic activity for periodontal bone regeneration. The biocompatible membranes induced the osteogenic differentiation of human periodontal membrane stem cells (hPDLSCs) by activating the expression of osteoblast-related genes (ALP, Runx2, OCN, and OPN) without additional inducers.

In addition to hydroxyapatite, sericin can be conjugated with other biomaterials for bone regeneration. Jiang et al. [114], in 2021, developed an injectable hydrogel for bone regeneration composed of alginate/sericin/graphene oxide. The sericin and graphene oxide contributed synergistically to bone regeneration, i.e., graphene oxide significantly enhanced the spreading, osteogenic differentiation and mineralization of encapsulated rat bone marrow-derived stem/stromal cells (BMSC). In contrast, the sericin promoted the activation of signaling pathways (e.g., MAPK), contributing to the M2 polarization of macrophages that further induced osteogenic differentiation of BMSC cells via several secreted cytokines [114]. Overall, the hydrogel contributed to the successful repair of distal femoral defects in rats.

Silk has been extensively used in suturing incisional wounds and skin injuries since ancient times [115]. Sericin's inherent property to stimulate cell migration and proliferation seems to be directly related to the accelerated wound healing properties. It is considered a good choice of biomaterial for developing wound dressings and bioartificial skin grafts [116–118]. The mitogenic effect of sericin on mammalian cells is well-established in numerous studies, especially on fibroblasts and keratinocytes, which are majorly involved in the wound-healing process [117].

Baptista-Silva et al. [119] developed an in situ enzyme-mediated forming sericin hydrogel that demonstrated the potential to regenerate the skin both in vitro and in vivo. The hydrogel was non-toxic to the L929 fibroblast cell line and contributed to cell adhesion, colonization and proliferation. The wounds in diabetic mice treated with the hydrogel for 21 days demonstrated lower granulation tissue and inflammatory cells and a reduction in wound size compared to those treated with a dressing commonly used in the clinic (Tegaderm).

In turn, Sapru et al. [120] produced non-mulberry sericin/chitosan/polyvinyl alcohol (PVA) nanofibrous matrices that supported the adhesion, proliferation and cellular interconnection of human keratinocytes. Furthermore, the membranes could fully regenerate full-thickness wounds.

Tao et al. [121] demonstrated that the sponges composed of silver nanoparticles–sericin/PVA have the expected high porosity, biocompatibility towards NIH/3T3, HEK-293, and RAW264.7 cells, good wettability, hygroscopicity, mechanical properties and an effective antibacterial activity against *S. aureus*, *E. coli* and *P. aeruginosa*. The following

in vivo experiments, using Wistar rats, suggested that the composite dressing could be helpful for re-epithelialization and collagen deposition to promote wound healing, which is crucial for skin regeneration.

In 2018, Chen et al. [122] prepared a 3D-printed hydrogel scaffold composed of sericin and methacrylic anhydride-modified gelatin (GelMA) by 3D printing and observed that it has the potential to regenerate skin wounds. The authors referred to hydrogel's transparency as its most notable property, which allowed wound care visualization.

In terms of cardiac tissue regeneration, Song et al. [123] prepared sericin as an injectable hydrogel that, when administrated into an acute myocardial infarction area in mouse models, reduces scar formation and infarct size, increases wall thickness and neovascularization, and inhibits the induced inflammatory responses and apoptosis, thereby leading to a significant functional improvement. Moreover, sericin exhibits angiogenic activity by promoting migration and tubular formation of human umbilical vessel endothelial cells.

To date, the application of sericin for tissue engineering purposes is still mainly investigated in in vitro and in vivo studies. Nevertheless, some clinical studies have been conducted on products that contain sericin. As summarized by Liu et al. [11], some clinical studies have shown the potential of sericin (film, cream, scaffold) for wound healing and skin regeneration [124–127]. In a clinical study by Aramwit et al. [125], the efficacy of sericin added to a standard antimicrobial cream (silver zinc sulfadiazine) for open wound care was evaluated in the treatment of second-degree burn wounds in a total of 29 patients with 65 burn wounds. The results showed that sericin was safe, as no infection or severe reaction was detected in any wound. Moreover, sericin contributed to wound healing, i.e., the complete healing of the control group (wounds treated with silver zinc sulfadiazine cream only) occurred after 29.28 ± 9.27 days, while it took 22.42 ± 6.33 days in the group treated with sericin.

A clinical trial performed by Siritientong et al. [126] also showed that sericin-releasing bioactive wound dressing was more efficient in the treatment of split-thickness skin graft donor sites compared to the clinically available wound dressing Bactigras® (complete healing at 14 ± 5.2 days versus 12 ± 5.0 days), and also reduced significantly the pain. In another clinical study, a sericin-based cream improved hydration and reduced pruritus after 6 weeks in hemodialysis patients with uremic pruritus compared to a cream without sericin [124]. For bone regeneration purposes, to the best of our knowledge, only silk (and not sericin isolated) from the cocoon of the silkworm was investigated in a clinical trial [128].

5.3. Other Applications

Metabolism corresponds to the various chemical reactions that guarantee the energy and structural needs of the human organism. When there is a change in metabolism, metabolic disease can occur [129]. Sericin is composed of 18 types of amino acids, out of which 8 types of amino acids play a significant role in human metabolic pathways [13].

In 2010, Okazaki et al. [130] analyzed the impact of sericin on sugar and lipid metabolism in mice fed with a high-fat diet. The body weight, nourishment utilization and fat weight were not modified by adding 4% sericin to the eating routine for 5 weeks. Yet, this addition reduced serum concentration of free unsaturated fats, cholesterol, phospholipids, triglycerides, very low-density lipoproteins (VLDL) and low-density lipoprotein (LDL).

A study from 2019 investigating the effects of sericin extracted from silkworm *B. mori* cocoon on morphophysiological parameters in mice with obesity induced by a high-fat diet suggested that the physiological changes caused by obesity were not 100% reverted by sericin [131]. However, treatment with sericin restored jejunal morphometry and increased lipid excretion in feces in obese mice, suggesting potential anti-obesity effects.

Another metabolic dysfunction is diabetes mellitus which is a chronic health condition. Several therapies are available to treat diabetes, although they are associated with severe side effects [132]. Thus, the continuing need for more effective and safer antidiabetic agents is evident [133].

Several recent studies have attempted to prove sericin's antidiabetic activity [130]. In 2020, Dong et al. [134] investigated silk sericin's in vivo hypoglycaemic effect on Type II diabetic mice. The results demonstrated that sericin significantly decreased fasting blood glucose, fasting plasma insulin and glycosylated serum protein levels. The protein also improved oral glucose and insulin tolerance and enhanced antioxidative activities. Sericin could help maintain normal glucose levels, regulate insulin secretion, insulin and lipid metabolism, and inhibit inflammation. Thus, sericin could be developed into a novel functional health food with a significant hypoglycaemic effect. More recently, in 2022, sericin's effect on liver injury in Type II diabetic rats was investigated [135]. After 4 weeks of dietary supplementation with sericin, the liver masses and organ coefficients of Type II diabetic rats improved compared with those of the rats not fed with sericin. These results demonstrate that sericin might improve glycogen synthesis, accelerate glycolysis, and inhibit gluconeogenesis by enhancing the anti-oxidation capability and reducing inflammatory reactions. Therefore, sericin could potentially be used to develop functional health foods that can lower blood sugar. Furthermore, the ability of sericin to retain water and unfermented fibers may be indicated to improve constipation [12].

Silk sericin has also been described as an ingredient for skin and hair care cosmetic formulations due to its singular physical-chemical composition [136]. The Expert Panel for Cosmetic Ingredient Safety reviewed the safety of hydrolyzed silk and nine other silk protein ingredients, which function primarily as skin and hair conditioning agents and bulking agents in cosmetic products, and concluded that sericin is among the eight silk protein ingredients considered as safe for cosmetics development [137].

The use of sericin in cosmetics, namely in creams and shampoos, increased hydration, elasticity, cleaning with lower irritancy, and anti-aging and anti-wrinkle effects [12]. For example, it was demonstrated that silk sericin has antioxidant and tyrosinase inhibition activity, indicating its whitening potential, which could be a plus to cosmetic applications [138], as well as the protective effects of a hair care treatment based on silk proteins (fibroin, sericin and other proteins) against the damage induced by hair bleaching and coloring [139].

6. Conclusions

The cost, availability and resources required to raise silkworms and process silk threaten the future of this industry. Yet, it is often not recognized that various renewable and sustainable by-products can be obtained during silk production, which has considerable economic value. In the textile industry, silk sericin is usually considered a waste which seriously impacts the environment. However, sericin has been recognized as a biomaterial with outstanding potential for biomedical and pharmaceutical applications due to its promising biological activities. Thus, sericin recovery from silk industry wastewaters has great economic, social and environmental importance.

As mentioned in this review, sericin has unique properties that make it a suitable material for several biomedical and pharmaceutical applications. Developing drug delivery systems incorporating sericin in films, nanoparticles, foams, hydrogels, fibers and other materials is one of the most explored strategies to explore and use sericin in biomedicine. Sericin also has been vastly applied in tissue engineering, mainly in bone tissue engineering and wound healing, to promote cell adhesion and proliferation, tissue reconstruction and repair, and skin re-epithelialization. Overall, silk sericin enables the design and development of economically viable, biocompatible and biodegradable products. In addition, the use of sericin is more advantageous compared to the use of other natural biomaterials because it can be easily functionalized (blended with other natural and synthetic polymers) and is approved by the FDA.

Besides all the beneficial effects and possible applications of silk sericin, it presents some limitations that restrict its application for biomedical purposes. Firstly, the selection of the extraction method assures a standard physicochemical profile and improved biological performance, which should be sustainable and scalable to be applied at the industrial

level and even consider the potential contribution to the maintenance of the economic competitiveness of the silk industry. Moreover, silk sericin has a high solubility in water and is an animal-derived product, which can restrict its applications in some biomedical fields [140]. Furthermore, silk sericin cannot be used per se as drug delivery systems or scaffolds for tissue engineering due to its rheological and low mechanical properties. Therefore, sericin-based scaffolds with desirable properties are usually prepared in combination with other components [141].

Considering the sustainability challenge and the promising biomedical potential of sericin as well as other wastes from silkworm rearing, sericulture could be turned into biofactories that generate proteins, lipids and polysaccharides in the future for biomedical and pharmaceutical applications.

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References

1. Cramer, E. Ueber die Bestandtheile der Seide. *J. Prakt. Chem.* **1865**, *96*, 76–98.
2. Huang, N.; Chen, J. *7000 Year of Chinese Silk Science and Technology*; Chinese Textile Press: Beijing, China, 2002.
3. Gong, Y.; Li, L.; Gong, D.; Yin, H.; Zhang, J. Biomolecular Evidence of Silk from 8500 Years Ago. *PLoS ONE* **2016**, *11*, e0168042.
4. Rheinberg, L. THE ROMANCE OF SILK: A Review of Sericulture and the Silk Industry. *Text. Prog.* **1991**, *21*, 1–43.
5. Ingle, S.; Bagde, N.; Ansari, R.F.; Kayarwar, A.B. Analysis of growth and instability of silk production in India. *J. Pharmacogn. Phytochem.* **2022**, *11*, 195–201. [CrossRef]
6. Babu, K.M. 1-Silk production and the future of natural silk manufacture. In *Handbook of Natural Fibres*; Kozłowski, R.M., Ed.; Woodhead Publishing: Cambridge, UK, 2012; pp. 3–29.
7. Aramwit, P.; Siritientong, T.; Srichana, T. Potential applications of silk sericin, a natural protein from textile industry by-products. *Waste Manag. Res.* **2012**, *30*, 217–224. [CrossRef] [PubMed]
8. Giacomin, A.M.; Garcia, J.B.; Zonatti, W.F.; Silva-Santos, M.C.; Laktim, M.C.; Baruque-Ramos, J. Silk industry and carbon footprint mitigation. *IOP Conf. Ser. Mater. Sci. Eng.* **2017**, *254*, 192008.
9. Lu, L.; Fan, W.; Ge, S.; Liew, R.K.; Shi, Y.; Dou, H.; Wang, S.; Lam, S.S. Progress in recycling and valorization of waste silk. *Sci. Total Environ.* **2022**, *830*, 154812. [PubMed]
10. Fabiani, C.; Pizzichini, M.; Spadoni, M.; Zeddita, G. Treatment of waste water from silk degumming processes for protein recovery and water reuse. *Desalination* **1996**, *105*, 1–9.
11. Liu, J.; Shi, L.; Deng, Y.; Zou, M.; Cai, B.; Song, Y.; Wang, Z.; Wang, L. Silk sericin-based materials for biomedical applications. *Biomaterials* **2022**, *287*, 121638.
12. Kunz, R.I.; Brancalhão, R.M.C.; Ribeiro, L.D.F.C.; Natali, M.R.M. Silkworm Sericin: Properties and Biomedical Applications. *Biomed Res. Int.* **2016**, *2016*, 8175701. [CrossRef]
13. Suryawanshi, R.; Kanoujia, J.; Parashar, P.; Saraf, S. Sericin: A versatile protein biopolymer with therapeutic significance. *Curr. Pharm. Des.* **2020**, *26*, 5414–5429.
14. Arango, M.C.; Montoya, Y.; Peresin, M.S.; Bustamante, J.; Álvarez-López, C. Silk sericin as a biomaterial for tissue engineering: A review. *Int. J. Polym. Mater.* **2021**, *70*, 1115–1129. [CrossRef]
15. Ahsan, F.; Ansari, T.M.; Usmani, S.; Bagga, P. An Insight on Silk Protein Sericin: From Processing to Biomedical Application. *Drug Dev. Res.* **2018**, *68*, 317–327. [CrossRef] [PubMed]
16. Kumar, J.P.; Mandal, B.B. Antioxidant potential of mulberry and non-mulberry silk sericin and its implications in biomedicine. *Free Radic. Biol. Med.* **2017**, *108*, 803–818. [PubMed]
17. Zhang, Y.Q. Applications of natural silk protein sericin in biomaterials. *Biotechnol. Adv.* **2002**, *20*, 91–100. [CrossRef]

18. Dash, B.C.; Mandal, B.B.; Kundu, S.C. Silk gland sericin protein membranes: Fabrication and characterization for potential biotechnological applications. *J. Biotechnol.* **2009**, *144*, 321–329. [CrossRef] [PubMed]
19. Elahi, M.; Ali, S.; Tahir, H.M.; Mushtaq, R.; Bhatti, M.F. Sericin and fibroin nanoparticles—natural product for cancer therapy: A comprehensive review. *Int. J. Polym. Mater.* **2021**, *70*, 256–269.
20. Ghosh, S.; Rao, R.S.; Nambiar, K.S.; Haragannavar, V.C.; Augustine, D.; Sowmya, S.V. Sericin, a dietary additive: Mini review. *J. Med. Radiol.* **2019**, *6*, 4–8.
21. Rocha, L.K.; Favaro, L.I.; Rios, A.C.; Silva, E.C.; Silva, W.F.; Stigliani, T.P.; Guilger, M.; Lima, R.; Oliveira, J.M., Jr.; Aranha, N. Sericin from Bombyx mori cocoons. Part I: Extraction and physicochemical-biological characterization for biopharmaceutical applications. *Process Biochem.* **2017**, *61*, 163–177. [CrossRef]
22. Cao, T.T.; Zhang, Y.Q. Processing and characterization of silk sericin from Bombyx mori and its application in biomaterials and biomedicines. *Mater. Sci. Eng. C* **2016**, *61*, 940–952. [CrossRef]
23. Kundu, S.C.; Dash, B.C.; Dash, R.; Kaplan, D.L. Natural protective glue protein, sericin bioengineered by silkworms: Potential for biomedical and biotechnological applications. *Prog. Polym. Sci.* **2008**, *33*, 998–1012.
24. Silva, S.S.; Fernandes, E.M.; Pina, S.; Silva-Correia, J.; Vieira, S.; Oliveira, J.M.; Reis, R.L. 2.11 Polymers of Biological Origin. In *Comprehensive Biomaterials II*; Ducheyne, P., Ed.; Elsevier: Oxford, UK, 2017; pp. 228–252.
25. Qi, Y.; Wang, H.; Wei, K.; Yang, Y.; Zheng, R.Y.; Kim, I.S.; Zhang, K.Q. A Review of Structure Construction of Silk Fibroin Biomaterials from Single Structures to Multi-Level Structures. *Int. J. Mol. Sci.* **2017**, *18*, 237.
26. Fatahian, R.; Fatahian, A.; Fatahian, E.; Fatahian, H. A critical review on application of silk sericin and its mechanical properties in various industries. *J. Res. Appl. Mech. Eng.* **2021**, *9*, 1–11.
27. Fatahian, R.; Hosseini, E.; Fatahian, A.; Fatahian, E.; Fatahian, H. A Review on Potential Applications of Sericin, and its Biological, Mechanical, and Thermal Stability Characteristics. *Eng. Sci. Technol. Int. J.* **2022**, *9*, 1–9.
28. Lamboni, L.; Gauthier, M.; Yang, G.; Wang, Q. Silk sericin: A versatile material for tissue engineering and drug delivery. *Biotechnol. Adv.* **2015**, *33*, 1855–1867.
29. Gilotra, S.; Chouhan, D.; Bhardwaj, N.; Nandi, S.K.; Mandal, B.B. Potential of silk sericin based nanofibrous mats for wound dressing applications. *Mater. Sci. Eng. C* **2018**, *90*, 420–432. [CrossRef]
30. Kumar, S.S.D.; Abrahamse, H. Sericin-based nanomaterials and their applications in drug delivery. In *Bio-Based Nanomaterials*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 211–229.
31. Shitole, M.; Dugam, S.; Tade, R.; Nangare, S. Pharmaceutical applications of silk sericin. *Ann. Pharm. Fr.* **2020**, *78*, 469–486. [CrossRef]
32. Sehnal, F.; Sutherland, T. Silks produced by insect labial glands. *Prion* **2008**, *2*, 145–153. [CrossRef]
33. Hurst, G.H. Silk dyeing, printing, and finishing. *Nature* **1892**, *46*, 75.
34. Lee, K.H. Silk Sericin Retards the Crystallization of Silk Fibroin. *Macromol. Rapid Commun.* **2004**, *25*, 1792–1796.
35. Akai, H. The ultrastructure and functions of the silk gland cells of Bombyx mori. In *Insect Ultrastructure*; Springer: Berlin/Heidelberg, Germany, 1984; pp. 323–364.
36. Dhawan, S.; Gopinathan, K.P. Cell cycle events during the development of the silk glands in the mulberry silkworm Bombyx mori. *Dev. Genes Evol.* **2003**, *213*, 435–444. [PubMed]
37. Mondal, M.; Trivedy, K.; Kumar, S.N.I. The silk proteins, sericin and fibroin in silkworm, Bombyx mori Linn., A review. *Casp. J. Environ. Sci.* **2007**, *5*, 63–76.
38. Michaille, J.J.; Garel, A.; Prudhomme, J.C. The expression of five middle silk gland specific genes is territorially regulated during the larval development of Bombyx mori. *Insect Biochem.* **1989**, *19*, 19–27. [CrossRef]
39. Grzelak, K. Control of expression of silk protein genes. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **1995**, *110*, 671–681.
40. Takasu, Y.; Yamada, H.; Tsubouchi, K. Isolation of three main sericin components from the cocoon of the silkworm, Bombyx mori. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2715–2758. [CrossRef] [PubMed]
41. Barajas-Gamboa, J.A.; Serpa-Guerra, A.M.; Restrepo-Osorio, A.; Ivarez-López, C.Ã. Sericin applications: A globular silk protein. *Ing. Competitividad.* **2016**, *18*, 193–206. [CrossRef]
42. Shaw, J.T.B.; Smith, S.G. Amino-acids of Silk Sericin. *Nature* **1951**, *168*, 745.
43. Zhu, L.J.; Arai, M.; Hirabayashi, K. Gelation of silk sericin and physical properties of the gel. *J. Sericult. Sci. Jpn.* **1995**, *64*, 415–419.
44. Hirabayashi, K.; Arai, M.; Chu, L.J. Gelation of silk sericin. *J. Sericult. Sci. Jpn.* **1989**, *58*, 81–82.
45. Padamwar, M.; Pawar, A. Silk sericin and its applications: A review. *J. Sci. Ind. Res.* **2004**, *63*, 323–329.
46. Freddi, G.; Mossotti, R.; Innocenti, R. Degumming of silk fabric with several proteases. *J. Biotechnol.* **2003**, *106*, 101–112. [PubMed]
47. Yun, H.; Oh, H.; Kim, M.K.; Kwak, H.W.; Lee, J.Y.; Um, I.C.; Vootla, S.K.; Lee, K.H. Extraction conditions of Antheraea mylitta sericin with high yields and minimum molecular weight degradation. *Int. J. Biol. Macromol.* **2013**, *52*, 59–65. [PubMed]
48. Biswal, B.; Dan, A.K.; Sengupta, A.; Das, M.; Bindhani, B.K.; Das, D.; Parhi, P.K. Extraction of Silk Fibroin with Several Sericin Removal Processes and its Importance in Tissue Engineering: A Review. *J. Polym. Environ.* **2022**, *30*, 2222–2253. [CrossRef]
49. Wang, W.; Pan, Y.; Gong, K.; Zhou, Q.; Zhang, T.; Li, Q. A comparative study of ultrasonic degumming of silk sericin using citric acid, sodium carbonate and papain. *Color. Technol.* **2019**, *135*, 195–201.
50. More, S.V.; Chavan, S.; Prabhune, A.A. Silk Degumming and Utilization of Silk Sericin by Hydrolysis Using Alkaline Protease from Beauveria Sp. (MTCC 5184): A Green Approach. *J. Nat. Fibers* **2018**, *15*, 373–383.

