INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Chemical and biological studies of Portuguese Bee venom

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Abbreviations

2D - Two dimensions

AAPH – 2,2 – azobis – 2 – methyl – propanimidamide, dihydrochloride

AChE - Acetylcholinesterase

AD - Alzheimers disease

AgCl – Silver chloride

Al - Aluminium

Pt – Platinum working electrode

OD - Outside diameter

ID - Interior diameter

ANOVA – Analysis of variance

As - Arsenic

ASDPV - Anodic stripping differential pulse voltametry

ATCC - American type culture collection

B - Boron

Ba - Barium

Ca - Calcium

Cd - Cadmium

Co - Cobalt

Cr - Chromium

Cu - Copper

$CuSO_4.5H_2O$ - Sulfate of copper penta hidrated

DMSO - Dimethyl sulphoxide

DPV - Differential Pulse Voltammetry

DTNB - 5,5'- dithiobis-2-nitrobenzoic acid

GCE - Glassy carbon electrode

ELISA - Enzyme linked immunosorbent assay

Epeak – Peak potential

ESA – Escola Superior Agrária

ESI - Electrospray ionization

EUR - Euro

GC-MS – Gas chromatography with mass spectrometry detector

H_2SO_4 - Sulfuric acid

HCl – Hydrochloric acid

HPLC - High-Performance Liquid Chromatography

IC50 - Half Maximal Inhibitory Concentration

ICP-MS - Inductively coupled plasma with mass spectrometry detector

IPB - Instituto Politécnico de Bragança

IPPG - Ion Permeability Porous Glass

IST – Instituto Superior Técnico

K - Potassium

KI - Potassium iodide

LC - Liquid Chromatography

LD50 – Lethal dose required to kill 50% of microorganisms

LOX - Lipoxygenase

MALDI - Matrix Assisted Laser Desorption Ionization

MCD - Mast Cell Degranulating

Mg - Magnesium

MHB - Mueller Hinton Broth

MIC - Minimum inhibitory concentration

Mn - Manganese

Mo - Molibidium

MRSA - Methicillin resistant *Staphilococcus Aureus*

MS - Mass spectrometry

Na - Sodium

NaOH - Sodium hydroxide

NDGA - Nordihydroguaiaretic Acid

Ni - Nickel

NPB - Nucleolus precursor body

O.D - Optical density

Pb - Lead

PBS - Phosphate buffered saline

PDMAB - p-dimethylaminobenzaldehyde

PEEK – polyether ether ketone

pH – Scale to specify the acidity/basicity of an aqueous solution; equal to $-\log_{10}[H3O^{+}]$

PLA2 - Phospholipase A2

PNPG - p-Nitrophenyl-β-D-glucopyranoside

Q – Quadrupole

QqQ – Triple quadrupole mass spectrometry

QToF - Quadrupole time of flight

ReD - Red Blood Cells

RSE – Relative standard error

Sb - Antimonium

SDS - Sodium Dodecyl Sulfate

Sr - Strontium

Tof - Time of flight

UV - Ultra Violet

V - Vanadium

Zn - Zinc

Abstract

This research aimed to develop analytical methodologies for the quality control of apitoxin, as well as evaluating its biological activities. A spectrophotometer method was established to estimate the total protein content, equivalent to albumin, in the apitoxin samples. Its advantage lies in preserving sample integrity. Additionally, electrochemical methodologies were developed, allowing confirmation that apitoxin lacks antioxidant activity and implementing quality control measures for four heavy metals (Zn, Cd, Pb, and Cu).

The antimicrobial activity of the five apitoxin samples revealed bactericidal action against all tested strains of *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, except for *Pseudomonas aeruginosa* strains, which remained unaffected. Among the samples, Sample 1 exhibited the lowest MIC values (9.2 µg/mL) against *Staphylococcus aureus*, meaning that this bacteria was the most sensitive to the negative effects of apitoxine. The highest MIC (41.8 µg/mL) was observed against a strain of *Klebsiella pneumoniae* in the presence of Sample 5, denoting increased resistance to apitoxin.

Portuguese apitoxin demonstrated significant inhibitory effects against various enzymes: xanthine oxidase (IC50 values ranged between 1.7± 0.4 and 6.3± 0.85 µg/mL), lipase (IC50 values ranged from 0.05 ± 0.01µg/mL to 0.16 ±0, 0.03 µg/mL), α-amylase (IC50 values between 0.08 ± 0.02 and 0.4 ± 0.12 µg/mL), α-glucosidase (IC50 values between 0.03 ± 0.01 and 0.14 ± 0.01 µg/mL), lipoxygenase (IC50 values in the range of 0.06± 0.01 to 0.25± 0.04 µg /mL), acetylcholinesterase (IC50 values ranged from 0.78 ± 0.2 to 3.75 ± 0.2 µg/mL), tyrosinase (IC50 values between 1.6 ± 0.1 to 4.8 ± 0.24 µg/mL), and hyaluronidase (inhibition percentage between 85±5.4 % and 75.0±3.3%).

In conclusion, apitoxin possesses valuable antimicrobial and enzyme-inhibiting. These findings suggest that apitoxin could hold potential significance in addressing various health conditions and diseases, warranting further scientific investigations due to its potential application in clinical or pharmaceutical settings.

Resumo

Esta investigação teve como objetivo o desenvolvimento de metodologias analíticas para o controlo de qualidade da apitoxina, bem como a avaliação das suas actividades biológicas. Foi estabelecido um método espetrofotométrico para estimar o teor de proteína total, equivalente à albumina, nas amostras de apitoxina. A sua vantagem reside na preservação da integridade da amostra. Adicionalmente, foram desenvolvidas metodologias electroquímicas que permitiram confirmar a ausência de atividade antioxidante da apitoxina e implementar medidas de controlo de qualidade para quatro metais pesados (Zn, Cd, Pb e Cu).

A atividade antimicrobiana das cinco amostras de apitoxina revelou uma ação bactericida contra todas as estirpes testadas de *Escherichia coli*, *Staphylococcus aureus* e *Klebsiella pneumoniae*, com exceção das estirpes de *Pseudomonas aeruginosa*, que não foram afectadas. Entre as amostras, a Amostra 1 apresentou os valores mais baixos de CIM (9,2 µg/mL) contra *Staphylococcus aureus*, o que significa que esta bactéria foi a mais sensível aos efeitos negativos da apitoxina. A CIM mais elevada (41,8 µg/mL) foi observada contra uma estirpe de *Klebsiella pneumoniae* na presença da Amostra 5, denotando um aumento da resistência à apitoxina.

A apitoxina portuguesa demonstrou efeitos inibitórios significativos contra várias enzimas: xantina oxidase (os valores de IC50 variaram entre 1,7± 0,4 e 6,3± 0. 85 µg/mL), lipase (valores de IC50 variaram entre 0,05 ± 0,01µg/mL e 0,16 ±0, 0,03 µg/mL), α-amilase (valores de IC50 entre 0,08 ± 0,02 e 0,4 ± 0,12 µg/mL), α-glucosidase (valores de IC50 entre 0. 03 ± 0,01 e 0,14 ± 0,01 µg/mL), lipoxigenase (valores de IC50 na faixa de 0,06± 0,01 a 0,25± 0,04 µg /mL), acetilcolinesterase (valores de IC50 variaram de 0,78 ± 0. 2 a 3,75 ± 0,2 µg/mL), tirosinase (valores de IC50 entre 1,6 ± 0,1 a 4,8 ± 0,24 µg/mL) e hialuronidase (percentagem de inibição entre 85±5,4% e 75,0±3,3%).

Em conclusão, a apitoxina possui um valioso efeito antimicrobiano e inibidor de enzimas. Estes resultados sugerem que a apitoxina pode ter um significado potencial na abordagem de várias condições de saúde e doenças, justificando mais investigações científicas devido à sua potencial aplicação em contextos clínicos ou farmacêuticos.

Framing the issue

This work was part of the project: PHARMAPITOX - Development of an innovative collector and protocol for purifying apitoxin for use in the pharmaceutical and cosmetics industries, Co-promoted Business R&D Projects, NORTE-01-0247-FEDER-113540, Strengthening research, technological development and innovation, Region of intervention - NORTE, start date 01/05/21, end date 30/06/23, total eligible cost €691,818.37, European Union financial support €443,742.48 (FEDER), led at the Polytechnic Institute of Bragança by researchers L.G. Dias and Letícia Estevinho, and being the main leader the company ECOAPIS - UNIPESSOAL LDA.

Collector developed in the Pharmapitox project

The purpose was to study the Portuguese bee venom or apitoxin, which is one of the most valuable products in beekeeping and of interest to pharmaceutical and cosmetic industry. The venom can be an important source of income for beekeepers around the world. If it is of high quality and purified, the price can reach EUR 312/100mg for purified bee venom, and some compounds, such as purified melittin, can reach EUR 4240/100mg (www.sigmaaldrich.com). The project involved developing easy and quick analytical methodologies for apitoxin quality control to transfer to the Ecoapis company and evaluate the biological properties of Portuguese apitoxin.

The objectives of this work were:

- to develop a precise and accurate method for quantifying total protein content in samples in an innocuous way, ensuring no contamination of the sample;

- to optimize the analysis of heavy metals through voltammetry, ensuring that the method can detect a several heavy metal ions that can confirm its absence, with good sensitivity and specificity;
- to apply an electrochemical methodology to evaluate the apitoxin antioxidation power;
- to assess the antimicrobial, anti-adherence, enzymatic, and anti-hemolytic activities of apitoxin samples, ensuring a holistic evaluation of their potential biological effects and therapeutic applications.

This thesis is structured into 4 chapters:

- **Introduction,** where it is introduced the research topic and its significance, highlighting the key aspects that will be explored in detail throughout the thesis.
- **Material and Methods** is the chapter that outlines the methodologies and tools employed to analyze bee venom. This chapter presents the experimental procedures for the total protein quantification; heavy metals analysis by voltammetry; antioxidation power by voltammetry; antimicrobial activity; anti-adherence activity; enzyme activities; anti-hemolytic activity.
- **Results and discussion** is the chapter that presents the results of the work. The results are divided into the sections: quantification of total protein; heavy metals analysis by voltammetry; antioxidant power by voltammetry; antimicrobial activity; anti-adherence activity; enzyme activity; anti-hemolytic activity.
- **Conclusions,** where the most important conclusions of the experimental results.

The research lies in these chapters, as they collectively unravel the mysteries of bee venom composition and its diverse biological activities, aiming to contribute to the understanding of bee venom's potential applications in medicine and beyond.

1. INTRODUCTION

Bees produce venom as a defense mechanism, to protect themselves and their hive (self-defense against predators, insects or not) and as a hygienic tool, to get rid of diseases and bacteria. When a bee stings, it releases venom through a gland in its abdomen and injects it into the skin of the attacker. In high doses, venom can be harmful to humans, causing pain, swelling, and in some cases, allergic reactions that can be lethal (LD50 is about 2.8 mg/Kg in an adult), but when used in low concentrations it is considered safe and presents actions desirable at a physiological and even therapeutic level (Ali et al., 2012).

1.1. Bee venom composition

Bee venom is a complex mixture of biologically active compounds, including enzymes, hormones, and other molecules. Table 1 presents several analytical results about the seven main chemical composition of the bee venom.

Table 1– Main chemical components of the bee venom (% in the bee venom)

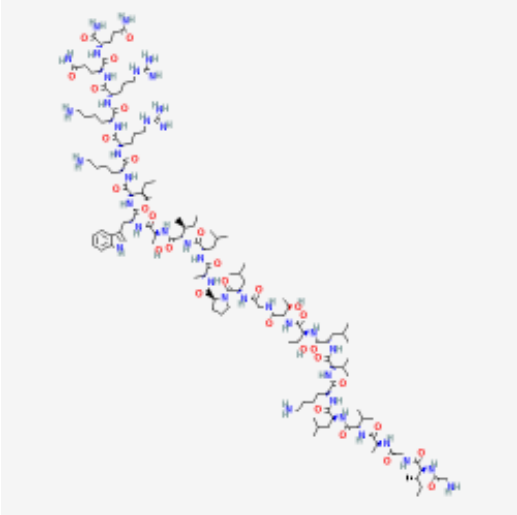
Studies	Melittin	PLA2	Apamin	MCD	Hyaluronidase	Secapin	Adolapin
Pascoal et al., 2019	40-60	10-12	2-3	3	1-2	0.5-2	0.5-1
Bogdanov et al, 2014	40-50	10-12	2-3	2-3	1-2	0.5-2	0.5-1
Carpena et al, 2020	50-60	10-12	1-3	1-3	1.5-2	1-2	0.1-0.8
Gajski et al, 2020	40-50	10-12	1-3	1-2	1-3	0.5-2	1.0
Eze et al, 2016	40-50	10-12	2-3	2-3	1-2	0.5-2	0.5-1

PLA2 - Phospholipase A2; MCD - Mast cell degranulating

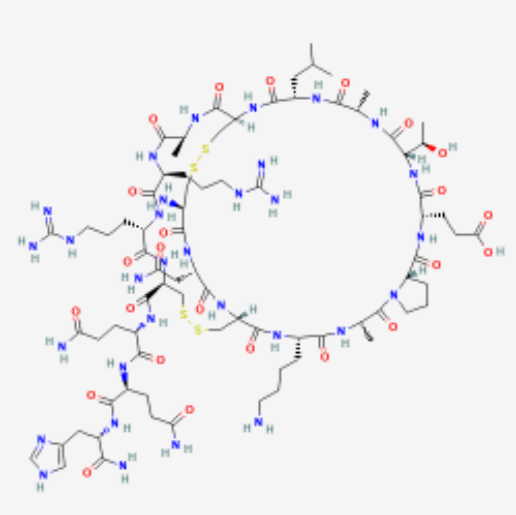
Considering the overall results presented in Table 1, the seven main components of bee venom are (Pascoal et al., 2019; Bogdanov et al, 2014; Carpena et al, 2020; Gajski et al, 2020; Eze et al, 2016, Lee et al, 2016, Shkenderov et al, 1982):

- Adolapin, constituting of 0.5 to 1% of the peptides, a basic peptide with 103 amino acid residues.
- Secapin, contributes with 0.5 to 2% of the peptides, composed of 25 amino acid residues that contain a disulfide link (Figure 1);
- Hyaluronidase, an enzyme that breaks down hyaluronic acid, a substance that helps to lubricate and protect tissues in the body, contributes with 1 to 3%; It has been shown to have anti- inflammatory effects and may also help to increase the absorption of other compounds in the venom.

