Universidade de Aveiro Departamento de Química
Ano 2019

André Jorge Teodoro da Silva

Avaliação do potencial antitumoral e antibacteriano do veneno de *Vipera latastei*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo em clínica, realizada sobre orientação científica do Investigador Júnior Sérgio Miguel Reis Luís Marques e da Doutora Helena Cristina Correia de Oliveira, Investigadores auxiliares, e do Professor Doutor Fernando J. Mendes Gonçalves, Professor associado com Agregação, todos do Departamento de Biologia e do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

“An understanding of the natural world is a source of not only great curiosity, but great fulfilment”

(David Attenborough)

o júri

presidente

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agradecimentos

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palavras-chave

Cancro; Bactérias multi-resistentes; Veneno de serpentes; Toxinas; *Vipera latastei*; Potencial antitumoral; Potencial antibacteriano

resumo

Atualmente, o cancro e a emergência de microrganismos multi-resistentes representam uma verdadeira ameaça à saúde humana e, por isso mesmo, são necessárias soluções urgentes que sejam uma mais valia no combate a estes problemas. Neste sentido, vários estudos têm-se focado na procura de compostos que possam vir a constituir novos agentes terapêuticos. A família Viperidae apresenta um veneno caracteristicamente hemotóxico, que embora possa ser letal ou resultar em danos consideráveis para os humanos, constitui igualmente uma fonte rica em componentes de elevado potencial terapêutico. Desta forma, procurando avaliar a bioatividade do veneno de *Vipera latastei*, este trabalho teve como principais objetivos: a) fornecer uma visão global relativamente ao veneno e toxinas produzidas por espécies pertencentes à família Viperidae, revendo o seu potencial antimicrobiano e antitumoral, b) avaliar o potencial antitumoral do veneno em bruto de *V. latastei* relativamente a células de melanoma, e c) avaliar o potencial antibacteriano do veneno da mesma espécie.

Através de uma extensa revisão bibliográfica foi evidenciado que os venenos de víbora apresentam um potencial antitumoral e antibacteriano promissor e que estas atividades estão associadas sobretudo a cinco famílias de proteínas – SV-LAAO, Desintegrinas, PLA2, SVMPs e lectinas do tipo C. Vários destes compostos são aqui reportados, destacando-se o seu potencial terapêutico e referindo alguns dos possíveis mecanismos de ação. Concluiu-se ainda que a composição dos venenos pode ser bastante variável entre espécies, o que pode contribuir para a descoberta de um maior número de compostos e ajudar na compreensão dos mecanismos de ação já conhecidos.

Relativamente ao potencial antitumoral, foi observado uma diminuição significativa da viabilidade celular das células de melanoma (MNT-1) e de queratinócitos (HaCaT) com o aumento da concentração do veneno em bruto de *V. latastei*. Contudo, esta atividade citotóxica não foi seletiva para as células tumorais. Adicionalmente, o veneno induziu alterações na dinâmica do ciclo celular das células tumorais, não se tendo observado alterações significativas nos queratinócitos não tumorais (HaCaT). Foi ainda observado um aumento da formação de ROS, sobretudo nas células tumorais expostas ao veneno.

Relativamente à atividade antibacteriana, verificou-se que o veneno inibiu o crescimento de todas as bactérias patogénicas testadas neste estudo, revelando uma interessante atividade antibacteriana.

De uma forma geral, esta dissertação suporta a ideia de que os venenos de serpente são uma fonte promissora de novos compostos com interesse terapêutico, apresentando os primeiros estudos relativos à bioatividade do veneno de *V.latastei*.

keywords

Cancer; Multi-resistant bacteria; Snake venom, Toxins; Vipera latastei; Antitumor potential; Antibacterial potential

abstract

Currently, cancer and the emergence of multi-resistant microorganisms represent a real threat to human health and therefore there is an urgent need for solutions that will be of added value in combating these problems. Thus, several studies have focused on the search for compounds that may constitute new therapeutic agents. Viperidae family presents a characteristic hemotoxic venom. Although it may be lethal or cause considerable damage to humans, venom is also a source rich in components with high therapeutic potential. In order to evaluate the bioactivity of *Vipera latastei* venom, the main objectives of this work were: a) to provide an overview of the venom and toxins produced by species belonging to the Viperidae family, reviewing its antimicrobial and antitumor potential, b) to evaluate the antitumor potential of *V. latastei* crude venom against melanoma cells, and c) to evaluate the antibacterial potential of the same venom.

Through an extensive literature review, it was evidenced that venoms of vipers present a promising antitumor and antibacterial properties, and these activities are mainly associated to five of these families – SV-LAAO, Disintegrins, PLA2, SVMPs and C-type lectins. Several of these compounds are reported here, highlighting their therapeutic potential and mentioning some of the possible mechanisms of action. Furthermore, the composition of the venoms can be variable among species, which may contribute to the discovery of more compounds, and help in understanding the already known mechanisms of action.

Regarding antitumor potential of the *V. latastei* crude venom, it was observed a significant decrease in cell viability of human melanoma MNT-1 and Human Keratinocyte HaCaT cells in a dose-dependent manner. However, this cytotoxic activity was not selective for tumor cells. Additionally, the venom induced changes in the cell cycle dynamics of the tumor cells. Nonetheless, no significant changes in the cell cycle of non-tumor keratinocytes (HaCaT) were observed. Furthermore, we also observed an increase in ROS generation, especially in tumor cells exposed to the venom.

Regarding antibacterial activity, the venom inhibited the growth of all pathogenic bacteria tested in this study, revealing an interesting antibacterial activity.

In general, this dissertation supports the idea that snake venoms are a promising source of new compounds of therapeutic interest, presenting the first studies on the bioactivity of *V. latastei* venom.

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List of Abbreviations

5-FU-5-fluorouracil

ATM- Ataxia telangiectasia

ATR- Ataxia telangiectasia – related protein

CASP8- Caspase 8

CDC- Centers for Disease Control and Prevention

CDK- Cyclin- dependent kinase

CHK- Checkpoint kinase

CKI- Cyclin- dependent kinase inhibitor

Cl- *Causus lichtensteinii*;

CNG- Cyclic nucleotide-gated;

Cr-*Causus rhombeatus*;

CRD- C-terminal cysteine-rich domain;

CRISPs- Cysteine-rich secretory proteins;

c-Src- Transforming protein Ras homolog family member A

DCF- 2',7' – dichlorofluorescein

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

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

ECM- Extracellular matrix proteins;

FBS- Fetal bovine serum

FGF- Fibroblast growth factor

JNK Pathway- c-Jun N-terminal kinase pathway

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

NGF- Nerve growth factor;

p53- Tumor protein p53

PBS- Phosphate-buffered saline

PDT- Photodynamic therapy

PI- Propidium iodide

PLA_2- Phospholipases A_2;

PR-1- N-terminal pathogenesis-related proteins of group 1;

RhoA- Transforming protein Ras homolog family member A

RNase- Ribonuclease

ROS- Reactive oxygen species;

SD- Standard deviation

SV-LAAOs-Snake venom L-amino acid oxidases;

