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Cytotoxic effect of wasp venom peptides in breast cancer cells

Efeito citotóxico de peptídeos de veneno de vespa em células do cancro da mama

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Verónica Isabel Correia Bastos, Investigadora do Departamento de Biologia da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro e, sobre coorientação da Doutora Helena Cristina Correia de Oliveira, Investigadora Auxiliar do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

“I am among those who think that science has great beauty”
Marie Curie

o júri

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palavras-chave

Cancro da mama; MCF-7; MDA-MB-231; Veneno de vespa; *Polybia dimorpha; Chartergellus communis*; Potencial antitumoral; Citotoxicidade celular; Prolistarina; Chartergellus-CP1.

resumo

Atualmente, o cancro é a segunda principal causa de morte no mundo e uma clara ameaça para a saúde pública, levando anualmente à morte de quase 10 milhões de pessoas. O cancro da mama, especificamente, continua a ser o mais frequente em mulheres tendo ainda muitas complicações e mortes associadas o que torna evidente a necessidade de desenvolver novos agentes terapêuticos para o combater. Os venenos são na sua maioria constituídos por uma panóplia extensa e complexa de compostos químicos naturais e biologicamente ativos, eficazes contra vários microrganismos patogénicos e algumas doenças, como o cancro. São vários os estudos que concluíram que diversas espécies de víboras, escorpiões e abelhas produzem venenos ricos em compostos com fortes propriedades antitumorais. No entanto, a família *Vespidae* está muito pouco estudada no que concerne ao seu potencial antitumoral, uma vez que existem muitas espécies por explorar, como é o caso da espécie de vespa social *Polybia dimorfa* e da *Chartergellus communis*.

Assim, com o objetivo de avaliar o potencial antitumoral de dois novos péptidos provenientes do veneno das vespas sociais *P.dimorpha* (Prolistarina) e *C. communis* (Chartergellus-CP1), foram realizados ensaios para estudar a sua citotoxicidade em duas linhas celulares de cancro da mama: a linha celular MCF-7 e a linha celular MDA-MB-231. Estas células foram expostas a diferentes concentrações de cada péptido, durante 24h e 48h, de modo a avaliar a dose resposta através da análise da viabilidade celular ao longo do tempo de exposição. A Prolistarina não apresentou citotoxicidade relevante nas linhas celulares estudadas. Em contraste, o péptido Chartergellus-CP1 revelou-se altamente citotóxico contra ambas as linhas celulares, promovendo uma elevada diminuição da viabilidade com o aumento da concentração. Assim, foram calculados o IC20 e IC50 deste peptido e estudados os mecanismos de citotoxicidade nas duas linhas celulares, através da análise da dinâmica do ciclo celular, da produção intracelular de espécies reativas de oxigénio (ROS) e da apoptose.

Os resultados mostraram que o Chartergellus-CP1 originou, em ambas as linhas celulares, um aumento significativo das células na fase S do ciclo celular assim como, um aumento de ROS (este mais evidente na linha celular MCF-7). Prevemos que esta produção de ROS esteja associada com a morte celular em ambas as linhas celulares, uma vez que as duas apresentaram um aumento significativo de células em apoptose inicial quando expostas ao IC50.

Esta dissertação demostrou, pela primeira vez, que o péptido Chartergellus-CP1 do veneno da vespa *Chartergellus communis* tem um elevado potencial biotecnológico no combate ao cancro da mama

keywords

Breast cancer; MCF-7; MDA-MB-231; Wasp venom*; Polybia dimorpha; Chartergellus communis*; Antitumor potential; Cellular Cytotoxicity; Prolistarin; Chartergellus-CP1.

abstract

Currently, cancer is the second leading cause of death in the world and a clear threat to public health, leading to the death of almost 10 million people per year. Breast cancer, specifically, remains the most common cancer in women. Their complications and associated deaths highlight the need to develop new therapeutic agents to combat the disease. Venoms are mostly composed of an extensive and complex panoply of natural and biologically active chemical compounds, that are effective against various pathogenic microorganisms and some diseases, such as cancer. Several studies reported that a wide variety of viper, scorpion, and bee species produce venoms with compounds that have strong anti-tumor properties. However, some species in the Vespidae family are still poorly studied in terms of their antitumor potential, for example the social wasp species *Polybia dimorpha* and *Chartergellus communis*, which has no associated studies on this subject.

Thus, in order to evaluate the antitumor potential of two new peptides from the social wasps *P. dimorpha* (Prolistarin) and *C. communis* (Chartergellus-CP1), assays were performed to determine their cytotoxicity against two breast cancer cell lines: the MCF-7 cell line and the MDA-MB-231 cell line. These lines were exposed to different concentrations of each peptide, for 24h and 48h, in order to evaluate their cell viability along time exposure. Prolistarin showed no relevant cytotoxicity against the studied cell lines. In contrast, the Chartergellus-CP1 peptide proved to be highly cytotoxic against both cell lines, promoting a high dose-dependent antitumor action.

Thus, IC20 and IC50 of Chartergellus-CP1 were used to assess cell cycle dynamics, intracellular reactive oxygen species (ROS) production and apoptosis in both breast cancer cell lines.

The results showed, for both cell lines, Chartergellus-CP1 leaded to a significant increase of cells in the S phase of the cell cycle, as well as to a high generation of ROS (being more evident in the MCF-7 cell line). We predict that this ROS production is associated with cell death in both cell lines, as both cells showed a significant increase of cells in early apoptosis when exposed to IC50.

This dissertation demonstrated, for the first time, that the Chartergellus-CP1 peptide from the venom of *Chartergellus communis* wasps has high potential in breast cancer treatment.

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List of Abbreviations

ACP: Anticancer peptide

AMP: Antimicrobial peptide

BVP: Bee venom therapy

CAR-T: Chimeric antigen receptor T-cells

CDK: Cyclin- dependent kinase

CHK: Checkpoint kinase

CKI: Cyclin- dependent kinase inhibitor

COVID-19: Severe Acute Respiratory Syndrome Coronavirus-2

DCF: 2',7' - dichlorofluorescein

DCFH-DA: 2',7'- dichlorofluorescein diacetate

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic

EDTA: 2,2',2",2'"-(Ethane-1,2-diyldinitrilo)tetraacetic acid

FBS: Fetal Bovine Serum

FCM: Flow Cytometry

FDA: Food and Drug Administration

HER2: Human Epidermal growth factor Receptor-type 2

HIV: Human Immunodeficiency Virus

HPLC: High Performance Liquid Chromatography

HPLC-MS/MS: High Performance Liquid Chromatography with tandem mass spectrometry

HR+/-: Hormone Receptor positive/negative

HUVECs: Primary Human Umbilical Vein Endothelial Cells

kDA: Kilodalton

MALDI-TOF/TOF: Matrix Assisted Laser Desorption Ionization Time-of-Flight mass spectrometry

MMP-2: Metalloproteinases-2

MPI: Polybia-mastoparan I

MRI: Magnetic Resonance Imaging

mRNA: Messenger RNA

MS: Mass Spectrum

MTT: 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide

NRU: Neutral Red Uptake

p53: Tumor protein p53

PARP: Poly (ADP-ribose) polymerase

PBS: Phosphate-buffered saline

PD-1: Programmed cell death-1

PI: Propidium Iodide

PD-L1: Programmed cell death ligand-1

RNA polymerase: Ribonucleic Acid Polymerase

RNase: Ribonuclease

ROS: Reactive Oxygen Species

SD: Standard Deviation

siRNA: Small interfering RNA

TAF: Tumor Angiogenesis Factors

TP53: p53 coding gene

USA: United States of America

WHO: World Health Organization

List of amino acids abbreviations

Amino acid	Three letter code	One letter code
Alanine	ala	A
Arginine	arg	R
Asparagine	asn	N
aspartic acid	asp	D
asparagine or aspartic acid	asx	B
Cysteine	cys	C
glutamic acid	glu	E
Glutamine	gln	Q
glutamine or glutamic acid	glx	Z
Glycine	gly	G
Histidine	his	H
Isoleucine	ile	I
Leucine	leu	L
Lysine	lys	K
Methionine	met	M
Phenylalanine	phe	F
Proline	pro	P
Serine	ser	S
Threonine	thr	T
Tryptophan	trp	W
Tyrosine	tyr	Y
Valine	Val	V

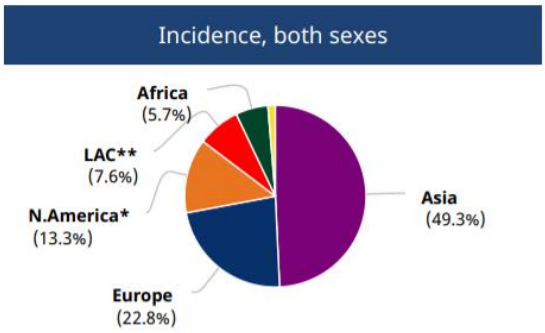
1 Introduction

1.1 Cancer

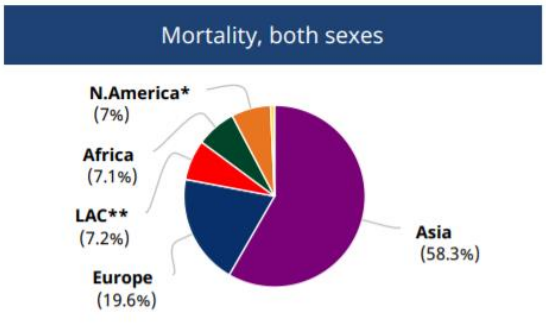
1.2 General Considerations

According to the World Health Organization (WHO), cancer is the second leading cause of mortality and morbidity worldwide and one of the most common diseases in Western society [1]. Cancer is characterized by the abnormal proliferation of different types of cells in the organism, and may begin in any organ or tissue and invade adjacent organs and other parts of the body [2]. This disease is defined as a multi-stage process that progresses from a precancerous lesion to a malignant tumor [3]. These progressions are essentially due to the interaction of the patient's genetic factors and external agents that may be physical, chemical, environmental or biological [2, 4]. Lifestyle, aging, eating and drinking habits (e.g. tobacco, alcohol, physical exercise and diet), ultraviolet radiation and exposure to biological agents (e.g., infections by micro-organisms such as viruses and bacteria) are some examples of carcinogens that influence and increase the risk of developing cancer [1, 2, 4].

Statistically, Europe is currently the second most affected continent by the disease, reporting in 2020 an incidence of 22.8% of cases and a rate of approximately 19.6% of reported cancer deaths in the world (Figure 1) [5].



Population	Number
Asia	9 503 710
Europe	4 398 443
*Northern America	2 556 862
**Latin America and the Caribbean	1 470 274
Africa	1 109 209
Oceania	254 291
Total	19 292 789



Population	Number
Asia	5 809 431
Europe	1 955 231
**Latin America and the Caribbean	713 414
Africa	711 429
*Northern America	699 274
Oceania	69 354
Total	9 958 133

Figure 1 - Cancer Cases vs Death Cases in 2020. Source: WHO [5].

Although the scientific efforts to fight cancer have been offering new perspectives and significant advantages in understanding the mechanism of action of the disease and new ways to genetically predict, early detect and treat cancer, the incidence has still increased around the word in the last decades [3, 6]. In early 2010, it was estimated that by the year 2030 the average annual number of cancer deaths would be approximately 7.6 million [7]. However, in 2020, of the approximately 19.3 million people diagnosed with the disease, the WHO has recorded almost 10 million deaths, i.e. an annual number that is already much higher than expected and which adds up to almost 52% of deaths to 48% of people cured by cancer [1, 4]. The most common cancers in 2020 were breast cancer (11.7% of the cases), lung cancer (11.4%) and colorectal cancer (10.0%). The cancers that caused the most mortality was respectively lung cancer (1.8 million deaths) and colorectal cancer (935 000 deaths) [1].

In March 2020, the WHO declared COVID-19 a global pandemic that has increased the worldwide mortality of cancer patients [8]. The pandemic – which by October 2021 caused more than 240 million infected people and over 4.9 million deaths – has been monopolizing health systems around the world and neglecting medical care for cancer patients [8, 9]. Based on available studies, cancer, increased the risk of a patient getting severely ill from COVID-19 once, as several treatments for many types of cancer can debilitate the patient body's and compromise the ability to fight back the disease [10]. According to research developed by Lai et al. in April 2020 [11] (one month after the pandemic state was declared by the WHO) it was already noticeable, a reduction of between 45-66% in chemotherapy sessions and a decreased between 70 to 89% in early cancer diagnoses which is extremely important for a more efficient and effective treatment. The authors also refer to an enormously alarming scenario of almost total postponement of scheduled surgeries for cancer patients [11]. So, the lack of preventive healthcare, chemotherapy treatments, surgeries that were constantly postponed, and being COVID-19 risk patients, results in an increase in cancer patient mortality that may reach 20% in some countries (although this value may be even higher, especially in the poorest countries with more difficult to access healthcare services) [12].

1.1.1 Cancer Hallmarks, disease progression and differences between tumors

Malignant cells exhibit structural, genetic and functional differences when compared with normal/healthy cells, that are associated with the triggering of tumorigenesis [13]. These differences, present in a large majority of cancer types, are well documented and are frequently used as markers for malignant cells [14, 15]. Douglas Hanahan and Robert Weinberg [14] described, in 2011, ten of these acquired hallmarks responsible for the proliferation, survival and dissemination of cancer cells represented in Figure 2. According to these authors, the tumor cells must be able:

(1) to maintain proliferative signaling. Tumor cells, unlike normal cells, need to maintain a complete signaling independent of external growth signals to sustain proliferation. These, result from activation of intrinsic signaling pathways factors and/or the interruption of negative feedback mechanisms [14, 16]. Thus, tumor cells promote a continuous stimulation of cell division, while contributing to their displacement even after contact with neighboring cells and to their disordered migration over adjacent cells [16].

(2) to promote genomic instability and mutations. To avoid cell death, cancer cells develop several cellular mechanisms: one mechanism and probably the most reported in the origin of cancer is based on the occurrence and accumulation of mutations - whether spontaneous or epigenetic (methylation, modification of histones, phosphorylation and acetylation of nucleotides) – in a gene with DNA repair function, such as proto-oncogenes and as tumor suppressor genes [17, 18].

(3) to evade growth suppressors. Tumor suppressor genes are responsible for regulating cell division, repairing DNA damage, and also promoting apoptosis to prevent mutations - which occur spontaneously or during meiosis/mitosis processes – from passing on to the following cell generations [14, 19]. The above mutations and genetic instability frequently result in the compromised or lost function of these genes, being unable to activate the machinery that prevents mutant cells from reproducing, thus allowing them to grow, proliferate uncontrollably and trigger, in many cases, a tumor [18, 20]. The most common gene to suffer loss of function is the TP53 gene (which codes for the p53 tumor suppressor protein) - mutations in this gene are found in 50 to 75% of known cancers - making it a good marker for cancer [20].