51. Silva, V.R.; Ribani, M.; Gimenes, M.L.; Scheer, A.P. High Molecular Weight Sericin Obtained by High Temperature and Ultrafiltration Process. *Procedia Eng.* **2012**, *42*, 833–841.
52. Rangi, A.; Jajpura, L. The Biopolymer Sericin: Extraction and Applications. *Int. J. Text. Sci.* **2015**, *5*, 1–5.
53. Çapar, G.; Aygün, S.S. Characterization of sericin protein recovered from silk wastewaters. *Turk hij. ve Deney. Biyol. Derg. Turk. Bull. Hyg. Exp. Biol.* **2015**, *72*, 219–234. [CrossRef]
54. Dou, H.; Zuo, B. Effect of sodium carbonate concentrations on the degumming and regeneration process of silk fibroin. *J. Text. Inst.* **2015**, *106*, 311–319.
55. Gulrajani, M.; Agarwal, R.S.; Chand, S. Degumming of silk with a fungal protease. *Indian J. Fibre Text. Res.* **2000**, *25*, 138–142.
56. Aramwit, P.; Kanokpanont, S.; Nakpheng, T.; Srichana, T. The effect of sericin from various extraction methods on cell viability and collagen production. *Int. J. Mol. Sci.* **2010**, *11*, 2200–2211. [PubMed]
57. Chirila, T.V.; Suzuki, S.; McKirdy, N.C. Further development of silk sericin as a biomaterial: Comparative investigation of the procedures for its isolation from Bombyx mori silk cocoons. *Prog. Biomater.* **2016**, *5*, 135–145. [CrossRef] [PubMed]
58. Aramwit, P.; Damrongsakkul, S.; Kanokpanont, S.; Srichana, T. Properties and antityrosinase activity of sericin from various extraction methods. *Biotechnol. Appl. Biochem.* **2010**, *55*, 91–98. [PubMed]
59. Das, G.; Shin, H.-S.; Campos, E.V.R.; Fraceto, L.F.; Rodriguez-Torres, M.D.; Mariano, K.C.F.; de Araujo, D.R.; Fernández-Luqueño, F.; Grillo, R.; Patra, J.K. Sericin based nanoformulations: A comprehensive review on molecular mechanisms of interaction with organisms to biological applications. *J. Nanobiotechnol.* **2021**, *19*, 30. [CrossRef] [PubMed]
60. Cacopardo, L. Chapter 18-Biomaterials and biocompatibility. In *Human Orthopaedic Biomechanics*; Innocenti, B., Galbusera, F., Eds.; Academic Press: Cambridge, MA, USA, 2022; pp. 341–359.
61. Terada, S.; Nishimura, T.; Sasaki, M.; Yamada, H.; Miki, M. Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including a hybridoma. *Cytotechnology* **2002**, *40*, 3–12. [PubMed]
62. Terada, S.; Sasaki, M.; Yanagihara, K.; Yamada, H. Preparation of silk protein sericin as mitogenic factor for better mammalian cell culture. *J. Biosci. Bioeng.* **2005**, *100*, 667–671. [CrossRef] [PubMed]
63. Gil, E.S.; Panilaitis, B.; Bellas, E.; Kaplan, D.L. Functionalized silk biomaterials for wound healing. *Adv. Healthcare Mater.* **2013**, *2*, 206–217. [CrossRef]
64. Chlapanidas, T.; Faragò, S.; Lucconi, G.; Perteghella, S.; Galuzzi, M.; Mantelli, M.; Avanzini, M.A.; Tosca, M.C.; Marazzi, M.; Vigo, D.; et al. Sericins exhibit ROS-scavenging, anti-tyrosinase, anti-elastase, and in vitro immunomodulatory activities. *Int. J. Biol. Macromol.* **2013**, *58*, 47–56.
65. Aramwit, P.; Kanokpanont, S.; De-Eknamkul, W.; Srichana, T. Monitoring of inflammatory mediators induced by silk sericin. *J. Biosci. Bioeng.* **2009**, *107*, 556–561. [CrossRef]
66. Mandal, B.B.; Priya, A.S.; Kundu, S.C. Novel silk sericin/gelatin 3-D scaffolds and 2-D films: Fabrication and characterization for potential tissue engineering applications. *Acta Biomater.* **2009**, *5*, 3007–3020.
67. Holland, C.; Numata, K.; Rnjak-Kovacina, J.; Seib, F.P. The Biomedical Use of Silk: Past, Present, Future. *Adv. Healthcare Mater.* **2019**, *8*, 1800465.
68. Cao, Y.; Wang, B. Biodegradation of silk biomaterials. *Int. J. Mol. Sci.* **2009**, *10*, 1514–1524. [PubMed]
69. Koh, T.J.; DiPietro, L.A. Inflammation and wound healing: The role of the macrophage. *Expert Rev. Mol. Diagn.* **2011**, *13*, e23.
70. Anderson, J.M.; Rodriguez, A.; Chang, D.T. Foreign body reaction to biomaterials. *Semin. Immunol.* **2008**, *29*, 86–100.
71. Zhao, X. 5-Antibacterial bioactive materials. In *Bioactive Materials in Medicine*; Zhao, X., Courtney, J.M., Qian, H., Eds.; Woodhead Publishing: Cambridge, UK, 2011; pp. 97–123.
72. Bowler, P.G.; Duerden, B.I.; Armstrong, D.G. Wound microbiology and associated approaches to wound management. *Clin. Microbiol. Rev.* **2001**, *14*, 244–269. [PubMed]
73. de Magalhães, A.P.S.A. *Atividade Antimicrobiana em Têxteis*; Faculty of Pharmacy-University of Porto: Porto, Portugal, 2015.
74. Caldeira, E.S.D.A. *Biofuncionalização do Algodão com L-Cisteína: Estudo do Efeito Antibacteriano e dos Mecanismos de Acção contra S. aureus e K. Pneumoniae*; University of Beira Interior: Covilhã, Portugal, 2012.
75. Ahamad, S.I.; KumarVootla, S. Extraction and evaluation of antimicrobial potential of antheraea mylitta silk sericin. *Int. J. Recent Sci. Res.* **2018**, *9*, 23019–23022.
76. Jassim, K.; Al-Saree, O. Study of the antimicrobial activity of silk sericin from silkworm bombyx mori. *Iraqi J. Med. Sci* **2010**, *23*, 130–133.
77. Aramwit, P.; Napavichayanum, S.; Pienpinijtham, P.; Rasmi, Y.; Bang, N. Antibiofilm activity and cytotoxicity of silk sericin against Streptococcus mutans bacteria in biofilm: An in vitro study. *J. Wound Care* **2020**, *29*, S25–S35. [CrossRef]
78. Takechi, T.; Wada, R.; Fukuda, T.; Harada, K.; Takamura, H. Antioxidant activities of two sericin proteins extracted from cocoon of silkworm (Bombyx mori) measured by DPPH, chemiluminescence, ORAC and ESR methods. *Biomed. Rep.* **2014**, *2*, 364–369.
79. Kato, N.; Sato, S.; Yamanaka, A.; Yamada, H.; Fuwa, N.; Nomura, M. Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 145–147.
80. Li, Y.G.; Ji, D.F.; Lin, T.B.; Zhong, S.; Hu, G.Y.; Chen, S. Protective effect of sericin peptide against alcohol-induced gastric injury in mice. *Chin. Med. J.* **2008**, *121*, 2083–2087. [CrossRef] [PubMed]
81. Zhaorigetu, S.; Yanaka, N.; Sasaki, M.; Watanabe, H.; Kato, N. Inhibitory effects of silk protein, sericin on UVB-induced acute damage and tumor promotion by reducing oxidative stress in the skin of hairless mouse. *J. Photochem. Photobiol. B.* **2003**, *71*, 11–17. [CrossRef]

82. Gore, P.M.; Naebe, M.; Wang, X.; Kandasubramanian, B. Progress in silk materials for integrated water treatments: Fabrication, modification and applications. *J. Chem. Eng.* **2019**, *374*, 437–470. [CrossRef]
83. Reddy, M.S.B.; Ponnamma, D.; Choudhary, R.; Sadasivuni, K.K. A Comparative Review of Natural and Synthetic Biopolymer Composite Scaffolds. *Polymers* **2021**, *13*, 1105. [CrossRef]
84. Bhatia, S. Natural Polymers vs. Synthetic Polymer. In *Natural Polymer Drug Delivery Systems: Nanoparticles, Plants, and Algae*; Bhatia, S., Ed.; Springer International Publishing: Cham, Switzerland, 2016; pp. 95–118.
85. Mogoşanu, G.D.; Grumezescu, A.M. Natural and synthetic polymers for wounds and burns dressing. *Int. J. Pharm.* **2014**, *463*, 127–136. [CrossRef]
86. Utech, S.; Boccaccini, A.R. A review of hydrogel-based composites for biomedical applications: Enhancement of hydrogel properties by addition of rigid inorganic fillers. *J. Mater. Sci.* **2016**, *51*, 271–310.
87. Chocholata, P.; Kulda, V.; Babuska, V. Fabrication of Scaffolds for Bone-Tissue Regeneration. *Materials* **2019**, *12*, 568.
88. Bhatt, K.; Eggermont, L.J.; Bencherif, S.A. Chapter Three-Polymeric scaffolds for antitumor immune cell priming. In *Engineering Technologies and Clinical Translation*; Amiji, M.M., Milane, L.S., Eds.; Academic Press: Cambridge, MA, USA, 2022; pp. 63–95.
89. Filippi, M.; Born, G.; Chaaban, M.; Scherberich, A. Natural Polymeric Scaffolds in Bone Regeneration. *Front. Bioeng. Biotechnol.* **2020**, *8*, 474. [CrossRef]
90. Nasr, S.M.; Rabiee, N. Biodegradable Nanopolymers in Cardiac Tissue Engineering: From Concept Towards Nanomedicine. *Int. J. Nanomed.* **2020**, *15*, 4205–4224. [CrossRef] [PubMed]
91. Riha, S.M.; Maarof, M.; Fauzi, M.B. Synergistic Effect of Biomaterial and Stem Cell for Skin Tissue Engineering in Cutaneous Wound Healing: A Concise Review. *Polymers* **2021**, *13*, 1546.
92. Lujerdean, C.; Baci, G.M. The Contribution of Silk Fibroin in Biomedical Engineering. *Insects* **2022**, *13*, 286. [PubMed]
93. Levis, H.J.; Kureshi, A.K.; Massie, I.; Morgan, L.; Vernon, A.J.; Daniels, J.T. Tissue Engineering the Cornea: The Evolution of RAFT. *J. Funct. Biomater.* **2015**, *6*, 50–65. [CrossRef] [PubMed]
94. Rouse, J.G.; van Dyke, M.E. A review of keratin-based biomaterials for biomedical applications. *Materials* **2010**, *3*, 999–1014. [CrossRef]
95. Nishida, A.; Yamada, M.; Kanazawa, T.; Takashima, Y.; Ouchi, K.; Okada, H. Sustained-release of protein from biodegradable sericin film, gel and sponge. *Int. J. Pharm.* **2011**, *407*, 44–52. [PubMed]
96. Yan, C.; Liang, J.; Fang, H.; Meng, X.; Chen, J.; Zhong, Z.; Liu, Q.; Hu, H.; Zhang, X. Fabrication and evaluation of silk sericin-derived hydrogel for the release of the model drug berberine. *Gels* **2021**, *7*, 23. [PubMed]
97. Liu, J.; Qi, C.; Tao, K.; Zhang, J.; Zhang, J.; Xu, L.; Jiang, X.; Zhang, Y.; Huang, L.; Li, Q.; et al. Sericin/Dextran Injectable Hydrogel as an Optically Trackable Drug Delivery System for Malignant Melanoma Treatment. *ACS Appl. Mater. Interfaces* **2016**, *8*, 6411–6422. [CrossRef] [PubMed]
98. Yalcin, E.; Kara, G.; Celik, E.; Pinarli, F.A.; Saylam, G.; Sucularli, C.; Ozturk, S.; Yilmaz, E.; Bayir, O.; Korkmaz, M.H.; et al. Preparation and characterization of novel albumin-sericin nanoparticles as siRNA delivery vehicle for laryngeal cancer treatment. *Prep. Biochem. Biotechnol.* **2019**, *49*, 659–670. [PubMed]
99. Deng, L.; Guo, W.; Li, G.; Hu, Y.; Zhang, L.M. Hydrophobic IR780 loaded sericin nanomicelles for phototherapy with enhanced antitumor efficiency. *Int. J. Pharm.* **2019**, *566*, 549–556. [CrossRef] [PubMed]
100. Xu, S.; Yang, Q.; Wang, R.; Tian, C.; Ji, Y.; Tan, H.; Zhao, P.; Kaplan, D.L.; Wang, F.; Xia, Q. Genetically engineered pH-responsive silk sericin nanospheres with efficient therapeutic effect on ulcerative colitis. *Acta Biomater.* **2022**, *144*, 81–95. [CrossRef]
101. Zhang, Y.; Kim, I.; Lu, Y.; Xu, Y.; Yu, D.-G.; Song, W. Intelligent poly(l-histidine)-based nanovehicles for controlled drug delivery. *J. Control. Release* **2022**, *349*, 963–982.
102. Tang, Y.; Varyambath, A.; Ding, Y.; Chen, B.; Huang, X.; Zhang, Y.; Yu, D.-G.; Kim, I.; Song, W. Porous organic polymers for drug delivery: Hierarchical pore structures, variable morphologies, and biological properties. *Biomater. Sci.* **2022**, *10*, 5369–5390. [CrossRef] [PubMed]
103. Wang, Z.; Zhang, Y.; Zhang, J.; Huang, L.; Liu, J.; Li, Y.; Zhang, G.; Kundu, S.C.; Wang, L. Exploring natural silk protein sericin for regenerative medicine: An injectable, photoluminescent, cell-adhesive 3D hydrogel. *Sci. Rep.* **2014**, *4*, 7064. [PubMed]
104. Ogino, M.; Tanaka, R.; Hattori, M.; Yoshida, T.; Yokote, Y.; Takahashi, K. Interfacial Behavior of Fatty-Acylated Sericin Prepared by Lipase-Catalyzed Solid-Phase Synthesis. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 66–75. [PubMed]
105. Huang, L.; Tao, K.; Liu, J.; Qi, C.; Xu, L.; Chang, P.; Gao, J.; Shuai, X.; Wang, G.; Wang, Z.; et al. Design and Fabrication of Multifunctional Sericin Nanoparticles for Tumor Targeting and pH-Responsive Subcellular Delivery of Cancer Chemotherapy Drugs. *ACS Appl. Mater. Interfaces* **2016**, *8*, 6577–6585.
106. Hu, D.; Xu, Z.; Hu, Z.; Hu, B.; Yang, M.; Zhu, L. pH-Triggered Charge-Reversal Silk Sericin-Based Nanoparticles for Enhanced Cellular Uptake and Doxorubicin Delivery. *ACS Sustain. Chem. Eng.* **2017**, *5*, 1638–1647.
107. Oh, H.; Lee, J.Y.; Kim, A.; Ki, C.S.; Kim, J.W.; Park, Y.H.; Lee, K.H. Preparation of silk sericin beads using LiCl/DMSO solvent and their potential as a drug carrier for oral administration. *Fibers Polym.* **2007**, *8*, 470–476.
108. Wang, P.; He, H.; Cai, R.; Tao, G.; Yang, M.; Zuo, H.; Umar, A.; Wang, Y. Cross-linking of dialdehyde carboxymethyl cellulose with silk sericin to reinforce sericin film for potential biomedical application. *Carbohydr. Polym.* **2019**, *212*, 403–411.
109. Vineis, C.; Maya, I.C.; Mowafi, S.; Varesano, A.; Ramírez, D.O.S.; Taleb, M.A.; Tonetti, C.; Guarino, V.; El-Sayed, H. Synergistic effect of sericin and keratin in gelatin based nanofibers for in vitro applications. *Int. J. Biol. Macromol.* **2021**, *190*, 375–381.

110. Noosak, C.; Jantorn, P.; Meesane, J.; Voravuthikunchai, S.; Saeloh, D. Dual-functional bioactive silk sericin for osteoblast responses and osteomyelitis treatment. *PLoS ONE* **2022**, *17*, e0264795.
111. Veiga, A.; Castro, F.; Rocha, F.; Oliveira, A.L. Protein-Based Hydroxyapatite Materials: Tuning Composition toward Biomedical Applications. *ACS Appl. Bio Mater.* **2020**, *3*, 3441–3455.
112. Ielo, I.; Calabrese, G.; Luca, G.D.; Conoci, S. Recent Advances in Hydroxyapatite-Based Biocomposites for Bone Tissue Regeneration in Orthopedics. *Int. J. Mol. Sci.* **2022**, *23*, 9721. [PubMed]
113. Ming, P.; Rao, P.; Wu, T.; Yang, J.; Lu, S.; Yang, B.; Xiao, J.; Tao, G. Biomimetic Design and Fabrication of Sericin-Hydroxyapatite Based Membranes With Osteogenic Activity for Periodontal Tissue Regeneration. *Front. Bioeng. Biotechnol.* **2022**, *10*, 899293. [PubMed]
114. Jiang, L.-B.; Ding, S.-L.; Ding, W.; Su, D.-H.; Zhang, F.-X.; Zhang, T.-W.; Yin, X.-F.; Xiao, L.; Li, Y.-L.; Yuan, F.-L.; et al. Injectable sericin based nanocomposite hydrogel for multi-modal imaging-guided immunomodulatory bone regeneration. *J. Chem. Eng.* **2021**, *418*, 129323.
115. Altman, G.H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R.L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D.L. Silk based biomaterials. *Biomaterials* **2003**, *24*, 401–416. [PubMed]
116. Lamboni, L.; Li, Y.; Liu, J.; Yang, G. Silk Sericin-Functionalized Bacterial Cellulose as a Potential Wound-Healing Biomaterial. *Biomacromolecules* **2016**, *17*, 3076–3084. [CrossRef] [PubMed]
117. Chouhan, D.; Mandal, B.B. Silk biomaterials in wound healing and skin regeneration therapeutics: From bench to bedside. *Acta Biomater.* **2020**, *103*, 24–51. [PubMed]
118. Ai, L.; He, H.; Wang, P.; Cai, R.; Tao, G.; Yang, M.; Liu, L.; Zuo, H.; Zhao, P.; Wang, Y. Rational design and fabrication of ZnONPs functionalized sericin/PVA antimicrobial sponge. *Int. J. Mol. Sci.* **2019**, *20*, 4796. [CrossRef] [PubMed]
119. Baptista-Silva, S.; Borges, S.; Costa-Pinto, A.R.; Costa, R.; Amorim, M.; Dias, J.R.; Ramos, Ó.; Alves, P.; Granja, P.L.; Soares, R.; et al. In Situ Forming Silk Sericin-Based Hydrogel: A Novel Wound Healing Biomaterial. *ACS Biomater. Sci. Eng.* **2021**, *7*, 1573–1586. [CrossRef]
120. Sapru, S.; Das, S.; Mandal, M.; Ghosh, A.K.; Kundu, S.C. Prospects of nonmulberry silk protein sericin-based nanofibrous matrices for wound healing–In vitro and in vivo investigations. *Acta Biomater.* **2018**, *78*, 137–150.
121. Tao, G.; Cai, R.; Wang, Y.; Liu, L.; Zuo, H.; Zhao, P.; Umar, A.; Mao, C.; Xia, Q.; He, H. Bioinspired design of AgNPs embedded silk sericin-based sponges for efficiently combating bacteria and promoting wound healing. *Mater. Des.* **2019**, *180*, 107940. [CrossRef]
122. Chen, C.S.; Zeng, F.; Xiao, X.; Wang, Z.; Li, X.L.; Tan, R.W.; Liu, W.Q.; Zhang, Y.S.; She, Z.D.; Li, S.J. Three-Dimensionally Printed Silk-Sericin-Based Hydrogel Scaffold: A Promising Visualized Dressing Material for Real-Time Monitoring of Wounds. *ACS Appl. Mater. Interfaces* **2018**, *10*, 33879–33890. [CrossRef] [PubMed]
123. Song, Y.; Zhang, C.; Zhang, J.; Sun, N.; Huang, K.; Li, H.; Wang, Z.; Huang, K.; Wang, L. An injectable silk sericin hydrogel promotes cardiac functional recovery after ischemic myocardial infarction. *Acta Biomater.* **2016**, *41*, 210–223. [PubMed]
124. Aramwit, P.; Keongamaroon, O.; Siritientong, T.; Bang, N.; Supasyndh, O. Sericin cream reduces pruritus in hemodialysis patients: A randomized, double-blind, placebo-controlled experimental study. *BMC Nephrol.* **2012**, *13*, 119. [CrossRef]
125. Aramwit, P.; Palapinyo, S.; Srichana, T.; Chottanapund, S.; Muangman, P. Silk sericin ameliorates wound healing and its clinical efficacy in burn wounds. *Arch. Dermatol. Res.* **2013**, *305*, 585–594.
126. Siritientong, T.; Angspatt, A.; Ratanavaraporn, J.; Aramwit, P. Clinical potential of a silk sericin-releasing bioactive wound dressing for the treatment of split-thickness skin graft donor sites. *Pharm. Res.* **2014**, *31*, 104–116. [CrossRef] [PubMed]
127. Napavichayanun, S.; Yamdech, R.; Aramwit, P. The safety and efficacy of bacterial nanocellulose wound dressing incorporating sericin and polyhexamethylene biguanide: In vitro, in vivo and clinical studies. *Arch. Dermatol. Res.* **2016**, *308*, 123–132.
128. Kim, J.-W.; Jo, Y.-Y.; Kim, J.-Y.; Oh, J.-h.; Yang, B.-E.; Kim, S.-G. Clinical Study for Silk Mat Application into Extraction Socket: A Split-Mouth, Randomized Clinical Trial. *Appl. Sci.* **2019**, *9*, 1208.
129. Huang, P.L. A comprehensive definition for metabolic syndrome. *Dis. Model Mech.* **2009**, *2*, 231–237.
130. Okazaki, Y.; Kakehi, S.; Xu, Y.; Tsujimoto, K.; Sasaki, M.; Ogawa, H.; Kato, N. Consumption of sericin reduces serum lipids, ameliorates glucose tolerance and elevates serum adiponectin in rats fed a high-fat diet. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 1534–1538.
131. Kunz, R.I.; Capelassi, A.N.; Alegre-Maller, A.C.P.; Bonfleur, M.L.; Ribeiro, L.d.F.C.; Costa, R.M.; Natali, M.R.M. Sericin as treatment of obesity: Morphophysiological effects in obese mice fed with high-fat diet. *Einstein* **2019**, *18*, 1–9.
132. Song, C.; Yang, Z.; Zhong, M.; Chen, Z. Sericin protects against diabetes-induced injuries in sciatic nerve and related nerve cells. *Neural Regen. Res.* **2013**, *8*, 506–513. [PubMed]
133. Zhao, J.G.; Wang, H.Y.; Wei, Z.G.; Zhang, Y.Q. Therapeutic effects of ethanolic extract from the green cocoon shell of silkworm Bombyx mori on type 2 diabetic mice and its hypoglycaemic mechanism. *Toxicol. Res.* **2019**, *8*, 407–420. [CrossRef] [PubMed]
134. Dong, X.; Zhao, S.X.; Yin, X.L.; Wang, H.Y.; Wei, Z.G.; Zhang, Y.Q. Silk sericin has significantly hypoglycaemic effect in type 2 diabetic mice via anti-oxidation and anti-inflammation. *Int. J. Biol. Macromol.* **2020**, *150*, 1061–1071. [PubMed]
135. Wei, Z.Z.; Weng, Y.J.; Zhang, Y.Q. Investigation of the Repairing Effect and Mechanism of Oral Degraded Sericin on Liver Injury in Type II Diabetic Rats. *Biomolecules* **2022**, *12*, 444. [PubMed]
136. Tengattini, S.; Orlandi, G.; Perteghella, S.; Bari, E.; Amadio, M.; Calleri, E.; Massolini, G.; Torre, M.L.; Temporini, C. Chromatographic profiling of silk sericin for biomedical and cosmetic use by complementary hydrophylic, reversed phase and size exclusion chromatographic methods. *J. Pharm. Biomed. Anal.* **2020**, *186*, 113291. [PubMed]