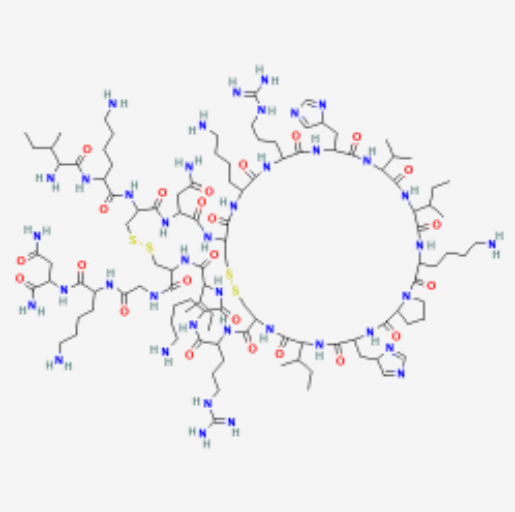
Melittin



Apamin



Mast cell degranulating peptide



Secapin

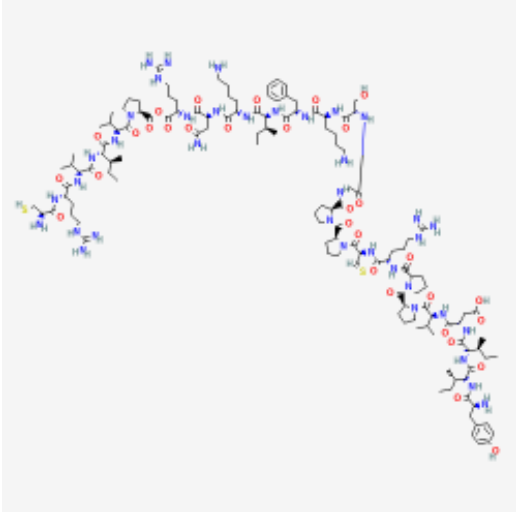


Figure 1 – Two-dimensional (2D) molecular structures representation of the compounds: melittin, phospholipase A2, mast cell degranulating peptide and secapin (download from PubChem Database).

- Mast cell degranulating (MCD), a cationic 22-amino acid residue peptide (Figure 1), represents 1 to 3% of the peptides;
- Apamin, a 18 amino acid globular peptide (Figure 1), contributes with ranges from 1 to 3%; It has a variety of effects on the nervous system, blocking the transmission of nerve impulses.

- Phospholipase A2 (PLA2), amounts more than 12% in bee venom, is an enzyme that breaks down phospholipids; It has been shown to have anti-inflammatory and analgesic effects.
- Melittin, composed of 26 amino polypeptides (Figure 1), being the main active component and is responsible for most of its toxic effects, constitutes over 40% of venom peptides; it is a powerful anti-inflammatory agent and has been shown to have pain-relieving properties.

These components collectively contribute to the wide range of biological effects attributed to bee venom, making it a subject of study for various biomedical and therapeutic applications.

In addition to these main components, bee venom also contains other biologically active molecules, including other enzymes and peptides. A more detailed compositional profile of bee venom was obtained in the works of Matysiak et al (2011) through the application of liquid chromatography - matrix-assisted laser desorption/ionization – time of flight/time of flight tandem mass spectrometry (hyphenated LC–MALDI–ToF/ToF–MS) and liquid chromatography - electrospray ionization - quadrupole time of flight tandem mass spectrometry (LC–ESI–QToF–MS) in proteomic characterization. With these techniques, it was possible to detect 269 proteins.

Several other studies were carried out to analyse several other minor compounds present in small amounts:

- Pawlak et al (2019) include seven organic acids analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS): citric acid (constituted at least 7.3% of the venom dry weight), fumaric acid, glutaric acid, kynurenic acid, malic acid, malonic acid and succinic acid.
- Wang et al (2019) analysed five biogenic amines bases on LC-MS/MS (histamine, 5-hydroxytryptamine, dopamine, adrenaline, and noradrenaline) in the range of μg/g of honeybee venom from different subspecies.
- Isidorov et al (2023) studied the composition of volatile and extractive components of dry and fresh bee venom by GC-MS. In this work, it was possible to identify 149 organic C1–C19 compounds of different classes in the volatile secretions of the studied bee venom samples, 152 organic C2–C36 compounds were registered in ether extracts, and 201 compounds were identified in methanol extracts.

- Kokot et al (2008) used the inductively coupled plasma mass spectrometry (ICP-MS) technique to analyze 20 metals/semi-metals in honeybee venom samples: Na, K, Ca, Mg, Al, Co, Cu, Zn, Mn, Mo, B, V, Sr, Ni and the toxic metals As, Ba, Pb, Cd, Sb and Cr. The metal levels were much lower than the tolerable upper intake levels for these elements.
- Klupczynska et al (2020) did an analysis of amino acids in honeybee venom samples was using various mass spectrometry techniques (LC-Q-Orbitrap-MS and LC-QqQ-MS/MS). The findings of this investigation revealed that the predominant amino acids present in the venom were alanine, arginine, aspartic acid, β-alanine, cystine, ethanolamine, glutamine, glutamic acid, histidine, proline, and taurine.

It is important to note that the concentrations of these minor compounds are usually very low in bee venom, and they are not the main biologically active compounds. But, these are just a few examples of the biologically active compounds that have been identified in bee venom. There are likely many other compounds present in bee venom that have yet to be fully characterized and understood.

1.2. Analytical methodologies

Several analytical methodologies are commonly used for the analysis of bee venom. Some of these include:

- High-performance liquid chromatography (HPLC): This is a powerful analytical technique that separates compounds based on their size, charge, and other physical properties. It can be used to identify and quantify the different components of bee venom, such as peptides, proteins, and enzymes (Dong et al., 2015; zókán et al., 1994; Kokot et al., 2009; Ionete et al., 2013).
- Mass spectrometry: This is a powerful analytical technique that measures the mass of molecules and ions. It can be used to identify and quantify the different components of bee venom, as well as to study the structure and function of these molecules. Usually, a mass spectrometry detector is coupled to a liquid or gas chromatography (Matysiak et al., 2016; Scaccabarozzi et al., 2021; Zhou et al., 2010; Tusiimire et al., 2015).
- Enzyme-linked immunosorbent assay (ELISA): This is a technique that uses antibodies to detect and quantify specific proteins or other molecules. It can be used

to measure the concentration of bee venom components, such as venom allergens or toxins (Xing et al., 2003).

- Western blotting: This is a technique that uses antibodies to detect and identify specific proteins. It can be used to study the components of bee venom and their function (Huang et al., 2021).
- Gel electrophoresis: This is a technique that separates molecules based on their size and charge. It can be used to study the molecular structure and composition of bee venom (Gajski et al., 2008; Peiren et al., 2005).

Electrochemical analysis is a type of analytical technique that uses an electrical current to measure the concentration of a substance or to study the chemical reactions that occur at an electrode. It can be applied to the analysis of bee venom in several ways:

- Cyclic voltammetry is a technique that measures the current produced by a substance as a function of the applied voltage. It can be used to study the electrochemical properties of bee venom components, such as the oxidation or reduction of proteins or enzymes (Becucci et al., 2017).
- Differential pulse voltammetry is a technique that measures the current produced by a substance in response to a small, pulsed voltage. It can be used to detect and quantify low concentrations of bee venom components (Nguyen et al., 2015).
- Square wave stripping voltammetric is also an electrochemical technique used to detect and quantify low concentrations of electroactive species in a solution. It uses a square wave potential, which is a series of rapid potential changes between a base value and a peak value at a fixed frequency (Yilmaz et al., 2016).
- Electrochemical impedance spectroscopy: This is a technique that measures the resistance and reactance of a substance to an applied electrical current. It can be used to study the electrical properties of bee venom and the interaction of venom components with electrodes (Becucci et al., 2017).

Overall, electrochemical techniques can be powerful tools for the analysis of proteins and other biomolecules, providing valuable information about their structure, function, and interaction with other molecules. The previous works showed that electrochemical tools have applicability in the analysis of bee venom, providing valuable information about its chemical and physical properties and the behavior of its components in different environments. In this study, it is also pretended to apply electrochemical analysis to

investigate the antioxidant properties of apitoxin and to carry out heavy metal analysis, for quality control of this natural product.

1.3. Toxic effects

The most common symptom of a bee sting is immediate pain and swelling at the site of the sting. The venom from the bee's sting contains enzymes that can break down tissues and cause inflammation. This can lead to redness, swelling, and warmth at the site of the sting. In some cases, a person may experience more severe allergic reactions to bee venom, which can include symptoms such as hives, difficulty in breathing, nausea, vomiting, dizziness, and fainting. The severity of the toxic effects of bee venom can vary depending on the species of bee, the amount of venom injected, and the individual's sensitivity to the venom. Some people may be more sensitive to bee venom and may experience more severe reactions than others (Hammoodi et al., 2021). In rare cases, a person may experience anaphylaxis, which is a life-threatening allergic reaction that requires immediate medical attention. It is important to seek medical attention if you experience any of these symptoms after being stung by a bee. Most bee stings can be treated with over-the-counter pain medications and other remedies, but more severe reactions may require more intensive treatment. (Lee et al., 2016; Hammoodi et al., 2021). The toxic effects of bee venom are due to a variety of biologically active compounds, including melittin (main active component of bee venom), apamin, and phospholipase A2. Melittin is a powerful anti-inflammatory agent and has been shown to have analgesic (pain-relieving) properties. However, it can also cause tissue damage and inflammation when injected into the skin (Brandão et al., 2020; Hammoodi et al,. 2021). Apamin is a small peptide that affects nerve transmission, and phospholipase A2 is an enzyme that breaks down phospholipids, which are a type of fat found in cell membranes (Brandão et al., 2020; Lee et al., 2016; Hammoodi et al., 2021).

1.4. Biological activities

In addition to its toxic effects, bee venom has been shown to have a variety of other biological activities, including antimicrobial, anti-tumor, and immune-modulatory effects. Bee venom is used in some traditional medicines and has been studied for its potential therapeutic effects in a variety of conditions, including arthritis, multiple

sclerosis, and cancer. However, more research is needed to fully understand the potential benefits and risks of using bee venom as a treatment.

Biological properties refer to the characteristics and functions of living organisms and their biological systems. These properties can be studied at various levels, including molecular, cellular, and physiological. Understanding the mechanisms of action behind these properties can provide insight into how living organisms function and interact with their environment.

One way to understand the mechanisms of action behind biological properties is to study the biochemical processes that occur within cells. These processes, such as metabolism and signaling pathways, involve the interactions of various biomolecules, including enzymes, hormones, and neurotransmitters. By understanding the role of these biomolecules and the pathways they participate in, we can gain a better understanding of how biological systems function and respond to various stimuli.

Another way to understand the mechanisms of action behind biological properties is to study the structure and function of cells and tissues. For example, understanding the structural and functional organization of tissues and organs can provide insight into how they carry out their specific roles within the body. This can also involve studying the interactions between different cell types and how they work together to maintain homeostasis within the body.

Overall, understanding the mechanisms of action behind biological properties requires a multidisciplinary approach that incorporates knowledge from various fields, including biochemistry, physiology, and cell biology. By studying these mechanisms, we can gain a deeper understanding of the complex processes that occur within living organisms and how they are regulated.

Bee venom has several biologically active compounds that can have a variety of effects on the body. Some of the main biological activities of bee venom include (Komi et al., 2018; Kim et al., 2019; Kim et al., 2020, Lee et al., 2016; Hammoodi et al., 2021):

- Anti-inflammatory effects: many of the biologically active compounds in bee venom, including melittin and phospholipase A2, have been shown to have anti-inflammatory effects. This means that they can help to reduce inflammation and swelling in the body.
- Analgesic (pain-relieving) effects: bee venom has been shown to have analgesic (pain-relieving) effects, which may be due to the presence of melittin and other biologically active compounds.

- Antimicrobial effects: some studies have suggested that bee venom may have antimicrobial properties and may be effective against a variety of bacteria and other microorganisms.
- Anti-tumor effects: some research has suggested that bee venom may have anti-tumor effects and may be effective against a variety of cancer cells. However, more research is needed to fully understand the potential benefits and risks of using bee venom as a cancer treatment.
- Immune-modulatory effects: bee venom has been shown to stimulate the immune system and increase the production of antibodies. This may have therapeutic effects in some conditions, such as autoimmune diseases.

It is important to note that more research is needed to fully understand the potential benefits and risks of using bee venom as a treatment for any condition. It is also important to use caution when handling bees or using bee venom, as it can be toxic and can cause allergic reactions in some individuals.

1.5. Enzymes activity

Enzymes are protein molecules that serve as biological catalysts, facilitating and accelerating essential chemical reactions in the human body. They regulate a wide array of processes, from digestion and energy production to detoxification and immune response. So, the study of compounds, termed enzyme modulators, that can modulate enzyme activity stands as a promising frontier in biomedical research, since it can have important implications for human health by enabling tailored therapies based on an individual's unique enzymatic profile. Examples from oncology, metabolic disorders, neurodegenerative diseases, infectious diseases, and drug development illustrate their potential applications.

In this work there was an exploration of the applications of eight enzymes for testing the apitoxin properties and gain comprehensive understanding of its therapeutic and pharmaceutical potential: xanthine oxidase, lipase, α-amylase, α-glucosidase, lipoxygenase, acetylcholinesterase, tyrosinase and hyaluronidase. The general purpose was to verify the apitoxin’s inhibitory effects on those enzymes, an expected result considering its biological activities.

Xanthine oxidase. It is an enzyme that plays an important role in the metabolism of purines. The final metabolite of purine degradation is uric acid. High levels of this compound in the blood can cause gout, a painful joint condition. This enzyme is also involved in the production of free radicals, which can contribute to oxidative stress and cell damage. A compound with ability to inhibit xanthine oxidase activity reveal potential anti-inflammatory properties, thus impacting the generation of reactive oxygen species and inflammatory responses (El-Guendouz et al., 2016).

Lipase. Pancreatic lipase is a lipophilic enzyme secreted by the pancreas which is responsible for hydrolyzing 50 to 70% of the total fat ingested with food and produced by the body. This enzyme can remove fatty acids from the C-1 and C-3 position of triglycerides. These acids are transported to the small intestine via micelles (chylomicrons), where they are absorbed and carried into the circulation. Inhibiting lipase activity intervenes in these processes, resulting in reduced fat absorption and, consequently, lower calorie consumption and weight loss (Jaradat et al., 2017).

α -amylase and α-glucosidase. α -amylase and α-glucosidase are enzymes involved in the digestion and absorption of complex carbohydrates in the gastrointestinal tract. They cleave the glycosidic bonds present in complex carbohydrates, converting them into smaller units that can be absorbed by the body. Deficiency or inhibition of this enzyme can lead to carbohydrate digestion problems, while pharmacological inhibition of these enzymes is used for regulating blood sugar, particularly in diabetes management (Uddin et al., 2022; Popova et al., 2015).

Lipoxygenase. It is an enzyme that catalyzes the oxidation of polyunsaturated fatty acids, mainly omega-6 fatty acids, to produce a variety of bioactive products such as leukotrienes and lipoxins. Leukotrienes are inflammatory mediators involved in the immune response and in the development of inflammatory conditions such as asthma and cardiovascular diseases. Lipoxins are molecules involved in controlling inflammation and modulating the immune response.