(4) to escape apoptosis. Tumorigenesis can trigger programmed cell death but, cancer cells can show resistance to apoptosis through different mechanisms as mentioned:

loss of function of the tumor suppressor TP53 is perhaps the most common pathway [20].

(5) to unlimited replication. Healthy cells have a limited replicative potential, at which point they stop growing and enter in senescence [19]. This is due to the natural shortening of telomeres - repeated nucleotide sequences at the 3' end of DNA - with each cell division cycle of a cell [2]. Nevertheless, the vast majority of cancer cells (between 85 and 90%) have also developed a genetic mechanism to overcome cellular senescence by synthesizing enzymes capable of prolonging their telomeres, the telomerases. These consist of reverse transcriptase whose function is to add the nucleotides that a normal cell loses throughout its life, making cancer cells with unlimited replication [21].

(6) to induce angiogenesis. Malignant cells secrete lytic factors often responsible for an important process for the growth and proliferation of a tumor: angiogenesis. This is a complex and normal process controlled by certain biomolecules that aim to form new blood vessels from pre-existing vessels in order to provide oxygen and nutrients to the cells, as well as ensure a pathway to eliminate carbon and other cellular surplus dioxide produced during cellular respiration. In cancer, malignant cells will take advantage of these new blood vessels to keep them viable and nourished [2, 22]. To do so, the tumor cells secrete "tumor angiogenesis factors", also called TAF that stimulate this process, in addition to cascades of cytokines, chemokines and angiogenic enzymes [22, 23].

(7) to deregulate cellular energetics. Tumor cells change their energy metabolism to support high cell proliferation and metabolic rate [24]. The main metabolic changes are dysregulation of pH and lipid metabolism, increased generation of reactive oxygen species (ROS), and increased aerobic glycolysis [25, 26].

(8) to promote an inflammatory response. The mentioned cellular energetic changes added to the inflammatory response generated by the immune system - production of molecules (enzymes, ROS, growth factors, etc.) - make an acidic microenvironment that is favorable for tumor cell survival and that sustains and supports the described tumorigenesis processes, the opposite of the immune system's goal in producing them [23, 24].

(9) to avoid immune system destruction. The ability to deregulate cellular metabolism and to use the inflammatory response to their advantage, cancer cells avoid immune system destruction [24].

(10) to activate tissue invasion and metastasis. In the last stage of cancer development, the malignant cells acquire the capacity to penetrate neighboring tissues and organs, to infiltrate the blood and lymphatic circulation and to settle in a new location and form a secondary or tertiary tumor - called metastatic cells [27]. The dissemination and proliferation of these cells to organs distant from the site of origin is called metastasis, and these have an enormous influence on the clinical complications and treatment of patients with this type of cancer [27, 28]. The activation of the invasion and the metastasis are associated with cellular alterations in the malignant cells - downregulation of E-cadherin, upregulation of N-cadherin - and the activation of extracellular proteases. These alterations promote the modification of the cell-cell contact and the very contact of the cell with the extracellular matrix [29]. Metastases and the inability to predict the action of these malignant tumors are what make them so difficult to treat and the real guilty of the high mortality associated with cancer - it is estimated that metastases lead to 90% of reported cancer deaths [28, 30]. It is, therefore essential to distinguish between malignant and benign tumor to understand the extent of this disease and, what are the best forms of treatment in each case [31]. A benign tumor is mainly characterized by remaining restricted to the organ or tissue of origin, not invading the bloodstream or the surrounding organs. These tumors can most often be removed surgically and are the easiest to treat [32]. On the other hand, malignant tumors are those that have acquired the ability to invade neighboring tissues and organs and to expand to other locations in the body through the lymphatic and blood system - called tumor metastases [7, 31, 32].

Figure 2 - Cancer markers described by Douglas Hanahan and Robert Weinberg in 2011. Image adapted from [14].

1.2 Breast Cancer

Breast cancer is a very common cancer in women and the second most common cause of death in the world [33]. In 2020, breast cancer was diagnosed in 2.26 million people, 685000 of whom died, i.e. about 30% of breast cancer cases resulted in the death of the patient [5]. DeSantis et al. [34]. , in 2019, reported that in the USA (United States of America), 1 in 8 women (approximately 13%) will be diagnosed with invasive breast cancer in their lifetime.. This disease develops silently, and most women discover they have breast cancer in routine examinations - usually in cancer screening programs that perform tests such as mammography [35, 36]. Generally, to diagnose breast cancer, attention is paid to small changes in the shape of the breast and the appearance of small nodules [37]. Physical examinations (e.g. patient's vital signs, habits, family and personal history), clinical examinations (palpation), mammography, ultrasound, magnetic resonance imaging (MRI), biochemical studies of the blood and a biopsy of the tissue suspected of being cancerous are used to diagnose this disease [37-39]. The effectiveness of the diagnosis and how quickly treatment is defined and put into practice is intrinsically related to the survival rate of the disease. Once the earlier breast cancer is diagnosed, the less likely it is that the tumor will tend to spread through the lymphatic system and lead metastases in other parts of the body [40, 41].

The increased incidence of breast cancer is related to genetic, physiological, behavioral and hormonal factors [42]. Breast cancer is more frequent in middle age woman – between 40 to 50 years old [43]. A personal history of cancer and a history of the disease in the immediate family are closely related to the likelihood of developing this particular cancer [44]. In addition to these risk factors, exposure of the female sex to the hormone estrogen - be it in pregnancies after the age of 30, late menopause after the age of 55, menarche before the age of 12 or taking a contraceptive pill composed of this hormone and progesterone – also increases the risk of developing breast cancer [45]. However, 5 to 10% of breast cancer cases are essentially due to genetic factors - the BRCA1 and BRCA2 genes are the two main genes involved in the increased susceptibility to develop breast cancer [44, 46]. BRCA1 and BRCA2 genes play a crucial role in DNA repair by promoting checkpoint activation and homologous recombination, respectively [46, 47]. These genes that code for tumor suppressor proteins, when mutated, can increase the risk of developing breast and other ovarian cancer [48].

1.2.1 Types of breast cancer

Breast cancer may be classified by its histological and by its molecular characteristics [49]. This is a cancer with high cellular heterogeneity, and it is classified in the histological branch according to the tissue where it originates and whether it is a carcinoma *in situ* (localized) or an invasive carcinoma (with metastasis) – usually carcinomas are mostly designated adenocarcinomas and originate from cells of the breast ducts and lobules [49–51]. Molecular classification is based on the presence/absence of hormone receptors (HR+/HR-) for progesterone and estrogen, along with the presence/absence of very high levels of the human epidermal growth factor receptor 2 protein (HER2) [52, 53]. Thus, at the molecular level, breast cancer is classified into four main subtypes: (1) luminal A, when it has HR+ and HER2-, progesterone and estrogen receptor-positive cells and absence of HER2; (2) HER2+, cells produce high levels of HER2 and don't have hormone receptors; (3) luminal B, characterized by HR+ and HER2+, cells with positive receptors and overexpression of HER2 levels - and; (4) triple negative (TNBC) characterized by being either HR- or HER2-, cells with no hormone receptors and no HER2 overexpression. These subtypes of cancers directly influence the treatment to be applied for, responses to treatment, disease progression and associated metastases [54]. The incidence of these types of cancer is not linear, 84% of patients diagnosed with the disease have estrogen and progesterone receptor positive which includes 71% luminal A and 12% luminal B. About 12% of breast cancer are triple negative [54, 55].

Cancers with the presence of the progesterone and estrogen receptors mentioned above are often dependent on disorders mediated by these hormones to be able to divide and proliferate [49, 56]. With this in mind, these receptors are seen as targets in therapy to stop cancer progression. There are currently drugs, such as tamoxifen, that perform an antagonist function to the specific receptors of receptor-positive cancers, preventing their hormone binding necessary for cancer cell proliferation [56]. In contrast to these cancers is TNBC, characterized essentially by the lack of gene expression of these receptors and no overexpression of the HER2 protein [53, 57]. Consequently, this cancer does not respond to anti-hormonal or anti-HER2 therapy and is thus considered to be the breast cancer with the most complex therapy [58, 59]. There are at least six subtypes of TNBC which in turn further complicates the effectiveness of a treatment [60]. For example, one subtype of this cancer is immunomodulatory TNBC (a target for immunotherapy as it is characterized by high expression of genes related to T-cell function), another subtype contains the androgen receptor and an additional subtype is derived from mutations of the BRCA-1 and BRCA-2

genes in somatic or germ cells - the latter is predicted to be inhibited by poly(ADP-ribose) polymerase, also known as PARP inhibition [60–62]. As a result, TNBC, and in contrast with luminal A which is slow growing and the less aggressive type, has the worst prognosis of all breast cancers, and has the lowest survival rate [55–58]. Currently, many doctors do not consider that there are appropriate and targeted molecular-based therapies for triple-negative breast cancer, and only chemotherapy treatments are applied, which fail in about 80% of cases. It is therefore essential to focus on new compounds and alternatives to increase the efficacy of treatment for this specific type of disease [57,58].

1.2.2 Therapies for breast cancer and future perspectives

The prognosis of the disease depends on (1) the type of cancer, (2) its stage of development, (3) the levels of hormone receptors, (4) the overexpression of human epidermal growth factor type 2, (5) the tumor tissue being triple negative or not, and (6) other epidemiological factors [42, 49]. Depending on these circumstances, a treatment strategy is defined in combating the disease [51]. Most patients have surgery to remove the tumor, a mastectomy (which can be partial, by removing the tumor and surrounding tissue, or total, when the entire breast is removed) [37, 63]. Despite this, the patient may be subjected to chemotherapy before or after surgery [64]. Chemotherapy consists of the administration of drugs orally, by infusion or by injection in order to stop the growth of cancer cells [37, 65]. Chemotherapy via oral or injection is more usual, since they enter the patient's bloodstream and act on cancer cells throughout the body with greater efficacy [37]. A total of eighty-two drugs are approved so far by the FDA (Food and Drug Administration) in the fight against breast cancer, four to prevent a recurrence of the same and six combinations of drugs (which have an increased effectiveness when used together) [66]. This therapy is often used in conjunction with other therapies such as hormone therapy and radiotherapy [67]. In radiotherapy, patients are exposed to high-energy radiation such as x-rays, to kill and prevent cancer cells that have survived surgical removal of the tumor from proliferating further [37, 68]. Regarding hormone therapy, which consists of blocking the action of the aforementioned hormones, it is particularly applied and effective against cancers that have hormone receptors [37, 69].

Targeted therapies, such as those using monoclonal antibodies, generally cause less damage to normal cells than chemotherapy and radiotherapy without, however, diminishing the effectiveness in fighting breast cancer [70]. An example of a monoclonal antibody in fighting breast cancer is trastuzumab that is used to treat cancers with HER2 protein

overexpression since it blocks the effects of the proteins [70, 71]. Targeted therapies can also use tyrosine kinase inhibitor compounds and cyclin-dependent inhibitors that block, respectively, the signals required for tumor growth and cyclin-dependent protein kinases - known as CDKs [72].

A very recent therapy in the fight against cancer consists of immunotherapy used to increase, direct and restore the defenses of the patient's immune system. This therapy takes advantage of the patient's own immune system in order to guarantee the effectiveness of the treatment in the fight against breast cancer, but without adding radiation, chemicals or foreign substances to the organism that always have side effects [37, 73]. This is where CAR-T cell therapy fits in, harvesting their T-lymphocytes (or those of compatible donors) and, manipulating them in the laboratory in order to recognize, bind and eliminate the cancer cells specifically [73, 74]. Another widely used type of immunotherapy consists of PD-1/PD-L1 therapy. This therapy is based on the premise that some malignant cells have the PD-L1 protein in their membrane, in addition to the T-lymphocyte cells that have the PD-1 surface protein, which binds to each other and prevents the immune system from triggering cell death of malignant cells [75]. Thus, the use of a PD-L1 inhibitor of T-lymphocytes, such as the compound atezolizumab, represents a high potential to treat cancer [37, 75].

One of the main problems in the treatment of cancer, and that nanomedicine has proposed to overcome, is that the disease is not homogeneous, but is treated as if it were [76]. Nanomedicine has increasingly presented in recent years a platform biocompatible and specific systems, capable of making a localized drug delivery to a particular type of tumor cells and controlling its release profile and its concentration in the tissue [77]. This targeted therapy, such as the use of nanoparticles that make an efficient drug delivery, greatly increases the specificity and efficacy of various treatments [37, 76].

There is currently a wide range of treatments under investigation and clinical trials, many of them already approved by the FDA, such as gene therapy, siRNA silencing, thermal ablation and therapeutic hyperthermia [37, 78]. However, despite these scientific efforts, the incidence of cancer is expected to increase by 47% in 2040 compared to 2020, i.e. 28.4 million new cases of cancer compared to the current year in which 19.3 million cases were reported, and one of the most common will continue to be breast cancer [79]. Thus, there is clear evidence of the need to discover new compounds and develop new therapeutic strategies that provide more efficient cancer-fighting alternatives.

1.3 Can venoms fight against breast cancer?

1.3.1 Animals Venoms and their composition

In nature, several animals have evolved and adapted in such a way that they produce venoms to protect themselves, to hunt or for both purposes [80, 81]. In essence, venoms consist in mixtures of natural compounds that biochemically deregulate the nervous, cardiovascular and muscular systems of the target animal [80]. Venom secretions are produced by specialized cells of each animal. Each species developed a different survival strategy and, depending on its physiology, the venom can be released in different ways: sting in the case of wasps and bees, fangs in the snakes and vipers, skin in the case of frogs, among many others [81]. It is widely known that many venoms from a wide range of animal's cause pain and potentially fatal harm to their victims, thus many cultures have taken advantage of these venoms to develop weapons and hunt their food over the centuries - for example, dipping weapons into the venoms of different animals causes immediate death of their prey [82, 83].

Venoms, although varying in composition from animal to animal, are mostly composed of an extensive and complex panoply of naturally and biologically active chemical compounds [84, 85]. This heterogeneous mixture of compounds is mainly divided into smaller peptides, proteins, organic molecules and mineral salts [80, 86]. A high number of protein isoforms are also found in venoms, i.e. extremely similar proteins originating from a single gene or gene family, but with distinct biological functions that may belong to distinct protein families - this process mostly results from alternative splicing that takes place after transcription of the gene(s) by RNA polymerase [87]. The complexity of venoms and their compounds has currently been the focus of study and development of new therapeutic drugs for a range of diseases such as hypertension, cardiovascular disease, diabetes, osteoporosis and even has the potential to treat cancer [88, 89].