SVMPs- Snake venom metalloproteases;

SVSPs- Snake venom serine proteases;

TNF- Tumor necrosis factor

TNFRSF10B- Tumor Necrosis Factor Receptor Superfamily Member 10B

TNFRSF1A- Tumor Necrosis Factor Receptor Superfamily Member 1A

TP53- p53 coding gene

TRAIL- Tumor necrosis factor-related apoptosis-inducing ligand

USA- United States of America

VEGF- Vascular endothelial growth factor

VEGF- Vascular endothelial growth factor;

WHO- World Health Organization;

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Chapter 1- General introduction and Objectives

1.1 Multi-resistant microorganisms and cancer – An urgent need for solutions

1.1.1 Multi-resistant microorganisms

The discovery of antibiotics was one of the most important scientific advances of the 20th century, which totally revolutionized modern medicine and contributed with enormous benefits to the human population [1]. When antibiotics were discovered, they presented a huge potential to treat various untreatable infectious diseases that presented high mortality rates [2,3]. Especially during their golden era, several types of antibiotics were developed and have since been used for diverse therapeutic purposes, leading to an increase in the population's life quality and expectancy [1,2]. However, the discovery of new antibiotics has declined since 1980 and over the last 80 years [Figure 1], the excessive use of these drugs has put to risk their use in the future [4–6]. This extreme use resulted in a natural selection of the microorganisms with resistance mechanisms. Antibiotic resistance occurs when a drug becomes unable to inhibit bacterial growth efficiently, causing bacteria replication to remain even in the presence of therapeutic doses. Thus, these bacteria are called resistant and may acquire multiple resistance against several drugs over time [2]. The resistance to β-lactams presented by bacteria such as *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli* is a common example of this problem [7–11].

Antibiotic resistance can be an intrinsic characteristic which is independent of antibiotic selective pressure and is not obtained by horizontal gene transfer. This results from inherent structural and/or functional characteristics [12]. A good example is the difference in susceptibility between Gram-negative and Gram-positive bacteria to some drugs [13]. The difference in the composition of the membrane of Gram-negative bacteria acts as a barrier, preventing many drugs from passing through the outer membrane, inhibiting its antimicrobial action [1]. Furthermore, antibiotic resistance can also be a trait acquired via mutations in chromosomal genes, which may promote changes in antibiotic targets. These mutations are then transmitted vertically [12]. A second way of acquired resistance results from obtaining exogenous genetic material, which encodes resistance characteristics. These genes are propagated by horizontal transfer mechanisms [12].

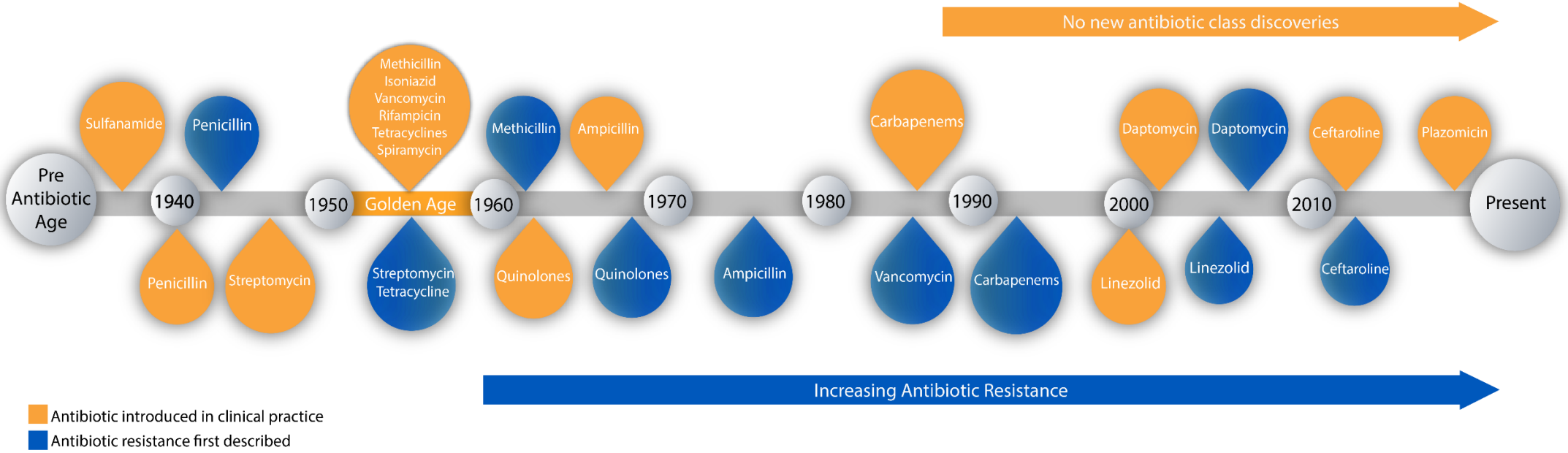


Figure 1– Antibiotic timeline, from their discovery to present. The discovery of penicillin in 1928 ushered in the age of antibiotics, revolutionizing modern medicine. Since its introduction for clinical use in the early 1940s, the discovery and introduction of new antibiotics has increased considerably, reaching its peak almost a decade after World War II. The period from 1950 to 1960 was baptized as the golden age, with the discovery of most of the antibiotics known and still used today. Between 1940 and 1970, 23 classes of antibiotic were discovered. However, since then there has been a decrease in the discovery of new classes and since 1987 no new class has been found. Simultaneously, with the introduction of antibiotics, the development of clinically significant resistance was observed in a short period of time [2,5,6].

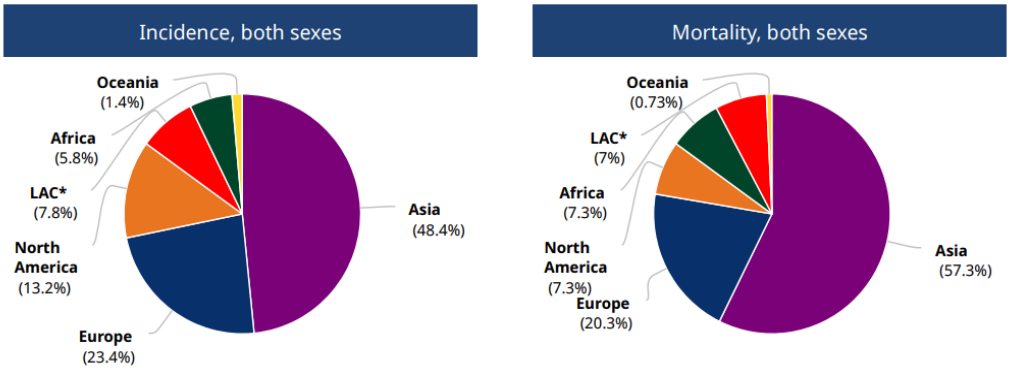
There are several mechanisms that contribute to the development of drug resistance. This may arise as a result of the permeability of some membranes [14,15], the increase of efflux pumps [16,17], the structural alteration of the targets by mutation [18,19], the modification without mutation of the target [20]. Furthermore, alteration of the antibiotic by hydrolysis or transfer of chemical groups is also one of the mechanisms already observed [21–23]. The production of β-lactamases is one of the main resistance mechanisms presented by *E.coli* and other Gram-negative bacteria. Among these enzymes the most important are plasmid-mediated AmpC β-lactamases – such as the CMY type, with CMY-2 being the most frequently observed [24]; CTX-M, an extended-spectrum β-lactamase that presents a high number of variants, emphasizing the CTX-M-15 for being the most common and dispersed in the world [10]; and carbapenemases like NDM-5 that present a great incidence in China [11]. It is also important to mention that the behaviour as well as the adaptive capacity presented by some bacteria can also contribute to the development of antibiotic resistance [25]. Adaptive resistance occurs in response to environmental stimulus (eg: pH, oxygen, temperature, nutrients) or due to the presence of antibiotics [8], leading to the modulation of gene expression [8,26–29]. The formation of biofilms by *P. aeruginosa* is also a characteristic that leads to the development of this type of resistance. Bacteria in biofilms have a transcriptome different from planktonic ones, which may lead to the expression of several mechanisms of resistance. The metabolic activity of the bacteria within the biofilm is also different, which may compromise the action of the antibiotic. Furthermore, among other mechanisms, these formations have an extracellular matrix which may be a barrier to antibiotics, as well as a site where some enzymes (eg: β-lactamases) may be concentrated [8].