Due to the role of this enzyme in different physiological and pathological processes, compounds that inhibit the activity of this enzyme can be promising for various therapeutic and clinical applications as: inflammation management; treatment of allergic reactions and asthma; reduce vascular inflammation and the development of

atherosclerosis; slow down the progression of certain types of cancer (Silva et al. 2012; El-Guendouz et al. 2016).

Acetylcholinesterase. It is a vital enzyme in the body that plays a central role in the regulation of neurotransmitters, particularly acetylcholine that is essential for the transmission of signals between nerve cells in various regions of the nervous system, including the central nervous system and the peripheral nervous system. It is primarily found in the neuromuscular junctions and cholinergic nerve synapses. Compounds that modulate its activity are crucial for assessing potential treatments for neurodegenerative disorders like Alzheimer's disease, a degenerative and progressive disease of the brain, since it is associated with a decrease in the neurotransmitter acetylcholine. Also, they can help prolonging the presence of acetylcholine in the synapses, making nerve impulses more effective and improving muscle function and have potential to enhance memory and cognitive function (Mata et al., 2007).

Tyrosinase. It is a crucial enzyme in the process of melanin synthesis, responsible to produce the pigment responsible for skin, hair, and eye color. While melanin plays essential roles in protecting the skin from UV radiation and determining its color, there are situations where inhibiting tyrosinase activity can be highly beneficial. Alterations or mutations in tyrosinase can also result in pigmentation disorders such as albinism, where there is reduced or absent melanin production. The accumulation of tyrosinase results in an increase in the content of this neurotransmitter and the reactive oxygen species located in the cytosol and mitochondria, leading to the production of melanin and cell apoptosis. Compounds that modulate tyrosinase activity can be applied in industry of cosmetics, as it can inhibit melanin production, helping to reduce dark spots and hyperpigmentation (El-Guendouz et al. 2016).

Hyaluronidase. It is an enzyme that exhibits the capacity to catalyze the degradation of hyaluronic acid, an important constituent within the extracellular matrix. This acid has various functions, including maintaining tissue hydration, joint lubrication, and contribution to skin elasticity. But excessive activity of this enzyme can have detrimental effects on the body due to degradation of hyaluronic acid and promotes inflammatory processes in human joints. So, natural products that induce negative effects on the action of this enzyme are important by preserving the integrity of hyaluronic acid and preventing

its excessive degradation. These compounds can enhance skin health, reduce inflammation, prolong the effectiveness of hyaluronic acid-based therapies, promote tissue repair, and prevent complications associated with hyaluronidase activity (Abhijit & Manjushree2010).

Regarding the biological activities, this study was justified not only by the few or practically non-existent studies on Portuguese apitoxin, particularly in the North of Portugal, as well as by its antimicrobial activity and inhibitory activity in several enzymes related to important metabolic processes in humans.

2. MATERIAL AND METHODS

This section presents the methodologies employed in the characterization of apitoxin. In the context of chemical characterization, a total of two distinct protein quantification methods were utilized, complemented by a single method for heavy metal analysis and an assessment of antioxidative capacity. In the assessment of apitoxin bioactive properties, assays were conducted including the assess of antimicrobial activity, anti-adherence properties and an evaluation of enzymatic inhibitions.

2.1. Sampling

The samples were supplied by the project's main leader, the company ECOAPIS - UNIPESSOAL LDA, and were taken for the work by IPB and IST. The apitoxin samples were received by the IPB for freeze-drying. As expected, the percentage of water present in the sample depends on the weather conditions at the time of collection. The percentage of water varied between 4.3% and 17.7%, with the lowest values obtained in the summer. After splitting the samples, the IPB was left with quantities of between 100-180 mg. The concentration of apitoxin in the aqueous solutions analyzed in this work is shown in the next table.

Table 2 – Apitoxin concentration in aqueous solution

Apitoxin sample	Concentration, mg/L
1	850
2	688
3	260
4	579
5	573

2.2. Total protein Quantification

To quantify the total protein in the sample, two analytical methodologies with complementary dynamic ranges were tested: the spectrophotometer method and the biuret method.

The **spectrophotometric method** is the most suitable for the analysis of total protein in apitoxin in all apitoxin harvesting/collection/storage processes, as well as quality control, as it results from the simple dilution of the sample in deionized water. This sample solution can then be used in further tests, such as chemical characterization of the sample and possible purification of melittin. The spectrophotometer method involves acquiring the ultraviolet spectrum in the wavelength range between 190 and 340 nm. Calibration used albumin as a standard and a dynamic range of 0.091 mg/L to 2.288 mg/L.

In the **Biuret method**, absorbance is measured at 545 nm (the wavelength selected because there are fewer interferences in the method) after adding the biuret reagent ($CuSO_4.5H_2O$; Na and K tartrate; NaOH; KI) and over a dynamic range from 0.091 mg/L to 2.288 mg/L of albumin. In this method, a purple color develops due to the reaction of copper with proteins in an alkaline medium.

2.3. Heavy metals analysis by voltammetry

The voltammetric method, optimized for the analysis of four heavy metals (Zn, Cd, Pb and Cu), proved to be a simple and effective approach for assessing quality and detecting heavy metal contamination in apitoxin. A portable Palmsens EmStat MUX8 (Netherlands) potentiostat controlled by Os Trace 5.2 software was used to determine these heavy metals. The voltammetric measurement system consisted of a reaction vessel with three electrodes: working electrode, reference electrode and auxiliary/counter electrode. The reference electrode used was Ag/AgCl, made of glass with an IPPG (Ion Permeability Porous Glass) junction, 70 mm high and 3.5 mm in diameter (RE-1B, Ag/AgCl, BAS Inc, Japan). The internal solution was 3.0 mol/L KCl, saturated with AgCl. The auxiliary electrode (counter electrode) was a 57 mm high platinum wire with a diameter of 0.5 mm, connected to a gold-plated pin, which was connected to the potentiostat using alligator clips. The working electrode used was a glassy carbon electrode (GCE) measuring 6 x 3 mm, coated with PEEK (BAS Inc, Japan), the surface of which was modified with a thin film of mercury.

The ASDPV (Anodic Stripping - Differential Pulse Voltammetry) technique was used to increase the detection and quantification limits. The analyses were carried out using the initial solution prepared by mixing 10 mL of deionized water with 0.2 mL of acetate buffer and KCl (pH = 4.60). To determine the concentrations of the 4 heavy metals, calibration by standard addition was carried out based on the 10 mL aqueous solution

resulting from the digestion of the apitoxin solution (2 mL of the aqueous solution of the bee venom sample was digested with 0.2 mL of concentrated H_2SO_4 to remove the organic load from the solution) and 0.2 mL of acetate and KCl buffer solution. Calibration by standard addition was carried out by adding two 0.1 mL volumes of the 10 mg/L standard mixture solution.

2.4. Antioxidation power by voltammetry

In the study of antioxidant properties, the redox capacity of the proteins present in apitoxin was tested against the standard gallic acid using the voltammetric method. Standard gallic acid solutions with concentrations of 0.80, 2.66, 5.30, 8.12, 39.9, 147, 388, 746 and 1207 mg/L were used for calibration. The reference electrode used was Ag/AgCl, the auxiliary electrode (counter electrode) was platinum wire and the working electrode was platinum (ALS Co., LDA, Pt 3 OD x 1.6 ID, Peek body, 110 cm long). A solution prepared by mixing 9 mL of deionized water and 1 mL of 0.33 mol/L tartaric acid buffer solution (pH=3.20) was used as a blank. DPV determinations were carried out at a temperature of 20ºC. The bee venom solution was analyzed using: 2 mL of the apitoxin solution, 1 mL of 0.33 mol/L tartarate buffer solution, pH=3.20, and 7 mL of deionized water.

2.5. Antimicrobial activity

The biological material used in this work was ATCC strains from the (LGC Standards SLU, Barcelona) ATCC collection. Clinical isolates provided by the Centro Hospitalar do Nordeste (Portugal) identified at the Escola Superior Agrária of the Instituto Politécnico de Bragança by molecular biology techniques were also used (Table 3).
Mueller Hinton Broth (MHB, Sigma) (bacteria) Mammalianl Cell Culture Media (RPMI, Sigma) were used to determine the minimum inhibitory concentration (MIC), the lowest concentration of the apitoxin extract capable of inhibiting microbial growth (bacteria and yeasts). The methodology used followed the protocol described by Morais et al. (2011), with some modifications. The apitoxin samples were dissolved in 50% DMSO and Mueller-Hinton medium for bacteria and in 50% DMSO and Mammalian Cell Culture Media for yeasts (final concentration 20 mg/ml).

Table 3 - Strains of bacteria utilized in the evaluation of the antimicrobial activity of apitoxin

Bacteria	Reference	Origin
Staphylococcus aureus	ATCC 6538™	Culture of reference
Methicillin-resistant *Staphylococcus aureus*	ESA 175	Pus
Methicillin-resistant *Staphylococcus aureus*	ESA 159	Expectoration
Escherichia coli	ATCC 29998™	Culture of reference
Cephalosporin-resistant *Escherichia coli*	ESA 37	Urine
Cephalosporin-resistant *Escherichia coli*	ESA 54	Blood culture
Klebiesella pneumoniae	ATTC 13883™	Culture of reference
Meropenem-resistant *Klebiesella pneumoniae*	ESA 356	Respiratory tract
Meropenem-resistant *Klebiesella pneumoniae*	ESA 234	Faeces
Pseudomonas aeruginosa	ATCC 15442™	Culture of reference
Imipenem-resistant *Pseudomonas aeruginosa*	ESA 22	Expectoration
Imipenem-resistant *Pseudomonas aeruginosa*	ESA 23	Vaginal exudate

In each well of the microplate, 100 µl of MHB and RPMI medium were placed, respectively for bacteria and yeasts. Next, 50 µl of the different concentrations of extract to be tested and 50 µl of the cell suspensions ($1x10^5$ for bacteria and $1x10^3$ for yeasts, grown in the appropriate medium overnight) were introduced into the various wells. After covering with sterile semi-permeable film, the microplates were incubated at 37 ºC for 24 hours. After this time, the optical density was determined at 600 nm and 540 nm for yeasts and bacteria respectively.

To determine the MIC, 20 µL was inoculated from the last well where there was a color change and from all the wells that remained unchanged onto plates with MHB for bacteria or RPMI for yeasts and incubated at 37 °C for 24 or 48 hours. The lowest concentration that did not result in growth after this subculture was considered the MIC. All tests were carried out in triplicate.

2.6. Anti-adherence activity

The anti-adhesion activity of apitoxin was evaluated as described by Apolónio et al. (2014) with slight modifications. The biological material used was *Staphylococcus aureus* ATCC® 6538™ and *Staphylococcus aureus* ESA 175, *Escherichia coli* ATCC® 29998™1077 and the multidrug-resistant strain *Escherichia coli* ESA 37 were exposed to the aqueous extract of apitoxin (0.1, 0.15 and 0.25 µL/ mL).

To do this, 200 µL of the culture were distributed over a 96-well flat-bottomed microplate and kept at room temperature in a flow chamber for 30 minutes. The bacterial suspension was then collected and the wells washed with phosphate buffered saline (PBS). The microplate was then dried at 80 ºC for 30 minutes to heat fix the bacterial cells. After cooling, the adherent cells were stained for 1 minute with 220 µL of crystal violet (0.1%). The stain was removed and the wells were washed twice with PBS, followed by dissolving the stain with 220 µL of ethanol-acetone (80:20) and, after 15 minutes, the O.D 595 nm was determined using a microplate reader. For comparison purposes, a standard solution of chlorohexidine (0.2 %, v/v) was applied.

2.7. Enzymes activity

In this study 8 enzymes were used to verify the apitoxin's inhibitory effects: xanthine oxidase, lipase, α-amylase, α-glucosidase, lipoxygenase, acetylcholinesterase, tyrosinase and hyaluronidase.

Xanthine oxidase inhibition: The inhibitory activity was determined using the method described by El-Guendouz et al. (2016) with slight modifications. For this, 50 µL of each apitoxin extract was mixed with 230 µL of phosphate buffer (50 mM, pH = 7.5) and 70 µL of xanthine oxidase enzyme (0.4 U/mL), after incubation at room temperature for 15 min, 330 µL of xanthine solution (substrate) (0.150 mM) were added and the mixture was incubated for 30 min. At the end, 100 µL of HCl (1 M) was added to stop the reaction and the reading was taken at 290 nm. The percentage inhibition of xanthine activity was calculated and the IC50 was determined. The tests were carried out in triplicate. Baicalin was used as a control (0.1 to 2.0 µL/mL).

Lipase inhibition. The porcine pancreatic lipase inhibition assay was adapted from Jaradat et al. (2017): 25 µL of aqueous apitoxin extract was mixed with 190 µL of Tris-HCl buffer (0.1 M; pH = 8) and 40 µL of lipase enzyme (200 U/mL) prepared before the assay. A first incubation was carried out at 37 ºC for 20 min, after which 20 µL of 5 mM NPB (p-nitrophenylbutyrate) substrate was added to the mixture. A second incubation was then carried out for one hour and 20 min. Nordihydroguaiaretic acid (NDGA) (0.03-1 mg/mL) was used as a control.

α-amilase inhibition. The α-amylase inhibition assay was carried out as described by (Uddin et al., 2022). The total assay mixture, consisting of 100 sodium phosphate buffer (0.02 M, pH 6.9 containing 6 mM sodium chloride), 50 µL of a-amylase (0.02 units) and apitoxin, was incubated at 37 °C for 10 min. After incubation, 200 µL of soluble starch (1%, w/v) was added to each test tube and the mixture was reincubated for 20 min at 37 °C. Approximately 300 µL of 10% HCl was added to stop the enzymatic reaction, followed by the addition of 300 µL of iodine reagent (5 mM I_2 and 5 mM KI) and then 8 mL of distilled water. The absorbance was read at 620 nm. The sample, substrate and blank were carried out under the same conditions. Each experiment was carried out in triplicate. The percentage of enzyme inhibition was calculated using the following formula:

Inibition % of α amilase: $$\left[1 - \frac{\left[\left(A^{-}_{\text{control}}\right) - \left(A^{+}_{\text{control}}\right)\right] - \left(A_{\text{sample}}\right)}{\left(A^{-}_{\text{control}}\right) - \left(A^{+}_{\text{control}}\right)}\right] \times 100$$

Where:
A- control represents the absorbance of 100% enzyme activity (70% ethanol with enzyme);
A+ control represents the absorbance of 0% enzyme activity (70% ethanol with enzyme);
A sample represents the absorbance of the sample.

Finally, the IC50 values were determined and compared with the control standard acarbose (0.025-1 µg/mL).