1.3.2 Venomics: the impact of "omics" and mass spectrometry

The study of venoms and their therapeutic biotechnological potentials has been gaining space in science, giving rise recently to a new branch: venomics [87, 90]. This branch, now presented as the solution to investigate in depth the high complexity of venoms has had a wide scientific growth mainly after the development of the so-called "omics" sciences: genomics, transcriptomics, proteomics and metabolomics. With the new "omics" technology even compounds that are found in small quantities and in small concentrations can be

identified, isolated and analyzed [80, 91, 92]. Genomics analyzes and studies the structure and function of the genome and specific genes that code for compounds of interest - using increasingly efficient sequencing technologies (e.g. Next Generation Sequencing) - while transcriptomics analyzes transcribed mRNA [87]. Proteomics, deals with the expression of peptides and cellular proteins, and metabolomics with the identification and quantification of all metabolites in the biological system [93, 94]. By analyzing the entire proteome and/or metabolome of a venomous species, it is possible to discover several compounds with biotechnological interest. Proteomics, in the study of venoms provide an advantage in the quantification and complete description of all components of a sample - this correlation of the components can not only provide important biological information regarding the effects of the venom, but also allows a quantification of them and their proportions [87, 95, 96]. Adding to this, proteomics allows establishing questions of homology with other families and, to establish biological relationships between them [96]. The relationship with the above sciences and venomics is shown in Figure 3 [80].

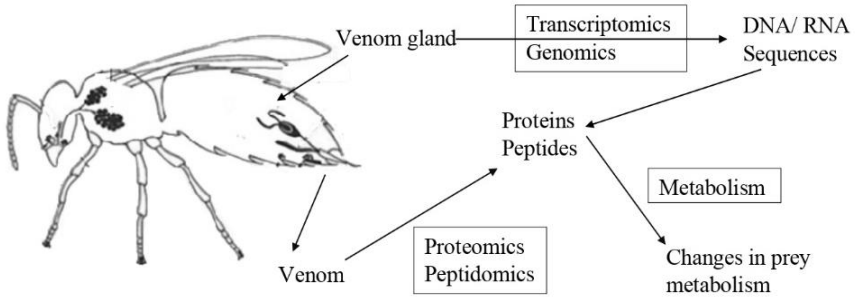


Figure 3 - Relationship between the "omics" sciences and venomics. Adapted from [80, 97].

There are several techniques used in these branches, and therefore techniques that help in the discovery, quantification and characterization of venoms [97]. However, venomics has been rather limited by the low amounts of venom sample available from some venomous species [87]. Standard chemical fractionation, which aims the biochemical study of venoms, has been replaced in recent decades by more promising and higher performance multidimensional techniques [80]. Of these, the most widely used is mass spectrometry, which is based on an analytical technique to measure the mass/charge ratio of ions and therefore to construct a mass spectrum and determine a protein signature [98]. These include, for example, high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) and matrix-assisted laser desorption ionization coupled to time-of-flight (MALDI-ToF/ToF) used in many studies of venoms [80, 100].

In the field of venomics, specifically mass spectrometry, the most widely used strategy to discover the proteome and protein profile of venoms is “bottom-up” [80]. This involves initial digestion of all the peptides and proteins of the sample venom by enzymes (trypsin is usually used), separated by HPLC and subsequent transfer to a mass spectrometer where a series of mass spectra are obtained and then analyzed [90, 92]. From the identified proteins it is possible to analyze the proteome from "bottom-up" until reaching the transcriptome and in turn the genome [80]. This strategy is important because it allows the identification of the genes that originate the proteins and therefore manage to synthesize, or genetically recombine, the proteins with therapeutic potential [101, 102]. Sanggaard et al. (2014) [101] used this strategy to assemble the genome and transcriptome of the spiders *Stegodyphus mimosarum* and *Acanthoscurria geniculata.*

1D or 2D gel electrophoresis although less common in venomics - as it is a technique used for the separation of proteins with higher molecular weights and venom peptides are small, usually between 500 Da and 7 kDa - is often used prior to liquid chromatography to increase the resolution of the protein profile. These are usually reduced and enzymatically digested in the gel before proceeding to HPLC [92].

In general, venomics follows a workflow as depicted in Figure 4 - important to note that for the identification of a new peptide it is essential to cross-reference the theoretical MS/MS spectrum with the practical one [81].

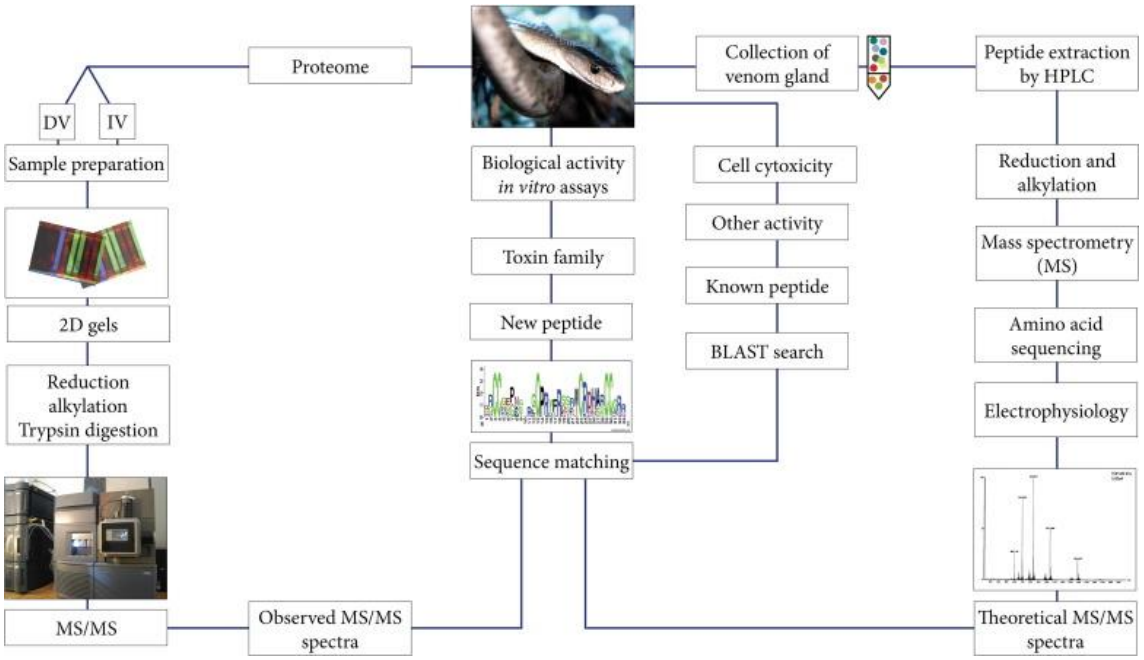


Figure 4 - Workflow commonly used in venomics for the analysis of a venom and the discovery of a new peptide. Taken from [81].

1.3.3 Animals that produce venoms with biotechnological potential and examples of compounds approved by the FDA for therapeutic purposes

Venomics is a scientific area with a lot of unexplored venoms, because besides the fact that venoms are extremely heterogeneous and complex as already mentioned, they are produced by a vast number of species - many of them difficult to capture and handle [87]. Venoms are produced by a wide range of animals, such as annelids, cnidarians (sea anemones), echinoderms (starfish) to mollusks (octopuses and snails), arthropods and vertebrates (frogs, toads, fish, lizards, snakes, birds and even some mammals) [81, 103]. The most studied in the area of cancer, due to their high antitumor potential, are the arthropods venoms, such as scorpions, bees, wasps, spiders and ants [103]. Nevertheless these facts, many vertebrates and other phyla mentioned above are being widely targeted for scientific studies in the area - as is the case of the huge studies with snakes [104].

Currently there are 6 compounds derived from the venom and approved by the FDA to treat some specific diseases, the first approved in 1981 and the last one already in 2005 [85]. These compounds are used to treat hypertension, chronic pain, coronary syndrome and the last one approved, exanatide, to treat type II diabetes [88, 106]. In the area of cancer, research is focused on overcoming the adverse effects (and often not very effective) of certain chemotherapeutics, as well as overcoming resistance to some of these compounds, which is already beginning to be demonstrated by many malignant cells [106].

1.3.3.1 <u>The Snake</u>

Snakes are among the best-known venomous animals and are, according to WHO estimates, responsible for 81,000 to 138,000 deaths annually and four-hundred amputations [81]. The venom of snakes and vipers has a high medicinal potential already well studied. They present compounds with antimicrobial, analgesic, anticoagulant and anticancer activity. These venoms are mostly composed by enzymes L-aminoacido oxidases, acetylcholinesterases, proteinases, metalloproteinases, phospholipases A2, nucleosidases and hyaluronisases, and also proteins/small peptides that inhibits proteases, disintegrins and cytotoxins [106]. Silva et al. [107], in a recent review published in 2021, analyzed the work of several authors with venom components from 30 different *Viperidae* species. The authors reported that the main antibacterial and antitumor mechanisms of the toxins from these venoms are channel and receptor blockade, activation of molecular pathways, interaction with integrins, and membrane damage [107]. As an example, the snake *Naja naja atra*, presents proteins that present both antimicrobial and antitumor activity – such a Cardiotoxin III [108].

This toxin, according to Chen et al. (2011) [109], damages the membranes of both gram-positive and gram-negative bacteria such as *Staphylococcus aureus* and *Escherichia coli*, respectively. It is also important to note the incredible ability of this toxin to prevent the migration and invasion of TNBC cell line MDA-MB-231, without inducing apoptosis in cells or stopping the cell cycle. Its mechanism of action consists briefly of attenuating the activity of Src kinase - a protein with an essential function in cell proliferation and cell adhesion - by phosphorylating it. The strong enzymatic activity, the simple peptides with proven pharmacological activity and the ease with which these peptides can be studied, optimized and recombined, make the economic and biotechnological interest in this growing area [106, 110]. To date, three peptides derived from snake and snake venom have obtained FDA approval to treat hypertension and to prevent the formation of blood clots (which can lead to strokes and heart attacks) - Captopril, Eptifibatide and Tirofiban - shown in Table I. However, there are already several other compounds, approved by other institutions, as well as other compounds that are in clinical trials. The number of these compounds is expected to increase exponentially in the coming decades [109].

Table I - FDA-approved snake venom-derived drugs. Adapted from [109].

Drug	Source	Application	State
CAPTOPRIL	Derived from bradykinin of the species *Botjrops jaracusa*	Treatment for hypertension	FDA Approved
EPTIFIBATIDE	Derived from the disintegrin of *Sistrurus miliarus barbourin*	Antiplatelet drug	
TIROFIBAN	Derived from the disintegrin of *Echis carinatus*	Antiplatelet drug	

1.3.3.2 The Scorpion

Scorpions are arthropods that produce extremely potent and dangerous venoms, stinging on average 1.2 million people per year and killing more than 3250 people as a consequence [111]. Despite this, scorpion venom has incredible promising prospects in the field of medicine and therapeutics, being a rich source of candidate compounds for therapeutic drugs [100, 112]. This is mainly due to its high complexity of nucleosides, proteins, amines, salts of small peptides and proteins added to its enormous target specificity

and its regulatory role in various biological mechanisms [112]. Scorpion peptides are currently being studied to combat cardiovascular and immunological diseases, especially from scorpions of the species *Pandinus imperator, Mesobuthus eupeus, and Heterometrus bengalensis* (Figure 5). The venom peptides with the greatest relevance in the treatment of these diseases pass through Ctriporine, Chlorotoxins (cltx), Neopladin I/II, Meucin 24, Meucin 25 and Hp 1090 [113]. In 2003, Deshane et al. [114] reported the activity of the peptide Chlorotoxin in the treatment of glioblastoma, a very common and severe brain cancer. Its mechanism of action is to bind to the receptor of metalloproteinases-2 (MMP-2) - a receptor not expressed in normal brain cells but expressed in glioblastoma tumor cells - and to inhibit its enzymatic activity and thus substantially reduce tumor cell expression [113, 114].

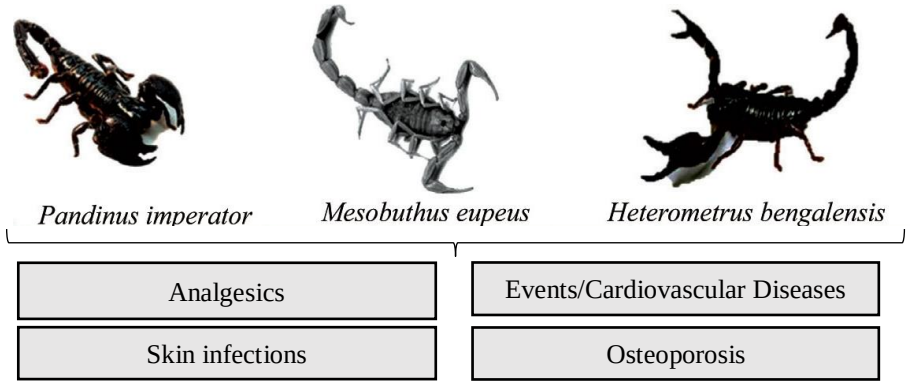


Figure 5 - Scorpion species that produce compounds that act in the referred diseases. Adapted from [113].

From the scorpion species *Parabuthus schlechteri* and *Opistophalmus carinatus*, were isolated three peptides with antimicrobial activity against gram-negative and gram-positive bacteria, namely opistoporin-I, parabutopotin and melittin [115]. Opistoporin-I also show high antifungal activity against several species of fungi, such as *Saccharomyces cerevisea* species. The antiviral activity of several compounds isolated from the venom of this species, especially the activity of compound Kn2-7 against the HIV (human immunodeficiency virus), which currently infects more than 34 million people, is being strongly investigated [116]. Compound Hp 1090, isolated from the species *Heterometrus peterssi*, has been proven to prevent hepatitis C infection and continues to be studied for future use as therapeutics [117].

1.3.3.3 The Bee

Bees are arthropods that produce a complex venom rich in peptides, enzymes, biologically active amines and other natural molecules - such as dopamine, histamine and norepinephrine - with a wide range of pharmaceutical properties [118, 119]. For over 3000 years, bee venom therapy (BVP) has been used to treat arthritis, rheumatism and muscle pain in Eastern Chinese culture. BVP therapy consists of the localized sting of the bee in areas specific to the symptoms or injection with the venom and the consequent anti-inflammatory activity and reduction of pain caused by inflammation [118]. The compounds in their venoms go on to exhibit various activities such as anti-inflammatory, antioxidant, antimicrobial and antifungal - as is the case of melittin [119]. About 40 to 60% of bee venom consists of melittin, a peptide of only 26 residues that, in addition to the activities mentioned, presents cytotoxic activity against breast and liver cancer cells, melanoma and leukemia [120, 121]. Their activity consists of attacking the phospholipid bilayer of cancer cells and activating caspases [122]. The use of this compound has been optimized either by combined use with gene therapy or with the use of nanoparticles [123]. The use of nanotechnology in the study and application of venoms to combat cancer and localized diseases is currently considered an innovative area with immense potential for efficient, rapid and biocompatible compound delivery [77, 123].