137. Johnson, W.; Bergfeld, W.F.; Belsito, D.V.; Hill, R.A.; Klaassen, C.D.; Liebler, D.C.; Marks, J.G.; Shank, R.C.; Slaga, T.J.; Snyder, P.W.; et al. Safety Assessment of Silk Protein Ingredients as Used in Cosmetics. *Int. J. Toxicol.* **2020**, *39*, 127–144.
138. Wang, W.H.; Lin, W.S.; Shih, C.H.; Chen, C.Y.; Kuo, S.H.; Li, W.L.; Lin, Y.S. Functionality of silk cocoon (*Bombyx mori* l.) sericin extracts obtained through high-temperature hydrothermal method. *Materials* **2021**, *14*, 5314. [CrossRef]
139. Camargo, F.B.; Minami, M.M.; Rossan, M.R.; Magalhães, W.V.; Ferreira, V.T.P.; Campos, P.M.B.G.M. Prevention of chemically induced hair damage by means of treatment based on proteins and polysaccharides. *J. Cosmet. Dermatol.* **2022**, *21*, 827–835.
140. He, M.; Hu, H.; Wang, P.; Fu, H.; Yuan, J.; Wang, Q.; Fan, X. Preparation of a bio-composite of sericin-g-PMMA via HRP-mediated graft copolymerization. *Int. J. Biol. Macromol.* **2018**, *117*, 323–330.
141. Hu, H.; Wang, L.; Xu, B.; Wang, P.; Yuan, J.; Yu, Y.; Wang, Q. Construction of a composite hydrogel of silk sericin via horseradish peroxidase-catalyzed graft polymerization of poly-PEGDMA. *J. Biomed. Mater. Res. Part B Appl. Biomater.* **2020**, *108*, 2643–2655. [CrossRef]

2. Objectives

This work aimed to characterize the biochemical and bioactivities of sericin extracted by hot water at 120 ºC for 30 minutes from *Bombyx mori* cocoons with different origins (Bragança and Castelo Branco, Portugal) and different seasons.

Thus, the specific objectives are:

- Determine the amino acid profile of each of the samples by high performance liquid chromatography-FLD (HPLC-FLD);
- Characterize the bioactivities of the samples, namely:
  - antiproliferative by SRB assay, using human tumor cell lines;
  - antioxidant by thiobarbituric acid reactive substances (TBARS) method and cellular antioxidant activity (CAA) assay;
  - anti-inflammatory by nitric oxide synthesis inhibition, using a macrophage cell line (RAW 264.7)
  - antimicrobial by microdilution method;
  - anticoagulant activities by activated partial thromboplastin clotting time (APTT) assays, using adult mice plasma.

Thus, the working plan of this work is schematized in Figure 1.

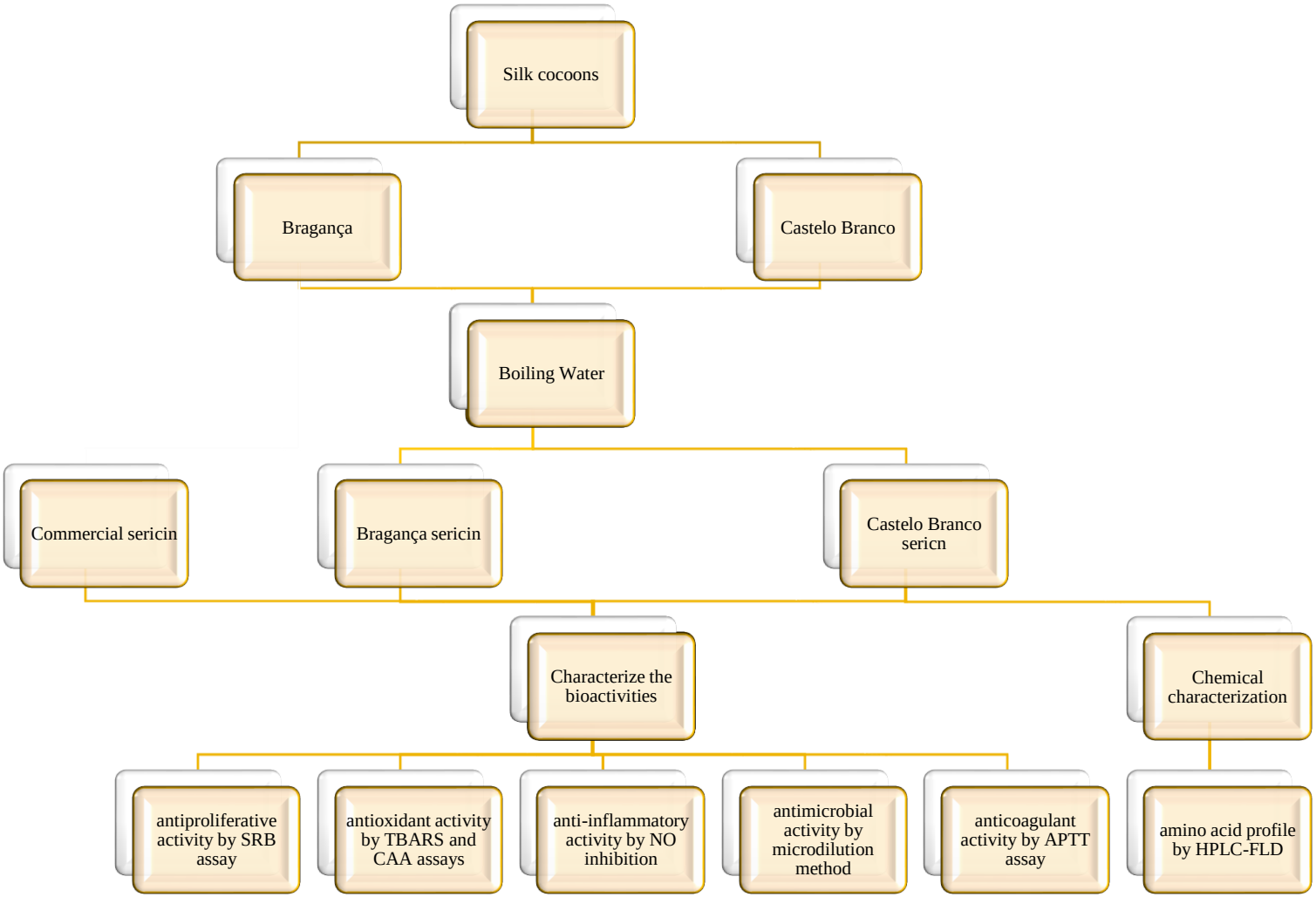

Figure 1- Schematic representation of the working plan.

3. Materials and methods

3.1 Standards and reagents

Fetal bovine serum (FBS), glutamine, penincillin solution, streptomycin solution, and RPMI-1640 medium, hanks' balanced salt solution (HBSS) were obtained from Hyclone (Logan, UT, USA). DMEM medium was obtained from Gibco Invitrogen Life Technologies (Carlsbad, Califórnia, EUA). 2.2 2'-azobis(2-methylpropionamide) dihydrochloride (AAPH) solution (600 μM) was purchased from Panreac Applichem (Barcelona, Spain). Acetic acid, FeSO4, ellipticine, sulforhodamine B (SRB), nitrite calibration curve, trypan blue, trichloroacetic acid (TCA) and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tris-(hidroximetil)-aminometano)-HCl (Tris-HCl), thiobarbituric acid (TBA), quercetin, liposaccharide solution (LPS), dexamethasone, dimethyl sulfoxide (DMSO), p-iodonitrotetrazolium chloride (INT), ascorbic acid methicillin and ampicillin were acquired from Sigma-Aldrich (St Louis, MO, USA). 2',7'-dichlorohydrofluorescein (DCFH) was obtained from Acros Organics (Geel, Belgium). The Griess Reagent System kit (nitrophenamide, ethylenediamine, and nitrite solutions) were from Promega, Madison,WI, USA. The fungicide ketoconazole, Tween 80 and Malt Extract Broth (MEB) were from Frilabo, Porto, Portugal. The Tryptic Soy Broth (TSB) was from Biomerieux (Marcy l'Etoile, France). The Blood agar (7% sheep blood) was obtained from Liofilchem (Roseto Degli Abruzzi, Italy). APTT and $CaCl_2$ was obtained from HORIBA ABX SAS, Portugal Branch. Ortho-phthalaldehyde (OPA), fluorenylmethyloxycarbonyl chloride (FMOC-Cl), 3-mercaptopropionic acid derivatizer (3-MPA), acetate buffer solution and sodium acetate were acquired from Sigma-Aldrich (Darmstad, Germany). The amino acid standards: Aspartic acid (Asp), Arginine (Arg), Asparagine (Asn), Cystine (Cys), Hydroxyproline (Hyp), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Proline (Pro), Serine (Ser), Taurine (Tau), Threonine (Thr) and Valine (Val) were purchased from Sigma-Aldrich. The amino acid standards: Glutamic acid (Glu), Alanine (Ala), Phenylanine (Phe) and Glycine (Gly) were purchased from Merck. And the amino acid standards Glutamine (Gln) and Tryptophan (Trp) were acquired from Fluka. The triethylamine (TEA) was obtained from Carlo Erba (Val de Reuil, France). Acetic acid was purchased from Panreac (Barcelona, Spain).

3.2 Sampling

The sericin was extracted from *Bombyx mori* cocoons that were collected in different places and dates in Portugal. The Casa da Seda (Centro de Ciência Viva de Bragança) and the Museu da Seda de Freixo de Espada à Cinta, provided cocoons originating from Bragança. Cocoons originating from Castelo Branco were furnished by the Associação Portuguesa de Pais e Amigos do Cidadão Deficiente Mental (APPACDM). The commercial samples (CS) of sericin were acquired on Sigma-Aldrich and FUJIFILM Wako Chemicals (Table 1).

Table 1- Coding information of sericin.

Date	Code	Location/ Provider
2019	S1	Castelo Branco
2019	S2	Bragança
2021	S3	Bragança
2022	S4	Bragança
2023	CS1	Sigma-Aldrich
2023	CS2	FUJIFILM Wako Chemicals

3.3 Extraction process

The extraction process was adapted from Da Silva et al., (2014). First, cocoons were manually cleaned and divided into fragments measuring around 1 cm^2. They were then rinsed three times with deionized water. For the degumming process, the cocoons were dried at 50 °C, weighed, and submerged in ultrapure water at a ratio of 1.5% w/V. A cycle of autoclave (120 ºC for 30 minutes) was used to extract the aqueous sericin solution. The sericin solution was then filtered to remove the fibers, stored in a sealed container, and then it was frozen at -80 °C. By lyophilizing the sericin solutions, sericin powders were obtained, and they were kept at 4° C until usage (Sahu et al., 2016). The procedure is shown in the Figure 2.

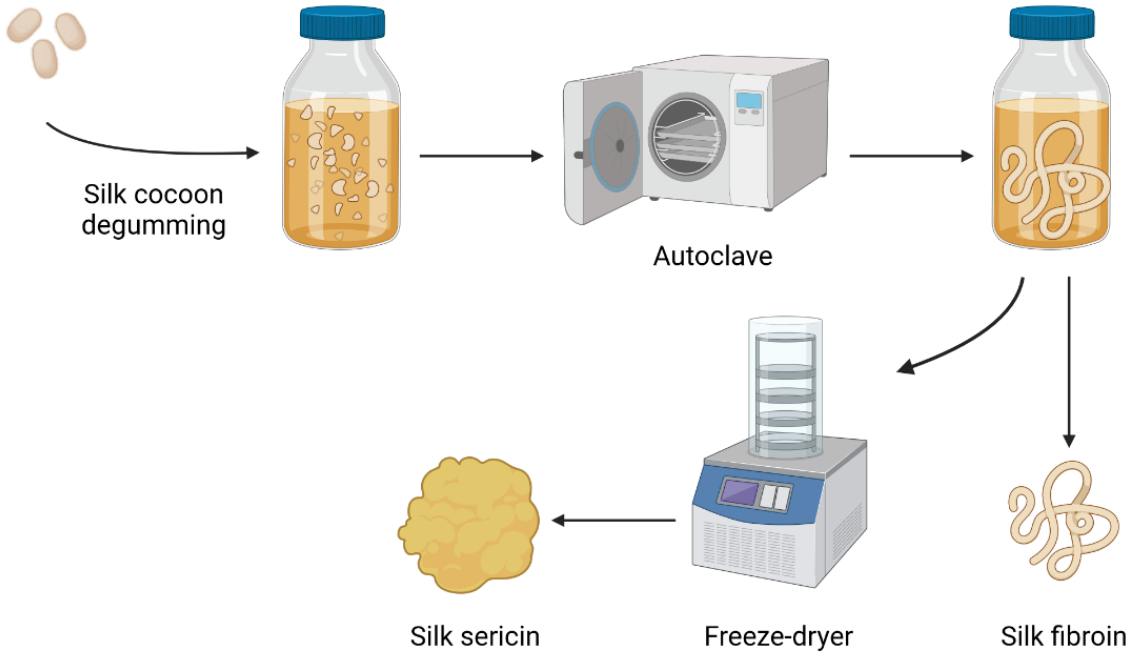

Figure 2- Extraction process of sericin, developed using © 2023 BioRender, own work.

3.4 Chemical characterization

Proteins are constituted of amino acids (Lv et al., 2022), which are classified as either nutritionally essential (indispensable) or non-essential (dispensable) for mammals, birds, and fish (Le Plénier et al., 2012; Liu et al., 2012; Obayashi et al., 2012). Nutritionally essential amino acids include lysine (Lys), tryptophan (Trp), phenylalanine (Phe), methionine (Met), threonine (Thr), isoleucine (Ile), leucine (Leu), and valine (Val) (Lv et al., 2022). These amino acids cannot be synthesized de novo in animal cells or are insufficiently synthesized by the organism for maintenance, growth, development, and health, and must be provided in the diet (Wu, 2010). On the other hand, nutritionally non-essential amino acids (NEAA) are those that can be synthesized de novo in sufficient amounts by the organism, meeting their needs for maintenance, growth, development, and health (Wu et al., 2013).

Amino acid analysis was performed using high-performance liquid chromatography (HPLC) on a Shimadzu system (Tokyo, Japan), which included an SCL-40 system controller, LC-40D pump, CTO-40C column oven, four-channel low-pressure gradient unit, SIL-40C automatic injector, DGU-405 5-channel degasser, and RF-20Axs fluorescence detector (FLD).

All the samples were analyzed with or without a hydrolysis process, as described by (Malmer & Schroeder, 1990). Two mL of methanesulfonic acid (MSA) 4 M with 0.2% tryptamine (to preserve the tryptophan concentration during acid hydrolysis) was added to 16 mm × 25 mm screw-cap tubes containing 20 mg of sample. The tubes were sealed with nitrogen, heated to 110 °C for a whole day in an electric oven, allowed to cool, and then their contents were vacuum-filtered through Whatman No. 4 paper. The filtrate was diluted to 4 mL in a glass vial with ultrapure water, and 1 mL of the resulting liquid was membrane-filtered (using Millipore® 0.45 µm regenerated cellulose membranes). Reverse phase high-performance liquid chromatography (RP-HPLC) with fluorescence detection was then used to determine the total amino acid content. Two hydrolyses of each sample were performed.

The HPLC-FLD analysis of total and free amino acids involves a derivatization step using OPA and FMOC-CI, as described by (Goodno et al., 1981) and adapted to a micro-well plate (Arrebas-Lorenzo & Morales, 2009). The derivatization process was carried out in a 1.5 mL amber vial and involves adding 80 µL of the sample or standard to 200 µL of the internal standard solution (norvaline), 200 µL of borate buffer (80 mM boric acid, pH = 10.4, VWR), 80 µL of the OPA/ 3-MPA derivatized, and 8 µL of the FMOC-Cl derivatized dissolved in acetonitrile.

The derivatized compounds were separated using a Kinetex Core-Shell C18 column (5 µm, 150 mm X 4.6 mm, Phenomenex, Torrance, CA, USA), with a 10 µL sample injected. The mobile phase was prepared from a gradient of two solutions: A (25 mM acetate buffer solution - 2.05 g sodium acetate in 1 L of water- and 0.05% TEA adjusted to pH 7.2 with concentrated acetic acid) and B (mixture of water: acetonitrile (Chromasolv, VWR): methanol (HPLC gradient, VWR) (20:40:40, v/v)).

Amino acid detection was carried out by fluorescence and was measured for amino acid derivatives and OPA-3MPA (λex = 340 nm, λem = 450 nm), FMOC-Cl derivatives (λex = 237 nm, λem = 340 nm, greater sensitivity for proline), with a change in λ at 26.25 min. The data were presented in mg/L, with norvaline serving as the standard. All samples were digested and extracted in duplicate and injected in duplicate.

The methodology was validated for analytical parameters such as retention times (RT), working range, linearity, limits of detection (LOD) and quantification (LOQ), with results of the parameters displayed in Table 2.

Table 2- Analytical parameters for the amino acids analyzed.