α-glucosidase inhibition. The α-glucosidase inhibition capacity was determined as described (Popova et al., 2015). The total assay mixture consisted of 10 µL of aqueous apitoxin extract added to 50 µL of yeast α-glucosidase (2.4 U/mL) prepared in phosphate buffer (100 mM; pH = 6.8), the mixture was incubated for 10 min. Next, 100 µL of a 5 mM solution of p-Nitrophenyl-β-D-glucopyranoside (PNPG) in phosphate buffer was added. This reaction solution was incubated at room temperature for 30 minutes and then 80 µL of sodium carbonate solution (0.4 mM) was added to stop the reaction. The reading was taken at 405 nm and the test was carried out in triplicate. The percentage of enzyme inhibition was calculated as follows:

$I\% = [(A0 - A1) / A0) \times 100]$

and the IC50 was determined. The tests were carried out in triplicate. A solution of Acarbose (0.025-1 µg/mL) was used as a control.

Lipoxygenase inhibition. The lipoxygenase assay was used as an indicator of anti-inflammatory and antioxidant activity (Silva et al. 2012). The inhibition action of the aqueous apitoxin solution was carried out as reported by (El-Guendouz et al. 2016), with some modifications. In short, 10 µL of the aqueous apitoxin solution was used in the tests. The inhibitory effect of the test was calculated by comparison with the negative control:

$$\text{Inhibition } \% = [(A0-A1)/A0] \times 100$$

Where:
A0 is the absorbance of the blank sample;
A1 is the absorbance of the sample.
The results were expressed as the IC50 value. Nordihydroguaiaretic acid (NDGA) was used as a standard (0.03-1 mg/mL).

Acetylcholinesterase inhibition. The enzyme acetylcholinesterase converts acetylcholine into acetic acid and thiocholine. The latter compound reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to form 5-thio-2-nitrobenzoic acid. The accumulation of 5-thio-2-nitrobenzoic acid was evaluated at 405 nm. The methodology used was that developed by Mata et al. (2007). Eserine (0.0025- 0.01 µg/mL) was used as a standard control. The percentage inhibition of acetylcholinesterase activity was then determined and the value (IC50) was calculated. The tests were carried out in triplicate.

Tyrosinase inhibition. Tyrosinase activity was determined based on the protocol described by (El-Guendouz et al. 2016) with a slight modification. The total assay mixture consisted of the addition of 25 µL of aqueous apitoxin extract, mixed with 170 µL of phosphate buffer (50 Mm; pH = 6.5) and 20 µL of tyrosinase enzyme (100 U/mL), the mixture was incubated for 40 min, after which 60 µL of substrate (5 mM L-Dopa) was added. The reading was carried out at 492 nm, the percentage of enzyme inhibition was calculated and finally the IC50 values were compared. The tests were carried out in triplicate. Kojic acid was used as a control (0.5- 2.0 µg/mL).

Hyaluronidase inhibition. The anti-inflammatory activity was assessed indirectly by inhibiting the hyaluronidase enzyme, using the method described by Abhijit &

Manjushree (2010). 150 µL of bovine hyaluronidase enzyme (7900 units/mL) was dissolved in acetate buffer (pH 3.6). A 25 mL of apitoxin extract (0.2-100 mg/mL) was then added and the mixture incubated for 30 min at 37 ºC. Then 50 µL of calcium chloride (12.5 mM) was added and incubated again under the same conditions. After this period, 250 µL of sodium hyaluronate (1.2 mg/mL) was added and the mixture was incubated again at 37 ºC for 1.5 hours. After this period, 50 mL of 0.4 M sodium hydroxide and 100 mL of 0.6 M sodium borate were added and placed in a water bath for 3 minutes. Finally, it was cooled on ice and 1.5 mL of PDMAB (p- dimethylaminobenzaldehyde) was added (4 g PDMAB dissolved in 50 mL of 10M HCl and 350 mL of glacial acetic acid). The absorbance was measured at 585 nm. The test was carried out in triplicate and epigallocatechin (0.010-0,30 mg/mL) was used as a standard control. The percentage inhibition of the hyaluronidase enzyme was calculated according to the following equation:

$$\text{hyaluronidase enzyme inhibition (\%)} = \frac{Abs600 controlo\ Abs600\ amostra)}{Abs600 controlo} \text{ x } 100$$

2.8. Antihemolytic activity

Following the procedure described in the work of Valente el al. (2011), the tests were carried out using a suspension of erythrocytes at 2% hematocrit. The cells were pre-incubated at 37 ºC for 30 min in the presence of the chosen apitoxin concentrations (0.5 to 1.0 µg apitoxin aqueous extract/ml), then a 50 mM AAPH solution was added. This reaction mixture was stirred gently during incubation at 37 ºC for 4 h. In all experiments, a negative control (erythrocytes in PBS), as well as extract controls (erythrocytes in PBS with each apitoxin extract) were used. The extent of hemolysis was determined spectrophotometrically according to a method every hour of the 4 h of incubation, diluted with saline solution and centrifuged at 4000 rpm for 10 min to separate the erythrocytes. The percentage of hemolysis was determined by measuring the absorbance of the supernatant (A) at 545 nm and compared with that of complete hemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water. The percentage of hemolysis was calculated using the formula A/B 100. The IC50 values at time 3 h were also determined from the concentration-response curve obtained through the percentage inhibition of hemolysis versus the concentration of the extract. Sodium

dodecyl sulfate (SDS) was used as the reference antioxidant compound (35.50 µg/mL). All the tests were carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1. Quantification of total protein

Two protein quantification methods were applied to determine the total protein content in apitoxin samples: spectrophotometric method and Biuret method.

The **spectrophotometric method** for total protein analysis was calibrated with albumin calibration standard solutions. Figure 2 shows the spectra of albumin in the calibration standard solutions.

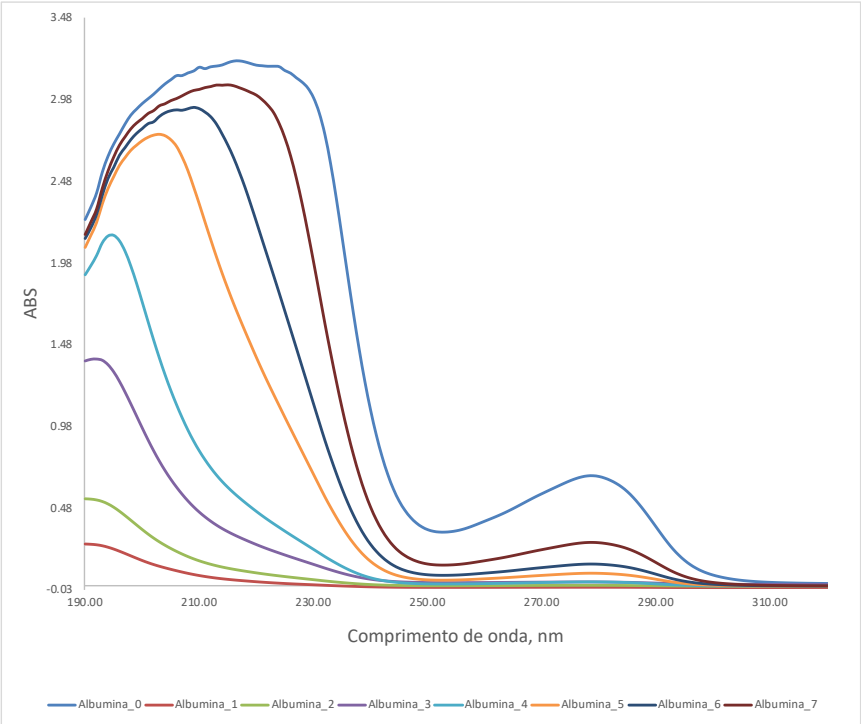


Figure 2 - Spectra of albumin in calibration standard solutions.

The absorbances were measured at a wavelength of 280 nm and a linear relationship was obtained with a slope of 0.589±0.004, an ordinate at the origin of -0.006±0.004 and a correlation coefficient of 0.99989 (Figure 3). The calculated limits of detection and quantification are 0.023 and 0.068 mg/mL, respectively. The limits are adequate for measuring the total protein of bee venom samples.

The aqueous solutions of bee venom, prepared at a ratio of 0.02 g to 10 mL of deionized water (clear solution), showed concentrations of over 45.9% total protein (albumin

equivalents). This new methodology should be compared with the data obtained in the protein characterization through HPLC analysis of many apitoxin samples, with the aim of establishing multivariate relationships between the model for predicting the total protein content equivalent to albumin and the apitoxin values. In addition to the great advantage of not contaminating the sample for this analysis (only, apitoxin and deionized water), which also enables the utilization of the diluted sample in other assays, the spectrophotometric method has greater sensitivity and lower detection and quantification limits. Moreover, this diluted sample can be analysed and used in the apitoxin purification process, for quantities greater than 5g of apitoxin, as described in patents from KR100758814B1, WO2010/134676 and DE924582 (all origin of South Korea, a simplified purification method of bee venom). A more recent method for purifying bee venom without inducing a change in the composition thereof, thereby producing highly pure bee venom on a mass scale was described in EP2800477 patent.

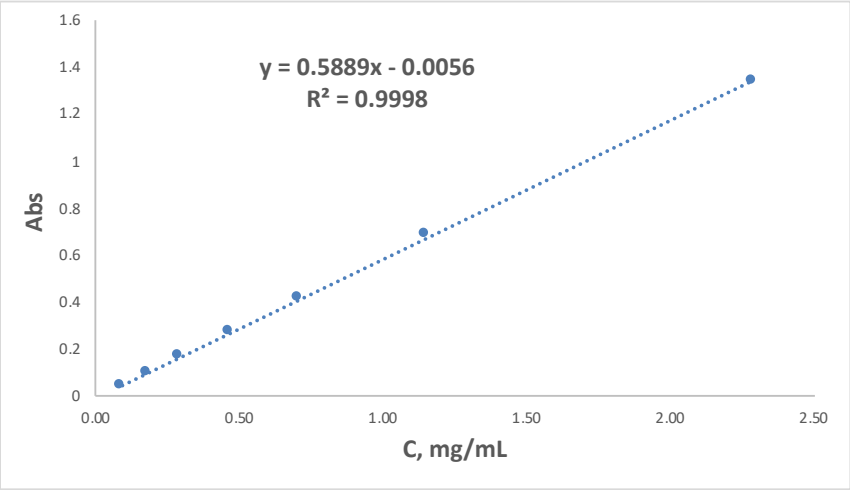


Figure 3 - Calibration curve obtained between the absorbance measured at 280 nm and the albumin concentration in the standard solution.

The **Biuret method** showed lower sensitivity for the same dynamic range. The linear relationship shows a slope of 0.247±0.002, an ordinate at the origin of 0.005±0.002 and a correlation coefficient of 0.9997 (Figure 4). The calculated detection and quantification limits are 0.033 and 0.101 mg/mL, respectively.

Although these limits are adequate for measuring total protein, the sample is contaminated with the Biuret reagent. So, this method was discarded because the color development did not allow the sample to be used in subsequent tests.

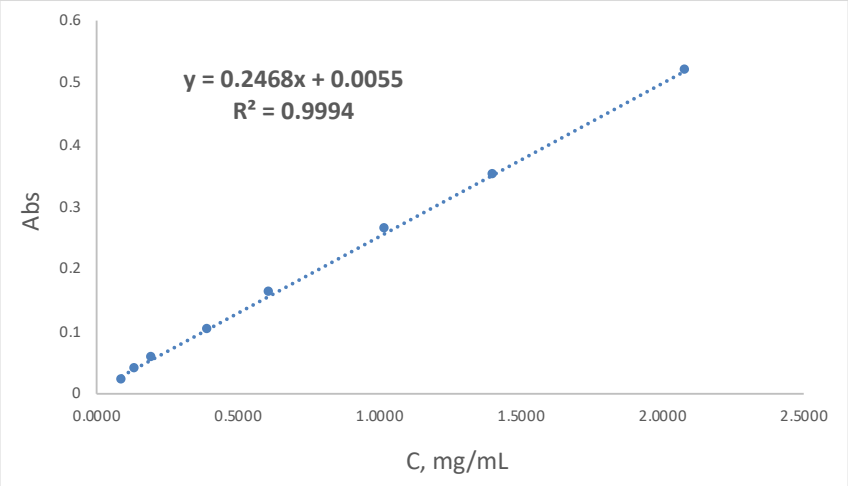


Figure 4 - Calibration curve obtained between the absorbance measured at 545 nm and the concentration of albumin in the standard solution using the Biuret method.

3.2. Heavy metals analysis by voltammetry

Figure 5 shows the visual appearance of the voltammograms obtained from the analysis of the 4 metals using the standard addition calibration method. The peaks from left to right on the potential axis represent the analysis of the metals Zn (Epeak = -1.0 V), Cd (Epeak = -0.65 V), Pb (Epeak = - 0.45 V) and Cu (Epeak < -0.14 V; the peak shifts to positive potentials with increasing concentration).

The first apitoxin sample contained the metals Zn and Cu, but at low concentration levels (Cd and Pb were not detected). In a bee venom solution prepared with 57 mg of freeze-dried bee venom in 25 mL of deionized water, a concentration of 0.64±0.03 mg/L of Zn was obtained and 0.25±0.02 mg/L of Cu. In terms of apitoxin mass, this corresponds to 27.9 mg/100 g and 10.9 mg/100 g of apitoxin, respectively, for Zn and Cu. These levels are not considered significant but indicate metal contamination due to beekeeping practices. Based on these results, metal utensils were avoided when taking apitoxin samples, which resulted in better quality samples (the four metals were not detected in subsequent samples).

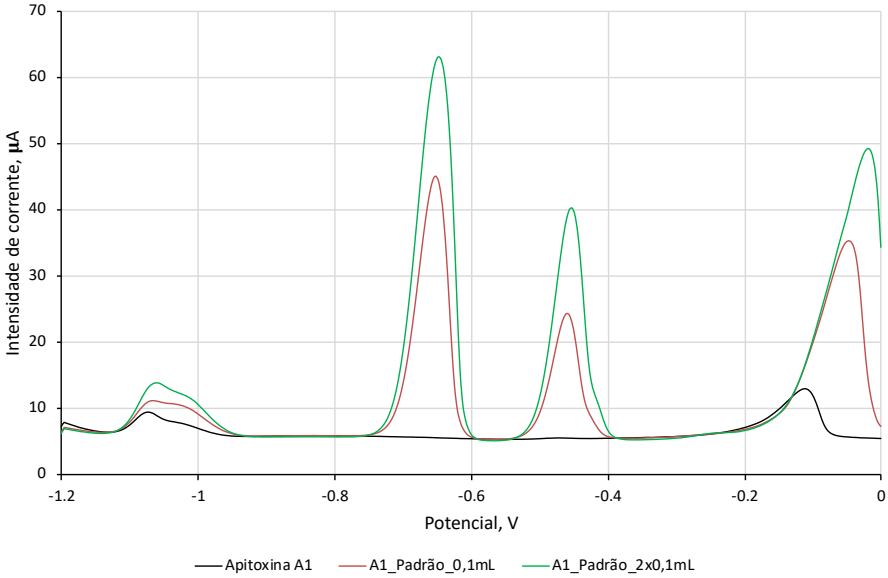


Figure 5 - Analysis of 4 metals in apitoxin using standard addition calibration.