1.4 Wasp Venoms and biotechnological potential

1.4.1 The wasps *Polybia dimorpha* and *Chartergellus communis* and its therapeutic potential

The species *Polybia dimorpha* and *Chartergellus communis* are part of subfamily *Epiponini,* family *Vespidae* and the superfamily *Vespoidea* belonging to the Hymenoptera order [124, 125]. The superfamily *Vespoidea* comprehends 10 families, with *Vespidae* being composed mostly of social wasp and the subfamily *Epiponini* comprises the majority of genera of the neotropical social wasp fauna, comprising 19 distinct genera of which. Genus *Polybia* is the most numerous and have the greatest morphological differences between species. The genus *Polybia* has 58 species, 44 of which have already been recorded in Brazil, one of which is *P. dimorpha* [126]. The *Chartergellus communis*, part of the genus *Chartergellus*, were firstly described by Richards in 1978 [127] and, although not very abundant, can be found in the Brazilian cerrado and in the caatinga biome [127, 128]

Wasps have developed their venom as a chemical weapon against their predators with the ultimate goal always being the defense of the swarm [130]. The wasps of this genus are widely used in pest control by farmers as they are known for their painful sting, with each sting releasing between 1.5 to 20 micrograms of venom on the prey [131]. The sting causes inflammation, pain and can result in neurotoxic effects, tachycardia/bradycardia and cardiac arrhythmia [132].

Wasp venom is one of the most variable venoms among species and consists of complex mixtures of biologically active compounds, mostly peptides, proteins and other small molecular mass compounds [133]. Bee and wasp venoms share several of these compounds such as phospholipases A2 and B, histamine, dopamine, noradrenaline, serotonin and adrenaline. Serotonin, dopamine/norepinephrine respectively promotes the pain felt on stinging and the speeding up of the victim's heartbeat. However, compounds such as mastoparan and bradykinin are unique to wasps, just as melittin and apamin are to bees [129, 130]. Wasp sting is also considered more painful than a bee sting, much due to the high levels of acetylcholine present in wasp venom that stimulate pain receptors [133].

The current literature allows to conclude that there is a huge and growing potential for the use of wasp venoms in medicine and therapy of several diseases. The high specificity and affinity of the compounds for specific targets makes them fascinating from a biotechnological perspective, in order to develop to a series of drugs for the treatment of various diseases [135].

The "omics" sciences and mass spectrometry has allowed the detection and identification of compounds of small molecular mass that are in small concentrations in wasp venom. Recently, in 2016, das Neves et al. obtained Chromatographic profile obtained by RP-HPLC Mass spectrum of the low molecular masses present in the venom of *Polybia dimorpha* by a combination of reverse phase HPLC and mass spectrometry where it is already possible to analyze the high molecular complexity of the venom in question – Figure 6 [136].

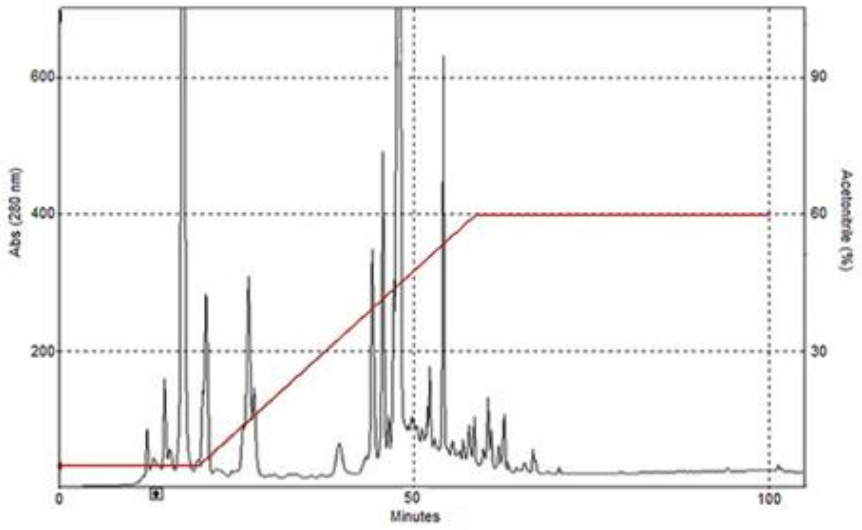


Figure 6 - Chromatographic profile obtained by RP-HPLC Mass spectrum of the low molecular masses present in the venom of *Polybia dimorpha*. Taken from [136].

Also, Lopes et al. (2017) [125] obtained the chromatographic profile of the *Chartergellus communis* venom fractionation by RP-HPLC (Figure 7), this being the first study published in literature with relevant information on the chemical, structural and biological characterization of some compounds isolated from this venom.

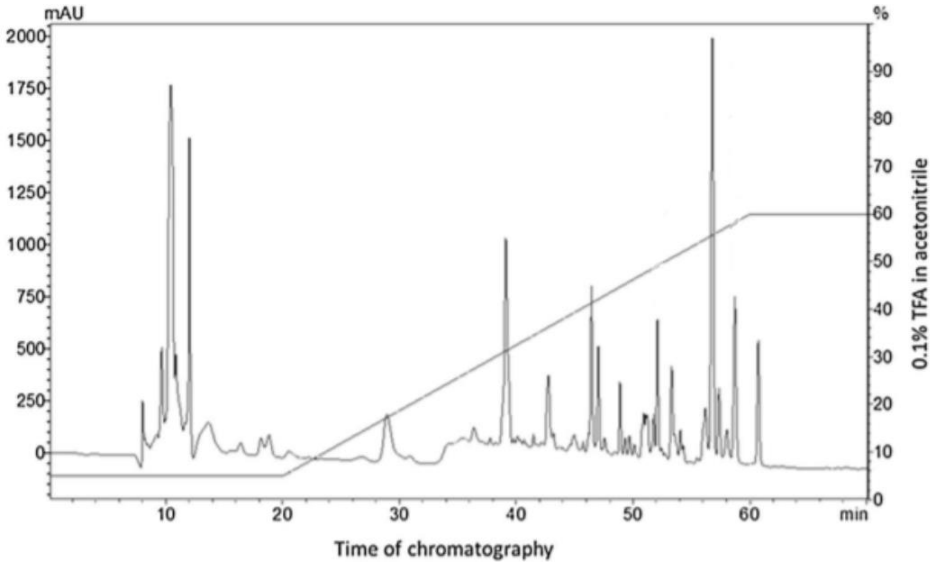


Figure 7 - Chromatographic profile obtained by RP-HPLC Mass spectrum of the low molecular masses present in the venom of *Chartergellus communis*. Taken from [125]

1.4.2 Classification of wasp venom peptides/proteins

In social wasp venom the peptides/proteins are generally classified according to the type of biological activity they exhibit [132]. They consist of:

(1) mastoparans, small amphipathic peptides with only 14 amino acids rich in hydrophobic and basic residues (such as lysine) capable of forming pores in cell membranes [128, 133]. Mastoparans exhibit a wide variety of biological effects: they induce a strong mitochondrial permeability transition (by Ca^{2+} mobilization) - which affects cell viability and can lead to cell lysis -, cause the secretion of histamine from mast cells, serotonin from platelets, catecholamines from chromaffin cells and prolactin from pituitary, stimulate phospholipase enzymes and promote cell death by necrosis/apoptosis [129, 134, 135]. In short, it exhibits antimicrobial, antiviral, hemolytic activity and induces cytotoxicity in tumor cells. Thus, this peptide is a viable alternative to antibiotic resistance and cancer treatment. In this regard, several studies are being developed to increase the selectivity of a molecule analogous to mastoparan, mitoparan, to cell adhesion molecules overexpressed in cancer cells thus mitigating the progression of metastasis [134, 135]. The ability of mastoparans and analogues to efficiently cross the plasma membrane and, their potential as cell-penetrating peptides, i.e. the ability to transport chemotherapeutics (such as doxorubicin) and other compounds through liposomes across the phospholipid bilayer of tumor cells and/or bacteria, has also been investigated [140]. Mastoparans are also currently being the focus of study for

neurological disorders, such as epilepsy, mood disorders, Parkinson's disease and Alzheimer's disease, due to their interaction with G-protein receptors (currently widely used in understanding these complex diseases and as a therapeutic target) and their ability to cross the blood-brain barrier [137–139]. To date, this peptide is the only one isolated from wasp venom that stimulates insulin release by increasing intracellular Ca^{2+} concentration and inhibiting potassium channels, making it a potential antidiabetic drug [142].

(2) Chemotactic peptides are peptides that induce chemotaxis, but also other cellular responses, such as promoting mast cell degranulation [144]. An example of this type of peptides isolated from wasps is the polyfunctional peptide, protonectin, which, in addition to the activities mentioned, presents antimicrobial activity against gram-positive and gram-negative bacteria and, promotes the release of enzymes such as lactate dehydrogenase in mast cells [145].

(3) Quinines, peptides of which bradykinin and analogues are part, are main responsible for the pain caused by wasp sting and for the paralyzing action used in the capture of prey. These neurotoxins are made up of only nine amino acids, but with an essential function in the species to maintain blood pressure, heart and kidney function and, promote the inflammation process. Several studies suggest that these peptides trigger a cascade of inflammatory events in the central nervous system, but also exhibits neuroprotective properties [137].

(4) Very short linear peptides exhibit chemotactic and inflammatory activity [132].

1.4.3 Antimicrobial peptides as peptides with high potential in the fight against cancer

The emerging problem of antibiotic resistance, which stems from the overuse of these compounds, is currently considered one of the biggest emerging public health problems around the globe [142, 143]. Currently, more than 25 000 people die due to infections caused by multidrug-resistant bacteria only in Europe, which indicates that the search for new antibiotics is increasingly important [148]. It is in this context that the search for natural alternatives, such as antimicrobial peptides (AMPs), that exhibit antimicrobial activity and are equally effective against pathogenic multidrug-resistant gram positive and gram-negative bacteria, emerges [149]. Many of these peptides are found in various organisms, including venomous organisms such as the snake, spider or scorpion [150]. There are increasingly evidences of a high correlation between natural peptides with antimicrobial activity (AMPs) with an anticancer activity (ACPs), which makes them at the vanguard in the innovative

treatment of cancer potentially replacing conventional treatments such as chemotherapy - overcoming resistance effects caused by chemotherapeutic compounds and, increasing their specificity [151]. In 2018, Torres et al. [152] referred the existence of more than 2500 AMPs, and of these at least 600 were ACPs, that is, they had proven activity against cancer. The different origin of these compounds leads to their homology being very small, but there are similar features that help to detect their potential as anticancer peptides: (1) positive charge (2) amphipathic structure (3) well-defined secondary structure in hydrophobic environments and (4) helix structures in most active compounds [148, 149]. This relationship has already promoted the immense research that initially focused on peptides with antimicrobial activity to investigate their activities as ACPs and even proceed to genetic manipulations in the peptide to make it able to specifically treat cancer without causing damage to normal cells of the body [118, 148]. For example, taking as a starting point the work developed by Konno et al. [154], in 2007, that described an AMP, decoralin, isolated from the wasp *Oreumenes decaratus*, with a high antimicrobial activity against gram-positive and gram-negative bacteria, fungi and protozoa. Nevertheless, there is a strong possibility of increased hemolytic activity induced by this decoralin - destruction of red blood cell membranes that prevented the use of this compound *in vivo* [149]. Torres et al. [152], in the year 2018, developed and tested a series of derivatives in which resulted a recombined peptide, Dec-NH_2, that decreases the hemolytic activity in red blood cells without losing efficacy against microorganisms and, mainly, with anticancer potential against MCF-7 breast cancer cells. Following this example, Guimarães et al. (2017) [155] revised the work developed by Gomes et al. (2015) [156] on the compound botropoidin and, proved it has antitumor and anti-angiogenic activity against breast cancer MDA-MB-231. This compound was described by the first authors as a metalloprotein with high proteolytic activity and it was evidenced with this work its specificity for breast cancer causing apoptosis in malignant cells, an inhibition in the adhesion of these cells and a dose-dependent relationship of their migration [156].

It should also be noted that the peptides reported above obtained from various venoms, such as wasps, act even at concentrations in the micromolar order [157]. Torres et al. (2018) [152] concluded that a concentration below only 12.5 μmol/L of Dec-NH_2 suffices to ensure its efficacy against MCF-7 breast cancer. Guimarães et al. (2017) [155] reported a treatment efficacy of MDA-MB-231 breast cancer with botropoidin concentrations between 10 and 40 μg/mL.

The interest on venomous species relies on their potential in antimicrobial and anticancer activity, especially on the few analyzed so far. In 2016, das Neves [136] analyzed

the mass spectrum (MS) of the social wasp *P. dimorpha* and identified by MALDI-TOF/TOF an abundance of peptides of the most varied m/z ratios and, the ion with m/z 2441.7 $[M+H]^+$ was identified as Polydim-I with only 22 amino acids [136]. The authors analyzed its antimicrobial activity against *Mycobacterium abcessus* species, a virulent, pathogenic and drug-resistant mycobacterium that is currently responsible for an increasing number of infections in humans. Extremely promising results were obtained, such as the reduction of the mycobacterium growth to 50% with a concentration of only 7.6 μg/mL of Polidym-I. It is concluded from this study that the compound extracted from the wasp venom, Polidym-I, causes disruption of the cell membrane of these mycobacteria, while, at the same time, exhibits a low hemolytic activity. The authors also concluded that Polidym-I is not cytotoxic to mammalian cells, making it a good AMP [132, 153]. It is important to analyze that this AMP has some good indications to be a good ACP in the fight against cancer - hydrophobic peptide with amphipathic structures, thus, research on their antitumor potential should be performed [153, 154].

1.5 Objectives and work to be developed in the thesis

Polybia dimorpha and *Chartergellus communis* are species of social wasp that produce venom containing peptides with high potential for antitumor activity. The venom of these species is still scarcely studied: only Polydim-I peptide was described by das Neves et al. (2016) [136] with high antimicrobial activity in *Polybia dimorpha* venom and, only this year 2021, Lopes et al. [159] reported a high potential of new peptides from the venom of the wasp *Chartergellus communis* in the pharmacotherapy of epilepsy. The high similarity of this peptide with chemotactic peptides with a proven antimicrobial and antitumoral activity makes Chartergellus-CP1 peptide biotechnology interesting in cancer research [159].