The emergence of multi-resistant microorganisms is presently one of the biggest problems concerning human health. According to Spellberg *et al* (2004) [30], despite the initial success of antimicrobial drugs against infectious diseases, nowadays these diseases are one of the main causes of death in the world, with few or without any treatment. According to WHO [31], the post-antibiotic periods will result in an increase in the rate of infections and small injuries can become lethal. It is currently estimated that in Europe 25,000 people die annually due to infections of multi-resistant bacteria [1]. Spellberg *et al* (2013) [4] exemplified the adaptation of bacteria to antibiotics referring that in the majority

of the bacteria isolated in the intensive care units in the USA, 10% of *Klebsiella spp.*, 20% of *Pseudomonas aeruginosa*, and 50% of *Acinetobacter baumannii* strains are resistant to carbapenems. With the evolution of resistance mechanisms to the antimicrobial drugs, it is necessary to keep searching for strategies to combat emerging and re-emerging resistance phenomena. The research is based on the discovery of new antibiotic compounds with new targets, inhibitors of resistance mechanisms such as β-lactamases and efflux pumps inhibitors, and agents capable of interfering with signs of virulence (eg: quorum quenching), blocking infection [32–34].

1.1.2 Cancer

Cancer is of great concern in current society, being the second leading cause of mortality worldwide [35]. Statistically, 18,078,957 new cancer cases and approximately 9,555,027 deaths were observed in 2018, of which 23.4% of incidences and 20.3% of deaths occurred in Europe, the second most affected continent [36] [Figure 2]. Cancer is a generic term to design a multi-cellular and multi-genic disease which is characterized by uncontrolled growth and proliferation of abnormal cells [37,38]. It can develop in almost all type of tissues, presenting a diverse etiology. Genetic factors, lifestyle (e.g diet, tobacco smoke, alcohol), radiation, ultraviolet rays exposure and infectious agents (e.g: human herpes virus, human immunodeficiency virus and *Helicobacter pylori*) are some of the key factors associated with an increased risk of developing cancer [38].



Population	Number
Asia	8 750 932
Europe	4 229 662
North America	2 378 785
*Latin America and the Caribbean	1 412 732
Africa	1 055 172
Oceania	251 674
Total	18 078 957

Population	Number
Asia	5 477 064
Europe	1 943 478
North America	698 266
Africa	693 487
*Latin America and the Caribbean	672 758
Oceania	69 974
Total	9 555 027

Figure 2 – Cancer incidence and mortality in 2018. Adapted from [36]

Tumor cells exhibit structural and functional characteristics substantially different from those presented by normal cells, resulting from genomic alterations that may promote tumorigenesis. The development of malignant cells is a process with several steps that leads to the acquisition of some capabilities shared by the vast majority of human cancers [39]. In 2011, Douglas Hanahan and Robert Weinberg [40] suggested the hallmarks of cancer, that include ten biological capabilities acquired during tumorigenesis by some cancer cells which allow their survival, proliferation and dissemination [Figure 3]. According to their review, tumor cells must be able (1) to maintain proliferative signalling, allowing to sustain an active proliferative state. Contrary to normal cells, cancer cells have reduced dependence on external growth signals, presenting several mechanisms that generate a pro-growth response [41,42]. (2) to have insensitivity to growth suppressors. Tumor suppressors are responsible for regulating cell division, repairing DNA damage and promoting apoptosis. Mutations in these genes are usually associated with loss of function, resulting in tumor suppressor proteins inactivation [43], allowing cancer cells to overcome strategies for blocking cell proliferation, such as the actions promoted by p53. (3) to escape apoptosis. Physiologic stress generated during tumorigenesis or therapy can trigger programmed cell death, however cancer cells may present resistance to apoptosis through different mechanisms. As previously mentioned, the loss of function of TP53 tumor suppressor is perhaps the most common pathway. In addition, cancer cells can resist apoptosis by downregulating or upregulating proapoptotic and antiapoptotic factors, respectively [44]. (4) Unlimited replication. Normal cells have a limited replicative potential, after which they stop growing and dividing, becoming senescent [45]. Replicative senescence occurs as a result of progressive telomere shortening during successive cycles of replication. However, cancer cells are able to overcome this event by processes that promote telomere lengths maintenance. In contrast to normal cells, telomerase, an enzyme responsible for the telomere prolongation, is functionally active in about 90% of all malignant tumors, allowing an unlimited replication of tumor cells [46]. (5) to induce angiogenesis, contributing to the survival of tumors. Progressive tumor growth requires a continuous activation of the angiogenesis, allowing the formation of new vessels capable of guaranteeing adequate blood supply to the tumor mass. Activation of angiogenesis by tumors is associated with alteration of angiogenesis inducer balance such as VEGF, FGFs, thrombospondin-1 [47]. (6) Tissue invasion and metastasis. Invasion and metastasis define the processes by which cancer cells

from a primary tumor mass spread and invade other tissues to form secondary tumors. This is one of the major clinical complications related to cancer, and it can lead to the death of most patients [48]. Activation of invasion and metastasis is associated with cellular changes – such as downregulation of E-cadherin, upregulation of N-cadherin, altered integrin expression - and activation of extracellular proteases, leading to modifications in cell-cell and cell-extracellular matrix contact [39,40,48]. (7) Genome instability and mutation, which describes the acquisition and accumulation of important mutations for tumorigenesis by cancer cells [49]. This may occur as a result of malfunctioning of genome repair programs, as well as increased susceptibility to mutagenic agents presented by these cells [40]. (8) to promote an inflammatory response important to tumor progression. Inflammation generated by the immune system to fight and eliminate the tumor has an opposite effect, releasing several molecules (ROS, growth factors, enzymes) that stimulate the processes of tumorigenesis [50]. Furthermore, the ability to deregulate cellular metabolism in order to support tumor proliferation and avoid immune destruction are referred as emerging hallmarks [40]. The synthesis of these ten characteristics acquired by cells to generate a cancer provides a useful tool that contributes to a better understanding of the biology of tumors. Nevertheless, these should be carefully considered in order to avoid misinterpretation, such as assuming that hallmarks occur in all cancer cells and are expressed continuously by cells [51]. Cellular characteristics can be influenced by the tumor microenvironment. In addition to cancer cells, a tumor is also constituted by stromal tissue, which includes different cell types – cancer stem cells, immune cells, fibroblasts, endothelial cells, pericytes - forming a complex and dynamic system called tumor microenvironment. This has an important role in modelling of tumor characteristics, influencing the initiation, progression, growth, invasion and metastasis of a cancer [40,52,53].