3.3. Antioxidant power by voltammetry

Figure 6 shows the DPV voltammograms obtained for the gallic acid solutions and for a sample of bee venom.

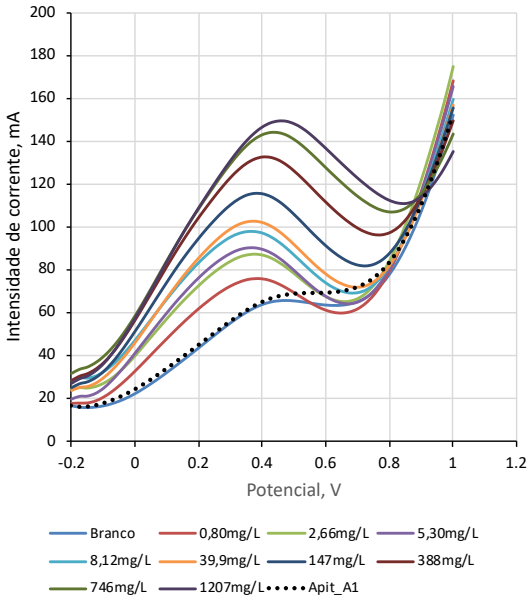


Figure 6 - DPV voltammograms obtained when analysing the blank, gallic acid and venom sample.

The figure shows that the oxidizing power of the poison solution is close to that of the blank solution, showing that it has no antioxidant capacity.

The calibration carried out with gallic acid, using the maximum current intensity obtained at the peak as the signal, showed a curvilinear relationship adjusted by a trend line with a power model. Figure 7 shows the calibration obtained by presenting two graphs with the same data, but with the difference of whether the abscissa axis had the logarithmic scale defined. The aim was to check that the fit obtained was acceptable, as expected considering the coefficient of determination (R^2 = 0.9975), meaning that the model allowed to explain 99.75% of the total variability.

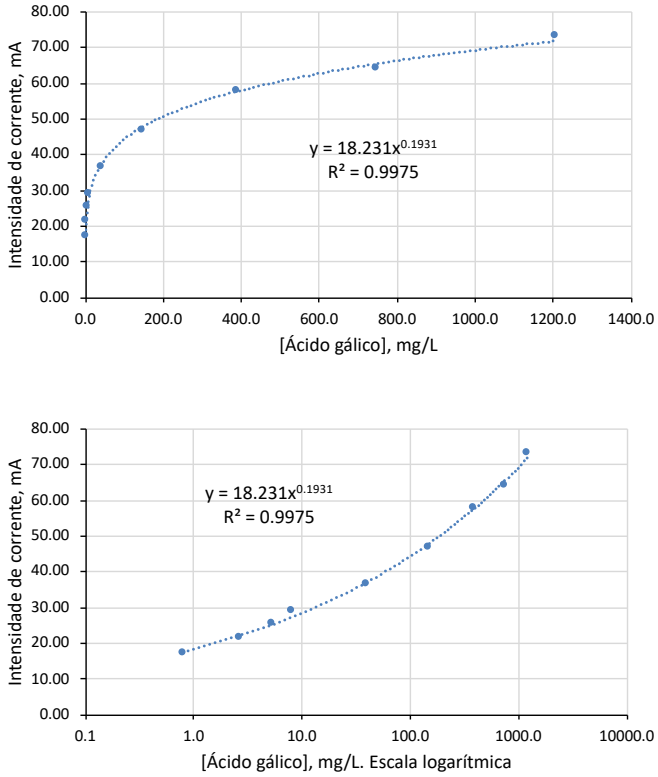


Figure 7 - Calibration of the maximum current intensity obtained in the DPV peak as a function of gallic acid concentration.

3.4. Antimicrobial activity

The increase in antibiotic resistance has led researchers to look for strategies to reduce the use of these drugs and prevent the rise of multi-resistant strains. In recent decades,

compounds isolated from natural products have shown promising activity against resistant bacteria. The composition of apitoxin includes several peptides, particularly melittin, which have shown high antimicrobial activity.

The results obtained for the antimicrobial activity of the 5 apitoxin samples tested indicate that the antimicrobial effect of apitoxin against the microorganisms tested (Gram-positive bacteria, Gram-negative reference strains and multidrug-resistant clinical isolates) depended on the composition of the apitoxin, particularly the melittin content, as well as the bacterial strain tested. This compound had bactericidal action against all strains of *Escherichia coli*, *Staphylococcus aureus* and *Klebiesella pneumoniae*, however, did not affect the growth of *Pseudomonas aeruginosa* ATCC® 15442™, *Pseudomonas aeruginosa* ESA 22, *Pseudomonas aeruginosa* ESA 23, both reference strains and hospital isolates. The graphs showing the minimum inhibitory concentration (MIC) of all strains of *Escherichia coli*, *Staphylococcus aureus* and *Klebiesella pneumoniae* for each sample analyzed are represented in Figure 8. The control (gentamicine) had higer inhibition than the apitoxin samples but, all have a similar behaviour regarding how the microorganism tested. As can be seen, the 3 strains of *S. aureus* were the ones less inhibited. On the contrary, *E. coli* and *K. pneumoniae* had a similar response to the presence of apitoxin. Sample 1 induced the lowest MIC values (9.2 µg/mL) against *Staphylococcus aureus*, meaning that this bacteria was the most sensitive to the negative effects of apitoxine. The highest MIC (41.8 µg/mL) was observed against *Klebsiella pneumonia* ESA 234 in the presence of sample 5, which means that this bacterium was the most resistant to apitoxin. To better evaluate these results, graphs showing the minimum inhibitory concentration (MIC) for all the samples and control analyzed against all strains of *Escherichia coli*, *Staphylococcus aureus* and *Klebiesella pneumoniae in* study, are represented in Figure 9 (illustrates the mean MIC values required for inhibiting the microorganisms by the samples and the control, along with their respective error intervals). The ANOVA results indicate that all models were statistically significant (with RSE < 4.105, $R^2 > 0.865$, and p-value < 0.001). The letters representing the results of the significance tests for the means were included in the plots in Figure 9. It is evident that sample 1 had the best MIC (lowest value) and was statistically equivalent to the control only in two cases, when the microorganisms under study were *E. coli* ESA 54 and *K. pneumoniae* ATCC 13883. In the E. coli assays, sample 1 exhibited similar results to sample 4 and 5, with the exception of sample 5 for *E. coli* ATCC. Samples 2, 4, and 5 were statistically equivalent, but in the case of *E. coli* ESA 37, samples 2 and 3 were also

statistically equivalent. The experiments with *S. aureus* yielded similar results, with the exception that samples 1, 4, and 5 were similar only for *E. coli* ESA 175.

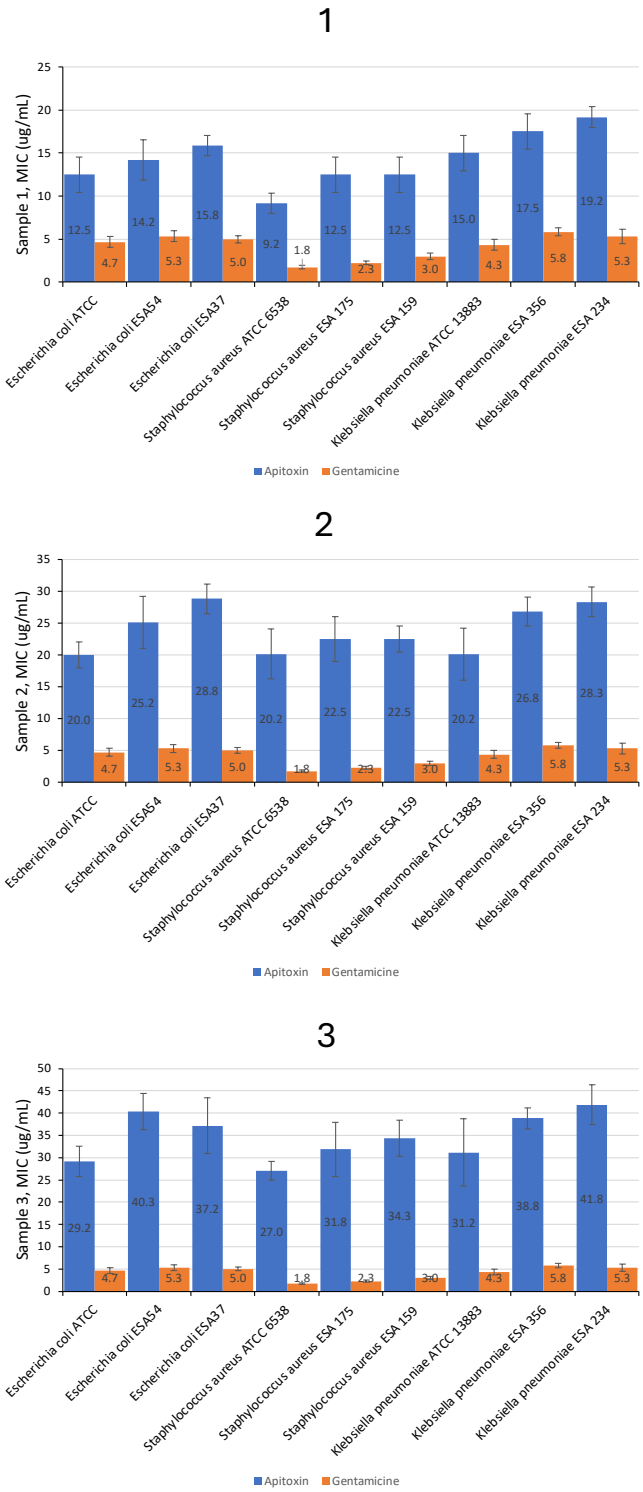


Figure 8 - Minimum inhibitory concentration (MIC) of all strains of Escherichia coli, Staphylococcus aureus and Klebiesella pneumoniae for each sample in the study. The numbering identifies the apitoxin sample.

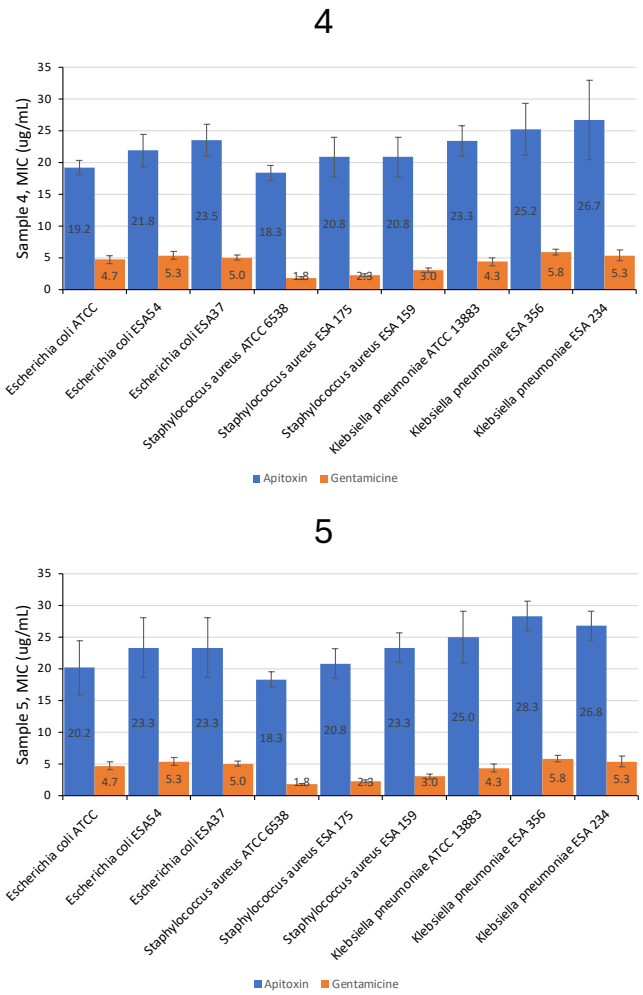


Figure 8 - Minimum inhibitory concentration (MIC) of all strains of *Escherichia coli*, *Staphylococcus aureus* and *Klebiesella pneumoniae* for each sample in the study. The numbering identifies the apitoxin sample. (continuation)

The results for *K. pneumoniae* were slightly different. *K. pneumoniae* ATCC 13883 showed that samples 1, 2, 4, and 5, as well as samples 2, 3, 4, and 5, could be considered statistically equivalent.

For *K. pneumoniae* ESA 356, the results indicate that sample 1 and 3 are different from the others. Concerning *K. pneumoniae* ESA 234, all apitoxin samples, except sample 3, exhibited statistically similar results. Overall, the behavior of all the bacteria studied in the presence of apitoxin samples 2, 3, 4 and 5 was identical.

Usually, the effect of antibacterials against Gram-positive bacteria compared to Gram-negative bacteria can be explained by the protection provided by the structure of the outer membrane of Gram-negative bacteria and the production of hydrolytic enzymes that block and break down the active ingredients of these compounds. Our results suggest that

this does not seem to be the case, since the growth of *Pseudomonas aeruginosa* was not affected by apitoxin, making it imperative to carry out further studies to assess the mechanisms of action of this apicola product on bacteria.

Other authors studied the antimicrobial activity of propolis hydroethanlic extracts:

- Campos et al. (2022) studied propolis from the Brazilian Stingless Bees *Melipona quadrifasciata anthidioides* and *Scaptotrigona depilis* using several bacteria (Staphylococcus aureus, Enterococcus faecalis, Escherichia coli and Pseudomonas aeruginosa e Cryptococcus neoformans. Overall, the results presented in the range of MIC of $1.67x10^{+3}\pm0.17x10^{+3}$ to $16.5x10^{+3}\pm0.28x10^{+3}$ µg/mL.
- Santos et al. (2017) studied the geopropolis of the stingless bee *Melipona orbignyi*, against bacteria and yeasts, isolated from reference strains and hospital origin. The obtained MIC values of the Gram positive bacteria (*Staphylococcus aureus* and *Enterococcus faecalis*) were ranging between 6.13±0.10 and 9.08±0.08 mg/mL; the Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), 10.5±0.82 and 13.4±1.22 mg/mL; the fungi (*Cryptococcus neoformans* and *Candida albicans*), 19.3±0.60 and 24.4±1.83 mg/mL.

Another work that should be mentioned is that of Lamas et al. (2020) that evaluated the antimicrobial activity of five bee venoms (*Apis mellifera*; Ecuador) against 50 strains of the Gram-negative pathogen Salmonella enterica and 8 strains of the Gram-positive pathogen Listeria monocytogenes. Overall, the apitoxin results showed similar MIC values having three levels: 256, 512 (more frequent; more than 68%) and 1025 µg/mL.