In this thesis, two new peptides – Prolistarin and Chartergellus-CP1 – bio-inspired from *P. dimorpha* and isolated from *C. communis* social wasps venom, respectively, and kindly provided by Dr. Márcia Mortari from University of Brasília (Brasil), were used to study the antitumor potential against breast cancer cell lines - in MDA-MB-231 triple negative breast cancer cells (one of the most complex breast cancers with the highest mortality rate) and in MCF-7 a Luminal A cell line derived from breast adenocarcinoma (the most common type of breast cancer) [160].

For this purpose, the work developed in this thesis can be divided in specific aims:

- To evaluate the effects of peptides on cell viability;
- To evaluate the effect of Chartergellus-CP1 peptide on cell morphology;
- To evaluate the role of Chartergellus-CP1 peptide on cell cycle dynamics;
- To characterize if and how Chartergellus-CP1 peptide induce oxidative stress;
- To characterize if and how Chartergellus-CP1 peptide induce apoptosis/necrosis.

2 Material and Methods

2.1 Peptides Solution Preparation

Prolistarin peptide (amino acid sequence: DKPRWNVKKPPRLL-NH_2; Molecular Weight: 1746.14 Da), a bio-inspired peptide, from the *Polybia dimorpha* wasp venom and Chartergellus-CP1 peptide (amino acid sequence: IIGTILGLLKSL–NH2; Molecular Weight: 1239.9 Da) isolated from *Chartergellus communis,* were kindly provided by Dr. Márcia Mortari from University of Brasília (Brasil) [159]. Before the experiences, the peptides were diluted to a concentration of 20 mg/mL in dimethyl sulfoxide (DMSO), and stored at -20 ºC.

2.2 Cell culture

In the present work two different breast cancer cell lines were used: MCF-7, derived from adenocarcinoma breast cancer cell line and MDA-MB-231, a triple negative breast cancer cell line. MDA-MB-231 cells were kindly provided by Dr. Fátima Duarte from CEBAL - Centre of agronomic and Agro-Industrial biotechnology of Alentejo, Beja, Portugal, while the MCF-7 cells were kindly provided by Dr. Liliana Monteiro from IBIMED, University of Aveiro.

Cells were maintained in cell culture flasks (SPL Life Sciences, South Korea), with complete Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/m streptomycin and 2.5 μg/mL fungizone at 37 °C in 5% CO2 humidified atmosphere. The cells were routinely observed under an inverted phase-contrast Eclipse TS100 microscope (Nikon, Tokyo, Japan) to check confluence, morphology and ensure the absence of possible contaminants.

Cell subcultures were routinely performed every 2 or 3 days, when the cultures reach about 80% of the confluence. To subculture cells, the old medium was removed, the cells were washed with phosphate-buffered saline (PBS) (pH 7.2) and incubated during 5 minutes with Trypsin-EDTA (0,25% trypsin, 1 mM EDTA) to detach the cells from the flasks. After the incubation, trypsin was inactivated with complete DMEM. Finally, cells were counted using a haemocytometer and the desired cell concentration was seeded in a new flask with new cell culture medium.

2.3 Cell Viability

Cell Viability of MCF-7 and MDA-MB-231 cells was determined by the colorimetric 3-(4,5- dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay described by Twentyman and Luscombe (1987) [161]. The Neutral Red Uptake (NRU) as described by Reppeto et al. (2008) [156], was also performed to analyse the cell viability of MDA-MB-231 cells.

2.3.1 MTT Assay

For the MTT Assay, cells were seeded in 96-well plates at 9.0 x 10^4 cells/mL and 7.0 x 10^4 (for both cell lines: MCF-7 and MDA-MB-231) during 24h and 48h exposure periods, respectively, in a final volume of 100 µL/well. After 24h, cells were incubated with 100 µL/well fresh medium as control or 100 µL/well medium containing the solution of Prolistarin or Chartergellus-CP1 with different concentrations (concentration range 3.1, 6.3, 13, 25, 50, 100 and 200 µg peptide/mL) at 37 ºC in 5% CO_2 humidified atmosphere. After exposure time, 50 µL of MTT (1 mg/mL in PBS) were added to each well and incubated for 4h at 37 ºC and 5% CO_2. After incubation, MTT solution was removed and replaced by 150 µL of DMSO. Finally, the plate was shaken in the dark for 2h at room temperature to dissolve the formazan crystals.

The absorbance value was measured with a microplate reader (Synergy HT Multi-Mode, BioTek, Vinooski, VT) at 570 nm and the percentage of viable cells was calculated (Formula 1). The concentration of both proteins that leads to a 20% and 50% decrease in cell viability, IC20 and IC50 respectively, was determined.

Three independent assays, with at least three replicates for both peptides, were performed and the results compared with control (no exposure).

$$\% \text{ Cell viability} = \frac{(\text{absorbance of Sample} - \text{absorbance of Blank})}{(\text{absorbance of Control} - \text{absorbance of Blank})} * 100$$

Formula 1 - Formula used to calculate % cell viability.

2.3.2 Neutral Red Uptake

For Neutral Red uptake, the stock solutions prepared were the Neutral red destain solution (1% glacial acetic acid, 49,5% Mili-Q water and 49,5% ethanol 96%) and the Neutral red stock solution (4 μg/mL in PBS). The Neutral Red stock solution was protected from sunlight and stored for a maximum of two/three weeks at room temperature. On the day before the assay, Neutral red medium was prepared (diluted 1:100 Neutral red in complete DMEM) and incubated overnight at 37 ºC.

For this assay, cells were seeded in 96-well plates at 9.0 x 10^4 cells/mL and 7.0 x 10^4 (for MDA-MB-231) during 24h and 48h exposure periods, respectively, in a final volume of 100 μL/well. After 24h, cells were incubated with 100 μL/well fresh medium as control or 100 μL/well medium containing the solution of Prolistarin or Chartergellus-CP1 with different concentrations (concentration range 3.1, 6.3, 13, 25, 50, 100 and 200 μg peptide/mL) at 37 ºC in 5% CO_2 humidified atmosphere. After exposure, the Neutral red medium was centrifuged for 10 min (600 *g*) and the solution was transferred to a new falcon to remove the crystals formed. The exposure medium was removed, and 100 mL of the freshly centrifuged solution (Neutral red medium) was added and incubated for 2h at 37 ºC in 5% CO_2. After this time, the solution was removed, 150 μL of Neutral red destain was added to each well, and stirred for 10 minutes. The absorbance value was measured with a microplate reader (Synergy HT Multi-Mode, BioTek, Vinooski, VT) at 540 nm and the percentage of viable cells was calculated with Formula 1.

Two independent assays, with at least three replicates for both peptides, were performed and the results compared with control (no exposure).

For the further experiments only the cytotoxic mechanisms of Chartergellus-CP1 peptide were studied.

2.4 Cell Cycle Analysis

Cell cycle was analyzed by flow cytometry (FCM) according to the method previously described by Oliveira et al. (2014) [163]. Cells were seeded in 12-well plates with a density of 1.5 x 10^5 cells/mL for both cell lines, during 24h. Then, cells were incubated at 37 ºC in 5% CO_2 humidified atmosphere for 24h with IC20 corresponding concentration of Chartergellus-CP1 (MCF-7: 7.361 μg peptide/mL; MDA-MB-231: 25.23 μg peptide/mL) and IC50 (MCF-7: 37.89 μg peptide/mL; MDA-MB-231: 44.36 μg peptide/mL). After exposure, cells were washed with PBS (pH 7.2), trypsinized used Trypsin-EDTA and centrifuged at

700 *g* for 5 minutes. Cells were then fixed with 85% cold ethanol and kept at -20 ºC until analysis.

For cell analysis, cells were centrifuged (3600 rpm, 6 minutes, 4 ºC) to remove ethanol and the pellets were resuspended with PBS. To remove any visible clusters, each sample was filtered with nylon membranes (nylon of 41 µm). Then, 50 µg/mL RNase and 50 µg/mL propidium iodide (PI) were added the suspensions were incubated for 20 min in the dark and at room temperature until analysis. Relative fluorescence intensity of PI was measured in an Attune® Acoustic Focusing Cytometer (Applied Biosystems) and the percentage of cells at G0/G1, S and G2/M phases was determined using FlowJo 10.7.2 software (Tree Star Inc., Ashland, Oregon, USA), applying the Dean-Jett Fox model. For each sample, the number of nuclei analyzed was approximately 10000.

Two independent assays, with at least three replicates for both cell lines, were performed and the results compared with control (no exposure to Chartergellus-CP1 peptide).

2.5 Analysis of intracellular ROS

The measurement of intracellular ROS was assessed by FCM using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as probe. This probe enters the cells and is deacetylated by cellular esterases producing non fluorescent DCFH. In the cytosol this molecule is quickly oxidized to fluorescent DCF by intracellular radioactive oxygen species (ROS), allowing their determination [164]. For this assay, cells were seeded in 12-well plates with a density of 1.5 x 10^5 cells/mL for both cell lines, during 24h. Cells were incubated at 37 ºC in 5% CO_2 for 24h with Chartergellus-CP1 IC20 (MCF-7: 7.361 µg peptide/mL; MDA-MB-231: 25.23 µg peptide/mL) and IC50 (MCF-7: 37.89 µg peptide/mL; MDA-MB-231: 44.36 µg peptide/mL). After exposure, the medium was discarded, cells were washed with PBS (pH 7.2), and incubated for 30 min, at 37 ºC, in the dark with DMEM containing 2% FBS and 10 µM DCFH-DA. Then, cells were washed again with PBS, trypsinized and collected for immediate analysis. The florescence intensity of DCF was measured in a flow cytometer (Attune® Acoustic Focusing Cytometer (Applied Biosystems)) and intracellular ROS formation was determined using the FlowJo 10.7.2 software (Tree Star Inc., Ashland, Oregon, USA). For each sample, the number of events was at least 10000.

Two independent assays, with at least three replicates for both cell lines, were performed and the results compared with control (no exposure to Chartergellus-CP1 peptide).

2.6 Annexin V Assay

Using the FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, San Diego, CA-USA) apoptosis and cell viability were measured by flow cytometry in a Attune® Acoustic Focusing Cytometer (Applied Biosystems). Cells were seeded in 6-well plates with a density of 2.0 x 10^5 cells/mL and 2.5 x 10^5 cells/mL, for MDA-MB-231 and MCF-7, respectively, for 24h. Cells were then incubated at 37 ºC in 5% CO_2 humidified atmosphere for 24h with Chartergellus-CP1 IC20 (MCF-7: 7.361 µg peptide/mL; MDA-MB-231: 25.23 µg peptide/mL) and IC50 (MCF-7: 37.89 µg peptide/mL; MDA-MB-231: 44.36 µg peptide/mL). After exposure time, cells were washed with PBS, trypsinized, and centrifuged at 400 *g* for 5 minutes at 4 °C. The cell pellets were resuspended with 1000 µL of cold PBS and a new centrifugation was performed at the same conditions as described above. The cells were resuspended in diluted binding buffer provided with the kit (1:10 in distilled water) at 1.0×10^6 cells/mL. To stain the cell suspension, 5 µL of FITC-Annexin V and 5 µL of PI were added for 15 minutes at room temperature in the dark, after which each sample was diluted in 400 µL binding buffer. For each sample, 10000 events were analyzed. The cytogram of FITC florescence versus PI fluorescence were analyzed by FlowJo 10.7.2 software (Tree Star Inc., Ashland, OR). The cytograms allows the identification of live intact cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic/necrotic cells (Annexin V-FITC positive, PI positive), and dead cells (Annexin V-FITC negative, PI positive) [165].

Two independent assays, with at least three replicates for both cell lines, were performed and the results compared with control (no exposure to Chartergellus-CP1 peptide).

2.7 Statistical analysis

For all experiments, at least three replicates and two independent assays were performed. All data were expressed as mean ± standard deviation (SD). SigmaPlot version 14.0 for Windows (Systat Software Inc.) was used for the statistical analysis. For MTT and Neutral Red Uptake assays, data were analyzed by one-way ANOVA, followed by Dunnet or Dunn's method (as parametric and non-parametric test, respectively). For the other assays, results were compared using one-way ANOVA, followed by Holm-Sidak's. The differences were considered statistically significant for $p<0.05$.

3 Results

3.1 Cell viability Assays

MTT assay was performed to measure cytotoxicity induced by Prolistarin and Chartergellus-CP1 peptides to MCF-7 derived from adenocarcinoma breast cancer cells and in MDA-MB-231 triple negative breast cancer cells after 24h and 48h of exposure. The NRU was only performed on MDA-MB-231 triple negative breast cancer cells to confirm the cytotoxic effects obtained through MTT assay after 24h and 48h exposure to Prolistarin and Chartergellus-CP1 peptide. The results were expressed by the percentage of viable cells and the concentration of both peptides that leads to a 20% and 50% decrease in cell viability corresponding to IC20 and IC50 respectively, was calculated.

3.1.1 Viability on MCF-7 and MDA-MB-231 cell lines– MTT assay

The results obtained presented in Figure 8 and 9 demonstrate that both peptides (Prolistarin and Chartergellus-CP1) from the wasps' venom exhibit different cytotoxicity in MCF-7 cells and in MDA-MB-231 cells.

3.1.1.1 Viability of cells after exposure to Prolistarin peptide

The effect of MCF-7 cells exposure to Prolistarin is presented in Figure 8. In this cell line, it is possible to note that for the Prolistarin peptide there was a significant decrease in cell viability, at the highest concentrations (200 µg peptide/mL) after 24h of exposure ($p<0.05$). In 48h of exposure, the concentration of 100 µg peptide /mL and 200 peptide µg/mL shows a significant decrease in cell viability ($p<0.05$). The reduction in cell viability at the highest peptide concentration (200 µg peptide/mL) doesn't reach more than 30% compared to the control, thus only IC20 was calculated, which corresponds to the 20% cell viability reduction. The IC20 for 24h and 48h was 125.2 µg peptide/mL and 53.78 µg peptide/mL, respectively.

In MDA-MB-231 cell line the viability of cells does not change at concentrations from 3.1 to 50 µg peptide/mL presenting values very close to 100%. Still, the viability of these cells significatively decrease after 48h of exposure at the concentration of 100 µg peptide/mL and, at both time exposures (24h and 48h) at the highest concentration (200 µg peptide/mL). In this case, it was only possible to calculate the IC20 value at 48h of exposure, which corresponded to 101.3 µg peptide/mL.