Despite the high incidence in society, there is still no efficient treatment. Although conventional therapies, such as chemotherapy and radiotherapy, show some success, they are not a viable treatment since they also affect normal cells leading to exacerbated side effects which can condition the life quality of patients [37,54]. Other strategies have been tested and immunotherapy has proven to be a good alternative in cancer treatment. Tumor-immune system interactions are complex and cancer cells are capable to evade immune response through several mechanisms. Acquired knowledge about these strategies led to the emergence of new targets and drugs that allow to generate antitumor immunity action [55].

The most relevant example of this scientific advance is the development of immune checkpoint therapy by James P. Allinson and Tasuku Honjo [56–59], recently awarded the 2018 Nobel Prize in Medicine. Nevertheless, immunotherapy is also related with adverse side effects, which can be particularly worrying [60]. In addition, there are several concepts that need to be elucidated, being necessary to optimize the existing immunotherapeutic approaches in order to improve therapies and decrease side effects. [61]. Furthermore, other hallmarks of cancer can be used as reference of new targets for future drugs. Thus, there is a need to discover new compounds and develop new therapeutic strategies that allow not only to provide more efficient alternatives in the treatment of cancer, but also to improve the clinical outcomes for patients.

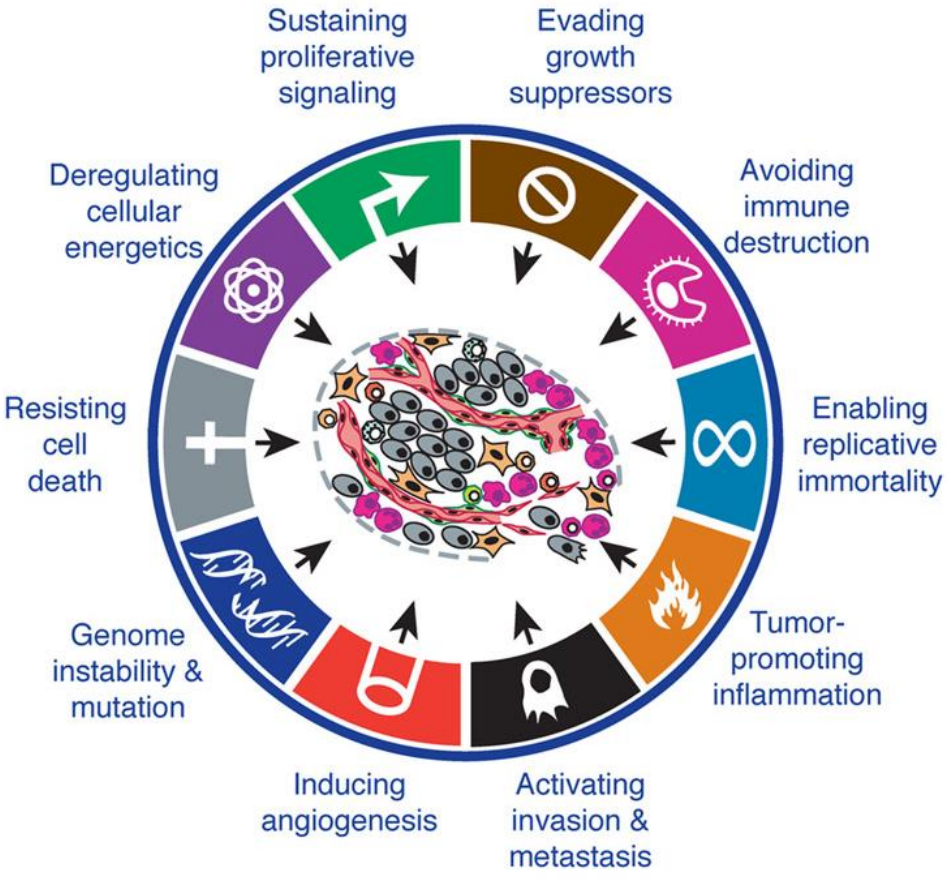


Figure 3 – The hallmarks of cancer suggested by Douglas Hanahan and Robert Weinberg [40].

1.1.3 Can snake venom be a solution?

Venom can be defined as a set of secretions, produced in specialized cells of one animal and delivered to another through an inflicted wound, for example, by a bite. These secretions are complex mixtures of proteins, small peptides, salts and other organic molecules, which once in the target animal will act in the vital systems of the organism, promoting endophysiological and biochemical dysregulations in order to facilitate hunting, feeding, defense or competition with other animals [62,63]. Furthermore, in the same venom different isoforms can occur as well as proteins from the same family but with different effects. Hence, this complexity in the constitution of venoms leads to their effects being equally complex. Venom production is shared by several species in the Kingdom Animallia, from anemones, jellyfish and arthropods to even some mammals. In fact, only about 20% of snakes are, according to the traditional denomination, truly venomous [64]. From the pharmaceutical point of view, the venom represents a two-edged sword. On one hand, it is cocktail rich in toxins, many of which are lethal to humans [63]. According to World Health Organization (WHO), it is estimated that about 5 million snake bites occur each year, resulting in about 2,7 million envenomings. These result in between 81,000 and 138,000 deaths and 400,000 amputations and other deficiencies [65]. On the other hand, the venom constituents have a high specificity and affinity for a particular target, making these secretions a rich source of proteins and other compounds with potential for application in medicine, like in the diagnosis and treatment of several diseases [66–68]. In the past, the venoms of snakes and other animals were already used as therapies, for example, in traditional Chinese medicine [69]. Nowadays, drugs have also been commercialized with proteins isolated from the venom of snakes, such as captopril, a drug used in the treatment of renovascular hypertension, and whose active principle is a peptide isolated from the venom of *Bothrops jararaca* [66,70–73]. Moreover, there is an enormous number of species whose venom can be studied, since the profile of this secretion can vary between species of the same genus and even between individuals of the same species, which increases the probability of discovering new peptides and proteins with potential interest [62].