The findings across these studies emphasize the variability in antimicrobial efficacy among bee-derived substances against different microorganisms. While apitoxin demonstrated specific bactericidal effects against certain bacterial strains in the present study, other investigations revealed a wide array of microorganisms when exposed to different bee-derived compounds. Regarding the apitoxin results, the present work had better antimicrobial activity than the reported by Lamas et al. (2020). Further understanding of the antimicrobial potential of bee-derived substances is of considerable importance in the context of combatting multidrug-resistant bacteria and managing infectious diseases.

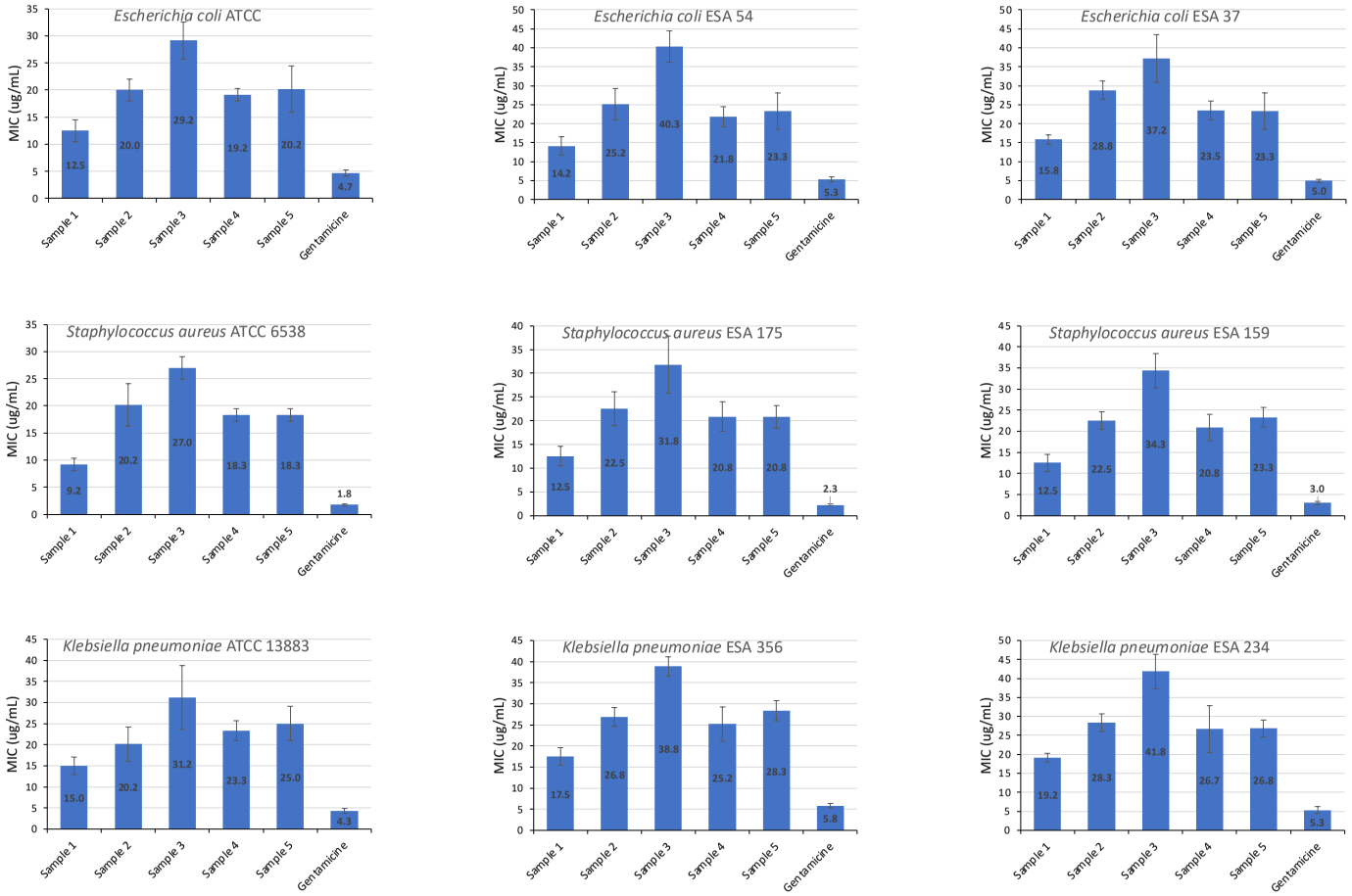


Figure 9 - Minimum inhibitory concentration (MIC) for all the samples analyzed against all strains of Escherichia coli, Staphylococcus aureus and Klebiesella pneumoniaeno under study. Equal letters represent statistically equal means.

3.5. Anti-adherence activity

The inhibition of adherence to surfaces evaluates the ability to prevent the establishment of bacterial communities that will form biofilms that are difficult to eliminate.

The results showed that apitoxin samples 1, 2, 4 and 5 tested at a concentration of 12.5 µL/mL were able to reduce the adherence of *Staphylococcus aureus* ATCC and both methicillin-resistant *S. aureus* (ESA 175 and ESA 159) as well as all *Escherichia coli* strains compared to chlorohexidine solution (0.2 % v/v). Sample 1 of apitoxin had the strongest effect in inhibiting adherence, while sample 3 only inhibited *S. aureus* adherence. These results suggest that apitoxin inhibited biofilm formation, which is significant as biofilms are known to be difficult to eliminate and can cause persistent infections. However, further studies may be necessary to explore and validate the mechanisms behind the inhibitory effects of apitoxin on biofilm formation, its potential applications, optimal concentrations, and safety considerations for practical use in preventing bacterial colonization and biofilm-related infections.

3.6. Enzymes activity

The next section presents the results of the inhibitory effects of apitoxin on eight enzymes: xanthine oxidase, lipase, α-amylase, α-glucosidase, lipoxygenase, acetylcholinesterase, tyrosinase, and hyaluronidase.

Xanthine oxidase inhibition. Xanthine oxidase is an enzyme that plays an important role in the metabolism of purines. The final metabolite of purine degradation is uric acid. High levels of this compound in the blood can cause gout, a painful joint condition. This enzyme is also involved in the production of free radicals, which can contribute to oxidative stress and cell damage. Proper control of xanthine oxidase activity is important for the body's general health. The maximum value required to inhibit this enzyme was IC50=6.3± 0.85 µL/mL, observed in the presence of sample 3 and the minimum when evaluating sample 1 (IC50=1.7± 0.4 µL/mL).

The linear model in the one-way ANOVA analysis demonstrated a significant fit, explaining 91.4% of the variability in the original data (RSE = 0.626; R^2 = 0.914; p-value < 0.001). The ANOVA results revealed significant differences between the samples and the control standard (baicalin). Upon comparing the means, it was observed that the

results of the control were statistically equivalent to those of sample 1, which yielded the most favorable outcome. Additionally, samples 2, 3, and 5 were statistically equivalent. Notably, sample 3 exhibited a statistically significant difference and displayed the highest IC50 value at 6.3 μg/mL, which was 4.5 times higher than that of the baicalin control.

Figure 10 illustrates the mean IC50 values required for inhibiting the xanthine oxidase enzyme by the samples and the control, along with their respective error intervals. The associated letters represent the outcomes of the significance test for the means.

The xanthine oxidase enzyme inhibition demonstrated notable results considering results from works with other with other aplicultura products. For instance, El Guendouz et al. (2016) reported values ranging from 8±52 to 3116±52 μg/mL in the study assessing the inhibition of xanthine oxidase enzyme using 24 different samples of Moroccan propolis. regarding the evaluation of the ability of honeys to inhibit enzyme activity, Sahin (2016) demonstrated IC50 values ranging between $28x10^{+3}$ and $452x10^{+3}$ μg/mL in tests conducted with chestnut, oak, and polyfloral honeys diluted in water. In the study by Di Petrillo et al. (2018), Sardinian honeys derived from various floral sources (Arbutus, Asphodelus, Eucalyptus, Thistle, and Sulla) exhibited IC50 values ranging between $4.2x10^{+3}$±0.2 $x10^{+3}$ and $57.8x10^{+3}$±4.4 $x10^{+3}$ μg/mL. The considerably lower IC50 values observed in this study highlight the potential of the tested samples in inhibiting the enzyme compared to the reported values in these other works.

Considering the importance of xanthine oxidase in purine metabolism and its association with diseases like gout and cardiovascular conditions, the efficacy of the samples in inhibiting this enzyme is promising. These findings emphasize the potential health benefits and therapeutic implications of natural products such as propolis and honey derivatives in managing conditions associated with xanthine oxidase activity, further underscoring the significance of these inhibitory results in promoting human health.

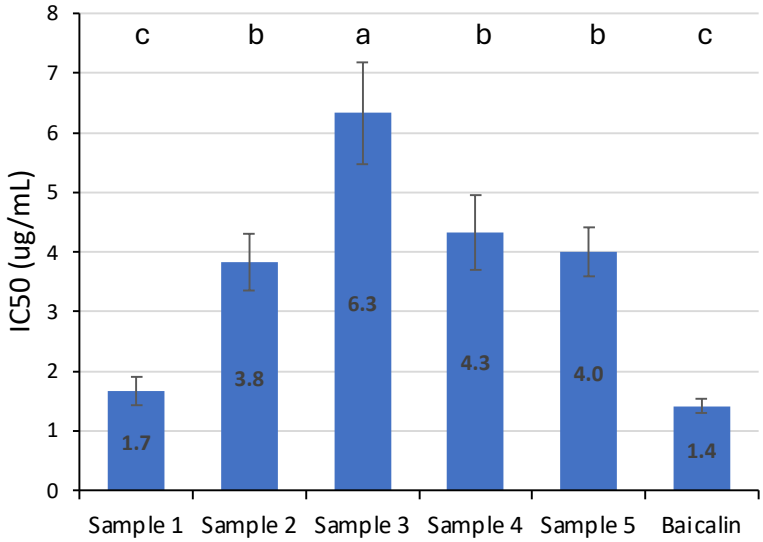


Figure 10 – IC50 values required to inhibit the xanthine oxidase enzyme. Equal letters represent statistically equal means.

Lipase inhibition. Pancreatic lipase is a lipophilic enzyme secreted by the pancreas which is responsible for hydrolyzing 50 to 70% of the total fat ingested with food and also produced by the body. This enzyme is capable of removing fatty acids from the C-1 and C-3 position of triglycerides. These acids are transported to the small intestine via micelles (chylomicrons), where they are absorbed and carried into the circulation. Inhibiting lipase activity intervenes in these processes, resulting in reduced fat absorption and, consequently, lower calorie consumption and weight loss. All the samples demonstrated the ability to inhibit pancreatic lipase. Among the samples analyzed, 1 was the most efficient (IC50: 0.05 ± 0.01µg/mL) and 3 showed the least inhibition (IC50: 0.16 ±0, 0.03 µg/mL). O modelo linear de ajuste dos dados IC50 da lipase em função das 5 amostras de apitoxina e controlo (composto orlistate) explicou 89.3% da variabilidade dos dados (RSE = 0.019; R^2 = 0.893; p-value =< 0.001)

The linear model that correlates the lipase IC50 data with the 5 samples of apitoxin and the control compound (orlistat) in the ANOVA explained 89.3 percent of the variability in the data (RSE = 0.019; R^2 = 0.893; p-value < 0.001). The ANOVA analysis revealed significant differences between the samples and the control. Specifically, the IC50 values for the control and sample 1 yielded the most effective inhibition results and were statistically equivalent, with values of 0.02 and 0.05 µg/mL, respectively. However, when comparing the means, it was evident that sample 1 was also statistically equivalent to

samples 2 and 4. On the other hand, samples 2, 4, and 5, as well as samples 3 and 5, were statistically equivalent.

Figure 11 illustrates the mean IC50 values required for lipase enzyme inhibition by samples and the control, along with their respective error intervals. Additionally, the associated letters indicate the results of significance tests for the means. The inhibitory effect of the control was higher than that induced by any of the samples analyzed.

Some examples of IC50 values obtained in studies with bee pollen and can be presented:

- Khongkarat et al. (2022) investigated six distinct bee pollen samples collected by Apis mellifera from various monoculture flowering crops. The methanolic extracts were evaluated for their in vitro porcine pancreatic lipase inhibitory activity, displaying IC50 values ranging from 7.74±0.56 to 38.51±0.13 µg/mL.
- Balogun and Liu (2023) employed ethanolic extracts of propolis sourced from southwest Nigeria, revealing IC50 values of 100.38±4.17 and 263.78±2.76 µg/mL.
- Salas et al. (2020) investigated the effects of hydro-ethanolic extracts of Argentine propolis (80% ethanol) obtained between 2005 and 2018, observing IC50 values ranging from 13±3 to 28±1 µg/mL.

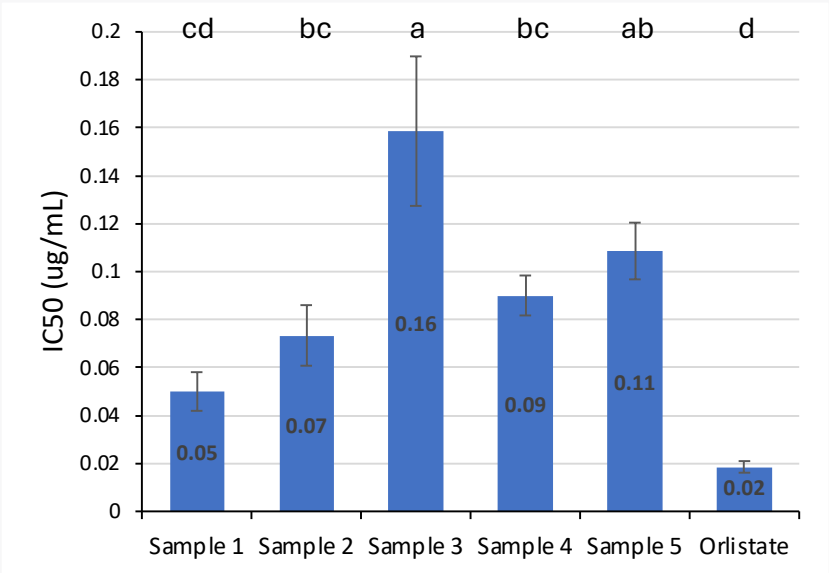


Figure 11 – IC50 values required to inhibit the lipase enzyme. Equal letters represent statistically equal means.

The apitoxin results showed higher inhibition in relation to the bee pollen and propolis works that were presented. However, these other studies revealed varying degrees of

inhibitory effects, highlighting the influence of geographical origin and extraction method. Since, effective inhibition of lipase can potentially mitigate excessive fat absorption and aid in managing metabolic disorders, further research can be relevant.