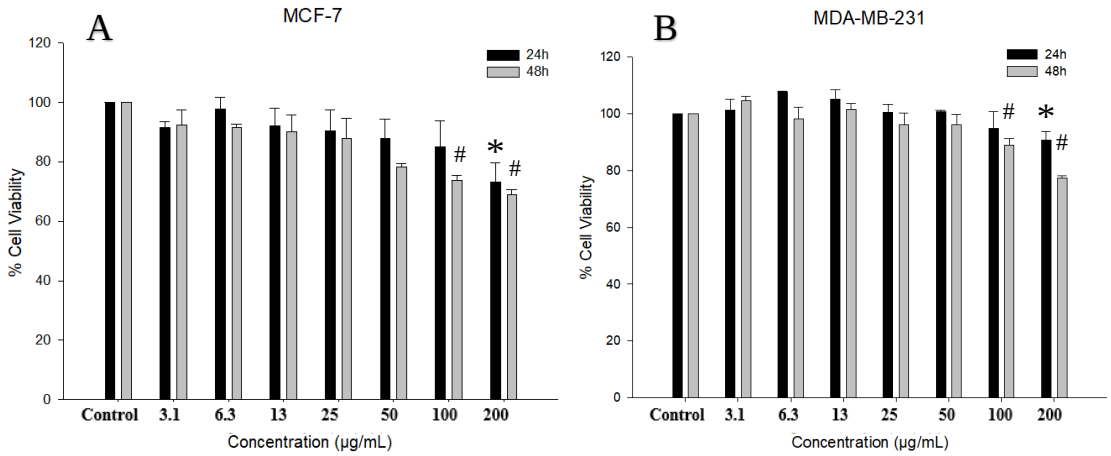


Figure 8 - Effect of Prolistarin peptide on the viability of (A) MCF-7 at 24h and 48h of exposure and (B) MDA-MB-231 at 24h and 48h of exposure. Results are shown as average ± standard deviation (SD). * (24h) or # (48h) indicates a significant statistical difference in relation to the control condition ($p<0.05$).

3.1.1.2 Viability of cells after exposure to Chartergellus-CP1 peptide

The effect of MCF-7 cells exposure to Chartergellus-CP1 is presented in Figure 9A. Upon exposure to Chartergellus-CP1 the MCF-7 cell's viability decreased significantly in a dose-dependent manner ($p<0.05$) from 6.3 µg peptide/mL, however a time-dependent manner was not detected, since the results for 24h and 48h of exposure are very similar. In the case of Chartergellus-CP1, it was possible to calculate for both time exposures the IC20 and IC50 values. For 24h, the IC20 was 7.361 µg peptide/mL and the IC50 was 37.89 µg peptide/mL, and for 48h the IC20 and IC50 were 6.168 µg peptide/mL and 44.17 µg/mL, respectively [Table II].

In MDA-MB-231 cell line, exposure to Chartergellus-CP1 at the studied concentrations led to a significant dose-dependent decrease in cell viability ($p<0.05$) (Figure 9B). The toxicity, however, was not dependent on the exposure time, with the values for 24h and 48h exposure times showing a similar trend. At the concentration of 50 µg peptide/mL, cell viability decreased drastically and significantly to values of around 30% cell viability ($p<0.05$). For 24h of exposure, the calculated IC20 and IC50 were 26.99 µg peptide/mL and 44.83 µg peptide/mL, respectively, while for 48h of exposure the IC20 was 25.23 µg peptide/mL and the IC50 was 44.36 µg peptide/mL [Table II].

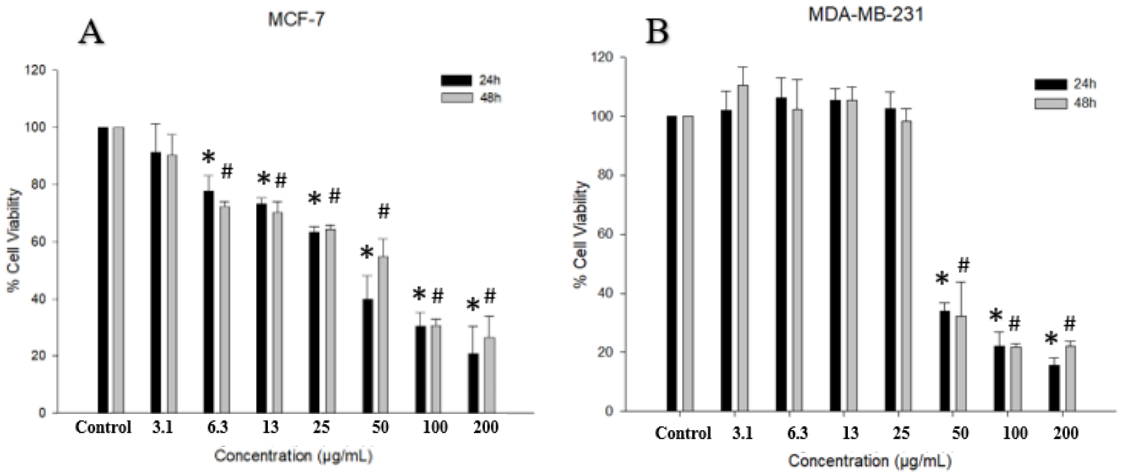


Figure 9 - Effect of Chartergellus-CP1 peptide on the viability of (A) MCF-7 at 24h and 48h of exposure and (B) MDA-MB-231 at 24h and 48h of exposure. Results are shown as average ± standard deviation (SD). * (24h) or # (48h) indicates a significant statistical difference in relation to the control condition ($p<0.05$).

3.1.2 Viability on MDA-MB-231 triple negative breast cancer cells – Neutral Red Uptake

3.1.2.1 Viability of MDA-MB-231 after exposure to Prolistarin peptide

In Neutral Red Uptake assay it was possible to note that for the Prolistarin peptide (Figure 10) there was only a significant decrease in cell viability, at 24h of exposure, in the concentrations of 13, 25, 50 and 200 µg peptide/mL ($p<0.05$). When comparing both time exposures, there was no significant difference between cells viability after exposure to 24 and 48h.

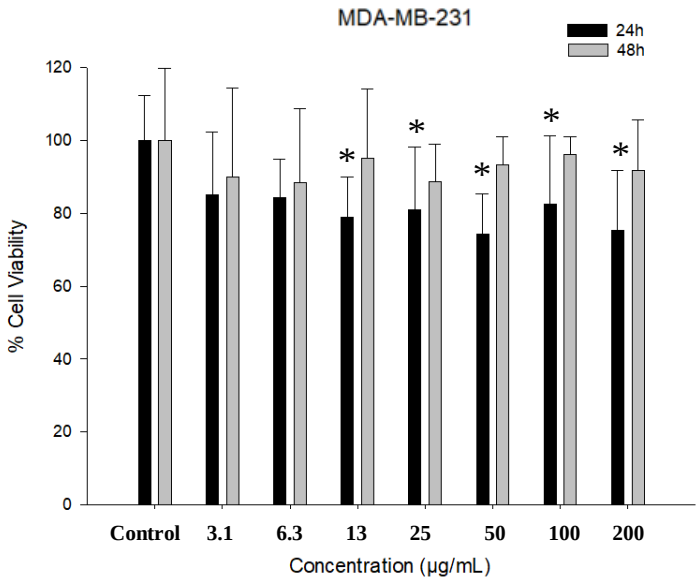


Figure 10 – Effect of Prolistarin peptide on the viability of MDA-MB-231 cells at 24h and 48h of exposure – Neutral Red uptake. Results are shown as average ± standard deviation (SD). * (24h) or # (48h) indicates a significant statistical difference in relation to the control condition ($p<0.05$).

3.1.2 Viability of MDA-MB-231 after exposure to Chartergellus-CP1 peptide

According to the results presented in Figure 11, after 24h of exposure to Chartergellus-CP1 peptide, the studied concentrations led to a significant dose-dependent decrease in cell viability of MDA-MB-231 cells ($p<0.05$). For the 48h of exposure, this significant dose-dependent decrease was only observed for concentrations of 25, 50, 100 and 200 µg peptide/mL. At lower concentrations of 3.1, 6.3, 13 and 25 µg peptide/mL the cells showed no cytotoxicity and viability remained basically constant and very close to 100%. However, from the concentration of 50 µg peptide/mL cell viability decreased drastically and significantly to values of around 30% cell viability ($p<0.05$).

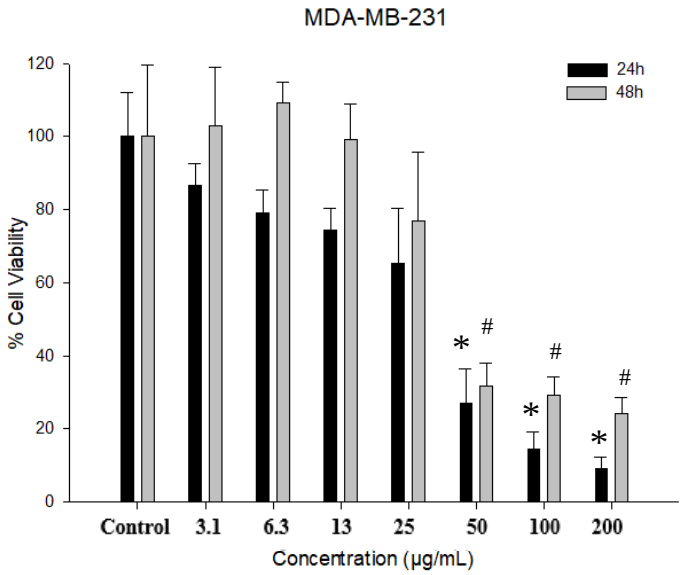


Figure 11 - Effect of Chartergellus-CP1 peptide on the viability of MDA-MB-231 cells at 24h and 48h of exposure assessed trough Neutral Red uptake. Results are shown as average ± standard deviation (SD). * (24h) or # (48h) indicates a significant statistical difference in relation to the control condition ($p<0.05$).

3.1.3 Comparation of Prolistarin and Chartergellus-CP1 peptides cytotoxicity in both cell lines (MCF-7 and MDA-MB-231)

As demonstrated in Figure 12, MCF-7 cells proved to be more sensitive to both peptides from wasps' venom when compared to MDA-MB-231 cells under all conditions studied, however the cytotoxicity to both cells had a similar trend after the two times of exposure.

For Prolistarin peptide, the cytotoxicity remained high in MCF-7 at all concentrations when compared to MDA-MB-231. At 48h exposure, this peptide showed significant differences between cell lines ($p<0.05$). For these cells, it was possible to calculate the IC20 at both exposure times in contrast to MDA-MB-231 where this was not possible.

The IC50 values of both cell lines described in 3.1.1, for Chartergellus-CP1 peptide, at 24h and 48h of exposure, are presented in Table II and were calculated according to the MTT test results mentioned above.

Table II – IC50 values (μg peptide/mL) of Chartergellus-CP1 peptide from *Chartergellus communis* wasp venom on MCF-7 and MDA-MB-231 breast cancer cell lines. Results are shown as average ± standard deviation (SD).

Cell Lines			**MCF-7**	**MDA-MB-231**
Peptide			Chartergellus-CP1	Chartergellus-CP1
Exposure time	24h	IC50	37.89 ± 5.495	44.36 ± 5.767
	48h	IC50	44.17 ± 9.872	44.83 ± 7.668

Comparing the action of Chartergellus-CP1 peptide in both cell lines, at 24h of exposure, there was a significant decrease in cell viability and a higher cytotoxicity in MCF-7 when compared to the MDA-MB-231 cell line at concentrations of 6.3, 13 and 25 μg peptide/mL ($p<0.05$). At 48h exposure, at the lowest concentrations up to 25 μg peptide/mL there was a significant decrease in cell viability of MCF-7 compared to the viability of MDA-MB-231 cells. However, at concentrations of 50 and 100 μg peptide/mL, the situation reversed and the cytotoxicity in MDA-MB-231 was significantly higher.

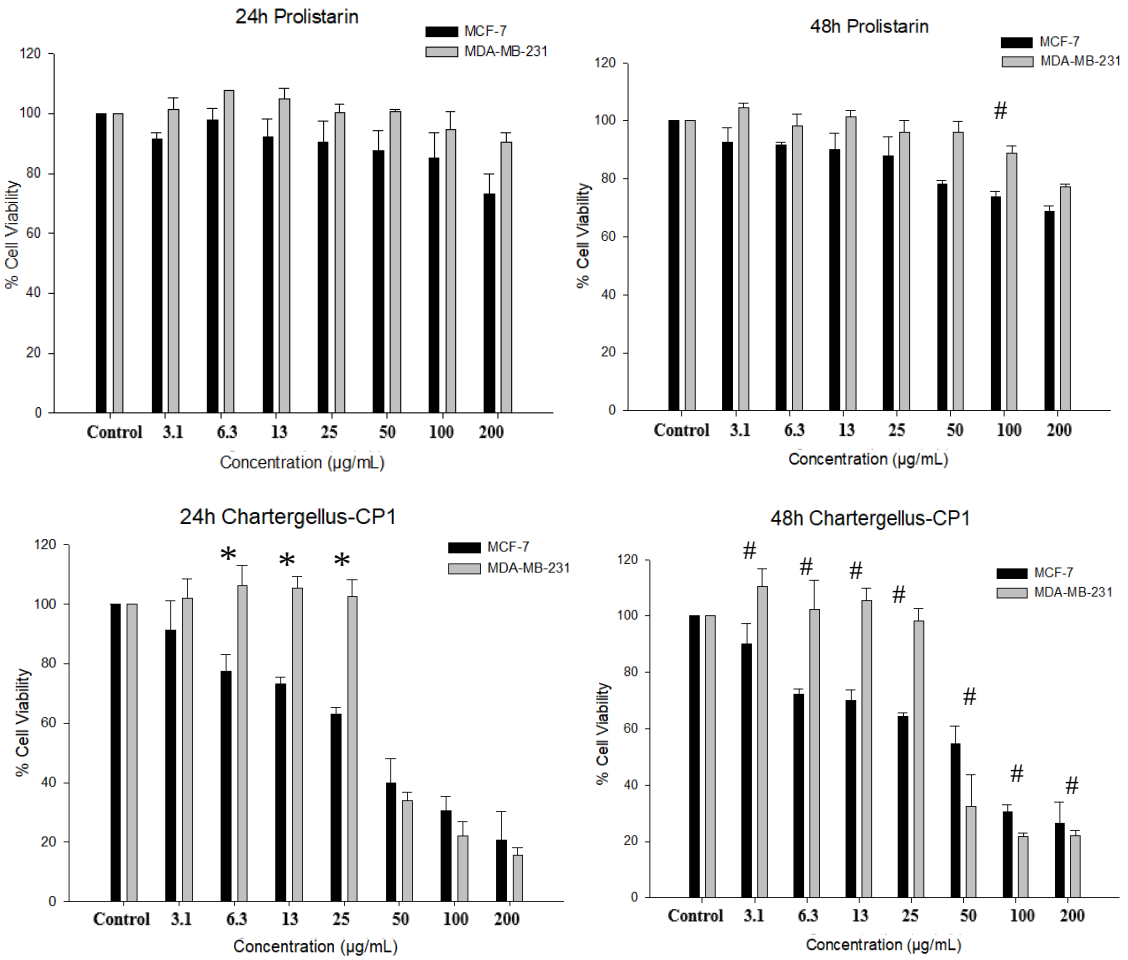


Figure 12 - Effect of Prolistarin and Chartergellus-CP1 peptides on the viability of MCF-7 and MDA-MB-231 cells at 24h and 48h. Results are expressed as mean ± SD of three independent experiments. * (24h) or # (48h) indicates a significant statistical difference between the viability of cell lines ($p<0.05$).