1.2 Vipers, venom and toxins

The family Viperidae comprise about 270 species of venomous snakes, standing out two main subfamilies: the Viperinae (true vipers or pitless vipers) and the Crotalinae (pit vipers). Although vipers represent only 9% of colubroid snakes, the species belonging to this family inhabit all continents except Australia and Antarctica and are found in the most diverse types of ecosystems (eg: deserts, tropical rainforests, and mountains), being often the dominant species [74,75]. All viperid snakes are solenoglyphous, presenting the most complex system, which is characterized by the possession of long retractable and hollow front fangs in a mobile maxilla. Viper venom system comprises four major regions [Figure 4]. The main gland is divided into several lobes, which converge in a large central lumen that stores venom and then is connected to an accessory gland by a primary duct. This accessory gland is responsible for the production of mucus and is connected to the fang via the secondary duct. Then, venom is injected quickly and controlled by the muscles of venom glands [62,76,77].

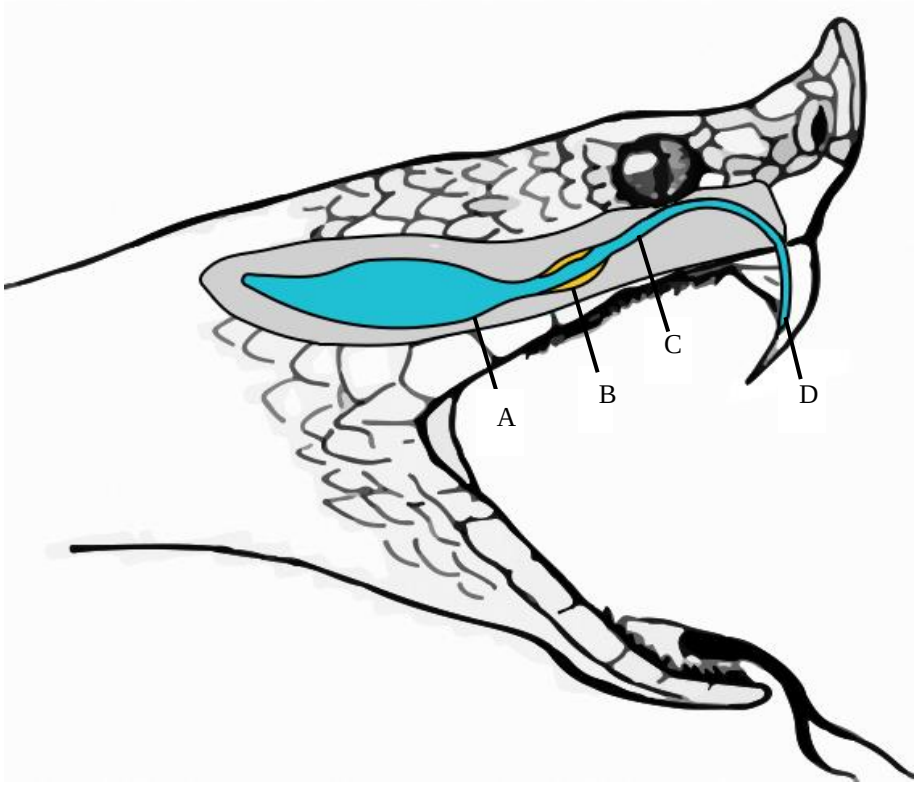


Figure 4- Venom system of vipers. A - Main gland; B - accessory gland; C - Duct; D – Fang. Adapted [77].

The venom of vipers is characterized by having a haemotoxic action. These venoms are complex mixtures of proteins, which interfere with the normal functioning of the haemostatic system, with the coagulation cascade and tissue integrity [78]. Although they present many components, venom toxins belong essentially to a few main protein families [63,67]. They can be part of enzymatic families - as serine proteases, metalloproteases, L-amino acid oxidase and phospholipases A_2 - or can belong to classes without enzymatic activity – such as disintegrins, C-type lectin-like proteins, natriuretic peptides, myotoxins, cysteine-rich secretory protein toxins, nerve and vascular endothelium growth factors, cystatin and Kunitz-type protease inhibitors [66,67]. For example, [Figure 5] shows a 1D non-reduced SDS-PAGE gel used to investigate the proteomic variation between the venoms of *Causus rhombeatus* and *Causus lichtensteinii* [79], and in [Figure 6] two pie charts with the abundance of each family in the venom of the species *Montivipera raddei* and *Montivipera bulgardaghica* are presented [80]. These two bibliographical examples show that although there is a variation in abundance and, sometimes, in the presence of certain classes of proteins, they generally share a set of major protein families.

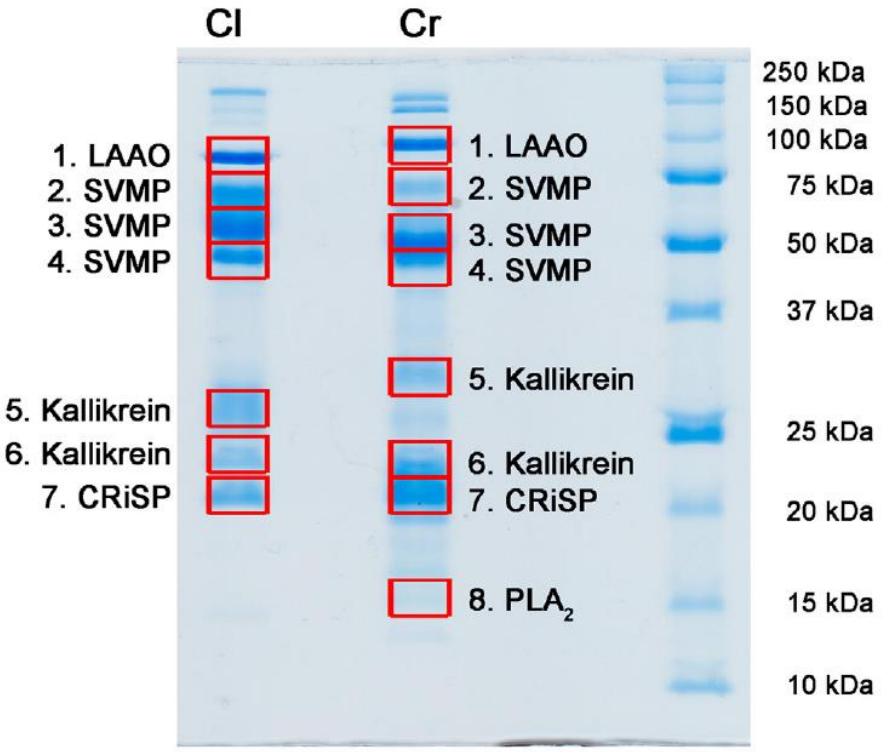


Figure 5- 1D non-reduced SDS-PAGE gel of *C. rhombeatus* (Cr) and *C. lichtensteinii* (Cl) venoms. Adapted from [79].