α-amilase and α-glucosidase. α -amylase and α-glucosidase are enzymes involved in the digestion and absorption of complex carbohydrates in the gastrointestinal tract. They cleave the glycosidic bonds present in complex carbohydrates, converting them into smaller units that can be absorbed by the body. Deficiency or inhibition of this enzyme can lead to carbohydrate digestion problems, while pharmacological inhibition of these enzymes is used in the treatment of type 2 diabetes.

Regarding the α-amylase inhibitory activity, it was observed that sample 1 exhibited the highest effectiveness, while sample 3 showed the least effectiveness, with IC50 values of 0.08 ± 0.02 and 0.4 ± 0.12 μg/mL, respectively. As for the inhibitory activity of α-glucosidase, the IC50 values for the samples, ranked from least to most inhibitory, were as follows: sample 3 (0.14 ± 0.01 μg/mL), sample 2 (0.07 ± 0.01 μg/mL), sample 4 (0.06 ± 0.01 μg/mL), sample 5 (0.05 ± 0.01 μg/mL), and sample 1 (0.03 ± 0.01 μg/mL). It's worth noting that the behavior of these two enzymes in the presence of apitoxin was highly similar. The control (acarbose) exhibited significantly lower inhibitory effects in both cases. In both enzyme assays, ANOVA analysis revealed significant differences. For α-amylase, the model yielded values of RSE = 0.083, R^2 = 0.782, and p-value = 0.0012, while for α-glucosidase, the model yielded values of RSE = 0.011, R^2 = 0.945, and a p-value of less than 0.001.

In the α-amylase tests, sample 3, which exhibited the lowest capability to inhibit the enzyme, was statistically equivalent to sample 4. Additionally, the control and all samples (except for sample 3) were also statistically equivalent. In the case of the α-glucosidase tests, samples 3 and 5 were statistically equivalent to the control (acarbose), demonstrating the most favorable inhibitory results.

Figures 12 and 13 present the IC50 values required to inhibit the α-amylase and α-glucosidase enzymes, respectively, for both samples and the control, along with their respective error intervals. The associated letters represent the results of significance tests for the means.

Contrasting findings reported by various authors regarding α-amylase inhibition using bee products reveal a wide spectrum of inhibitory potency. Salas et al. (2020) reported values ranging from 37±2 to 48±1 μg/mL for three distinct lyophilized Argentine propolis

extracts (20 g of propolis in hydro-ethanolic solution; 80% ethanol); Alaribe et al. (2021) demonstrated an IC50 of 368±55 µg/mL for a polar extract of Nigerian propolis; El Guendouz et al. (2016) revealed IC50 results varying between 18±9 µg/mL and 3632±97 µg/mL for 24 samples of Moroccan propolis (hydro-ethanolic extracts with 70% ethanol). Qasem et al. (2022) obtained an IC50 of 981.44±0.05 µg/mL using Moroccan honey. Khongkarat et al. (2022) investigated methanolic extracts from six distinct bee pollen types collected by *Apis mellifera* in various monoculture flowering crops, presenting *in vitro* α-amylase inhibitory activity with IC50 values ranging from zero to 14.31±0.99 µg/mL.

The variation observed in α-amylase inhibitory activity among different natural products, including propolis, honey, and bee pollen, suggests diverse bioactive compositions and potency levels. The present study demonstrates remarkably potent inhibitory effects within a lower IC50 range, indicating promising potential for these samples in α-amylase inhibition.

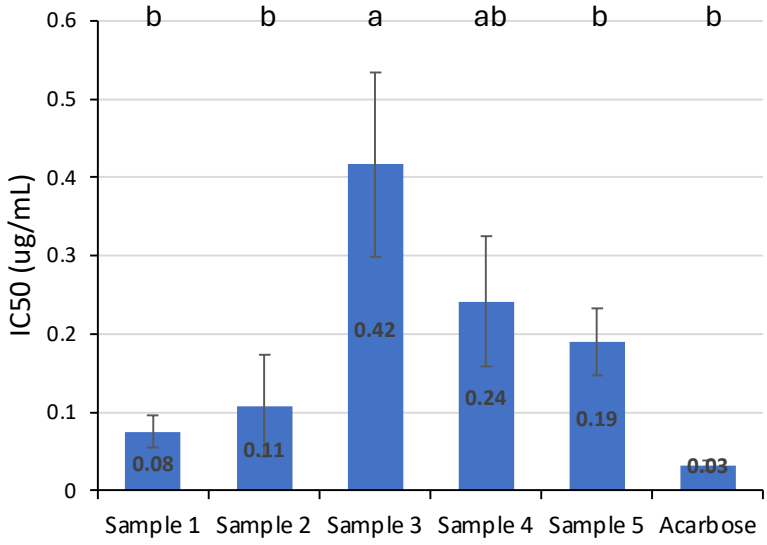


Figure 12 – IC50 values required to inhibit the α-amylase enzyme. Equal letters represent statistically equal means.

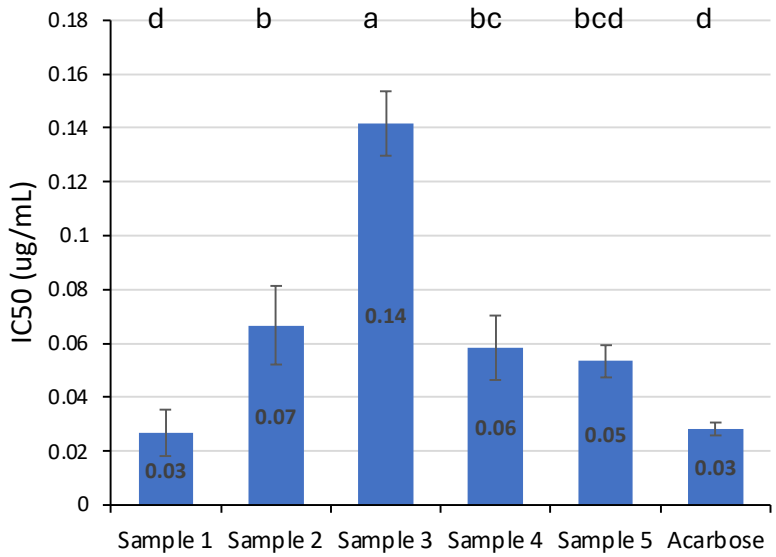


Figure 13 – IC50 values required to inhibit the α-glucosidase enzyme. Equal letters represent statistically equal means.

Given the significance of α-amylase in carbohydrate metabolism and its implications in managing conditions like diabetes and obesity, the observed potent inhibitory effects of these samples could hold substantial importance in developing therapeutic interventions or dietary supplements targeting α-amylase inhibition, thus contributing to potential human health benefits. Further exploration and understanding of the bioactive compounds responsible for this inhibitory activity could pave the way for novel treatments or preventive strategies for metabolic disorders associated with α-amylase dysregulation.

Regarding α-glucosidase inhibition with other bee products, as honey and propolis extracts, some works can be presented. Inhibiting the α-glucosidase enzyme with a honey sample revealed a high IC50 value of approximately 1971.31 µg/mL in the methanolic extract (Rahmawati et al., 2019). Qasem et al. (2022) demonstrated that Moroccan honey led to an IC50 of 845.31±0.02 µg/mL. Studies involving propolis showed lower IC50 values. For instance:

- El Guendouz et al. (2016) observed hydro-ethanolic extracts of Moroccan propolis displaying IC50 values ranging between 2±10 and 746±16 µg/mL.
- Taleb et al. (2020) revealed that the hydro-ethanolic extract (70% ethanol) of Anatolian propolis exhibited an IC50 value of 40.40±0.09 µg/mL.

- Boulechfar et al. (2022) investigated hydro-methanolic extracts (80% methanol) from four Algerian propolis samples, presenting IC50 values ranging from 11.4±0.6 to 41.66 ± 0.32 µg/mL.
- Balogun and Liu (2023) utilized ethanolic extracts of propolis from southwest Nigeria and obtained IC50 values ranging between 25.4±0.5 and 116.0±0.2 µg/mL.
- Salas et al. (2020) reported IC50 values ranging from 7±0 to 12±1 µg/mL with Argentine propolis hydro-ethanolic extracts (80% ethanol) obtained between 2005 and 2018.

The α-glucosidase inhibitory activity results of this work had significantly lower IC50 values, indicating a remarkable potency of the samples tested in inhibiting α-glucosidase compared to the examples given by other bee products. Since α-glucosidase has a role in carbohydrate metabolism with its implications in managing diabetes, the potent inhibitory effects of the apitoxin could hold significant importance in the development of therapeutic strategies or dietary interventions targeting α-glucosidase inhibition, as prevention of hyperglycemia-related conditions in human health.

Lipoxygenase inhibition. Lipoxygenase (LOX) is an enzyme that catalyzes the oxidation of polyunsaturated fatty acids, mainly omega-6 fatty acids, to produce a variety of bioactive products such as leukotrienes and lipoxins. Leukotrienes are inflammatory mediators involved in the immune response and the development of inflammatory conditions such as asthma and cardiovascular diseases. Lipoxins are molecules involved in controlling inflammation and modulating the immune response. Due to the role of this enzyme in different physiological and pathological processes, as well as in the development of therapies for inflammatory and immune response-related conditions, compounds have been investigated, including natural products, which inhibit the activity of this enzyme.

The activity of lipoxygenase inhibition by apitoxin, expressed in IC50 values, is inversely correlated with melittin concentration. Increasingly, IC50 values varied as follows (Figure 14): sample 3 (0.25± 0.04 µg/mL), sample 5 (0.17± 0.06 µg /mL), sample 4 (0.15± 0.03µg /mL), sample 2 (0.12± 0.03µg/mL) and sample 1 (0.06± 0.01µg /mL).

The ANOVA model indicated statistical significance (RSE = 0.042, R^2 = 0.783, and p-value = 0.0013), revealing significant differences between the samples and the control group. The mean comparison revealed that the control group and samples 3, 4, and 5 can

be considered statistically equivalent. Similarly, samples 1, 2, 4, and 5 can also be considered statistically equivalent. Based on these results, it can be concluded that the latter group exhibited more pronounced inhibitory effects on the enzyme lipoxygenase compared to the control (baicalin, IC50 0.23± 0.02 µg /mL).

To compare the results obtained, works on inhibiting this enzyme with other bee products are presented:

- El Guendouz et al. (2016) reported values ranging from 0.020±0.019 to 0.653±0.019 µg/mL in the study assessing the inhibition of Lipoxygenase enzyme using hydroalcoholic extracts of 24 different samples of Moroccan propolis.
- Sambou et al. (2020) studied the efficiency to extract phenolic and flavonoid antioxidants from Eastern Canada propolis by Soxhlet, microwave-assisted and ultrasound-assisted extraction procedures when using ten extraction solvents. These extracts were compared by their anti-inflammatory activity through inhibition of lipoxygenase enzyme and the IC50 mean values ranged between 59.03 to 590.6 µg/mL in Soxhlet extraction, 6.69 to 480.8 µg/mL in microwave-assisted extraction and 48.11 to 770.4 µg/mL in ultrasound-assisted extraction.
- Boutoub et al. (2021) studied the capacity of monofloral honeys of *Euphorbia resinifera* (48.7±1.1% pollen) and *Euphorbia officinarum* (52.1±1.6%) aqueous extracts to inhibit lipoxygenase enzyme, which gave the IC50 values of 32.7±0.4 and 46.8±0.4 mg/ml, respectively.

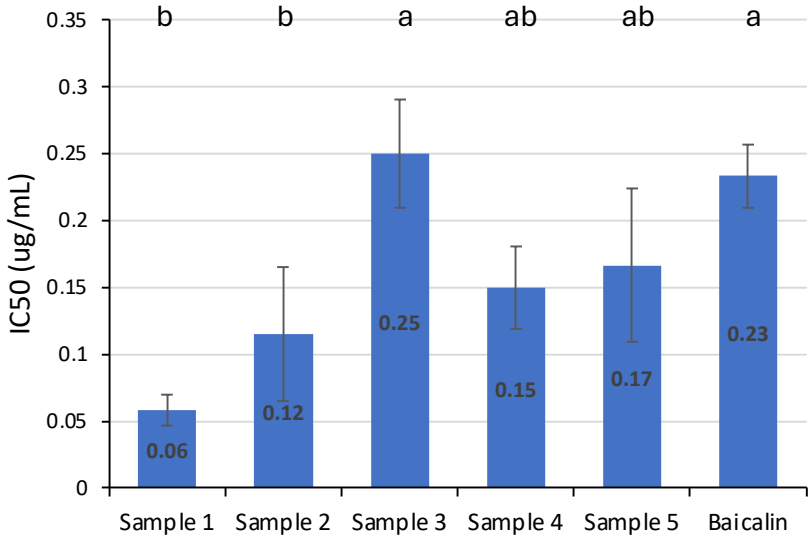


Figure 14 – IC50 values required to inhibit the lipoxygenase enzyme. Equal letters represent statistically equal means.

While the Portuguese apitoxin results demonstrated potent inhibition, some results with propolis samples were also comparable, although these results showed the great influence of geographical origins and extraction methods on the inhibitory potential against lipoxygenase. Since, the inhibition of lipoxygenase can potentially modulate inflammation, the apitoxin could present opportunities for managing various inflammatory conditions.

Acetylcholinesterase inhibition. Alzheimer's disease (AD) is known as a degenerative and progressive brain disease, considered one of the most common types of dementia affecting elderly people. It is associated with a decrease in the neurotransmitter acetylcholine, which is essential for the transmission of signals between nerve cells in various regions of the nervous system, including the central nervous system and the peripheral nervous system. Thus, interruption of the activity of this enzyme leads to the accumulation of acetylcholine, with hyperstimulation of nicotinic and muscarinic receptors, and continuous neurotransmitter processes. The IC50 values recorded for AChE inhibition ranged from 0.78 ± 0.2 to 3.75 ± 0.2 µg/mL for samples 1 and 3, respectively (Figure 15). The one-way ANOVA analysis, with a significant model (RSE = 0.274, R^2 = 0.963, and p-value < 0.001), indicated the presence of significant differences between the results of samples and control. Mean multiple comparisons revealed that samples 1 and 2, as well as samples 2, 4, and 5, were statistically equivalent. Notably, the control (eserine) exhibited the lowest IC50 among the assays, followed by sample 1, which was 75 times higher than the control.

For comparison purposes, several examples of AChE inhibition studies using bee products such as honey, propolis, and bee pollen are presented. Karatas et al. (2021) investigated Anatolia's monofloral honeys (chaste, thyme, citrus, and heather), obtaining IC50 values exceeding $25.24x10^{+3}$±$1.67x10^{+3}$ µg/mL. Boulechfar et al. (2022) examined hydro-methanolic extracts (80% methanol) from propolis of various origins in Algeria, resulting in IC50 values surpassing 71±3 µg/mL. Similarly, El Guendouz et al. (2016) reported IC50 values ranging between 2±51 and 2034±51 µg/mL for 24 samples of Moroccan propolis (hydro-ethanolic extracts with 70% ethanol). Khongkarat et al. (2022) investigated the methanolic extracts of six distinct monofloral bee pollen samples

collected by Apis mellifera, demonstrating in vitro acetylcholinesterase inhibitory activity with IC50 values ranging from 4.13±0.47 to 13.95±1.51 µg/mL.