3.1.4 Effects on cell growth and morphology after exposure to Chartergellus-CP1 peptide

MCF-7 and MDA-MB-231 cells exposed to cell culture medium (control) showed a typical morphology as can be seen in Figure 13 and in Figure 14, respectively.

MCF-7 cells when exposed to the IC20 and IC50 calculated earlier, for 24h, showed a high decrease in their confluence (Figure 13B and Figure 13C). As shown in Figure 13, MCF-7 cells do not appear to undergo morphological changes.

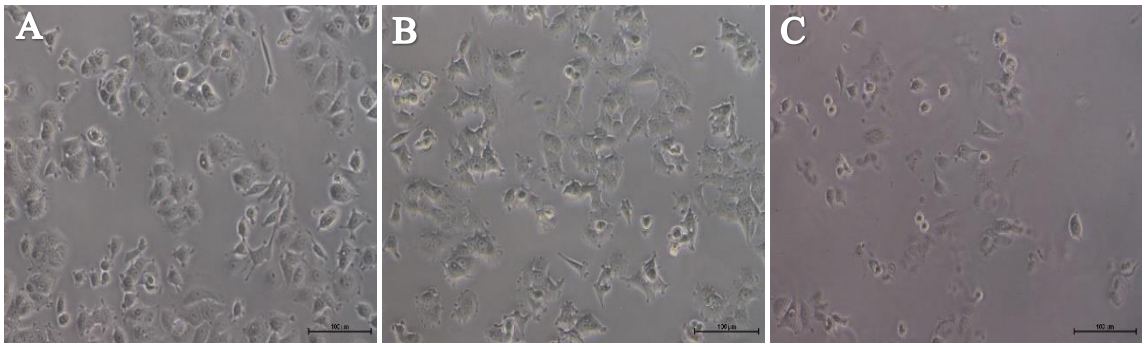


Figure 13 - Light microscopy images (100X) of MCF-7 cells: (A) control conditions; (B) cells exposed to IC20 of Chartergellus-CP1 after 24h; (C) cells exposed to IC50 of Chartergellus-CP1 after 24h.

In the case of MDA-MB-231 cells, at 24h exposure, a decrease in confluence occurs when exposed to IC20 with no apparent change in cell morphology. However, when exposed to IC50, not only the confluence decreases substantially, but also the cells undergo morphological change.

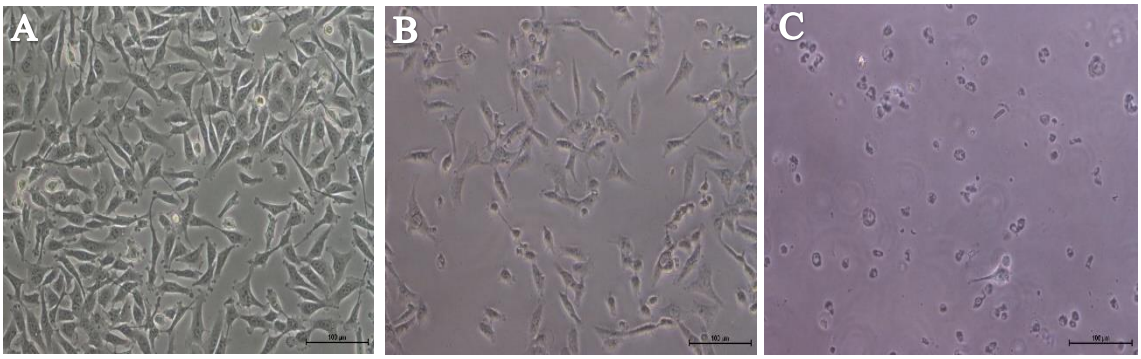


Figure 4 - Light microscopy images (100X) of MDA-MB-231 cells: (A) control conditions; (B) cells exposed to IC20 of Chartergellus-CP1 after 24h; (C) cells exposed to IC50 of Chartergellus-CP1 after 24h.

3.2 Cell Cycle analysis

3.2.1 Effect of Chartergellus-CP1 peptide in cell cycle profile after 24h exposure

The cell cycle dynamics of MCF-7 cells exposed to Chartergellus-CP1 is represented in Figure 15A. Cells exposed to IC50 showed a significant decrease of cells at G0/G1 phase from 52.8% to 36.2% ($p<0.05$). Furthermore, cells exposed to IC50 resulted in a significant increase in cells in S phase from 32.7% to 48.9% ($p<0.05$). A small increase in percentage of cell population in G2/M phase was observed when exposed to IC20 and IC50.

The cell cycle dynamics of MDA-MB-231 cells exposed to Chartergellus-CP1 is represented in Figure 15B. The results showed that IC20 induced a significant increase in the percentage of cells at G0/G1 (from 41.3% in the control group to 43.6% in IC20), and a significant decrease to 37.1% in IC50 ($p<0.05$). Treatment with IC50 induced a significant increase in phase S from 31.9% to 41.9% and, a significant decrease from 24.4% to 18.8% in the percentage of cells at phase G2/M ($p<0.05$).

The results are consistent with the example histograms obtained after exposure of both cell lines to the respective IC20 and IC50 of the Chartergellus-CP1 peptide (Figure 15), where significant changes when exposed to IC50 are noticeable in MCF-7 cells (significant decrease of cells in G0/G1 and increase of cells in S phase) and in MDA-MB-231 (significant decrease of cells in G0/G1 and G2/M phase and increase of cells in S phase) ($p<0.05$).

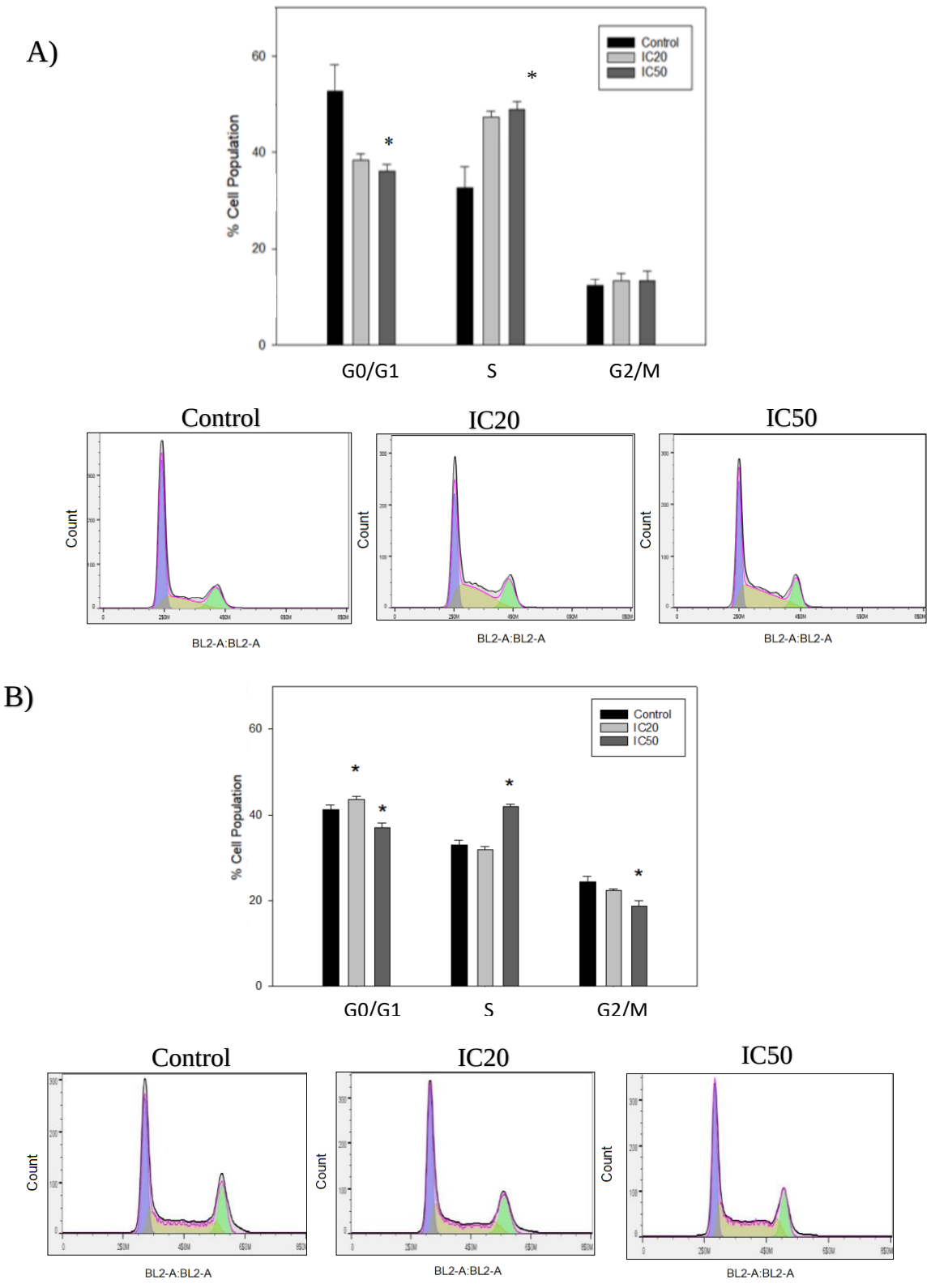


Figure 15 - Cell cycle dynamics and example histograms of A) MCF-7 cells; and (B) MDA-MB-231 cells, exposed to of Chartergellus-CP1 peptide after 24h exposure. Results are expressed as mean ± SD of two independent experiments. * $p<0.05$ indicates statistically significant differences compared to the control.

3.3 Intracellular ROS levels analysis

3.3.1 Effect of Chartergellus-CP1 peptide on intracellular ROS levels in cells after 24h exposure

The quantification of intracellular ROS in MCF-7 cells exposed to Chartergellus-CP1 peptide for 24h is presented in Figure 16. In these cells, the levels of ROS increased in a dose dependent manner upon exposure to IC20 (1.4) and IC50 (2.2) when compared to control levels (1.0) ($p<0.05$). Regarding MDA-MB-231 cells, the levels of ROS also increased in a dose-dependent manner, however only for cells exposed to the IC50 a significant increase (1.3) was observed when compared to control levels (1.0) ($p<0.05$).

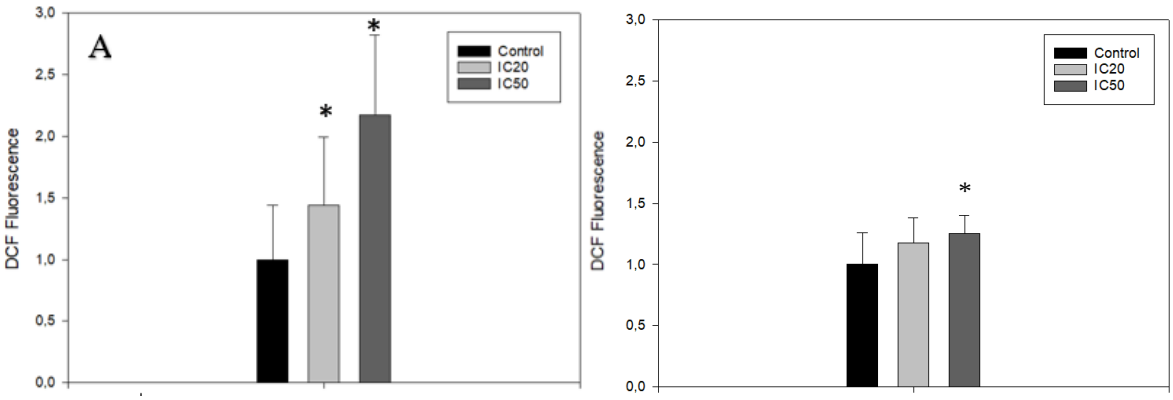


Figure 16 - Characterization of intracellular ROS production after exposure to IC20 and IC50 Chartergellus-CP1 peptide for 24h in (A) MCF-7 cells and (B) MDA-MB-231 cells. Results are expressed as mean ± SD of two independent experiments. * $p<0.05$ indicates statistically significant difference compared to the control.

3.4 Annexin V Analysis

3.4.1 Effect of Chartergellus-CP1 peptide in cells death after 24h of exposure

The annexin V apoptosis results of MCF-7 and MDA-MB-231 cells when exposed to IC20 and IC50 of Chartergellus-CP1 are shown in Figure 17.

For MCF-7 cells derived from breast adenocarcinoma, Chartergellus-CP1 exposure resulted in a non-significant increase of cells in late apoptosis from 30.7% to 32.3% (IC20) and to 35.1% (IC50) ($p<0.05$). Exposure to IC50 induced a significant increase in cells in early apoptosis (from 8.66% in control to 13.1% in Chartergellus-CP1 IC50 treatment). Live cells correspond to a percentage of 58.9% in the control and significantly decrease when cells are exposed to Chartergellus-CP1 IC50 treatment (where it takes values of 51.3%) ($p<0.05$).

In triple negative breast cancer cells, MDA-MB-231, exposure to IC50 with the Chartergellus-CP1 peptide resulted in a significant increase of early apoptotic cells from 11.4% (control) to 23% (IC50) ($p<0.05$). Concerning live cells, treatment with Chartergellus-CP1 IC50 resulted in a significant decrease in live cells from control (from 81.3% in control to 62.7% when exposed to Chartergellus-CP1 IC50) ($p<0.05$). No significant differences were observed in late apoptosis.