Amino acids	**RT (min)**	**Working range (mg/L)**	**Equation of the line (n= 12)**	**R^2**	**LOD (mg/L)**	**LOQ (mg/L)**
Cys	5.0	0.029-60	y = 644x - 1147	0.9996	3.91E-02	1.30E-01
Asp	5.4	0.015-30	y = 1315753x - 195542	0.9994	6.44E-04	2.15E-03
Glu	5.6	0.015-30	y = 1061700x - 59384	0.9997	7.98E-04	2.66E-03
Gln	6.5	0.015-15	y = 1517318x - 557190	0.9970	3.95E-04	1.32E-03
Ser	7.5	0.015-30	y = 248937x + 116436	0.9964	3.40E-03	1.13E-02
Asn	8.5	0.015-30	y = 1517318x - 557190	0.9970	5.58E-04	1.86E-03
Gly1	9.4	0.015-15	y = 669116x + 184582	0.9967	1.27E-03	4.22E-03
His	10.7	0.029-60	y = 599260x + 4919	0.9999	1.41E-03	4.71E-03
Thr	11.3	0.029-30	y = 1903888x - 730794	0.9967	4.45E-04	1.48E-03
Tau	11.9	0.015-15	y = 683418x + 178817	0.9979	1.24E-03	4.13E-03
Arg	12.9	0.015-30	y = 1619230x - 289464	0.9996	5.23E-04	1.74E-03
Ala	13.4	0.015-15	y = 3317934x - 450259	0.9989	2.55E-04	8.51E-04
Lys	14.8	0.12-30	y = 78752x + 44609	0.9976	1.08E-02	3.59E-02
Tyr	16.4	0.47-30	y = 692276x - 7926	0.9966	1.22E-03	4.08E-03
Val	19.6	0.015-15	y = 4353865x - 288779	0.9988	1.95E-04	6.49E-04
Met	20.1	0.015-30	y = 2206023x - 598103	0.9984	3.84E-04	1.28E-03
Trp	22.2	0.015-30	y = 1804957x + 154070	0.9993	4.69E-04	1.56E-03
Phe	22.8	0.015-30	y = 2201652x - 186046	0.9987	3.85E-04	1.28E-03
Ile	23.2	0.015-30	y = 2043967x - 204665	0.9997	4.14E-04	1.38E-03
Leu	24.5	0.015-15	y = 2997934x - 174370	0.9990	2.83E-04	9.42E-04
Hyp	26.7	0.029-60	y = 52663x + 46186	0.9978	1.61E-02	5.36E-02
Pro	30.6	0.029-60	y = 40629x + 8195	0.9991	2.09E-02	6.95E-02

3.5 Bioactivity assessment

3.5.1 Cell lines and culture conditions for cellular assays

In order to realize the different in vitro bioactivities, were used tumor cell lines and non-tumor cell lines: AGS (epithelial cells from the stomach tissue with gastric adeno-carcinoma), CaCo-2 (epithelial cells isolated from colon tissue derived from a male with colorectal adenocarcinoma), Vero (epithelial cells isolated from kidney tissue derived from a normal, adult African green monkey), RAW 264.7 (macrophage cell line,

established from a tumor in a male mouse induced with the Abelson murine leukemia virus), were purchased from the European Collection of Authenticated Cell Cultures (ECACC), MCF-7 (epithelial cells from the breasts with ductal carcinoma), HeLa (epithelial cell from the cervix with adenocarci-noma), NCI-H460 (epithelial cells from the pleural fluid with large cell lung cancer), were acquired from the Leibniz-Institute DSMZ– German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany). All the cell lines were maintained in RPMI-1640 medium, supplemented with 10% FBS, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL), individually, with the exception of Vero and RAW 246.7 maintained in DMEM medium, obtained from Gibco Invitrogen Life Technologies (Carlsbad, Califórnia, EUA), supplemented with FBS (10%), glutamine and antibiotics. Only when the cells had achieved 70 to 80% confluence could they be utilized, until that, the culture flasks were incubated in an incubator under a humid atmosphere, at 37ºC and with 5% CO_2 (Heal Force CO_2 Incubator, Shanghai Lishen Scientific Equioment Co, Ltd.).

3.5.2 Antiproliferative activity

To prepare stock solutions with a 16 mg/mL concentration, 8 mg of each sericin sample was weighed and dissolved in 0.5 mL of sterilizable H_2O. From stock solutions, four concentrations between 1.6 and 0.025 mg/mL were achieved through a series of dilutions. In 96-well microplates were incubated 20 µL of each sample, in duplicate, with 190 µL of cell suspension of the cell lines tested, for 72 hours, at 37ºC and with 5% CO_2, in a humid atmosphere, after checking the adherence of the cells. The concentration tested for cell suspension was 10,000 cells/well.

The antiproliferative activities of sericin samples have been evaluated by SRB method as described by (Skehan et al., 1990) and compared with commercial standards. Five human tumor cell lines were used: AGS, Caco-2, HeLa, MCF-7 and NCI-H460. A non-tumor cell line, Vero, was also used.

To fix the cells, 100 µL of previously cooled TCA (10% w/v) was added, and the plates were incubated at 4 ºC for 1 hour. The SRB solution (0.057%, w/v; 100 µL), which was added after the plates had been washed and dried with water, was allowed to stand at room temperature for 30 minutes. Plates were cleaned three times with a 1% v/v acetic

acid solution to removed non-adhered SRB, and then they were taken to dry. A microplate reader (Synergy H1, BioTek Instruments, Winooski, Vermont, USA) was used to measure the absorbance at a wavelength of 540 nm after solubilizing an adhered SRB with Tris (10 mM, 200 µL). Ellipticine (10 mM) was used as a positive control. The percentage of extract concentration that inhibits cell growth in 50% (GI_{50} in µg/mL) represents the way the results are discussed. In Figure 3 is possible to see the procedure used.

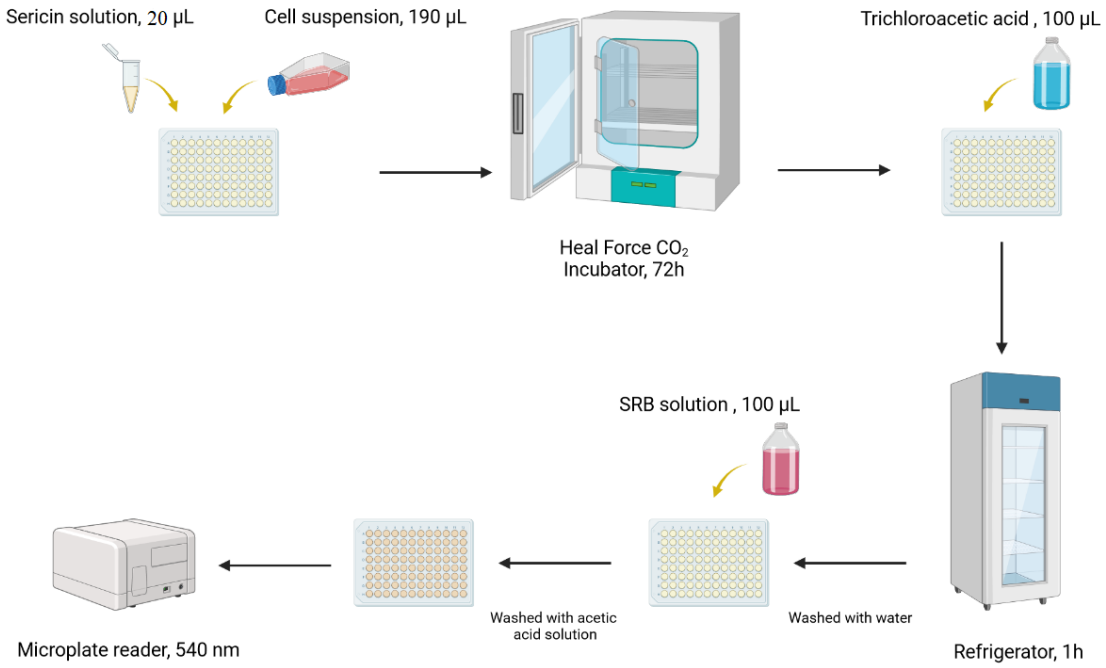

Figure 3- The SRB assay for analyze antiproliferative activity, developed using © 2023 BioRender, own work.

3.5.3 Antioxidant activity

The definition of antioxidant activity includes the ability to protect the biological target, at low concentration (Halliwell et al., 1995). Antioxidant substances are divided into primary and secondary categories. Primary antioxidants can inhibit or retard oxidation by neutralizing free radicals through the donation of hydrogen atoms or electrons, transforming them into more stable compounds. On the other hand, secondary antioxidants operate through various mechanisms, such as binding to metal ions, scavenging oxygen, transforming hydroperoxides into non-radical substances, absorbing UV radiation, or deactivating singlet oxygen (Maisuthisakul et al., 2007).

A variety of methods are used to evaluate the antioxidant capacity of compounds, including colorimetry, spectrophotometry, chromatography, fluorimetry, and photometry (S. Kumar et al., 2017). Three processes are used in Chemical Antioxidant Methods: chelation of transition metals, hydrogen atom transfer (HAT), and single electron transfer (SET). Ferric reducing antioxidant power (FRAP), iron-reducing capacity (IRC), total reducing capacity (TRC), hydroxyl radical antioxidant capacity (HORAC), and cupric reducing antioxidant capacity (CUPRAC) are some of the SET techniques. HAT techniques including the β-carotene bleaching test, lipid peroxidation inhibition (such as TBARS), and oxygen radical absorbance capacity (ORAC) (Romulo, 2020). Certain techniques, such radical scavenging of DPPH and ABTS radicals, combine both HAT and SET processes (Granato et al., 2018). By other hand, the cellular antioxidant activity (CAA) method is a method that evaluates the antioxidant activity of a compound by analyzing its interaction with complex enzymatic reactions in a biological system (Honzel et al., 2008).

In this study, the antioxidant activity of sericin samples was determined based on the inhibition of lipid peroxidation and by thiobarbituric acid reactive substances (TBARS) method, as described by Barros et al., (2008), that allows evaluate sericin potential to react with malondialdehyde (MDA), a breakdown product of unsaturated fatty acid endoperoxide resulting from lipid substrate oxidation, and the prevention the oxidation of intracellular dihydrodichlorofluorescein (DCFH2) by cellular antioxidant activity (CAA) method, as described by Wolfe & Liu, (2007), to our knowledge these methods have not yet been tested.

3.5.3.1 Thiobarbituric acid reactive substances (TBARS)

The extracts' capacity to prevent the production of malondialdehyde and other TBARS is assessed using the TBARS method. Porcine (Sus scrofa) brain tissues were used as the oxidizable substrate in the present study (Mandim et al., 2020).

The pig brain, obtained from official slaughtering animals (Local butcher shop, Bragança, Portugal), was homogenized with a Tris-HCl buffer (20 mM, pH 7.4) to obtain a 1:2 (w/v) brain tissue homogenate. This homogenate was then centrifuged at 3500 rpm for 10 minutes.

Sericin samples weighing 140 mg each were diluted in 2 mL of distilled water to create stock solutions with a concentration of 70 mg/mL. After a series of dilutions, six concentrations between 2.18 mg/mL and 70 mg/mL were obtained from stock solutions.

At 200 µL of each dilution was added 100 µL of brain suspension supernatant, 100 µL of ascorbic acid (0.1 mM) and 100 µL of $FeSO_4$ (10 µm) at 37 °C for 1 h. TCA (28% w/v, 500 µL) was added to stop the reaction, followed by TBA (2%, w/v, 380 µL), the mixture was heated for 20 minutes at 80 °C. To determine the color intensity of the MDA-TBA complex in the supernatant, its absorbance at 532 nm was measured after centrifugation at 3000 rpm for 10 min to remove the precipitated protein. The following formula was used to obtain the inhibition percent:

$$\%\ lipid\ peroxidation\ inhibition = \frac{A - B}{A}\ x\ 100\%$$

where A and B were the absorbance of the control and the compound solution, respectively. Tris-HCl buffer (20 mM, pH 7.4) and distilled water was used as a control. In Figure 4 is possible to see the procedure used.

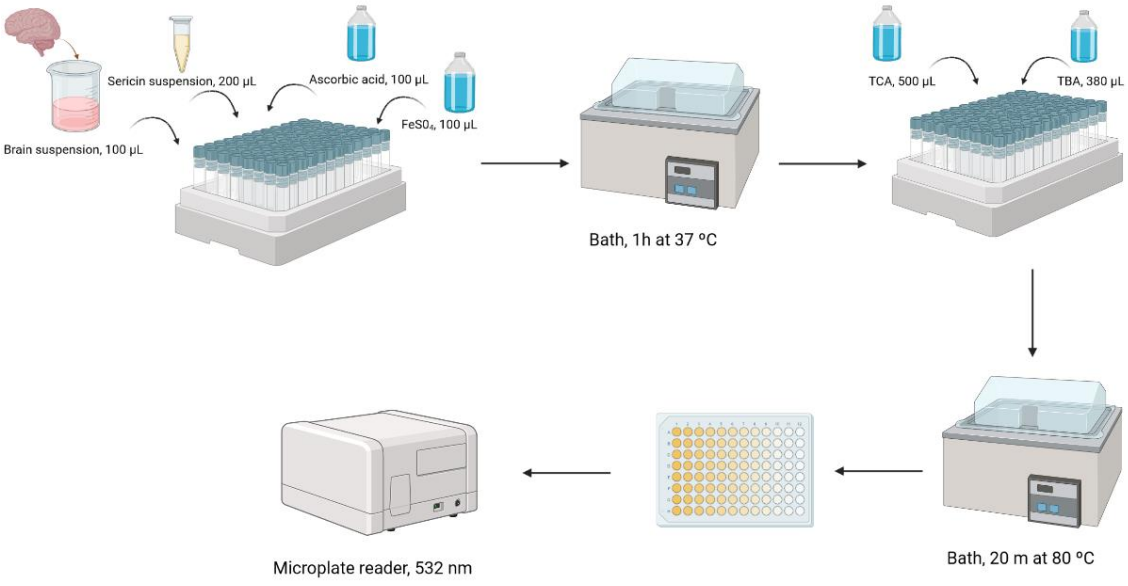

Figure 4- TBARS assay for analyze antioxidant potential, developed using © 2023 BioRender, own work.

3.5.3.2 Cellular antioxidant activity (CAA)

By other hand, the CAA method is based on measuring the ability of compounds to prevent the oxidation of intracellular dihydrodichlorofluorescein (DCFH2), which is easily oxidizable to fluorescent dichlorofluorescein (DCF) by peroxyl radicals (ROO•), in order to identify intracellular reactive oxygen and nitrogen species (ROS/RNS) (de la Fuente et al., 2022).

The samples were dissolved in H_2O to a concentration of 8 mg/mL and then successive dilutions were made with DCFH prepared with ethanol and diluted with HBSS (50 μM) to obtain the concentrations to be tested (500 - 2000 μg/ mL).

A cell scraper was used to separate mouse macrophages (RAW 246.7 cell line), and the content was transferred to a falcon. The solution was centrifuged for 5 minutes at 1200 rpm. The medium was discarded, and the amount of new medium was added to obtain a cell density of 70,000 cells/mL. A black microplate with clear-bottom (SPL Lifesciences) was used to inoculate an aliquot of the prepared solution (300 μL) and incubated for 48 h. After the incubation period, the medium was discarded and the cells were washed with 100 μL HBSS twice, treated with 200 μL of the different extract concentrations, and incubated for 1 hour. Afterward, the cells were washed once more with 100 μL HBSS twice and 100 μL of AAPH solution (600 μM) was added. Fluorescence was read every 5 minutes for 1 hour (Biotek Synergy H1 microplate reader) at 470 nm excitation and 530 nm emission. Quercetin (0.3 μg/mL) was used as a positive control, and dichlorohydroflurescein and DMEM culture medium were used as a negative control. The following formula was used to obtain the inhibition of the oxidation reaction percentage:

$$CAA\ unit = 100 - \left(\frac{\int AUCs}{\int AUCc}\right) x\ 100$$

where ∫ AUCs is the integrated area under the sample fluorescence versus time curve and ∫ AUCc is the integrated area from the control curve. In Figure 5 is possible to see the procedure used.

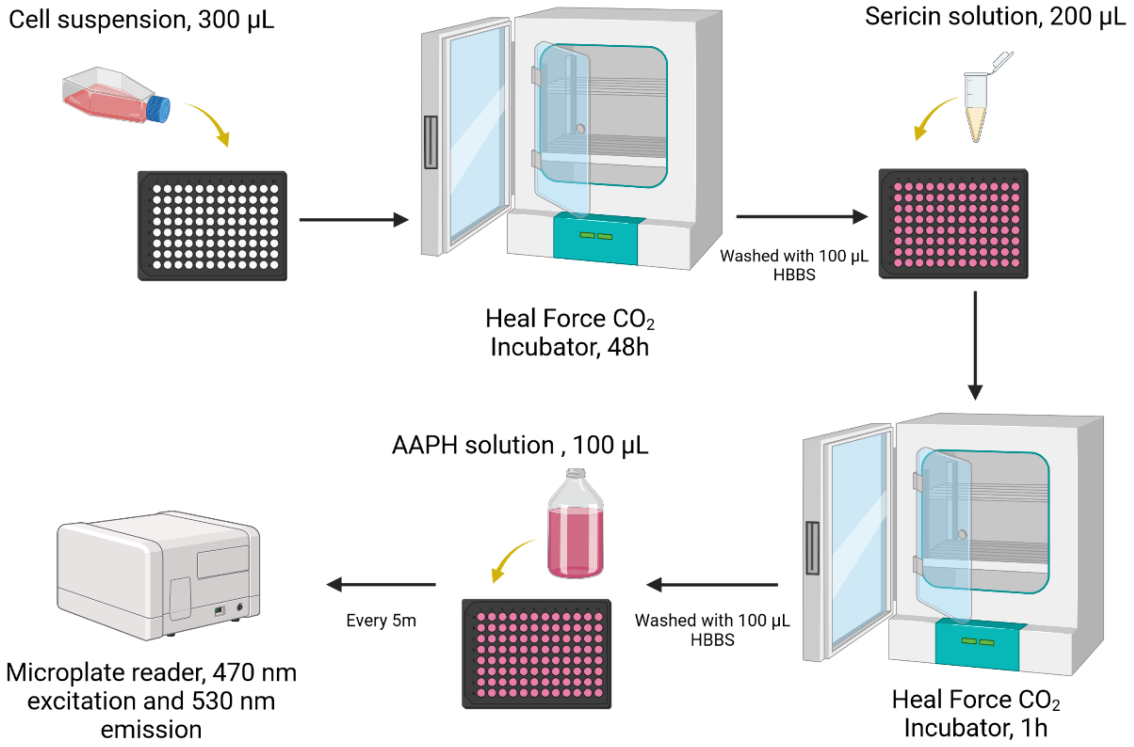

Figure 5- CAA procedure, developed using © 2023 BioRender, own work.

3.5.4 Anti-inflammatory activity

Inflammation is a biological process that occur if tissue structures are disturbed by physical, chemical, or biological stressors. The inflammatory response is a crucial part of the immune system, activated in response to infection and tissue damage to maintain a balanced internal environment. It serves as an adaptive reaction to various harmful stimuli that the human body frequently encounters (Olajide & Sarker, 2020). Inflammatory cells release growth factors and cytokines that attract the cells needed to build new tissue. The expression of metalloproteinases, which oversee degrading the extracellular matrix, is promoted by the unchecked, exuberant expression of inflammatory cytokines, therefore this phase must be managed. In this regard, biomaterials created for the treatment of wounds must have the ability to manage the inflammatory response (Anderson et al., 2008).

Nitric oxide production (NO) inhibition, following the procedure described by Sobral et al., (2016), was used to evaluate the anti-inflammatory potential of sericin. Four concentrations between 1.6 and 0.025 mg/mL were tested.

The RAW 264.7 cell line was used in this analysis. A cell scraper was used to remove the cells. In each well was added 300 µL of cell suspension of macrophages with a cell density of 5 x 10^5 cells/mL and with a proportion of dead cells below 5% according to the Trypan blue exclusion test. To ensure that the cells adhered properly and multiplied, the microplate was incubated in the incubator for 24 hours under the previously mentioned conditions. Following that time, 30 µL of each concentration of each simple were administered to the cells. After adding 30 µL of the LPS (1 mg/mL) stimulation was performed out, and the incubation period was extended by another 24 hours.

Nitric oxide was measured using a Griess reagent system kit and the nitrite calibration curve (100 mM sodium nitrite at 1.6 mM) generated in a 96-well plate. Nitric oxide production was measured by reading absorbances at 540 nm in a microplate reader (Synergy H1, BioTek Instruments, Winooski, Vermont, USA) and comparing the results to the reference calibration line.

The proportion of nitric oxide production inhibition against sample concentration was plotted graphically to determine the results, which were then stated in relation to the concentration of each extract that results in a 50% inhibition of nitric oxide production (IC_{50} values at µg/mL). Samples without LPS were utilized as a negative control, and dexamethasone (50 mM) was used as a positive control. In Figure 6 is possible to see the procedure used.