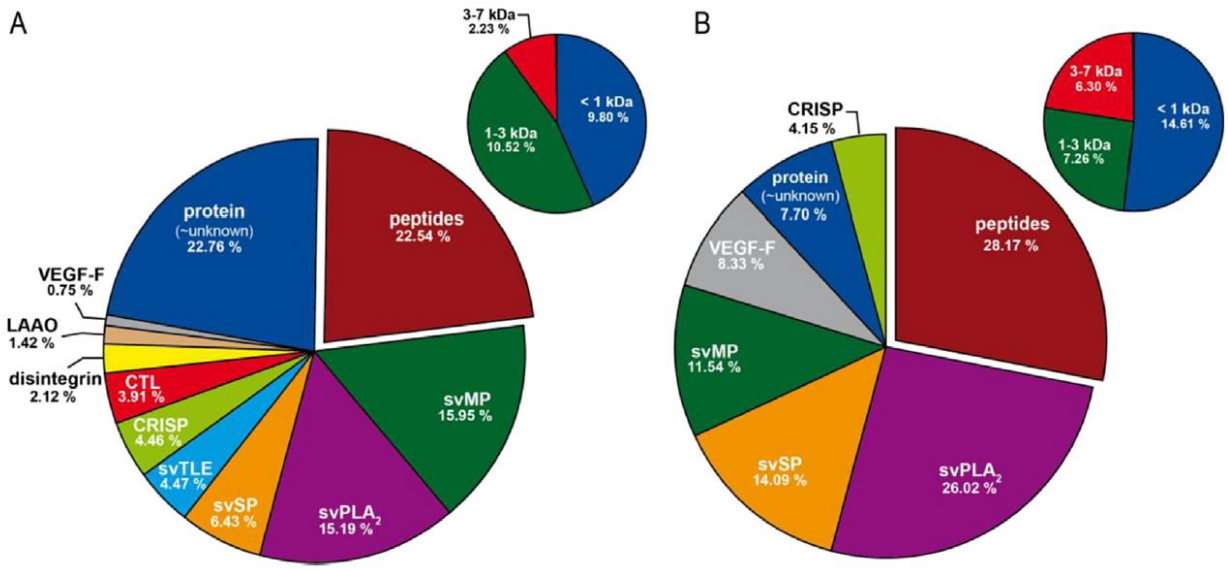


Figure 6 - Relative abundance of different toxins families in the venom of the species *Montivipera bulgardaghica* (A) and *Montivipera raddei* (B). Adapted from [80].

1.2.1 Snake venom serine proteases

Snake venom serine proteases (SVSPs) represent a group of toxins that belong to the clan PA, subclan PA(S), family S1 (chymotrypsin) and subfamily A [81]. The catalytic mechanism of SVSPs includes a reactive serine residue, which forms a catalytic triad with histidine and aspartic acid residues (His^{57}-Asp^{102}-Ser^{195}) [82,83]. SVSPs present twelve cysteine residues, with five disulfide bonds formed by ten residues, according to the homology with trypsin. Furthermore, most proteins of this family have different glycosylation patterns, presenting a diverse N- or O- glycosylation sites in sequence, which varies between SVSPs [66,81]. Although they have diverse biological functions and are able to cleave different extracellular matrix proteins (ECM), SVSPs have a higher specificity for substrates related to the haemostatic system. In fact, SVSPs are functionally similar to enzymes of this system, which includes thrombin-like enzymes or factor XA enzymes, among others [Table I]. They interfere in the pathways of the coagulation cascade by activating proteins involved in coagulation, fibrinolysis, platelet aggregation and the kallikrein-kinin system [81,83–85] . However, not all of these activities are present in the same venom and are not necessarily synonymous of massive thrombosis. Actually, proteins that can act as procoagulants cause consumption of coagulation factors, which can result in intense bleeding. Most of these activities are related to viperid envenomings [81,85,86].

1.2.2 Snake venom metalloproteases

Regarding snake venom metalloproteases (SVMPs), they represent the major components of the Viperidae venoms [87,88]. SVMPs belong to the M12 reprolysin family of metalloproteases and are classified into three groups, according to their size represented by different domain structures [89,90]. P-I SVMPs class consists of the most basic structure, having only a metalloproteinase domain. In contrast to this, P-II SVMPs class has a C-terminal disintegrin domain, in addition to the catalytic metalloproteinase domain. Finally, P-III SVMPs class has the most complex structure, presenting a metalloproteinase domain, a disintegrin-like domain and a cysteine-rich domain [89,91]. In addition, P-II and P-III classes can release their catalytic domain without losing their activity. For example, SVMPs which belong to P-II class, can release a functional disintegrin [89]. As a result of these different domain structures, SVMPs present several biological activities. SVMPs degrade ECM and cause haemorrhages, if they digest ECM of the vessel wall, allowing other venom components to enter the tissue [92]. Although haemorrhagic activity is the main activity performed by the major part of SVMPs, they are responsible for other activities such as fibrinogenolysis, prothrombin activation, factor X activation, apoptosis, platelet aggregation inhibition, pro-inflammation and inactivation of blood serine proteinase inhibitors [Table I] [87,88]. It is important to refer that these enzymes cleave the fibrinogen α-chain[93], acting on fibrin, in contrast to serine proteinases, which activate plasminogen to induce fibrinolysis [94].

1.2.3 Snake venom L-amino acid oxidases

Snake venom L-amino acid oxidases (SV-LAAOs) represent 1-9% of the Viperidae venoms total protein and are responsible for the yellowish colour of many venoms[95–97]. SV-LAAOs are flavoenzymes which catalyse the oxidative deamination of an L-amino acid to form α-keto acids, ammonia and hydrogen peroxide (H_2O_2) [98]. They are usually homodimeric glycoproteins, whose subunit is formed by three domains: a FAD-binding domain, a substrate-binding domain and a helical domain [95,99]. Furthermore, SV-LAAOs are thermo-labile enzymes with molecular masses of 110-150 KDa and have isoelectric points ranged in 4.4-8.5 [96]. Most of them exhibit high specificities for hydrophobic or aromatic amino acids – eg: L-Phe, L-Met, L-Leu and L-Ile- but some SV-LAAOs present affinity to other amino acids, such as L-Lys, L-Glu, L-Asp and L-Lys [95]. Diverse

biological effects have been associated to SV-LAAO activity such as platelet aggregation (activation or inhibition), edema formation, haemorrhage and hemolysis [Table I]. However, the mechanism of action is not yet well understood, although some authors point out that it may to some extent related to the production of H_2O_2 [66,95,96,100].

1.2.4 Phospholipases A2

Phospholipases A_2 (EC 3.1.1.4; PLA_2) are esterolytic enzymes that catalyze the hydrolysis of ester bond at the sn2 position in glycerophospholipids, resulting in the formation of lysophospholipids and free fatty acids (eg: arachidonic acid) [62,101,102]. Their activity was first observed in snake venoms and pancreatic juice and was then proposed to serve a digestive function [103,104]. Snake venom PLA_2s belong to the secreted phospholipases A_2 and are 14-18 kDa Ca^{2+}-dependent enzymes, which utilize a catalytic histidine at their active site. Adjacent to the catalytic histidine is an aspartate residue, important for the Ca^{2+} binding, forming the His/Asp dyad. Snake venom PLA_2s contain 120-135 amino acids with five to eight disulfide bonds and have a conserved 3D-structure [102,103]. They were classified into two groups: Group I, found in elapid and hydrophid snakes, and Group II, found in viperid and crotalid snakes [62,103]. These groups are well conserved, exhibiting highly conserved active site residues and both share six disulfide bonds. However, they are distinguished by one unique disulfide bond and by the presence or absence of a C-terminal extension [101,103,105]. PLA_2s exhibit a wide variety of biological effects, which include neurotoxic (pre and postsynaptic), cardiotoxic, myotoxic, hemolytic, convulsive, anticoagulant, initiation or inhibition of platelet aggregation, edema inducing and tissue damaging activities [Table I] [101,102,104]. No PLA_2 exhibits all these effects, displaying high specificity both in terms of the site of action and in terms of the resulting effect. This is explained by the presence of (glycol)protein or (glycol)lipid receptors on the surface of target tissues or cells, recognized by specific phospholipases. These bind with high affinity to these receptors triggering a set of particular mechanisms. Nevertheless, different mechanisms generated by different phospholipases that bind to different receptors may lead to the same effect [101,102].