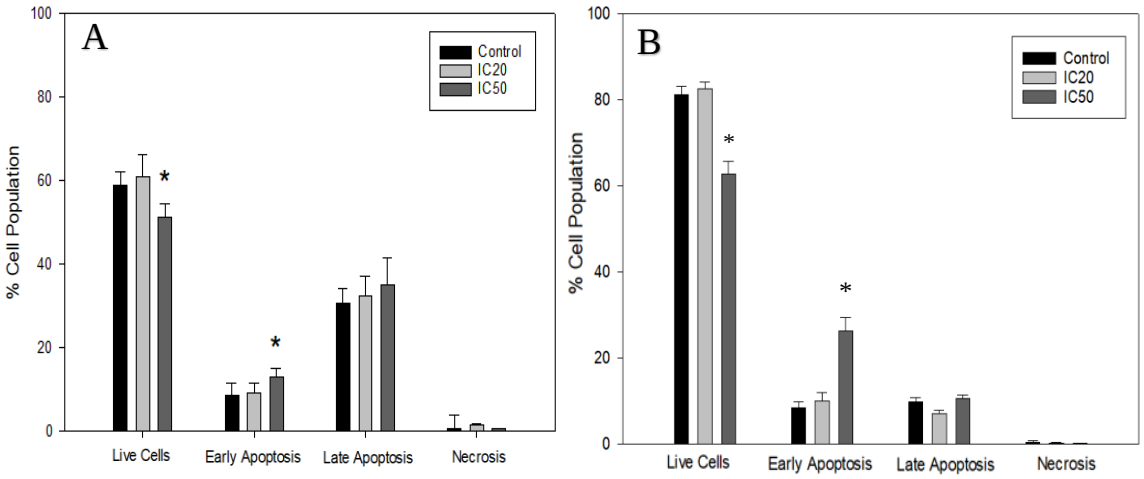


Figure 17 - Annexin V apoptosis analysis of (A) MCF-7 cells and (B) MDA-MB-231 cells, after exposure to Chartergellus-CP1 peptide during 24h. Results are expressed as mean ± SD of two independent experiments. * $p<0.05$ indicates statistically significant difference compared to the control.

4 Discussion

Venoms are a cocktail of proteins and peptides produced naturally by a wide variety of animal species, many of which have proven efficacy in treating a variety of different diseases such as cancer [89]. The most studied venoms for treating these diseases are snakes and scorpion venoms. For example, in 2000, Zhou et al. [166] described contortrostatin, a disintegrin isolated from the venom of the snake *Agkistrodon contortrix*, with a high antitumor action against MDA-MB-435, a melanoma cell line. Another example, focuses on the work developed by Qin et al. (2014) [167], which proved that Chlorotoxin peptide, isolated from the venom of the scorpion *Leiurus quinquestriatus* inhibited aggressive metastatic 4T1 breast cancer cells. Studies of venoms from other animal species such as wasps are still rare, however, they show very promising results and a high biotechnological potential in the development of new therapies to fight cancer [80]. One of the studies, for example, was developed by Wang et al. (2008) [168] who analyzed peptide polybia-mastoparan I (MPI) isolated from the venom of the Brazilian wasp *Polybia paulista* and, confirmed that it is effective against bladder (Biu87 and EJ) and prostate cancer (PC-3) cell lines. More recently, Torres et al. (2018) [152] following the work of Konno et al. (2007) [154], developed a derivative of the peptide decoralin (isolated from the wasp *Oreumenes decaratus*), Dec-NH2, with high antitumor potential against MCF-7 breast cancer cells.

The venom of the wasp *Polybia dimorpha* started to be studied recently by das Neves et al. (2016) [135]. The authors analyzed the antimicrobial potential of the Polydim-I peptide isolated from the venom of this wasp and, obtained very promising results against mycobacteria [136]. However, there are still no published studies on the antitumor potential of the venom of this particular wasp. Regarding the wasp *Chartergellus communis*, up to our knowledge, there are only two studies in the literature reporting the characterization and bioactivity of the peptides isolated from their venom [124, 167]. Lopes et al (2017) [125] studied the hyperalgesic, edematogenic and hemolytic effects of two peptides isolated from *C. communis* venom demonstrating their different bioactivity. The study conducted by Lopes et al. (2021) [159], reported the potential of new peptides from the venom of the social wasp *Chartergellus communis* on the pharmacotherapy of epilepsy. The authors found that, Chartergellus-CP-1, isolated from *C. communis* venom, showed improvement in latency, quantity, and percentage of protection against generalized electroencephalographic seizures in an *in vivo* model. Furthermore, the authors also reported that the peptide has significant similarities with compounds belonging to the chemotactic class, presenting a structural

identity (92%) with the molecule named Polybia-CP, isolated from the venom of the wasp *Polybia paulista*, which presents a high antitumor potential against prostate cancer cell line (PC-3) and blander cancer cell line (BIU87) and a high antibacterial potential against several gram positive (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram negative bacteria (*Bacillus subtilis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*) by disrupting the integrity of cell membrane [166–169].

Thus, in the present work, several methodologies were used to evaluate the antitumor potential of two new peptides: Prolistarin peptide, a bio-inspired peptide from *P. dimorpha* venom; and Chartergellus-CP1 peptide, a peptide isolated from *C. communis* venom.

In this work two cell lines, MCF-7 and MDA-MB-231, were used as both are ductal/breast carcinoma cells, but with a very different phenotype and genotype. MCF-7 cell lines are Luminal A type and estrogen and progesterone receptor positive while MDA-MB-231 are triple negative, estrogen and progesterone receptor negative. Furthermore MCF-7 and MDA-MB-231 are non-metastatic and metastatic tumor cell lines, respectively, and all these differences will affect drug sensitivity [172]. For example, MDA-MB-231 cells are usually more susceptible to chemotherapeutics than MCF-7 cells, as exemplified by the work developed by Ferreto et al. (2016) [173] with these cell lines and the strong chemotherapeutic, doxorubicin. On the other hand, there are treatments that are only efficient for MCF-7 since these cells are hormone receptor positive, as is the case of tamoxifen [56, 166]. Therefore, and since there is still no information regarding the bioactive potential of the peptides Prolistarin and Chartergellus-CP1, we analyzed their cytotoxic effect on both breast cancer cell lines mentioned.

To complete this analysis on the designated peptides two different cell viability assays were performed: the MTT and the Neutral Red uptake assay. The first one is widely used in science to analyze cell viability. However, this is less sensitive than the Neutral Red uptake assay since its approach involves the accumulation of neutral red dye in the lysosomes of viable cells while the MTT is based on the reduction of the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple formazan by viable cells [156, 167, 168]. So, some authors choose to perform both cell viability tests, as for instance Zhang et al. (2016) [169].

Regarding Prolistarin peptide, the results of the cell viability assays reveal a low cytotoxicity in MCF-7 assessed by MTT and low cytotoxicity in MDA-MB-321 assessed through MTT and Neutral Red. The maximum decrease in viability found at the highest concentration of 200 µg peptide/mL did not reach 20% in the case of MDA-MB-231 cell line

or the 30% in MCF-7 cell line, for the 24h exposure. Thus, no further trials were conducted with this peptide due to presenting a low antitumor potential for both cell lines up to the maximum concentration used 200 µg peptide/mL.

Chartergellus-CP1 peptide, on the other hand, showed significant concentration-dependent cytotoxic activity for both cell lines, and at both exposure times of 24h and 48h. This evidence is supported simultaneously by the results of cell viability in MCF-7, via the MTT assay, and in MDA-MB-231 by both the MTT and the Neutral Red uptake. It is not possible to compare these results with the literature, since this is the first study performed on an antitumor potential with this peptide or even with the venom of the wasp *C. communis*. Nevertheless, the cytotoxic and antitumor effects reported above can be observed in other studies performed with other peptides isolated from wasp venoms of the family *Vespidae* [147, 163, 170]. Azevedo et al. (2015) [178] developed a study with *Vespula lewisii* wasp venom in which shows that mastoparan peptide induced tumor cell cytotoxicity in various breast cancer cell lines such as SK-BR3, MCF-7 and MDA-MB-231 cell lines (with IC50 values of 320.3 µM, 432.5 µM and 251.25 µM, respectively). Furthermore, the work developed by Kakanj et.al. (2015) [179] also used MTT and Neutral Red uptake in the study of the cytotoxicity effect of snake venom *Vipera lebetina* in which reconfirmed cytotoxicity in both assays against human umbilical vein endothelial cells (HUVECs).

Despite the significant concentration-dependent cytotoxic activity for both cell lines, it is possible to find some differences in the impact of this peptide on these, according to MTT assay. The MCF-7 cell line shows a linear and significant dose-dependent cytotoxic activity from the low concentration of 6.1 µg peptide/mL to the highest concentration of 200 µg peptide/mL. While in MDA-MB-231 cells, at both exposure times, when exposed to higher concentrations (50, 100 and 200 µg peptide/mL) a significant and drastic decrease in cell viability occurs. This results for the MDA-MB-231 cells at lower concentrations of Chartergellus-CP1, suggests a hormesis effect, an adaptive biphasic dose–response to moderate stress in which the cell system improves its functionality and tolerance to drug/peptide [172]. This effect is characterized by exhibiting stimulating effects at low doses and inhibitory/toxic effects at high doses [172, 173]. Similar effect was also reported by Bao et al. (2015) [182] in which demonstrated that berberine, an active component of *Rhizoma coptidis*, at the low dose range promoted cell proliferation, while at the high dose range inhibited cell proliferation in MCF-7 and MDA-MB-231.

Therefore, the MDA-MB-231 cell line is more resistant to Chartergellus-CP1 than MCF-7 cell line. The calculated IC20 and IC50 take lower values in MCF-7 cells when

compared to MDA-MB-231 at both exposure times. For example, at 24h exposure, in MCF-7 cells, a concentration of 7.312 µg peptide/mL is sufficient to decrease cell viability by 20%, while in MDA-MB-231 cells a concentration of 25.23 µg peptide/mL would be required. These results are in agreement with the work done by Kisaki et al. (2021) [183], in which the authors studied the cytotoxicity in the same cell lines we studied, MCF-7 and MDA-MB-231, using venom from *Bothrops Jararaca* snake. In this study, the cell lines were treated with 0, 5, 10, 15 and 20 µg/mL of *Bothrops Jararaca* venom for 24 h and, the results showed IC50 values of 4.50 µg/mL venom for MCF-7 and 4.76 µg/mL venom for MDA-MB-231 cells. Similar results are also found in the work devolved by Malekara et al. (2020) [184] since, when exposing for 24, 48, 72, and 96h breast cancer cell lines (MDA-MB-231 and MCF-7) with snake venom from *Vipera raddei kurdistanica* noted that MCF-7 cells were more sensitive to the venom according to IC50 values. On the other hand, the work of Erzumumlu et al. (2016) [185] proved that MCF-7 are more resistant to desert black snake venom (*Walterinnesia morgani*) since the obtained IC50 values are 2.17 and 3.23 µg/mL in MDA-MB-231 and MCF-7 cells, respectively.

Cell cycle analysis is essential to analyze cell proliferation and the mechanisms responsible for the cytotoxic activity of the Chartergellus-CP1 peptide in cell lines. Significant cycle changes are evident in both cell lines. In MDA-MB-231 there was a significant increase of cells in S phase and a consequent significant reduction of cells at G2/M phase and at G1/G0 when exposed to IC50. MCF-7 cells show a significant increase in phase S, a significant decrease in phase G0/G1 and no changes in the cell cycle in phase G2/M when exposed to respectively IC50. These results indicate that Chartergellus-CP1 peptide promotes a dose-dependent cytotoxic effect and a genotoxic stress during DNA replication leading to cell cycle arrest at the S phase, which may be related to DNA damage and an activation of S phase checkpoint proteins (involving CDK's, CKI's and CHK) [185, 186]. The literature reports some examples of venoms with this action on various cell lines as is the case of the work developed by Antolikova et al. (2019) [188] on this cell line with *Naja ashei* venom. In contrast, Bernardes-Oliveira et al (2016) [189] reported that *Bothrops jararaca* and *Bothrops erythromelas* venoms led to cell cycle block in the G0/G1 phase in SiHa human cell carcinoma. Similar results were obtained by Gao et al. (2007) [190] in MCF-7 cell lines with venom from the spider *Macrothele raven*.

Increased ROS levels are associated with increased oxidative stress in cells and with cell death, as they damage lipids, proteins, and DNA [191]. In our work a significant increase in ROS production in both cell lines caused by Chartergellus-CP1 peptide was observed. The

amount of ROS increased in a dose-dependent manner in both cell lines, but the increase in the MCF-7 line is much higher than the one found in MDA-MB-231. Despite this, both had ROS significantly increased when exposed to IC50, while MCF-7 showed significant results already when exposed to IC20. Several studies have pointed out that increased ROS production is one of the main causes for the toxicity provoked by venoms in various cell types [191]. Al-Asmari et al. (2016) [192] analyzed the venom of four different snake species (*Bitis arietans*, *Cerastes gasperettii*, *Echis coloratus* and *Echis pyramidum*) and concluded that the elevated ROS production caused by these, leads to apoptosis in colorectal and breast cancer cell lines (HCT-8 and MDA-MB-231). We also demonstrate in this work, by Annexin-V assay, that both cell lines showed a significant increase of cells in early apoptosis when exposed to IC50. The cytotoxicity of the *C. communis* wasp venom peptide is also notable for the significant decrease in live cells in both cell lines. The results are similar with those obtained by Wu et al. (2018) [193] who analyzed the potential of VACP1 peptide isolated from *Vespa ducalis* venom and proved that these caused high levels of apoptosis in MG-63 osteosarcoma cell lines.

5 Conclusions

In summary, our study demonstrated that the novel peptide bio-inspired from the wasp venom of *Polybia dimorpha*, Prolistarin shows no major cytotoxic effects against MCF-7 and MDA-MB-231 cells, while the peptide isolated from *Chartergellus Communis* wasp venom, Chartergellus-CP1 was highly cytotoxic to both breast cancer cell lines. Chartergellus-CP1 peptide promoted a high dose-dependent antitumor effect that was not dependent on exposure time, in both cell lines. Despite these results, MCF-7 cells were more sensitive to the peptide than MDA-MB-231 cells, and the latter appeared to suffer the hormesis effect when exposed to the peptide - stimulating effects at low doses and inhibitory/toxic effects at high doses. Additionally, our results showed that Chartergellus-CP1 led to an S-phase arrest in the cell cycle of both cell lines. A significant increase in ROS generation occurred, also in both cell lines upon exposure to Chartergellus-CP1, but with higher values in MCF-7 cell line. We predict that high production of ROS may be one of the possible mechanisms that may lead to cell death, since in both MCF-7 and MDA-MB-231 a significant increase in cells at early apoptosis were seen when exposed to IC50.

Thus, this work shows that the venom of the wasp *Chartergellus communis* has components with high potential in fighting breast cancer, more specifically, the Chartergellus-CP1 peptide. However, further research is required, such as the study of the potential cytotoxic effects of peptide on non-tumor cells. Furthermore, in order to better understand its antitumor potential, other complementary assays could be performed as for instance the assessment of its antiangiogenic activity.

6 References

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