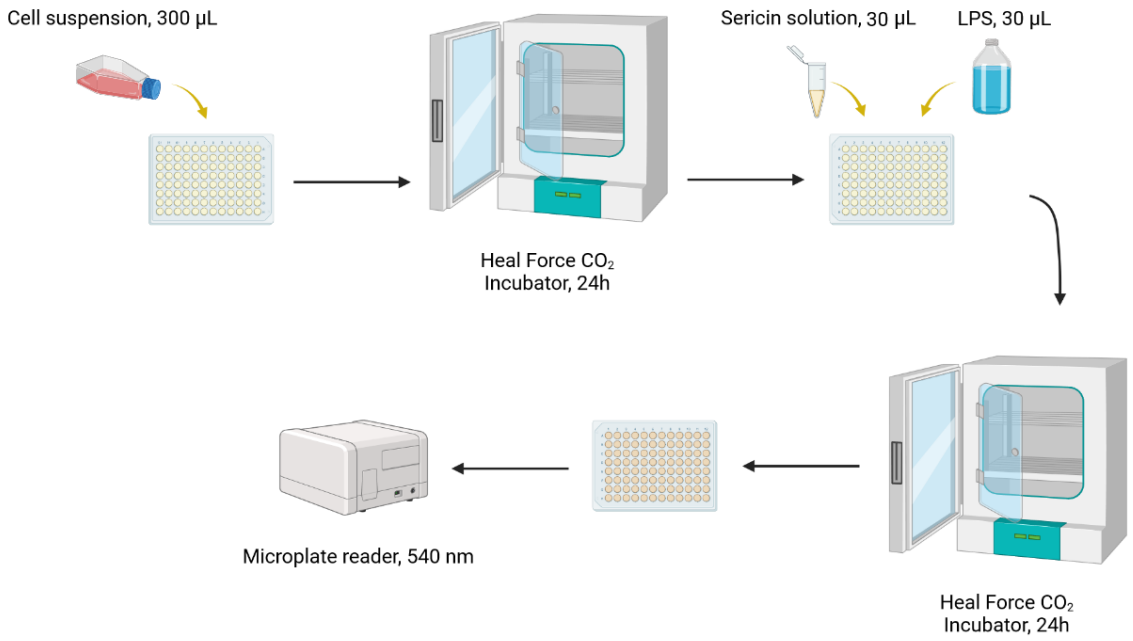

Figure 6- NO production inhibition method for anti-inflammatory activity, developed using © 2023 BioRender, own work.

3.5.5 Antimicrobial activity

Antibiotic resistance in clinical isolates has led to the development of innovative techniques for preventing and controlling infectious diseases due to their reduced effectiveness (Chifiriuc et al., 2011). This involves the development of new antimicrobial products (Sakkas & Papadopoulou, 2017).

In addition to other biological activities, the antibacterial and antifungal potential of sericin samples has been evaluated.

3.5.5.1 Antibacterial activity

The sericin samples, at concentration of 20 mg/mL, were tested against food contaminants, including three Gram-positive bacteria like *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), and *Staphylococcus aureus*, as well as four Gram-negative bacteria: *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027) and *Salmonella enterica* subs (ATCC 25923). All bacteria were acquired from Frilabo in Porto, Portugal.

Clinical isolates have been obtained from clinical samples at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal) namely five Gram-negative bacteria: *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, isolated from urine,

Pseudomonas aeruginosa and *Morganella morganii*, isolated from sputum, and three Gram-positive bacteria *Enterococcus faecalis* (isolated from urine), *Listeria monocytogenes* (isolated from cerebrospinal cord fluid), and methicillin-resistant *Staphylococcus aureus (MRSA)* isolated from sputum.

Bacteria were incubated at 37 ºC in fresh culture medium for 24 hours prior to the assays performed to maintain cultures in the exponential growth phase. The Minimum Inhibitory Concentration (MIC) was determined by microdilution test, as described by (*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, n.d.; Tsukatani et al., 2012). The procedure is shown in the Figure 7.

To produce a stock solution with a final concentration of 20 mg/mL, the samples were first dissolved in a solution consisting of 5% (v/v) DMSO and 95% autoclaved distilled water. A 96-well microplate was prepared by adding 100 µL of this concentration and 90 µL of TSB in duplicate to the first well, and 90 µL of TSB medium in the remaining wells. The concentration ranges were then obtained by serial dilution of the samples (10 to 0.031 mg/mL). The process was completed by adding 10 µL of inoculum, which was standardized at 1.5×10^6 Colony Forming Units (CFU) /mL, to each well, establishing the presence of 1.5×10^5 CFU. TSB and the sample were used as negative control. Two positive controls were produced, one with medium, bacteria, and antibiotics, and the other with TSB and each inoculum. All the examined microorganisms were treated with ampicillin and streptomycin, whereas *Staphylococcus aureus* was treated with methicillin. For 24 hours, the microplates were incubated at 37°C. After adding (40 µl) of 0.2 mg/mL INT to the samples and incubating them at 37°C for 30 min, the MIC of the samples was found.

A colorimetric test, described by Kuete, Ango, et al., (2011) and Kuete, Kamga, et al., (2011), was used to determine if the microorganisms are alive, by a change in color from yellow to pink. The MIC was defined as the lowest concentration that suppresses the visible bacterial growth.

In order to determine the Minimum Bactericidal Concentration (MBC), following the procedure indicated by (*Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, n.d.; Tsukatani et al., 2012), 10 µL of liquid from each well that failed to demonstrate a change in colour, showing antibacterial potential, was inoculated on solid medium, Blood agar (7% sheep blood), and incubated at 37°C for 24

h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. Streptomycin and ampicillin were used as positive controls (1 mg/mL in sterile physiological saline). Five percent DMSO was used as a negative control.

The MBC is determined by the lowest concentration at which there was no growth. MBC is the smallest concentration necessary to eradicate bacteria.

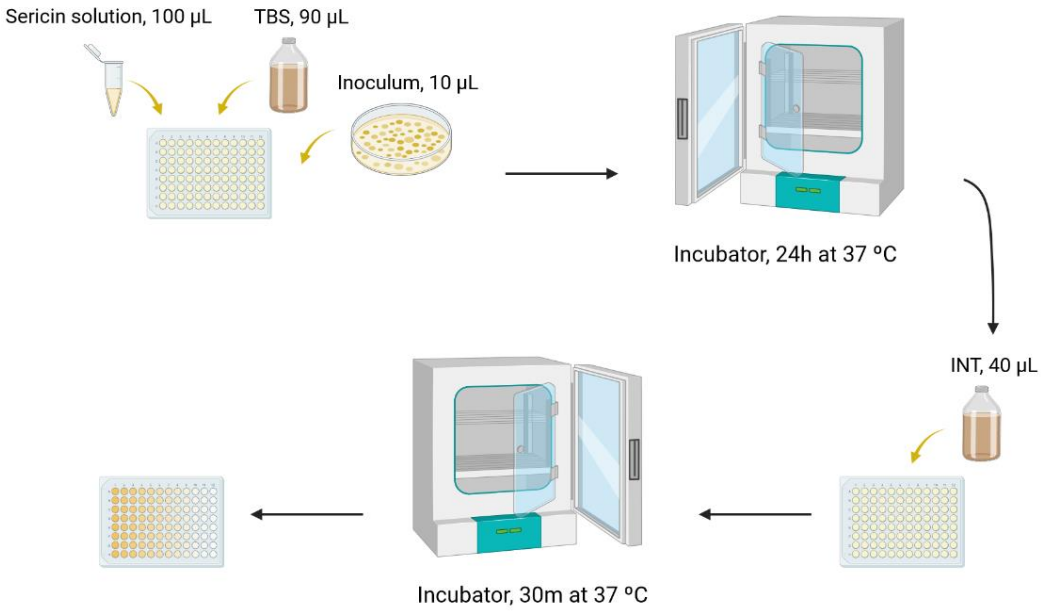

Figure 7- Colorimetric test to determine the bacterial activity, developed using © 2023 BioRender, own work.

3.5.5.2 Antifungal activity

As indicated by (Espinel-Ingroff, 2001; Hänel & Raether, 1988) the antifungal activity was performed using *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404), acquired from Frilabo in Porto, Portugal. The cultures of the micromycetes were maintained on malt agar and stored at 4 °C until being transferred to fresh media and incubated at 25 °C for 72 hours.

Fungal spores were removed from the surface of agar plates using sterile 0.85% saline containing 0.1% Tween 80 (v/v) in order to study the antifungal activity. In a final volume of 100 L per well, the spore suspension was adjusted with sterile saline to a concentration of roughly 1.0×10^5. The samples were prepared in the same way as for the

determination of antibacterial activity adding 100 μL of each sample in the first well (96-well microplate) in duplicate with 90 μL of MEB. In the remaining wells 90 μL of medium MEB were placed. Then the samples were serial diluted to obtain the concentration ranges (10 to 0.031 mg/mL).

MICs were established as the lowest concentrations at which there was no observable growth (under the binocular microscope). The minimum fungicidal concentration (MFC) was determined by serial subcultivation of a 2 μL of tested compounds dissolved in medium and inoculated for 72 h, into microplates containing 100 μL of MEB per well and further incubation 72 h at 26 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. As a positive control, commercial fungicide ketoconazole was used.

3.6 Anticoagulant activity

Hemostasis is a biological process that regulates blood flow in blood vessels and prevents bleeding from injured ones. It balances pro-coagulant and anticoagulant factors, and genetic or acquired conditions can alter this balance, leading to increased thrombus formation (Lamponi & Jung, 2021). An excess of coagulation can result in thrombosis, which can induce arteriovenous blockage and cause significant damage such pulmonary and brain embolism, heart attacks, and even death (Engelmann & Massberg, 2013; Guglietta et al., 2016). In addition, anticoagulant activity plays an increasingly important function in artificial organs and clinical medical devices that come into contact with blood (Biran & Pond, 2017; Xu et al., 2014).

To prevent the formation of the thrombus, diverse studies have been carried out to development of suitable anticoagulant molecules, such as the synthesis of anticoagulant polymers and the modification of natural macromolecules (Ma et al., 2019).

The procedure was followed according to the guidelines in the manual of the Yumizen G200 coagulation analyzer. Intrinsic anticoagulant activity was evaluated by APTT methods.

For testing, the highest concentration (1.6 mg/mL) was achieved by dissolving a known quantity of each extract (0.8 mg) in 0.5 mL of plasma, obtained from adult mice,

and centrifuged at 500× *g* for 5 min at 4◦C. Subsequently, dilutions were prepared to obtain the concentration of 0.025 mg/mL for assessment.

The activated partial thromboplastin clotting time (APTT) assay was evaluated using 50 µL sample and 50 µL APTT mixed in a test tube and incubated for 3 min at 37º C. Then, 100 µL $CaCl_2$, was added to the mixture. The anticoagulant potential was determined by the time required for clot formation. The procedure is shown in the Figure 8.

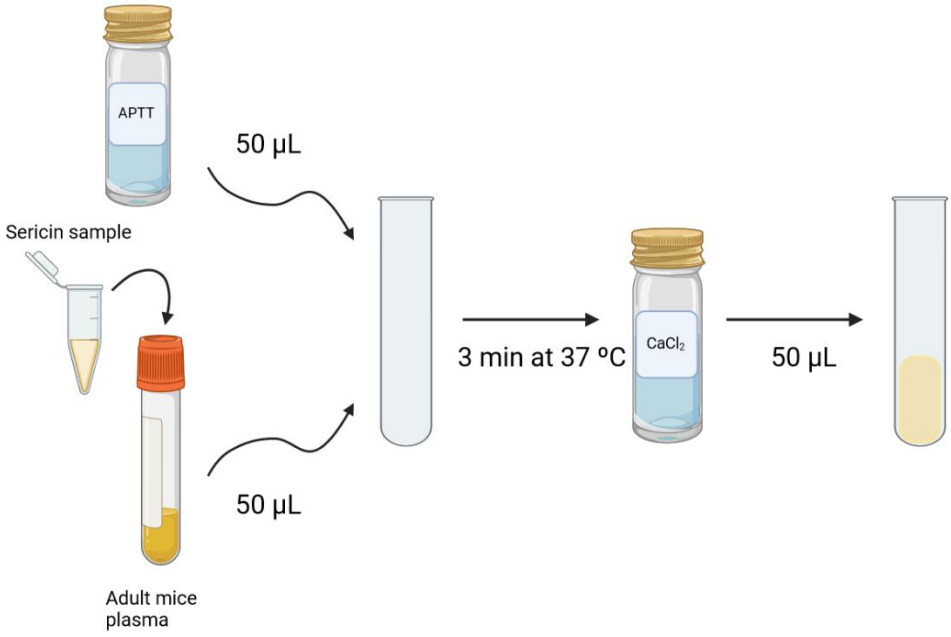

Figure 8- APTT for anticoagulant activity, developed using © 2023 BioRender, own work.

3.7 Statistical analysis

Results are presented as mean values ± standard deviation. The statistical analysis of the obtained results was performed using a one-way analysis of variance (ANOVA) with the Turkey post hoc test. A p-value lower than 0.05 ($p < 0.05$) was considered statistically significant. The SPSS v. 23.0 software was used to conduct the statistical analyses.

4. Results and discussion

4.1 Extraction yield

Silkworm cocoons are mainly composed of fibroin and sericin. Sericin was extracted by autoclave at 120 °C for 30 minutes. The sericin of S1 and S2 was extracted in February 2022 and sericin of S3 and S4 was extracted in September 2022. The results are presented in the following Table 3.

Table 3- Yield of the sericin extraction.

Code	Cocoon weight (g)	Fibroin weight (g)	Yield of fibroin (%)	Sericin weight (g)	Yield of sericin (%)
S1	7.62	5.86	77%	1.45	19.0%
S2	5.93	4.57	77%	1.13	19.0%
S3	5.48	4.00	73%	1.46	26.7%
S4	2.51	1.7	68.9%	0.76	30.2%

S4, a sample of the cocoons collected in Bragança in 2022, has the highest extraction yield. Sample S3 presented the second-highest yield of sericin (26.7%) and was a representative of the cocoons collected in Bragança in 2021. Finally, samples S1 and S2 from Castelo Branco and Bragança in 2019 exhibited identical extraction yields, with the lowest yield.

Our results are in accordance with the yield obtained by Gimenes et al., (2014), with 21.99 ±0.96% yield of sericin extracted from *Bombyx mori* cocoons using hot water at 120 ºC. At the same time, Gimenes and collaborators achieved higher yield (30.04 ± 0.83%) for extraction of sericin with 0.5% Na2CO3. Also, Aramwit et al., (2010) obtained yields ranging from 17.00 ± 3.14 to 21.47 ± 0.62 for extractions from *Bombyx mori* cocoons of different strains (white shell, greenish shell, and yellow shell) using the same method used in this work.

Indeed, the effect of the extraction methods influence the final yield of sericin was previously reported (Yun et al., 2013), as well as highlighted the impact of the origin of the cocoon and the strain of silkworm (Kunz et al., 2016). In the present work, differences in extraction yield may be attributed to the location and harvest period, and once the extraction method and Bombyx species remained consistent across samples.

4.2 Chemical characterization

The total and free amino acids were analyzed by HPLC-FLD, after a derivatization step, with or without a hydrolysis process. In non-hydrolyzed commercial sericin were identified seven amino acids, while 10 to 13 amino acids were found in extracted sericin. In contrast, 13 to 16 amino acids were identified in all hydrolyzed samples.

The following Figures (9-11) represent the chromatograms of the standards used in the analysis, and an example of the samples tested. Only a chromatogram of the sericin samples is presented since the rest are identical, only the content of the amino acid present varies.

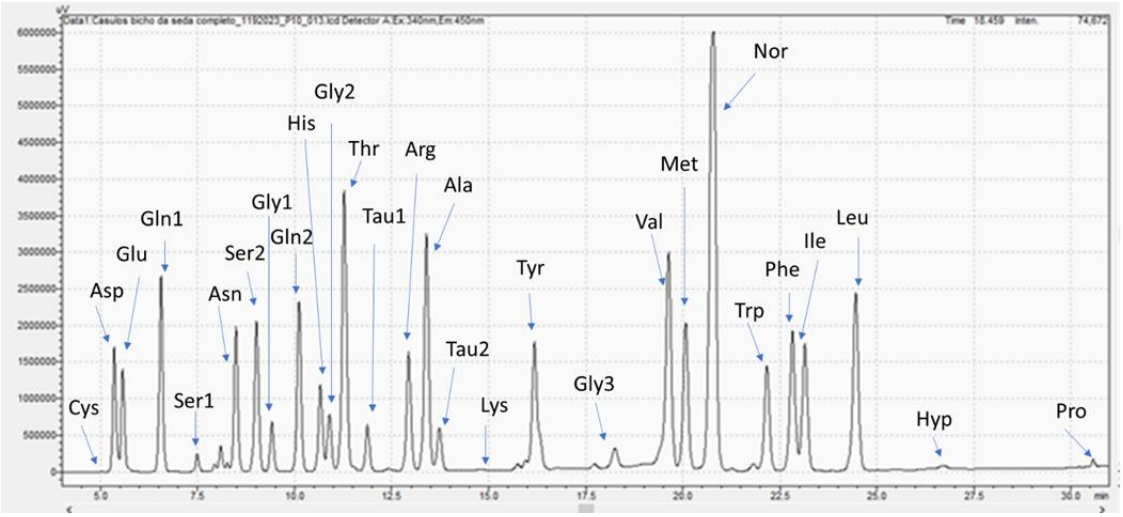

Figure 9- Chromatograms of the amino acid standards.

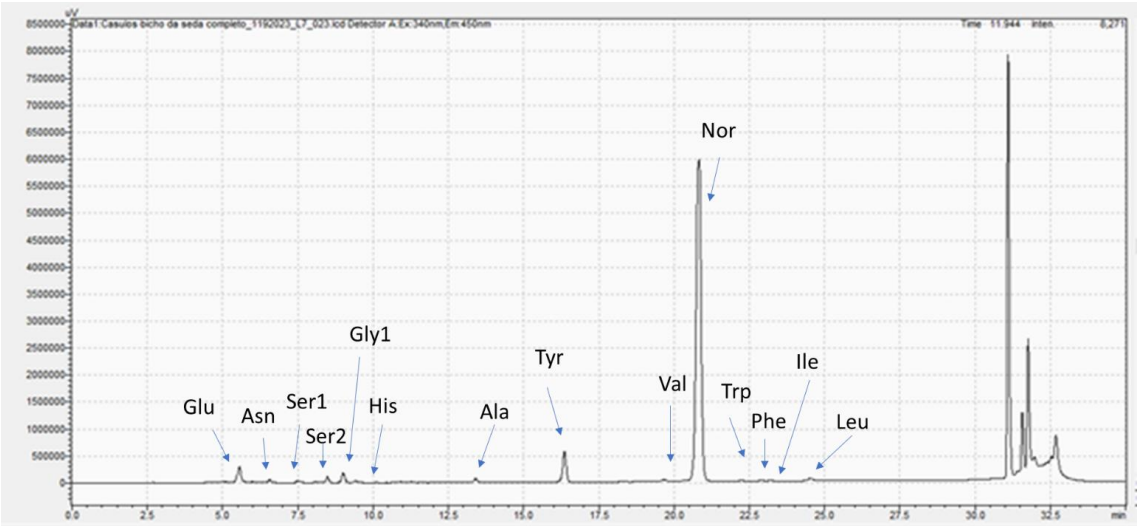

Figure 10- Free amino acids sample S2, without dilution.

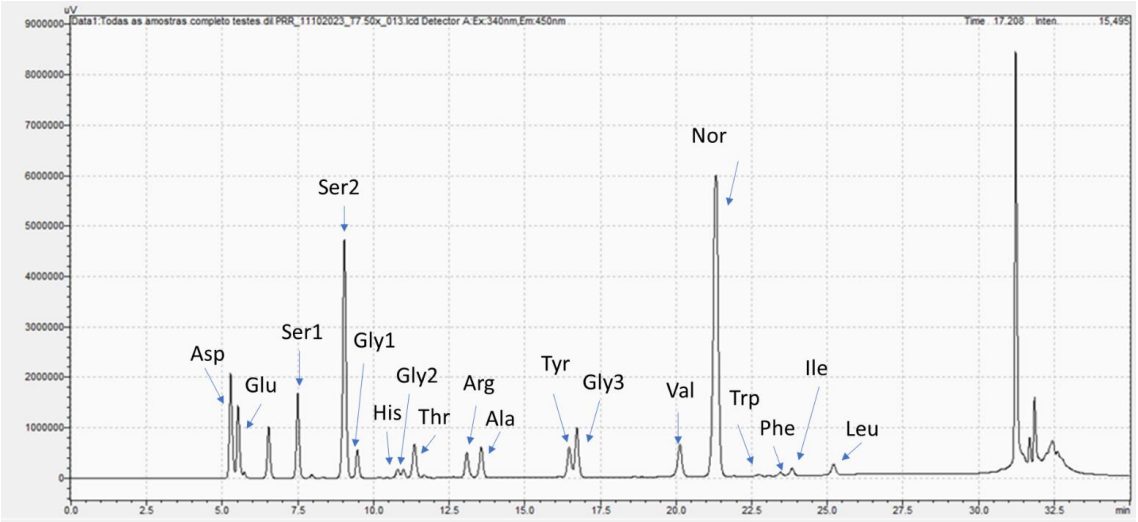

Figure 11- Sample S2 hydrolyzed (total amino acids), diluted 50 times.