1.2.5 Disintegrins

Disintegrins are a family of small (40-100 amino acids) cysteine-rich polypeptides with low molecular weight (4-15 kDa), which block the integrin receptors [106,107]. According to their length and number of disulfide bonds, disintegrins are classified into five groups: (1) small-sized, formed by 49-51 residues and four disulfide bonds; (2) medium-size, which are composed of about 70 amino acids and six disulfide bonds; (3) long-sized, which contain approximately 84 amino acids and seven disulfide bonds; (4) disintegrin domains from PIII SVMPs, containing about 100 amino acids with eight disulfide bonds; (5) Dimeric disintegrins (homo and heterodimers), which are composed of 67 amino acids with ten cysteine residues, involved in the formation of four disulfide bonds [66,106,108]. Disintegrins inhibitory activity is related to the inhibitory loop, which contains an active sequence that mimics the recognition site [66]. The most frequent sequence is the tripeptide RGD, formed by three amino acids: arginine, glycine and aspartic acid (Arg-Gly-Asp). However, there may be some exceptions obtained by changes in these amino acids. In fact, aspartate is conserved in most variations, which includes KGD, MGD, VGD, WGD, MLD, MVD [107,109]. All these domains will interact with different targets. Thus, disintegrins can also be classified according to the variability in their integrin-binding domains. They are divided into three main functional classes: (a) RGD, which block the $\alpha_8\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ integrins and includes KGD, VGD, MVD, MGD and WGD; (b) Leukocyte integrin binding disintegrins are represented by MLD disintegrins, which block $\alpha_4\beta_1$, $\alpha_4\beta_7$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, $\alpha_9\beta_1$ integrins and (c) R/KTS inhibit $\alpha_1\beta_1$ [107,109–111]. Regarding the biological effects, since disintegrins specifically inhibit platelet and other cell-expressed integrins, they may lead to inhibition of platelet aggregation, as well as to inhibition of cell adhesion, migration and angiogenesis, among others [Table I] [66,106,112].

1.2.6 C-type lectins

Snake venoms are composed of two types of C-type lectins: sugar binding C-type lectins and C-type lectin like proteins. However, the latter class is the most frequent in snake venoms and is commonly referred to as snaclecs (Snake venom C-type lectins) [113,114]. They are structurally different, while C-type lectin are non-enzymatic Ca^{2+} - dependent proteins, which bind sugar residues and can form homodimers, snaclecs are heterodimers, which do not contain the calcium/sugar-binding loop [115]. Heterodimeric structure is

composed of two subunits, α and β, with molecular weights of 14-15 and 13-14 kDa, respectively. Heterodimers are sometimes multimerized, resulting in a variety of oligomeric forms such as αβ, $(\alpha\beta)_2$ and $(\alpha\beta)_4$. Furthermore, the basic αβ dimer is formed by domain-swapping, in which a domain from one subunit replaces the corresponding domain of the other subunit [115,116]. Nevertheless, snaclecs have a sequence of amino acids which is 15-40% identical to the carbohydrate recognition domain of C-type lectins [116]. Snaclecs exhibit a wide variety of biological activities. In contrast to C-type lectins, C-type lectin like proteins have no lectin activity and act mainly in receptors, coagulation factors and proteins essential to haemostasis. These effects include activation and inhibition of platelet adhesion and/or blood coagulation factors [Table I] [115–118].

Table I: Main protein families of viper venoms that act on haemostatic system.
+: activation; -: inhibition; N: not observed

Target/Function		**Protein Family**					
		SVSP	SVMP	SV-LAAO	PLA_2	Disintegrins	Snaclecs
Platelets aggregation		+	-	+/-	+/-	-	+/-
Coagulation factor	FII	+	+	N	N	N	+
	FIIa	N	N	N	-	N	-
	FV	+	N	N	N	N	N
	FVII	+	N	N	N	N	N
	TF/FVII complex	N	N	N	-	N	N
	FIX	N	N	N	N	N	-
	FX	+	+	N	N	N	+/-
	FXa	N	N	N	-	N	N
	Protein C	+	N	N	N	N	N
Fibrin(ogen)		Clotting Degradation	Degradation	N	N	N	N
Plasminogen		+	N	N	N	N	N
Haemorrhage		N	+	+	N	N	N

1.2.7 Other components

Natriuretic peptides are modulators of the body fluid volume, which exhibit diuretic, natriuretic and vasodilatory properties [119]. All the natriuretic peptides described so far share a common structure consisting of a highly conserved ring formed by 17 amino acids and bridged by a disulfide bond [120,121]. Biological activity of snake venom natriuretic peptides has already been verified. For example, Evangelista (2008) observed that

NP2_Casca, a natriuretic isolated from *Crotalus durissus cascavella* venom, increased sodium excretion and leading to a hypotensive effect [122]. Coa_NP2 isolated from *Crotalus oreganus abyssus* also demonstrated a hypotensive effect [123]. This effect becomes relevant as it contributes to a rapid loss of consciousness in envenomated animals [119].

Cysteine-rich secretory proteins (CRISPs) are a large family of single chain polypeptides with molecular weights of approximately 20-30 kDa, found in several vertebrates [124]. Their sequences are highly similar, and all have 16 conserved cysteine residues, which form eight disulfide bonds. CRISPs found in snake venoms usually have molecular weights of 23-26 kDa and consist of two domains - N-terminal pathogenesis-related proteins of group 1 (PR-1) and C-terminal cysteine-rich domain (CRD) - linked by a compact hinge region [125,126]. Biological activity of these proteins remains unclear. Nonetheless, some studies have described that CRISPs target several ion channels, suggesting that they may act as ion channel blockers. Thus, it was verified that some of these toxins block L-type Ca^{2+} channels - leading to inhibition of smooth muscle contraction - and/or cyclic nucleotide-gated (CNG) channels in sensory neurons, while other are capable of blocking K^{+} and Ca^{2+} channels in neurons or ryanodine receptors in the sarcoplasmic reticulum of heart and skeletal muscles [127–130]. However, these effects are not common to all CRISPs. Some of these toxins found in vipers exhibit only few of the amino acid residues thought to participate in the interaction with the ion channels, suggesting the absence of channel-blocking effect [126,131]. Despite this, CRISPs found in venom of vipers may play a different biological activity, such as inducing inflammatory responses [132]. Nevertheless, it is not yet fully understood how all these processes occur[66,132].