The present apitoxin study reveals notably potent AChE inhibitory effects within a lower IC50 range compared to the reported values in these studies investigating honey, propolis, and bee pollen. Given the important role of AChE in neurotransmission and its implications in neurodegenerative conditions like Alzheimer's disease, the observed potent inhibitory effects of our tested samples are promising.

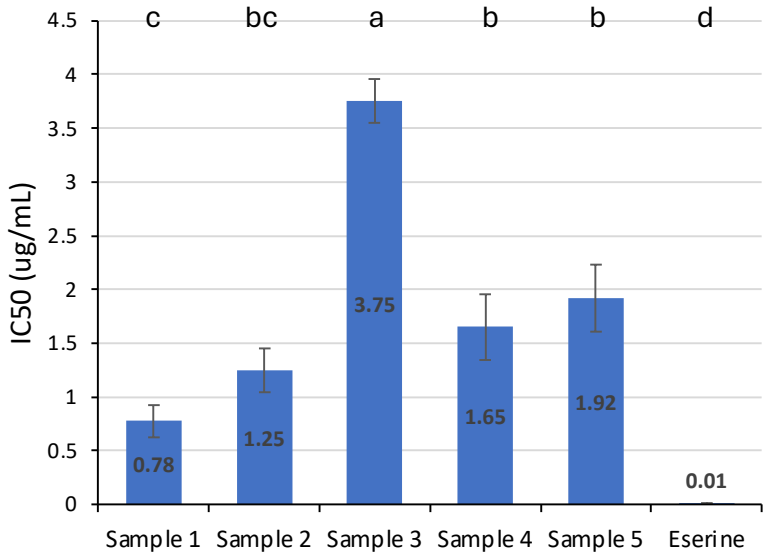


Figure 15 – IC50 values required to inhibit the acetylcholinesterase enzyme. Equal letters represent statistically equal means.

Tirosinase inhibition. Parkinson's disease is a neurodegenerative disease resulting from a deficiency of the neurotransmitter dopamine in the brain. The abundant accumulation of tyrosinase results in an increase in the content of this neurotransmitter and reactive oxygen species located in the cytosol and mitochondria, leading to the production of melanin and cell apoptosis. Changes or mutations in tyrosinase can also result in pigmentation disorders, such as albinism, where there is a reduced or absent production of melanin. It can also be applied in industry and cosmetics, being used in skin lightening products, as it can inhibit the production of melanin, helping to reduce dark spots and hyperpigmentation. In this context, the discovery of tyrosinase inhibitors is extremely important for the treatment of melanogenesis and Parkinson's disease. From the analysis of our results (Figure 16), a very pronounced inhibition effect of this enzyme was

observed in the presence of sample 1 (IC50: 1.6µg/mL ± 0.1) compared to sample 3 (IC50: 4.8µg/mL ± 0.24). In accordance with the significant ANOVA model (RSE = 0.428, $R^2 = 0.918$, and p-value < 0.001), the best inhibitory result observed in the control assay can be deemed statistically equivalent to samples 1 and 2. However, samples 1, 2, 3, and 4 can also be considered statistically equivalent to each other, except for sample 3, which yielded the least favorable inhibitory results. Comparing the IC50 values obtained for the kojic acid control (1.17± 0.10 µg/mL), all tested samples had acceptable efficient in inhibiting tyrosinase, except the sample 3.

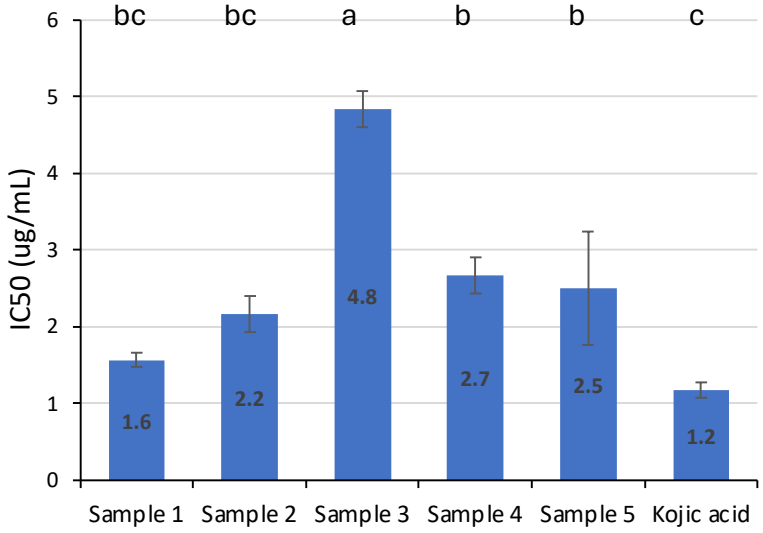


Figure 16 – IC50 values required to inhibit the tirosinase enzyme. Equal letters represent statistically equal means.

For a comparison of results, examples of other bee products studies exploring the tyrosinase enzyme inhibitory potential are presented. Samples of propolis were utilized to assess their inhibitory effect on the tyrosinase enzyme. For instance, 24 samples of Moroccan propolis (hydro-ethanolic extracts with 70% ethanol) displayed IC50 values ranging between 37±192 and 4286±192 µg/mL (El Guendouz et al., 2016). Gheibi et al. (2016) evaluated Iranian propolis samples using different extracting solvents (ethanol, methanol, and hexane), yielding IC50 values of 19.8±0.9, 28.6±2.3, and 40.6±2.5 µg/mL, respectively. Regarding studies with honey, Aumeeruddy et al. (2019) investigated the effect of raw unifloral Mauritian eucalyptus honey and a commercially available honey (without specification of floral source) dissolved in DMSO on the inhibition of the

tyrosinase enzyme, but no inhibitory activity was observed. However, in the work of Di Petrillo et al. (2018), IC50 values equal to or greater than $64.3x10^{+3}\pm1.6x10^{+3}$ µg/mL were obtained for Sardinian honeys derived from various floral sources (Arbutus, Asphodelus, Eucalyptus, Thistle, and Sulla).

In this study, the Portuguese apitoxin demonstrated high tyrosinase enzyme inhibitory effects within a relatively low IC50 range compared to findings from studies involving propolis and honey. Considering the role of tyrosinase in melanin production and its implications in skin pigmentation disorders, the observed potent inhibitory effects of the tested samples are promising in possible applications in skincare products or treatments targeting hyperpigmentation disorders.

Hialuronydase inhibition. Hyaluronidase is an enzyme that degrades hyaluronic acid, an important component of the extracellular matrix. He acid performs several functions, including maintaining tissue hydration, lubricating joints and contributing to skin elasticity. Therefore, its activity is involved in physiological and pathological processes, such as embryonic development, tumor dissemination and the absorption of anesthetics. The use of this enzyme in a clinical context can help in the dispersion of substances and improve the absorption of medications. Hence the relevance of finding natural products that induce negative effects on the action of this enzyme.

All samples were less effective in inhibiting hyaluronidase activity than the positive epigallocatechin-3-gallate standard (% inhibition: 88.5±6.4 %), as can be seen in Figure 17. Sample 1 was the most efficient (% inhibition: 85±5.4 %) followed by sample 2 (% inhibition: 75.0±3.3%). On the other hand, sample 3 presented the lowest values (49.5± 4.2%). Concerning the ANOVA analysis (significant model: RSE = 7.69, $R^2 = 0.830$, and p-value < 0.001), the mean control inhibition value was statistically equivalent to those obtained from samples 1, 2, and 4. Furthermore, statistically, the mean values of samples 1, 2, 3, and 5, as well as samples 3 and 5, could be considered as the same.

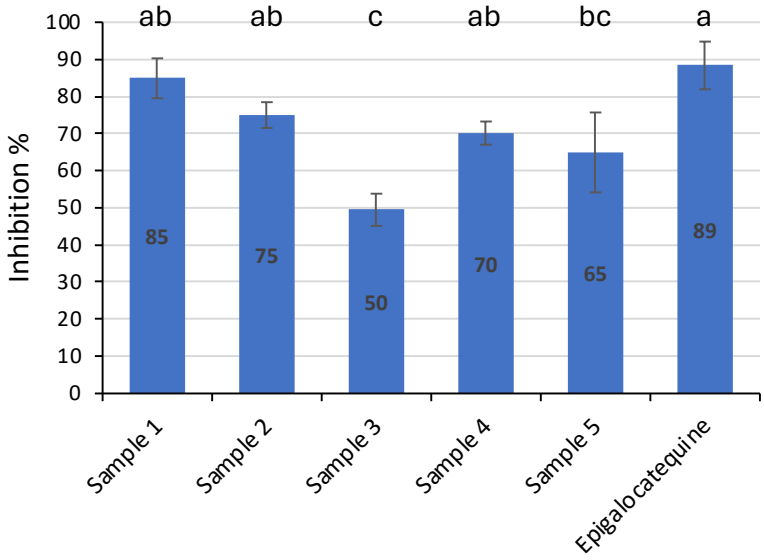


Figure 17 – IC50 values required to inhibit the hyaluronidase enzyme. Equal letters represent statistically equal means.

In contrast with results from propolis extracts, Osés et al. (2020) investigated thirteen bee propolis samples sourced from diverse geographical regions (6 from North-East European countries, 2 from the South American tropical zone, and 5 from South-West European countries). They obtained hyaluronidase inhibitory activity ranging from 0 to 68.20% at a propolis concentration of 10 mg/mL. Silva et al. (2012) demonstrated that Portuguese propolis exhibited inhibitory values ranging between 10 and 20% at a propolis concentration of 10 mg/mL, while concentrations of 25 mg/mL revealed values between 53.75 and 75.79%. Campos et al. (2015) obtained an average IC50 value of 119.6±20.5 µg/mL (with maximum inhibition of 86.5±2.8% at 500 µg/mL) for six hydro-ethanolic extracts (80% ethanol) of propolis sourced from the stingless bee *Tetragonisca fiebrigi* (Jataí).

Overall, the variability in the inhibitory potential of these bee products (apitoxin and propolis) showed promising inhibitory effects with potential therapeutic benefits, including inflammation, tissue degradation, and the progression of certain diseases.

3.7. Antihemolytic activity

Antihemolytic activity is an important area of research and development, as it focuses on protecting erythrocytes against hemolysis, thus contributing to the health and safety of

Humans. The antihemolytic activity induced by all apitoxin samples was greatly reduced 8.5± 0.5% (Figure 18). Corroborating the results obtained for the antioxidant activity of this product. The ANOVA analysis showed to have significant differences between the treatments (p-value < 0.001). Furthermore, statistically, the mean values of samples 1, 2, 3, 4 and 5 were considered as the same.

This is the activity where apitoxin has the least pronounced inhibitory effect compared to the other hive products. For instance, the work of Santos et al. (2017) verified that the hydroalcoholic extract of geopropolis from the Stingless Bee *Melipona orbignyi*, with concentrations of 25 and 50 µg/mL, reduced hemolysis by 40.9±8.0% and 93.2±0.8%, respectively. This discrepancy emphasizes the variation in the bioactive properties of different bee-derived substances.

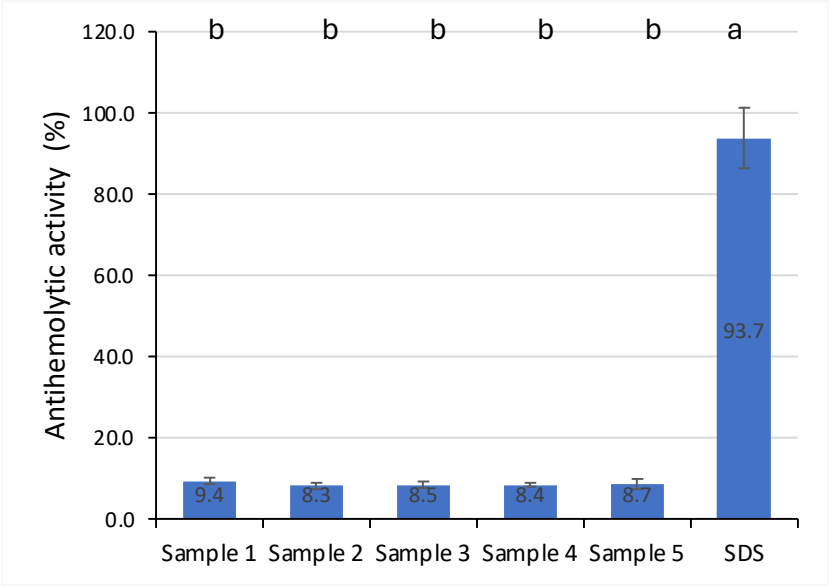


Figure 18 – Antihemolytic activity of SDS and apitoxin samples. Equal letters represent statistically equal means.

The Portuguese apitoxin demonstrates a notably diminished capacity in safeguarding erythrocytes against hemolysis compared to the desired level for effective cellular protection.

4. CONCLUSIONS

This work was carried out as part of the Pharmapitox project with the aim of developing analytical methodologies for the quality control of apitoxin, as well as evaluating its biological activities.

Regarding the analytical methodologies, it was developed a spectrophotometer method established to predict the total protein content, equivalent to albumin, within the apitoxin samples that had an advantage that lies in its ability to maintain sample integrity. Moreover, this spectrophotometric method exhibits superior sensitivity and lower detection and quantification limits. However, due to the low number of samples and the low quantity of samples collected, it was not possible to optimise this methodology in order to relate it to melittin and other peptides present in the samples. The electrochemical methodologies developed in this study facilitated the verification that apitoxin lacks antioxidant activity and enabled the implementation of quality control measures for apitoxin concerning four heavy metals (Zn, Cd, PB e Cu). The latter technique allowed for the assessment ensuring that the materials used for handling apitoxin were non-metallic in nature.

Regarding the biological activities, apitoxin exhibited bactericidal action against all strains of *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, with the exception of *Pseudomonas aeruginosa* ATCC® 15442™, *Pseudomonas aeruginosa* ESA 22, and *Pseudomonas aeruginosa* ESA 23, which remained unaffected.

The Portuguese apitoxin demonstrated significant inhibitory effects against the xanthine oxidase, lipase, α-amilase, α-glucosidase, lipoxygenase, acetylcholinesterase, tyrosinase and hialuronydase enzymes. The evaluation of apitoxin across these enzymatic inhibitions highlights its multifaceted potential in its applications in metabolic pathways and cellular processes studies.

These findings suggest that apitoxin could have potential significance in various health conditions and diseases, thereby supporting further scientific studies due to its potential utilization in clinical or pharmaceutical settings.

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