Chromatograms shown differences in the amount of free and total amino acids in protein. Samples of sericin not submitted to hydrolysis showed lower levels of free amino acids, while in hydrolysis sericin, it was possible to identify different and greater amounts of amino acids. This could occur since the hydrolysis process breaks the glycosidic bonds (Harun et al., 2010), aiming for the quantitative liberation of all amino acids from the substrate (Fountoulakis & Lahm, 1998).

When we analyze Figure 12, we can observe that all samples have common amino acids Ser, Glu, Gly, and Trp. Sample S4 shows levels of Asp and Cys, while samples S1 and S2 have the highest levels of Glu. Gln is common to S1 and S4, while Asn, Ala, Leu, and Val are common to S1, S2, and S3. Sample S1 does not contain His and Tau, while S3 only presents Thr. Lys is common to SC2 and S4, Phe to S1, and Ile to S1 and S3. Thus, the samples extracted from the cocoons stand out for having a higher amino acid content than the commercial samples.

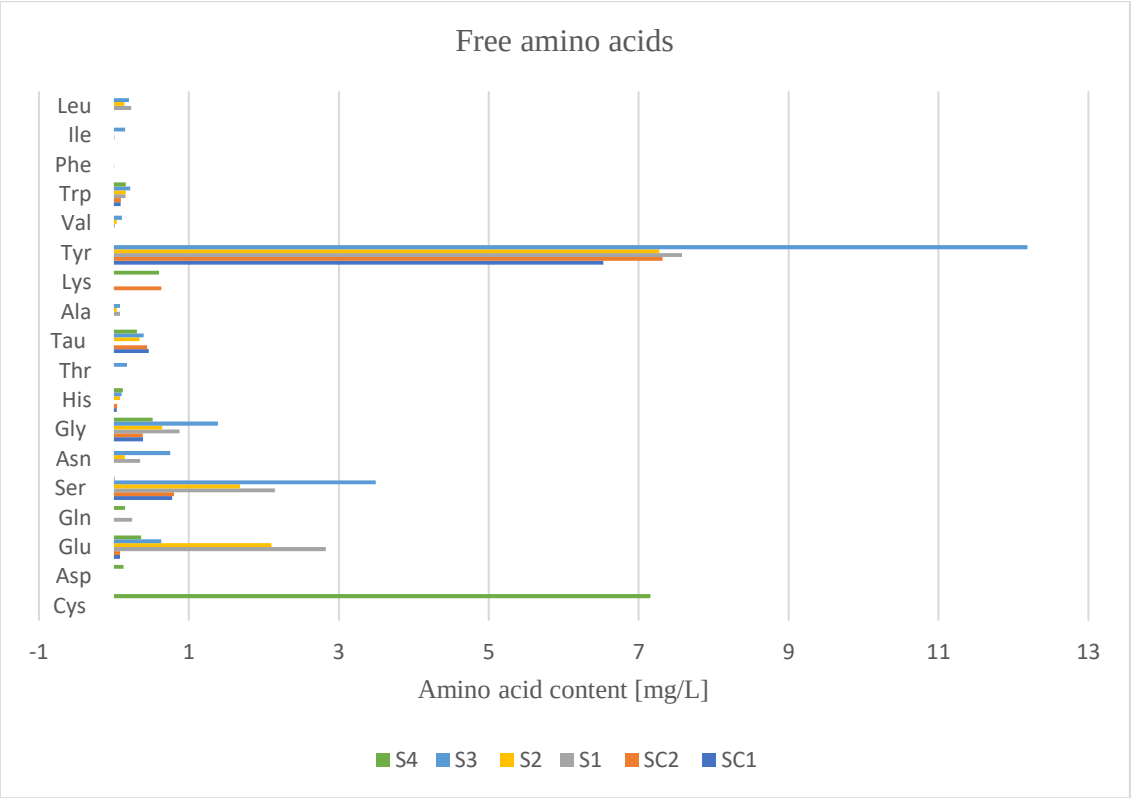

Figure 12- Amino acid profile for sericin samples of free amino acids.

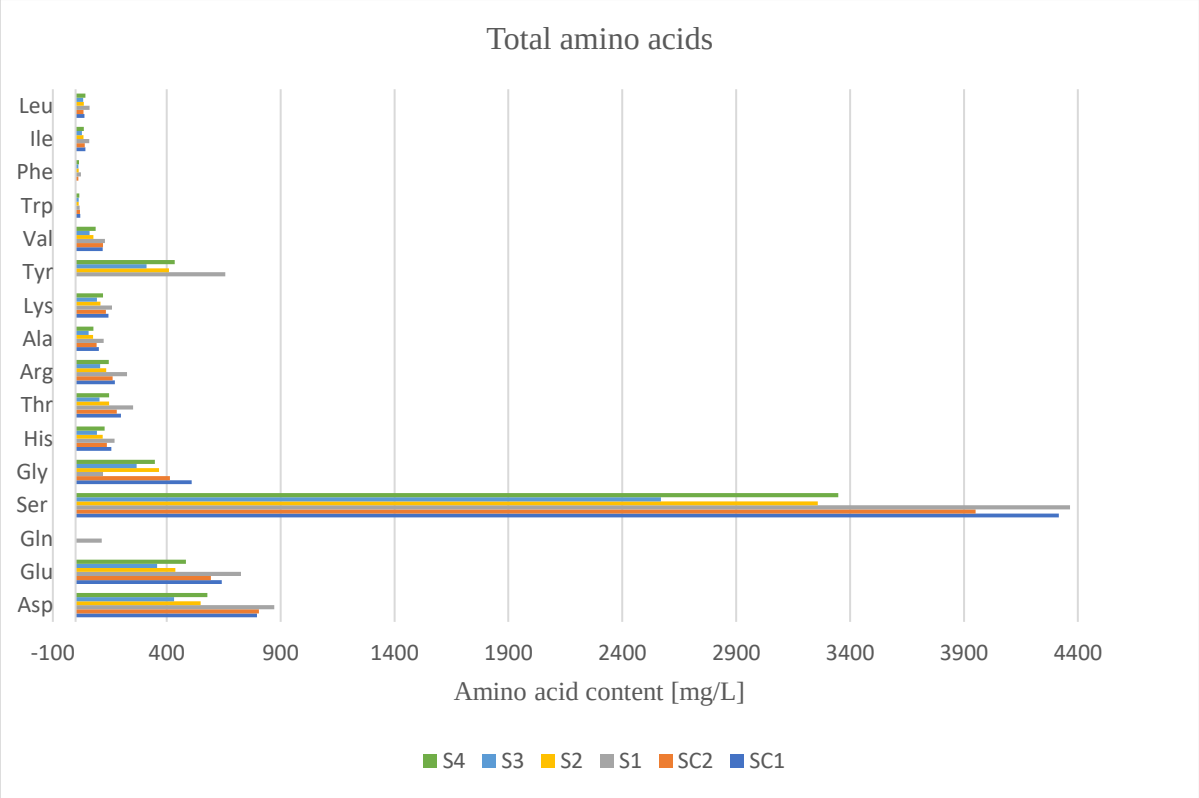

Figure 13- Amino acid profile for sericin samples of total amino acids.

Samples of sericin that did not submit to hydrolysis generally exhibit a higher content of the amino acid Tyr, except for sample S4, which had a higher content of the amino acid Cys. However, the amino acid content present in hydrolyzed sericin is most similar to studies by other authors for sericin extracted by the same method used in this work, such as (Ampawong et al., 2017; Kato et al., 1998; Keawkorn et al., 2013; Kunz et al., 2020), who demonstrated that serine is the most prevalent amino acid. Thus, the variety of cocoon and its origin could provide different amino acid composition, which can reflect in biological properties (Kunz et al., 2016).

4.3 Bioactivity assessment

4.3.1 Antiproliferative activity

The results for antiproliferative activity are presented in Table 4.

Table 4- Antiproliferative activity of sericin extracts determined by SRB assay.

	S1	**S2**	**S3**	**S4**	**CS1**	**CS2**
Tumoral cells (GI_{50}, mg/ mL)						
AGS	1.13 ± 0.09[a]	1.24 ± 0.02[a]	0.83 ± 0.01[b]	0.79 ± 0.02[b]	>1.6	>1.6
Caco-2	>1.6	>1.6	1.03 ± 0.09[a]	0.81 ± 0.03[b]	>1.6	>1.6
MCF-7	1.52 ± 0.03[a]	>1.6	0.94 ± 0.06[b]	0.52 ± 0,03[c]	>1.6	>1.6
NCI-H460	0.80 ± 0.00[a]	0.68 ± 0.15[a]	0.66 ± 0.07[a]	0.67 ± 0.04[a]	>1.6	>1.6
HeLa	>1.6	>1.6	1.07 ± 0.01[a]	1.14 ± 0.1[a]	>1.6	>1.6
Non tumoral cell (GI_{50}, mg/ mL)						
Vero	0.98 ± 0.16[a]	0.625 ± 0.04[a, b]	0.51 ± 0.24[b]	0.33 ± 0.05[b]	>1.6	>1.6

Results are presented as mean ± standard deviation. According to Tukey's test at a level of significance of $p<0.05$, averages that are followed by the same letters on the same line do not statistically different.

The sericin samples evaluated demonstrated decrease cell growth against normal cell line (Vero), in a range of concentrations from 0.33 to 0.98 mg/ mL, while this was not registered for the commercial samples. Sample S4 exhibited the lowest GI_{50} value for non-tumor cells, resulting in the highest antiproliferative potential. This tendency was also registered for tumoral cells. The different samples exhibited anti-proliferative activity in a range of concentrations from 0.52 to 1.53 mg/ mL in tumoral cells. In

contrast, commercial samples did not inhibit cell proliferation and for all the cell lines considered in this work, even at the maximum concentration tested.

All the samples were able to inhibit the AGS cell line proliferation, in which S4 having the lowest GI_{50}, an indication of higher antiproliferative activity. Only samples S3 and S4 had the ability to inhibit the proliferation of the Caco-2 cell line, with the last one demonstrating a lower (0.81 ± 0.03) and statistically more significant GI_{50} value. Among the samples, only sample S2 did not inhibit the proliferation of the MCF-7 cell line. The other samples, however, indicated significant differences, with sample S4 providing the lowest GI_{50} value. The NCI-H460 cell line's proliferation was inhibited by all four samples, although there were no significant differences statistically. Finally, samples S3 and S4 demonstrated antiproliferative potential against the HeLa cell line but did not differ in GI_{50} values ($p < 0.05$).

The results reported above are consistent with Kaewkorn et al., (2012), which showed that, at a concentration of 1.6 mg/mL, sericin reduced the proliferation of human colorectal cancer (SW480) and normal colonic mucosal (FHC) cell lines. Although with a higher concentration, Kumar & Mandal, (2019a) induced the decrease of cell growth of sericin at a concentration of 4 mg/mL in keratinocyte (HaCaT), non-tumorigenic epithelial (MCF-10), human squamous cell carcinoma (A431) and human tongue carcinoma (SAS) cell lines. However, at a concentration of 1 mg/mL, it had no effect on the cells.

On the other hand, Hakimi et al., (2010) studied the antiproliferative effects of sericin extracted from different species (*Bombyx mori* and *Antheraea pernyi*) and observed that the sericin extracted from *A. pernyi* showed powerful toxic effects. The authors suggest that enzymatic treatment of *A. pernyi* abolished this toxic effect. This could indicate that the release of toxic peptides in cocoons could be a defense against predators or microorganism digestion, with serum supplementation of medium components potentiating the effect. This aligns with the idea that toxic peptides in cocoons could serve as a defense against predators (Gauthier et al., 2004; Nirmala et al., 2001). Thus, the decrease cell growth capacity determined in our samples may also be related to toxic peptides present in the cocoons.

The antiproliferative activities could be related with the amino acid residue composition (Roudi et al., 2017). Thus, amino acids such as lysine, arginine and histidine

could induce cancer cytotoxicity via membrane permeability under acidic condition (Midoux et al., 1998; Navarro et al., 2008), while glutamic acid and aspartic acid could have antiproliferative activity on tumor cells (Yamaguchi et al., 2016). Moreover, cysteine, proline, glycine, and phenylalanine could interact with the membrane of target cells and may be able to increase cytotoxic activity (Ahmaditaba et al., 2017; Dennison et al., 2006; Oancea et al., 1998; Shamova et al., 2009). Finally, tyrosine may be able to increase cytotoxic activity (Ahmaditaba et al., 2017), and tryptophan could enter cancer cells following an endocytic pathway (Bhunia et al., 2018; Harris et al., 2013).

Some of the previously listed amino acids were found for our samples. The amino acids Glu, Gly, His, Tyr, and Trp have been identified for the commercial samples. Besides these, the amino acid Lys was also found in the SC2 sample. Common amino acids found in the extracted samples were Glu, Gly, and Trp. In S1, Tyr and Phe residues were also identified. S2 and S3 also identified the amino acid His and Tyr. Additionally, S4 identified Lys, Asp, and Cys. These amino acids could be related to the antiproliferative potential of the samples extracted from cocoons. Nonetheless, the fewer amino acids identified in commercial samples may have decreased their interaction and antiproliferative potential. Sample S4 showed greater antiproliferative capacity, possibly due to the presence of Cys and Asp amino acids.

4.3.2 Antioxidant activity

The antioxidant activity of sericin was evaluated using TBARS, with a range concentration of 2.18 at 70 mg/mL, and CAA, using a range concentration of 0.5 at 2 mg/mL, methods (Table 5).

Table 5- Antioxidant activity of sericin extracts and sericin commercial.

	S1	**S2**	**S3**	**S4**	**SC1**	**SC2**
Antioxidant activity						
TBARS (Inhibition percentage at maximum concentration)	33. 97 ± 0.61	22.92 ± 0.70	46.27 ± 1.11	20.19 ± 0.97	>70	>70
CAA (% inhibition by 2 mg/mL)	>2	>2	>2	>2	>2	>2

Results are presented as mean ± standard deviation.

The different sericin samples at concentrations of 70 mg/mL didn't show antioxidant activity. Higher concentrations have not been tested since are irrelevant for both pharmaceutical and biotechnology applications due its cytotoxicity.

To the best of our knowledge, there are no reports of evaluation of antioxidant activity of sericin using the TBARS method. Nevertheless, other studies reported the antioxidant activity of sericin by 2,2-difenil-1-picrylhydrazyl (DPPH) assay, namely Manosroi et al., (2010), for five varieties of Thai native silk cocoons, with SC_{50} values of free radical scavenging activity from 13.65 ± 0.20 (mg/mL) to 54.49 ± 0.59 (mg/mL), as well as Saha et al., (2019) shown antioxidant potential of sericin, from *Bombyx mori* waste silk cocoons, at concentrations of 20 mg/ ml and 40 mg/ ml. Also, Kumar & Mandal, (2017) reported the antioxidant potential for sericin extracted from *Antheraea assamensis, Philosamia ricini* and *B. mori* by using ferric ion reducing antioxidant power (FRAP) assay and DPPH assay, and *in vitro* assays, such as Lactate dehydrogenase (LDH) assay and by determination of intracellular antioxidant activity. Finally, Manesa et al., (2020) shown antioxidant activity, for silk sericin extracted from cocoons collected at different places in Southern Africa, and by using DPPH, FRAP and 2,2′-Azinobis-(3-Ethylbenzothiazoline-6-Sulfonic Acid) ($ABTS^{\cdot+}$) methods. These studies reported the effect of the biochemical profile on the antioxidant activity of sericin and confirmed as critical the origin of the cocoons and the extraction method, and similarly to the results obtained in the present study. Furthermore, these authors recommended the use of complementary assays to determine the antioxidant potential, related to the ability to inhibit lipid oxidation and free radical scavenging.

So, in this study was also performed the evaluation of the antioxidant activity of sericin using CAA in RAW 246.7, at a concentration of 2 mg/mL (higher concentrations were demonstrated to be toxic to cells and were not considered for further assays). Results obtained in this assay demonstrated that sericin samples under study did not reveal antioxidant capacity. Few reports considered the evaluation of the antioxidant capacity of sericin in cellular models. Nevertheless, the sericin effect on the intracellular reactive oxygen species generation in human keratinocytes (HaCaT) cells was studied by Kumar et al., (2018), and in mouse melanoma cell line (B16F10) by Kumar & Mandal, (2019b). In these studies, cells were incubated with sericin or vitamin C for an hour to be exposed to UVA (8 J/cm^2) and UVB (120 mJ/cm^2) radiation. In both studies, sericin was isolated

from the cocoons of non-mulberry (*A. assamensis and P. ricini*) silk varieties. For HaCaT cells, sericin, at concentrations of 10 μg/mL, significantly reduced ROS production. For B16F10, the sericin extracted from *A. assamensis* reduced ROS concentration in both conditions (UVA and UVB irradiation), whereas sericin from *P. ricini* reduced ROS levels in UVB irradiated cells. In addition, the topical application of *B. mori* sericin prevented hairless female mice from UVB-induced sunburn and tumor development, demonstrating the photoprotective potential of sericin, as demonstrated by Zhaorigetu et al., (2003). The reason for this mechanism is due to the fact sericin contains several amine-based groups that are rich in nitrogen, oxygen, and hydrogen. These groups enable high absorption of UV radiation with wavelengths less than 200 nm (Gore et al., 2019). Baptista-Silva et al., (2022) also demonstrated the antioxidant potential of a sericin-based hydrogel, from Castelo Branco, Portugal, by ORAC method.

The antioxidant activity of sericin, according to Fatahian et al., (2021) and Lamboni et al., (2015), could be related with the amount of serine and threonine, which hydroxyl groups act as chelating trace elements such as copper and iron. Nevertheless, for our samples, the amino acids serine and threonine were not identified in large amounts.

4.3.3 Anti-inflammatory activity

The anti-inflammatory potential of sericin was analyzed by NO, at a maximum concentration of 1.6 mg/mL. The samples under study did not show anti-inflammatory activity, and in accordance with other reports. For example, Panilaitis and collaborators, who examined the reaction of macrophages (RAW 264.7 cell line) when stimulated with LPS to silk protein, extracted by 0.02 M Na_2CO_3, concluded that sericin, when present in soluble form, had no inflammatory activity. In addition, the authors reported that sericin, when attached to fibers, can cause inflammatory reactions (Panilaitis et al., 2003).

Several studies reported the anti-inflammatory activity of sericin based products, namely (Baptista-Silva et al., 2021) of sericin- based hydrogels, Munir et al., (2023) for sericin-based hydrogels combined with curcumin, banana peel powder and AgNPs, Tuentam et al., (2022) of a silk sericin-based poly(vinyl) alcohol hydrogel, and Gilotra et al., (2018) for sericin-based nanofibrous mats. In addition, sericin also reported anti-

inflammatory activity when combined with others compounds, such as (Chachlioutaki et al., 2022) for sericin and poly lactide-co-glycolic acid (PLGA) electrospun scaffolds, and Chuysinuan et al., (2023) for turmeric extract-loaded carboxymethyl cellulose/silk sericin dressings. And other studies shown anti-inflammatory activity of sericin when modified, including Deenonpoe et al., (2019) for combined naringin and sericin in human peripheral blood mononuclear cells (hPBMCs) isolated patients with psoriasis, and Song et al., (2020) and Choi et al., (2023) for sericin after being irradiated by gamma-irradiation.

Thus, Kundu and collaborators suggested that coated sericin fibers may increase macrophage adhesion or prepare them for subsequent stimulation due to their conformational change after binding to the silk fiber (Kundu et al., 2008).

4.3.4 Antimicrobial activity

The MIC, MBC and MFC obtained for the different sericin samples are shown in Table 6.

Table 6- Antibacterial and antifungal activities of sericin extracts and sericin commercial.

	Sericin samples												Positive Control					
	S1		S2		S3		S4		SC1		SC2		Streptomicin (1mg/mL)		Methicilin (1mg/mL)		Ampicillin (10mg/mL)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
	Clinical bacteria																	
Gram-negative bacteria																		
Enterobacter Cloacae	>10	>10	>10	>10	>10	>10	10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	10	>10	10	>10	5	>10	5	>10	10	>10	>10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	>10	>10	10	>10	10	>10	10	>10	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	>10	>10	>10	>10	10	>10	10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	10	>10	10	>10	10	>10	10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria																		
Bacillus cereus	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	>10	>10	>10	>10	10	>10	10	>10	>10	>10	>10	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	>10	>10	>10	>10	10	>10	10	>10	10	>10	>10	>10	0.007	0.007	0.007	0.007	0.15	0.15
	Food Contaminants												Ampicillin (10mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
Gram-negative bacteria																		
Escherichia coli	>10	>10	>10	>10	10	>10	10	>10	10	>10	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	>10	>10	>10	>10	>10	>10	10	>10	>10	>10	>10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	>10	>10	>10	>10	10	>10	10	>10	10	>10	>10	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria																		
Enterococcus faecalis	>10	>10	>10	>10	10	>10	10	>10	5	>10	>10	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	>10	>10	>10	>10	10	>10	10	>10	>10	>10	10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	>10	>10	>10	>10	10	>10	10	>10	10	>10	>10	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5
	Fungal strains												Ketoconazole					
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**		**MFC**			
Aspergillus brasiliensis	10	>10	5	>10	10	>10	>10	>10	5	>10	5	>10	0.06		0.125			
Aspergillus fumigatus	5	>10	10	>10	10	>10	>10	>10	10	>10	10	>10	0.5		1			

The studied samples, except sample S1 and S2 for food contaminants, and sample SC2 for clinical bacteria, had the capacity to inhibit bacterial growth of the studied bacterial strains. For clinical bacteria, the samples showed better MIC values for gram-negative bacteria, while for food contaminants the samples showed lower MIC values for gram-positive bacteria. Thus, the bacterial growth inhibition values for the sericin samples range from 5 to 10 mg/mL.

Our results are consistent with other studies reporting the antimicrobial activity of sericin (against gram-positive and gram-negative bacteria) from different sources and origins. Manesa et al., (2020) reported antimicrobial activity in gram-positive bacteria for sericin extracted from *Gonometa postica*, *Gonometa rufobrunnae* and *Argema mimosa* cocoons for concentrations between 5 and 10 mg/mL by agar well diffusion assay and against three gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*) and two gram-negative bacteria (*Escherichia coli*, *Salmonella enterica*). Noosak et al., (2022) also demonstrated antimicrobial capacity for silk sericin extracted using a chemical-free boiling method, at concentrations ranged from 40–320 μg/mL, by determination of minimum inhibitory concentration and minimum bactericidal concentration against *S. aureus* ATCC29213 and *S. aureus* clinical isolates DS18, DS27, DS84, DS87, DS88, DS92, and DS110. The findings revealed that all bacteria produced using sterile saline displayed MIC and MBC in the range of 160-320 μg/mL, in contrast to the inoculum in broth, which had MIC and MBC greater than 320 μg/mL. Baptista-Silva et al., (2022) also studied the antimicrobial potential of a sericin solution and sericin based hydrogel, from Castelo Branco, Portugal, by a well diffusion assay, using *S. aureus*, *Pseudomonas aeruginosa*, and *E coli*. This study found that both sericin solution and sericin based hydrogel no inhibit the bacteria growth. The same happened with sample S1, collected from Castelo Branco.

In addition, the antifungal activity was assessed against two strains of fungus. Sample S4 did not impede the growth of the fungi under test, in contrast to what happened in the antibacterial activity test. MFC values range from 5 mg/mL to 10 mg/mL to all sericin samples. Our results are consistent with Mumtaz et al., (2023), that conjugated silver nanoparticles with sericin, extracted by autoclave and shown its antifungal potential by agar well diffusion method, using *M. mycetes*, *A. flavus*, *C. albicans*, and *A. niger* fungus strains, at a range 10 to 20 mg/mL of concentrations. Also, Ahamad & KumarVootla, (2018) demonstrated antifungal activity of sericin, extracted by 0.02M

sodium carbonate extraction and at a range of 25-100 µg/mL of concentrations, against *C. albicans* and *A. flavus*.

The previous studies revealed that the extraction method has a significant impact on the antimicrobial activity of sericin, highlighting the importance of the origin of the cocoon. The antimicrobial activity of sericin, according to the state of the art, may be associated with the presence of cysteine, as it contains sulfhydryl groups as constituents (de Magalhães, 2015), only identified in sample S4. Sulfhydryl groups can form weak hydrogen bonds with oxygen or nitrogen, resulting in highly reactive compounds that impact enzymatic reactions and metabolic functions of microorganisms (Caldeira, 2012). However, our results and the studies analyzed could demonstrate that the harvested time of the cocoons and the extraction method of sericin could influence the antimicrobial potential of the samples.

4.3.5 Anticoagulant activity

Anticoagulant potential was evaluated by APTT assay, the results are presented in Figure 14.

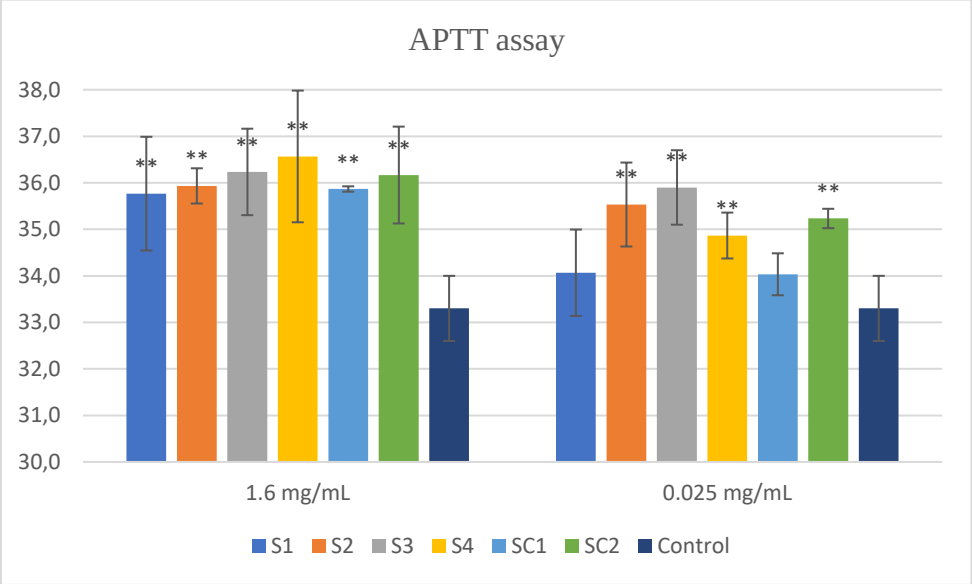

*Figure 14- Anticoagulant activity evaluated by APPT assay, results are expressed as mean ± standard deviation. According to Tukey's test at a level of significance of p<0.05, averages that are followed by ** on the same column do not statistically different.*

The presence of sericin in adult mice plasma prolonged the time until coagulation occurred in the APTT assay. The coagulation time was considerably extended by all

sericin’s at highest concentration compared to the control. Nevertheless, at the lowest concentration, all samples exceeded the controlled time, although S2, S3, S4, and SC2 exhibited significant differences in comparison to the control.

Our results suggest that sericin has the potential to prolong coagulation time, contrary to what is reported by Sano et al., (2009), which indicates that only sulfated sericin has higher values than the heparin control, a sulfated polysaccharide. Serine's hydroxyl groups play a major role in the binding of large quantities of sulfate via sericin's sulfation. In addition, Tamada et al., (2004) also showed that sulfated sericin has anticoagulant potential. However, the sulfated sericin's molecular weight determines the anticoagulant's potency (Monti et al., 2007).

For more than 90 years, heparin, a sulfated polysaccharide, has served as a therapeutic anticoagulant (Onishi et al., 2016). However, heparin has been associated with fatal illnesses, leading to the preference for its substitutes (Dhahri et al., 2020). Moreover, as an animal derived product (pigs' intestines and cows' lungs), certain religious groups are hesitant to use it (Onishi et al., 2016). Therefore, more studies are needed to understand the mechanism of action of sericin as a potential anticoagulant agent.

Anticoagulation or preventing blood clot formation can target various points along the coagulation pathway, often overlapping at multiple stages. Agents like direct thrombin inhibitors and direct factor Xa inhibitors can hinder the development of fibrin clots. Achieving anticoagulation can also involve inhibiting vitamin K-dependent factors by impeding their synthesis in the liver or altering their calcium-binding properties through modification (Umerah & Momodu, 2023).

5. Conclusion

Bombyx mori produce cocoons to provide the ideal conditions for the metamorphosis. The cocoon is constituted by fibroin that are attached by a protein, sericin, acting as a natural glue. Traditionally, in the textile industry, the cocoon is processed, and fibroin is converted into raw silk for the generation of numerous types of yarns and silk fabrics. The process of removing sericin is called degumming, and for many years this protein was devalued and discarded.

Sericin is characterized by being a glycoprotein and having different amino acids in its constitution, with polar groups, including amino, carboxyl and hydroxyl groups. In the constitution of the cocoons, sericin has a weight of 20-30% of the total weight.

In addition, sericin has been studied for biological activity. It has been observed that the biological properties of sericin seem to be directly related to the different extraction methods, origin, and variety of the cocoon, since it is possible to observe variations in protein size and amino acid content. Additionally, the potential applications of sericin have been studied for different industries, such as biomedical and pharmaceutical, food, textile, and other.

In this work, several samples of sericin from different origins in Portugal, Castelo Branco and Bragança, in different years, were extracted using the physical method (under high temperature and pressure) and for two commercial sericin were then evaluated the chemical characterization of the extracts and their bioactivities, such as antiproliferative and cytotoxic, antioxidant, anti-inflammatory, antimicrobial and anticoagulant activities.

The results obtained in this work revealed that the amino acid with the highest content in the non-hydrolyzed tested sericin samples is tyrosine. However, for hydrolyzed sericin, more acidic amino acids were identified, with serine having the highest content. Thus, the origin and seasonality of the samples revealed differences in the characterization of the protein. In this study, the objective was to investigate sericin as an industrial residue. Therefore, sericin underwent no processes that would modify it.

Nevertheless, the sericin tested has no anti-inflammatory effects at concentrations below 1.6 mg/mL. Similarly, it did not exhibit antioxidant effects at concentrations below 70 mg/mL according to the TBARS assay, nor at 2 mg/mL according to the CAA assay.

In addition, the antimicrobial effect of sericin was evaluated and the results showed that sericin inhibited the growth of the bacteria and fungi used. On the other hand, the extracted sericin showed cytotoxicity for tumor and non-tumor cells at a minimum concentration of 325 µg /mL. In contrast, commercially acquired sericin showed no cytotoxicity at a maximum concentration of 1.6 mg/mL. Finally, sericin was shown to have a potential anticoagulant effect as it prolonged the clotting time of mice plasma.

Although the sericin from Bragança and Castelo Branco, collected in 2019, did not differ greatly, differences in amino acid composition were identified. In addition, the sericin collected in Bragança in 2021 and 2022, S3 and S4, respectively, showed differences compared to the other samples and showed the best antiproliferative, antibacterial and anticoagulant potential. Additionally, there are also differences between extracted and commercial samples.

In the future, sericin may undergo a hydrolysis process and be the evaluation of the chemical and biological properties of sericin could be applied to sericin extracted using different methods and, in addition, determined the molecular weight of samples tested.

Additionally, since sericin has demonstrated anticoagulant activity, this potential will be assessed via extrinsic routes, including the Prothrombin Time (PT) assay, as well as to assessing sericin's potential as a platelet aggregation inhibitor. Furthermore, the anticoagulant potential of sericin will be investigated by testing individual amino acids as well as various combinations of amino acids.

In summary, Portuguese sericin showed better potential than commercial ones. We believe that that these findings will lead to further research on sericin's potential uses and value.

References

Ahamad, S. I., & KumarVootla, S. (2018). Extraction and evaluation of antimicrobial potential of antheraea mylitta silk sericin. *Int. J. Recent Sci. Res*, *9*, 23019–23022.

Ahmaditaba, M. A., Shahosseini, S., Daraei, B., Zarghi, A., & Houshdar Tehrani, M. H. (2017). Design, Synthesis, and Biological Evaluation of New Peptide Analogues as Selective COX-2 Inhibitors. *Archiv Der Pharmazie*, *350*(10). https://doi.org/10.1002/ARDP.201700158

Ampawong, S., Isarangkul, D., & Aramwit, P. (2017). Sericin improves heart and liver mitochondrial architecture in hypercholesterolaemic rats and maintains pancreatic and adrenal cell biosynthesis. *Experimental Cell Research*, *358*(2), 301–314. https://doi.org/10.1016/j.yexcr.2017.07.001

Anderson, J. M., Rodriguez, A., & Chang, D. T. (2008). Foreign body reaction to biomaterials. *Seminars in Immunology*, *20*(2), 86–100. https://doi.org/10.1016/j.smim.2007.11.004

Aramwit, P., Damrongsakkul, S., Kanokpanont, S., & Srichana, T. (2010). Properties and antityrosinase activity of sericin from various extraction methods. *Biotechnology and Applied Biochemistry*, *55*(2), 91–98. https://doi.org/10.1042/BA20090186

Arrebas-Lorenzo, G., & Morales, F. J. (2009). Effect of pyridoxamine on acrylamide formation in a glucose/asparagine model system. *Journal of Agricultural and Food Chemistry*, *57*(3), 901–909. https://doi.org/10.1021/JF802870T

Baptista-Silva, S., Bernardes, B. G., Borges, S., Rodrigues, I., Fernandes, R., Gomes-Guerreiro, S., Pinto, M. T., Pintado, M., Soares, R., Costa, R., & Oliveira, A. L. (2022). Exploring Silk Sericin for Diabetic Wounds: An In Situ-Forming Hydrogel to Protect against Oxidative Stress and Improve Tissue Healing and Regeneration. *Biomolecules*, *12*(6). https://doi.org/10.3390/BIOM12060801

Baptista-Silva, S., Borges, S., Costa-Pinto, A. R., Costa, R., Amorim, M., Dias, J. R., Ramos, Ó., Alves, P., Granja, P. L., Soares, R., Pintado, M., & Oliveira, A. L. (2021). *In Situ* Forming Silk Sericin-Based Hydrogel: A Novel Wound Healing Biomaterial. *ACS Biomaterials Science & Engineering*, *7*(4), 1573–1586. https://doi.org/10.1021/acsbiomaterials.0c01745

Barros, L., Venturini, B. A., Baptista, P., Estevinho, L. M., & Ferreira, I. C. F. R. (2008). Chemical Composition and Biological Properties of Portuguese Wild Mushrooms: A Comprehensive Study. *Journal of Agricultural and Food Chemistry*, *56*(10), 3856–3862. https://doi.org/10.1021/jf8003114

Bhunia, D., Mondal, P., Das, G., Saha, A., Sengupta, P., Jana, J., Mohapatra, S., Chatterjee, S., & Ghosh, S. (2018). Spatial position regulates power of tryptophan: Discovery of a major-groove-specific nuclear-localizing, cell-penetrating tetrapeptide. *Journal of the American Chemical Society*, *140*(5), 1697–1714. https://doi.org/10.1021/JACS.7B10254/SUPPL_FILE/JA7B10254_SI_001.PDF

Biran, R., & Pond, D. (2017). Heparin coatings for improving blood compatibility of medical devices. *Advanced Drug Delivery Reviews*, *112*, 12–23. https://doi.org/10.1016/J.ADDR.2016.12.002

Caldeira, E. S. D. A. (2012). *Biofuncionalização do Algodão com L-Cisteína: Estudo do Efeito Antibacteriano e dos Mecanismos de Acção contra S. aureus e K. Pneumoniae*. University of Beira Interior.

Chachlioutaki, K., Karavasili, C., Adamoudi, E., Bouropoulos, N., Tzetzis, D., Bakopoulou, A., & Fatouros, D. G. (2022). Silk sericin/PLGA electrospun scaffolds with anti-inflammatory drug-eluting properties for periodontal tissue engineering. *Biomaterials Advances*, *133*. https://doi.org/10.1016/J.MSEC.2022.112723

Chifiriuc, M. C., Cioaca, A. B., & Lazar, V. (2011). In vitro assay of the antimicrobial activity of kephir against bacterial and fungal strains. *Anaerobe*, *17*(6), 433–435. https://doi.org/10.1016/J.ANAEROBE.2011.04.020

Choi, J. H., Lee, S., Han, H. J., & Kwon, J. (2023). Antioxidation and anti-inflammatory effects of gamma-irradiated silk sericin and fibroin in H2O2-induced HaCaT Cell. *The Korean Journal of Physiology & Pharmacology : Official Journal of the Korean Physiological Society and the Korean Society of Pharmacology*, *27*(1), 105–112. https://doi.org/10.4196/KJPP.2023.27.1.105

Chuysinuan, P., Pengsuk, C., Lirdprapamongkol, K., Thanyacharoen, T., Techasakul, S., Svasti, J., & Nooeaid, P. (2023). Turmeric Herb Extract-Incorporated Biopolymer Dressings with Beneficial Antibacterial, Antioxidant and Anti-Inflammatory

Properties for Wound Healing. *Polymers*, *15*(5). https://doi.org/10.3390/POLYM15051090

Da Silva, T. L., Da Silva, A. C., Ribani, M., Vieira, M. G. A., Gimenes, M. L., & Da Silva, M. G. C. (2014). Evaluation of molecular weight distribution of sericin in solutions concentrated via precipitation by ethanol and precipitation by freezing/thawing. *Chemical Engineering Transactions*, *38*, 103–108. https://doi.org/10.3303/CET1438018

de la Fuente, B., Pinela, J., Mandim, F., Heleno, S. A., Ferreira, I. C. F. R., Barba, F. J., Berrada, H., Caleja, C., & Barros, L. (2022). Nutritional and bioactive oils from salmon (Salmo salar) side streams obtained by Soxhlet and optimized microwave-assisted extraction. *Food Chemistry*, *386*, 132778. https://doi.org/10.1016/j.foodchem.2022.132778

de Magalhães, A. P. S. A. (2015). *Atividade Antimicrobiana em Têxteis*. University of Porto.

Deenonpoe, R., Prayong, P., Thippamom, N., Meephansan, J., & Na-Bangchang, K. (2019). Anti-inflammatory effect of naringin and sericin combination on human peripheral blood mononuclear cells (hPBMCs) from patient with psoriasis. *BMC Complementary and Alternative Medicine*, *19*(1), 168. https://doi.org/10.1186/s12906-019-2535-3

Dennison, S., Whittaker, M., Harris, F., & Phoenix, D. (2006). Anticancer alpha-helical peptides and structure/function relationships underpinning their interactions with tumour cell membranes. *Current Protein & Peptide Science*, *7*(6), 487–499. https://doi.org/10.2174/138920306779025611

Dhahri, M., Sioud, S., Dridi, R., Hassine, M., Boughattas, N. A., Almulhim, F., Al Talla, Z., Jaremko, M., & Emwas, A. H. M. (2020). Extraction, Characterization, and Anticoagulant Activity of a Sulfated Polysaccharide from Bursatella leachii Viscera. *ACS Omega*, *5*(24), 14786–14795. https://doi.org/10.1021/ACSOMEGA.0C01724/ASSET/IMAGES/MEDIUM/AO0C01724_M002.GIF

Engelmann, B., & Massberg, S. (2013). Thrombosis as an intravascular effector of innate immunity. *Nature Reviews Immunology*, *13*(1), 34–45. https://doi.org/10.1038/NRI3345

Espinel-Ingroff, A. (2001). Comparison of the E-test with the NCCLS M38-P method for antifungal susceptibility testing of common and emerging pathogenic filamentous fungi. *Journal of Clinical Microbiology*, *39*(4), 1360–1367. https://doi.org/10.1128/JCM.39.4.1360-1367.2001/ASSET/FCA363A5-1D62-44BC-A0CB-AFDD518DC12B/ASSETS/GRAPHIC/JM0411110002.JPEG

Fatahian, R., Fatahian, A., Fatahian, E., & Fatahian, H. (2021). A critical review on application of silk sericin and its mechanical properties in various industries. *Journal of Research and Applications in Mechanical Engineering*, *9*(2), 2229–2152. https://doi.org/10.14456/jrame.2021.19

Fountoulakis, M., & Lahm, H. W. (1998). Hydrolysis and amino acid composition analysis of proteins. *Journal of Chromatography A*, *826*(2), 109–134. https://doi.org/10.1016/S0021-9673(98)00721-3

Gauthier, N., Mandon, N., Renault, S., & Bénédet, F. (2004). The Acrolepiopsis assectella silk cocoon: kairomonal function and chemical characterisation. *Journal of Insect Physiology*, *50*(11), 1065–1074. https://doi.org/10.1016/J.JINSPHYS.2004.09.008

Gilotra, S., Chouhan, D., Bhardwaj, N., Nandi, S. K., & Mandal, B. B. (2018). Potential of silk sericin based nanofibrous mats for wound dressing applications. *Materials Science & Engineering. C, Materials for Biological Applications*, *90*, 420–432. https://doi.org/10.1016/J.MSEC.2018.04.077

Gimenes, M. L., Silva, V. R., Vieira, M. G. A., Silva, M. G. C., & Scheer, A. P. (2014). High Molecular Sericin from Bombyx mori Cocoons: Extraction and Recovering by Ultrafiltration. *International Journal of Chemical Engineering and Applications*, *5*(3), 266–271. https://doi.org/10.7763/IJCEA.2014.V5.391

Goodno, C. C., Swaisgood, H. E., & Catignani, G. L. (1981). A fluorimetric assay for available lysine in proteins. *Analytical Biochemistry*, *115*(1), 203–211. https://doi.org/10.1016/0003-2697(81)90547-9

Gore, P. M., Naebe, M., Wang, X., & Kandasubramanian, B. (2019). Progress in silk materials for integrated water treatments: Fabrication, modification and applications. *Chemical Engineering Journal*, *374*, 437–470. https://doi.org/10.1016/J.CEJ.2019.05.163

Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L. D., Hidalgo, F. J., Miyashita, K., Camp, J. van, Alasalvar, C., Ismail, A. B., Elmore, S., Birch, G. G., Charalampopoulos, D., Astley, S. B., Pegg, R., Zhou, P., & Finglas, P. (2018). Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? *Food Chemistry*, *264*, 471–475. https://doi.org/10.1016/j.foodchem.2018.04.012

Guglietta, S., Chiavelli, A., Zagato, E., Krieg, C., Gandini, S., Ravenda, P. S., Bazolli, B., Lu, B., Penna, G., & Rescigno, M. (2016). Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis. *Nature Communications*, *7*. https://doi.org/10.1038/NCOMMS11037

Hakimi, O., Gheysens, T., Vollrath, F., Grahn, M. F., Knight, D. P., & Vadgama, P. (2010). Modulation of cell growth on exposure to silkworm and spider silk fibers. *Journal of Biomedical Materials Research Part A*, *92A*(4), 1366–1372. https://doi.org/10.1002/JBM.A.32462

Halliwell, B., Murcia, M. A., Chirico, S., & Aruoma, O. I. (1995). Free radicals and antioxidants in food and in vivo: what they do and how they work. *Critical Reviews in Food Science and Nutrition*, *35*(1–2), 7–20. https://doi.org/10.1080/10408399509527682

Hänel, H., & Raether, W. (1988). A More Sophisticated Method of Determining the Fungicidal Effect of Water-Insoluble Preparations with a Cell Harvester, Using Miconazole as an Example./Eine verbesserte Methode zur Bestimmung der Fungizidie von wasserunlöslichen Präparaten mit Hilfe eines Zellerntegerätes am Beispiel von Miconazol. *Mycoses*, *31*(3), 148–154. https://doi.org/10.1111/J.1439-0507.1988.TB03718.X

Harris, F., Dennison, S. R., Singh, J., & Phoenix, D. A. (2013). On the selectivity and efficacy of defense peptides with respect to cancer cells. *Medicinal Research Reviews*, *33*(1), 190–234. https://doi.org/10.1002/MED.20252

Harun, Z., Arsad, A., Pang, A. L., Abbas, M., Zaini, A., Abdurrahman, · Muslim, Awang, N., Junin, R., Rahmat Mohsin, ·, & My, A. (2010). *Acid Hydrolysis and Optimization Techniques for Nanoparticles Preparation: Current Review*. https://doi.org/10.1007/s12010-022-03932-6

Honzel, D., Carter, S. G., Redman, K. A., Schauss, A. G., Endres, J. R., & Jensen, G. S. (2008). Comparison of Chemical and Cell-Based Antioxidant Methods for Evaluation of Foods and Natural Products: Generating Multifaceted Data by Parallel Testing Using Erythrocytes and Polymorphonuclear Cells. *Journal of Agricultural and Food Chemistry*, *56*(18), 8319–8325. https://doi.org/10.1021/jf800401d

Kaewkorn, W., Limpeanchob, N., Tiyaboonchai, W., Pongcharoen, S., & Sutheerawattananonda, M. (2012). Effects of silk sericin on the proliferation and apoptosis of colon cancer cells. *Biological Research*, *45*(1), 45–50. https://doi.org/10.4067/S0716-97602012000100006

Kato, N., Sato, S., Yamanaka, A., Yamada, H., Fuwa, N., & Nomura, M. (1998). Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. *Bioscience, Biotechnology and Biochemistry*, *62*(1), 145–147. https://doi.org/10.1271/bbb.62.145

Keawkorn, W., Limpeanchob, N., Tiyaboonchai, W., Pongcharoen, S., & Sutheerawattananonda, M. (2013). The effect of dietary sericin on rats. *ScienceAsia*, *39*(3), 252. https://doi.org/10.2306/scienceasia1513-1874.2013.39.252

Kuete, V., Ango, P. Y., Fotso, G. W., Kapche, G. D., Dzoyem, J. P., Wouking, A. G., Ngadjui, B. T., & Abegaz, B. M. (2011). Antimicrobial activities of the methanol extract and compounds from Artocarpus communis (Moraceae). *BMC Complementary and Alternative Medicine*, *11*(1), 42. https://doi.org/10.1186/1472-6882-11-42

Kuete, V., Kamga, J., Sandjo, L. P., Ngameni, B., Poumale, H. M., Ambassa, P., & Ngadjui, B. T. (2011). Antimicrobial activities of the methanol extract, fractions and

compounds from Ficus polita Vahl. (Moraceae). *BMC Complementary and Alternative Medicine*, *11*(1), 6. https://doi.org/10.1186/1472-6882-11-6

Kumar, J. P., Alam, S., Jain, A. K., Ansari, K. M., & Mandal, B. B. (2018). Protective Activity of Silk Sericin against UV Radiation-Induced Skin Damage by Downregulating Oxidative Stress. *ACS Applied Bio Materials*, *1*(6), 2120–2132. https://doi.org/10.1021/ACSABM.8B00558

Kumar, J. P., & Mandal, B. B. (2017). Antioxidant potential of mulberry and non-mulberry silk sericin and its implications in biomedicine. *Free Radical Biology and Medicine*, *108*, 803–818. https://doi.org/10.1016/J.FREERADBIOMED.2017.05.002

Kumar, J. P., & Mandal, B. B. (2019a). Silk sericin induced pro-oxidative stress leads to apoptosis in human cancer cells. *Food and Chemical Toxicology*, *123*, 275–287. https://doi.org/10.1016/j.fct.2018.10.063

Kumar, J. P., & Mandal, B. B. (2019b). The inhibitory effect of silk sericin against ultraviolet-induced melanogenesis and its potential use in cosmeceutics as an anti-hyperpigmentation compound. *Photochemical & Photobiological Sciences : Official Journal of the European Photochemistry Association and the European Society for Photobiology*, *18*(10), 2497–2508. https://doi.org/10.1039/C9PP00059C

Kumar, S., Krishna Chaitanya, R., & Preedy, V. R. (2017). Assessment of Antioxidant Potential of Dietary Components. *HIV/AIDS: Oxidative Stress and Dietary Antioxidants*, 239–253. https://doi.org/10.1016/B978-0-12-809853-0.00020-1

Kundu, S. C., Dash, B. C., Dash, R., & Kaplan, D. L. (2008). Natural protective glue protein, sericin bioengineered by silkworms: Potential for biomedical and biotechnological applications. *Progress in Polymer Science*, *33*(10), 998–1012. https://doi.org/10.1016/J.PROGPOLYMSCI.2008.08.002

Kunz, R. I., Brancalhão, R. M. C., Ribeiro, L. D. F. C., & Natali, M. R. M. (2016). Silkworm Sericin: Properties and Biomedical Applications. *BioMed Research International*, *2016*. https://doi.org/10.1155/2016/8175701

Kunz, R. I., Capelassi, A. N., Alegre-Maller, A. C. P., Bonfleur, M. L., Ribeiro, L. de F. C., Costa, R. M., & Natali, M. R. M. (2020). Sericin as treatment of obesity:

morphophysiological effects in obese mice fed with high-fat diet. *Einstein (Sao Paulo, Brazil)*, *18*, eAO4876. https://doi.org/10.31744/einstein_journal/2020AO4876

Lamboni, L., Gauthier, M., Yang, G., & Wang, Q. (2015). Silk sericin: A versatile material for tissue engineering and drug delivery. *Biotechnology Advances*, *33*(8), 1855–1867. https://doi.org/10.1016/J.BIOTECHADV.2015.10.014

Lamponi, S., & Jung, F. (2021). Bioactive Natural Compounds with Antiplatelet and Anticoagulant Activity and Their Potential Role in the Treatment of Thrombotic Disorders. *Life 2021, Vol. 11, Page 1095*, *11*(10), 1095. https://doi.org/10.3390/LIFE11101095

Le Plénier, S., Walrand, S., Noirt, R., Cynober, L., & Moinard, C. (2012). Of leucine and citrulline versus non-essential amino acids on muscle protein synthesis in fasted rat: A common activation pathway? *Amino Acids*, *43*(3), 1171–1178. https://doi.org/10.1007/S00726-011-1172-Z/FIGURES/3

Liu, Z., Zhou, Y., Liu, S., Zhong, H., Zhang, C., Kang, X., & Liu, Y. (2012). Characterization and dietary regulation of glutamate dehydrogenase in different ploidy fishes. *Amino Acids*, *43*(6), 2339–2348. https://doi.org/10.1007/S00726-012-1313-Z

Lv, Z., Shi, W., & Zhang, Q. (2022). Role of Essential Amino Acids in Age-Induced Bone Loss. *International Journal of Molecular Sciences 2022, Vol. 23, Page 11281*, *23*(19), 11281. https://doi.org/10.3390/IJMS231911281

Ma, Y., Tong, X., Huang, Y., Zhou, X., Yang, C., Chen, J., Dai, F., & Xiao, B. (2019). Oral Administration of Hydrogel-Embedding Silk Sericin Alleviates Ulcerative Colitis through Wound Healing, Anti-Inflammation, and Anti-Oxidation. *ACS Biomaterials Science & Engineering*, *5*(11), 6231–6242. https://doi.org/10.1021/acsbiomaterials.9b00862

Maisuthisakul, P., Suttajit, M., & Pongsawatmanit, R. (2007). Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. *Food Chemistry*, *100*(4), 1409–1418. https://doi.org/10.1016/J.FOODCHEM.2005.11.032

Malmer, M. F., & Schroeder, L. A. (1990). Amino acid anslysis by high-performance liquid chromatography with methanesulfonic acid hydrolysis and 9-fluorenylmethylchloroformate derivatization. *Journal of Chromatography A*, *514*(C), 227–239. https://doi.org/10.1016/S0021-9673(01)89394-8

Mandim, F., Petropoulos, S. A., Dias, M. I., Pinela, J., Kostic, M., Soković, M., Santos-Buelga, C., Ferreira, I. C. F. R., & Barros, L. (2020). *Seasonal variation in bioactive properties and phenolic composition of cardoon (Cynara cardunculus var. altilis) bracts*. https://doi.org/10.1016/j.foodchem.2020.127744

Manesa, K. C., Kebede, T. G., Dube, S., & Nindi, M. M. (2020). Profiling of Silk Sericin from Cocoons of Three Southern African Wild Silk Moths with a Focus on Their Antimicrobial and Antioxidant Properties. *Materials (Basel, Switzerland)*, *13*(24), 1–16. https://doi.org/10.3390/MA13245706

Manosroi, A., Boonpisuttinant, K., Winitchai, S., Manosroi, W., & Manosroi, J. (2010). Free radical scavenging and tyrosinase inhibition activity of oils and sericin extracted from Thai native silkworms (*Bombyx mori*). *Pharmaceutical Biology*, *48*(8), 855–860. https://doi.org/10.3109/13880200903300212

Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. (n.d.). Retrieved December 7, 2023, from www.clsi.org.

Midoux, P., Kichler, A., Boutin, V., Maurizot, J. C., & Monsigny, M. (1998). Membrane permeabilization and efficient gene transfer by a peptide containing several histidines. *Bioconjugate Chemistry*, *9*(2), 260–267. https://doi.org/10.1021/BC9701611

Monti, P., Freddi, G., Arosio, C., Tsukada, M., Arai, T., & Taddei, P. (2007). Vibrational spectroscopic study of sulphated silk proteins. *Journal of Molecular Structure*, *834–836*(SPEC. ISS.), 202–206. https://doi.org/10.1016/J.MOLSTRUC.2006.11.009

Mumtaz, S., Ali, S., Kazmi, S. A. R., Mughal, T. A., Mumtaz, S., Tahir, H. M., Summer, M., Ara, C., & Rashid, M. I. (2023). Analysis of the antimicrobial potential of sericin-coated silver nanoparticles against human pathogens. *Microscopy Research and Technique*, *86*(3), 320–330. https://doi.org/10.1002/JEMT.24273

Munir, F., Tahir, H. M., Ali, S., Ali, A., Tehreem, A., Zaidi, S. D. E. S., Adnan, M., & Ijaz, F. (2023). Characterization and Evaluation of Silk Sericin-Based Hydrogel: A Promising Biomaterial for Efficient Healing of Acute Wounds. *ACS Omega*, *8*(35). https://doi.org/10.1021/ACSOMEGA.3C04178

Navarro, S., Aleu, J., Jiménez, M., Boix, E., Cuchillo, C. M., & Nogués, M. V. (2008). The cytotoxicity of eosinophil cationic protein/ribonuclease 3 on eukaryotic cell lines takes place through its aggregation on the cell membrane. *Cellular and Molecular Life Sciences : CMLS*, *65*(2), 324–337. https://doi.org/10.1007/S00018-007-7499-7

Nirmala, X., Mita, K., Vanisree, V., Žurovec, M., & Sehnal, F. (2001). Identification of four small molecular mass proteins in the silk of Bombyx mori. *Insect Molecular Biology*, *10*(5), 437–445. https://doi.org/10.1046/J.0962-1075.2001.00282.X

Noosak, C., Jantorn, P., Meesane, J., Voravuthikunchai, S., & Saeloh, D. (2022). Dual-functional bioactive silk sericin for osteoblast responses and osteomyelitis treatment. *PLoS ONE*, *17*(3 March). https://doi.org/10.1371/JOURNAL.PONE.0264795

Oancea, E., Teruel, M. N., Quest, A. F. G., & Meyer, T. (1998). Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells. *The Journal of Cell Biology*, *140*(3), 485–498. https://doi.org/10.1083/JCB.140.3.485

Obayashi, Y., Arisaka, H., Yoshida, S., Mori, M., & Takahashi, M. (2012). Proline protects liver from D-galactosamine hepatitis by activating the IL-6/STAT3 survival signaling pathway. *Amino Acids*, *43*(6), 2371–2380. https://doi.org/10.1007/S00726-012-1317-8

Olajide, O. A., & Sarker, S. D. (2020). Anti-inflammatory natural products. *Annual Reports in Medicinal Chemistry*, *55*, 153–177. https://doi.org/10.1016/BS.ARMC.2020.02.002

Onishi, A., St Ange, K., Dordick, J. S., & Linhardt, R. J. (2016). Heparin and anticoagulation. *Frontiers in Bioscience - Landmark*, *21*(7), 1372–1392. https://doi.org/10.2741/4462/PDF

Panilaitis, B., Altman, G. H., Chen, J., Jin, H. J., Karageorgiou, V., & Kaplan, D. L. (2003). Macrophage responses to silk. *Biomaterials*, *24*(18), 3079–3085. https://doi.org/10.1016/S0142-9612(03)00158-3

Romulo, A. (2020). The Principle of Some In vitro Antioxidant Activity Methods: Review. *IOP Conference Series: Earth and Environmental Science*, *426*(1), 012177. https://doi.org/10.1088/1755-1315/426/1/012177

Roudi, R., Syn, N. L., & Roudbary, M. (2017). Antimicrobial peptides as biologic and immunotherapeutic agents against cancer: A comprehensive overview. *Frontiers in Immunology*, *8*(OCT), 305858. https://doi.org/10.3389/FIMMU.2017.01320/BIBTEX

Saha, J., Ibrahim Mondal, M. H., Rezaul Karim Sheikh, M., Ahsan Habib, M., & Mondal, I. H. (2019). *Extraction, Structural and Functional Properties of Silk Sericin Biopolymer from Bombyx mori Silk Cocoon Waste*. https://doi.org/10.4172/2165-8064.1000390

Sahu, N., Pal, S., Sapru, S., Kundu, J., Talukdar, S., Singh, N. I., Yao, J., & Kundu, S. C. (2016). Non-Mulberry and Mulberry Silk Protein Sericins as Potential Media Supplement for Animal Cell Culture. *BioMed Research International*, *2016*, 1–13. https://doi.org/10.1155/2016/7461041

Sakkas, H., & Papadopoulou, C. (2017). Antimicrobial Activity of Basil, Oregano, and Thyme Essential Oils. *J. Microbiol. Biotechnol.*, *27*(3), 429–438. https://doi.org/10.4014/JMB.1608.08024

Sano, M., Tamada, Y., Niwa, K., Morita, T., & Yoshino, G. (2009). Sulfated sericin is a novel anticoagulant influencing the blood coagulation cascade. *Journal of Biomaterials Science. Polymer Edition*, *20*(5–6), 773–783. https://doi.org/10.1163/156856209X426853

Shamova, O., Orlov, D., Stegemann, C., Czihal, P., Hoffmann, R., Brogden, K., Kolodkin, N., Sakuta, G., Tossi, A., Sahl, H. G., Kokryakov, V., & Lehrer, R. I. (2009). ChBac3.4: A novel proline-rich antimicrobial peptide from goat leukocytes. *International Journal of Peptide Research and Therapeutics*, *15*(1), 31–42. https://doi.org/10.1007/S10989-008-9159-7/METRICS

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., & Boyd, M. R. (1990). New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. *JNCI Journal of the National Cancer Institute*, *82*(13), 1107–1112. https://doi.org/10.1093/jnci/82.13.1107

Sobral, F., Sampaio, A., Falcão, S., Queiroz, M. J. R. P., Calhelha, R. C., Vilas-Boas, M., & Ferreira, I. C. F. R. (2016). Chemical characterization, antioxidant, anti-inflammatory and cytotoxic properties of bee venom collected in Northeast Portugal. *Food and Chemical Toxicology*, *94*, 172–177. https://doi.org/10.1016/j.fct.2016.06.008

Song, I. B., Han, H. J., & Kwon, J. (2020). Immune-enhancing effects of gamma-irradiated sericin. *Food Science and Biotechnology*, *29*(7), 969–976. https://doi.org/10.1007/S10068-020-00734-6

Tamada, Y., Sano, M., Niwa, K., Imai, T., & Yoshino, G. (2004). Sulfation of silk sericin and anticoagulant activity of sulfated sericin. *Journal of Biomaterials Science, Polymer Edition*, *15*(8), 971–980. https://doi.org/10.1163/1568562041526469

Tsukatani, T., Suenaga, H., Shiga, M., Noguchi, K., Ishiyama, M., Ezoe, T., & Matsumoto, K. (2012). Comparison of the WST-8 colorimetric method and the CLSI broth microdilution method for susceptibility testing against drug-resistant bacteria. *Journal of Microbiological Methods*, *90*(3), 160–166. https://doi.org/10.1016/J.MIMET.2012.05.001

Tuentam, K., Aramwit, P., Reamtong, O., Supasai, S., Chaisri, U., Fongsodsri, K., Yamdech, R., Tirawanchai, N., Sukphopetch, P., & Ampawong, S. (2022). Sericin-Based Poly(Vinyl) Alcohol Relieves Plaque and Epidermal Lesions in Psoriasis; a Chance for Dressing Development in a Specific Area. *International Journal of Molecular Sciences*, *24*(1). https://doi.org/10.3390/IJMS24010145

Umerah, C. o., & Momodu, I. I. (2023). Anticoagulation. *Cardiology Board Review, Second Edition*, 615–626. https://doi.org/10.1002/9781119814979.ch30

Wolfe, K. L., & Liu, R. H. (2007). Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements. *Journal of Agricultural and Food Chemistry*, *55*(22), 8896–8907. https://doi.org/10.1021/jf0715166

Wu, G. (2010). Functional amino acids in growth, reproduction, and health. *Advances in Nutrition (Bethesda, Md.)*, *1*(1), 31–37. https://doi.org/10.3945/AN.110.1008

Wu, G., Wu, Z., Dai, Z., Yang, Y., Wang, W., Liu, C., Wang, B., Wang, J., & Yin, Y. (2013). Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. *Amino Acids*, *44*(4), 1107–1113. https://doi.org/10.1007/S00726-012-1444-2/TABLES/3

Xu, Y., Cai, C., Chandarajoti, K., Hsieh, P. H., Li, L., Pham, T. Q., Sparkenbaugh, E. M., Sheng, J., Key, N. S., Pawlinski, R., Harris, E. N., Linhardt, R. J., & Liu, J. (2014). Homogeneous low-molecular-weight heparins with reversible anticoagulant activity. *Nature Chemical Biology*, *10*(4), 248–250. https://doi.org/10.1038/NCHEMBIO.1459

Yamaguchi, Y., Yamamoto, K., Sato, Y., Inoue, S., Morinaga, T., & Hirano, E. (2016). Combination of aspartic acid and glutamic acid inhibits tumor cell proliferation. *Biomedical Research (Tokyo, Japan)*, *37*(2), 153–159. https://doi.org/10.2220/BIOMEDRES.37.153

Yun, H., Oh, H., Kim, M. K., Kwak, H. W., Lee, J. Y., Um, I. C., Vootla, S. K., & Lee, K. H. (2013). Extraction conditions of Antheraea mylitta sericin with high yields and minimum molecular weight degradation. *International Journal of Biological Macromolecules*, *52*(1), 59–65. https://doi.org/10.1016/J.IJBIOMAC.2012.09.017

Zhaorigetu, S., Yanaka, N., Sasaki, M., Watanabe, H., & Kato, N. (2003). Inhibitory effects of silk protein, sericin on UVB-induced acute damage and tumor promotion by reducing oxidative stress in the skin of hairless mouse. *Journal of Photochemistry and Photobiology B: Biology*, *71*(1–3), 11–17. https://doi.org/10.1016/S1011-1344(03)00092-7