Kunitz-type protease inhibitors usually correspond to a small percentage of total proteins in snake venom (about 0.3-7.5%) [63,127]. They are low molecular mass peptides and consist of a conserved fold of about 50-60 amino acids with three disulfide bonds, involved in the folded peptide stability [133–135]. Snake venom Kunitz-type protease inhibitors are structurally identical to bovine pancreatic trypsin inhibitor, being homologous with the conserved Kunitz motif present in this peptide [134,136]. Most of these display a conserved antiproteinase site (P1 site), which is responsible for binding and inhibition of specific proteases [136,137]. The Kunitz-type inhibitors exhibit a high functional diversity and, according to their functions, are classified into two groups: non-neurotoxic and neurotoxic [138]. In the first group is included the most known function of these peptides:

the inhibition of serine proteases such as trypsin, chymotrypsin, kallikreins and plasmin [62,127,133,138]. Furthermore, Kunitz-type inhibitors may interfere with blood coagulation and be involved in inflammation and fibrinolysis. The neurotoxic group includes peptides mainly isolated from elapid snakes, which act as K^+ and Ca^{2+} channel blockers [136,138].

Cystatin-type protease inhibitors belong to the cystatin superfamily and are classified into three different families: (a) Type 1 cystatins that consist of approximately 100 amino acids residues without disulfide bonds and carbohydrates (b) Type 2 cystatins, formed by about 120 residues with two disulfide bonds and (c) Type 3 cystatins which are composed of three type 2 domains [139,140]. Cystatin-type protease inhibitors are found in several animals, including mammals, reptiles, birds and some invertebrates [141–143]. Regarding viper venom, cystatins were initially found in *Vipera ammodytes* and *Bitis arietans* [144–146]. The relative abundance of these toxins is variable. For example, according to Calvete (2007), they correspond to 1.8% and 9.8% of total venom proteins of the species *Bitis gabonica gabonica* and *Bitis arietans* [147]. Snake cystatins isolated so far belong to type 2 cystatin class, with a high degree of similarity between them [145,148]. Nevertheless, their role in the envenoming process is unclear and studies are needed to elucidate their action [62,127,145].

Growth factors are also present in snake venoms, especially the nerve growth factor (NGF) and vascular endothelial growth factor (VEGF). Most NGFs isolated from snake venoms are non-covalently bound dimers, formed by two similar subunits. However, some have been identified as glycoproteins (10-20% carbohydrate) [149,150]. Despite the known functions of NGFs, their role as a component of venom has not yet been fully elucidated [127]. Some authors have suggested that they may be involved in prevention of venom autolysis or in an increasing the potency of the venom [62,151,152]. Furthermore, they may act on non-neuronal cells or tissues, leading to plasma extravasation and tissue vulnerability, for example [150]. Regarding VEGF family, it is classified into seven groups: VEGF-A to VEGF-F and placental growth factor [153]. VEGFs found in snake venoms mainly belong to VEGF-F class and according to their features and receptor-selectivity, they are divided into three subgroups: VEGF-F1 to VEGF-F3[127,153]. They bind to the corresponding receptors leading to an increase in vascular permeability resulting in hypotension and shock [62,66].

Some species of vipers may still contain myotoxins, which act on muscles, leading to muscle lesions in addition to hemotoxic action [62,154]. Myotoxic activity can be presented by two different types of components: (a) non-enzymatic polypeptides consisting of 43-45 amino acids residues and three disulfide bonds, such as *myotoxin a* isolated from *Crotalus viridis viridis* venom [154,155]; (b) phospholipases A_2 with myotoxic activity [154,156]. Myotoxins act by promoting skeletal muscle damage due to sarcolemma damage. Interaction with ion channels may be responsible for this effect. Furthermore, these muscle lesions may contribute to paralysis of prey [62,157].

1.3 Viper venom as a source of potential therapeutic agents against multi-resistant microorganisms and cancer

Since earlier, natural sources are used by humans as therapeutic agents for the treatment of several diseases. Plants have been the main source of compounds for pharmaceutical products [158]. At the beginning of the 20th century, 80% of all medicines came from plants, and currently about 25% of pharmaceutical products have vegetal origin [159]. Although there are still much to study on plants material, the search for new compounds has tried to include other sources like animals, fungi, algae, and even products produced by bacteria [160]. Regarding their unique traits, snake venoms present themselves as one of the most promising sources from which new drugs can be developed. Crude venoms of several viperid species have already been tested to verify their antimicrobial and antitumoral potential. Moridikia *et al* (2018) verified that different concentrations of crude venom of *Vipera latifii* inhibited the growth of *Bacillus subtilis* and *Staphilococcus aureus,* as well as, led to a significant decrease in survival of human liver cancer cell line (HepG2) in a concentration-dependent manner [161]. Furthermore, venoms from *Bothrops jararacussu* and *Bothrops jararaca* exhibit bactericidal activity against some Gram-positive and Gram-negative species such as *Eubacterium lentum, Peptostreptococcus anaerobius, Propionibacterium acnes, Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Salmonella typhimurium, Porphyromonas gingivalis* [162]. Sawan *et al* (2017) evaluate the cytotoxic effect of the *Montivipera bornmuelleri* venom on non-tumoral HaCat, benign A5 and malignant II4 keratinocytes and verified that crude venom was able to reduce cell viability depending on the concentration. In addition,

malignant II4 cells were the most affected compared to the other cell lines tested, while HaCat cells were the least affected [163]. Cytotoxic effects were also observed in SHY5Y, MCF-7, HeLa, A-549, CaCo-2, MDA-MB-231, 253-JBV and U87MG tumor cells and HEK-293 non-cancerous human cell line treated with crude venoms of *Cerastes cerastes* and *Cryptelytrops purpureomaculatus* [164]. In fact, these studies exemplify the potential of snake venoms and represent a good starting point. However, currently the research focuses on the isolation of toxins from the most diverse families present in the venom and evaluation of their activity.

Venom toxins promote their antimicrobial and antitumoral activities mainly through the induction of cell death [165]. In addition, antitumoral activity may also occur as a result of interference in different processes of metastasis and angiogenesis [66]. Common mechanisms of action include activation of apoptosis pathways, cell membrane damage, cell cycle interference, free radical generation and toxicity, DNA damage, interaction with integrins, among others [111,166,167]. These mechanisms are dependent on the type of venom component, target and type of interaction. Table II shows some examples of toxins isolated from viperid venoms and their mechanisms of action. In fact, most of the isolated toxins which exhibit therapeutic potential belong mostly to a set of five protein families: SV-LAAO, disintegrins, PLA_2, SVMPs and C-type lectin.

1.3.1 Snake venom L-amino acid oxidases

As may occur in the envenoming process, the production of H_2O_2 and consequent effects may be the main responsible for the antimicrobial and antitumoral activities evidenced by some SV-LAAOs. Casca LAO isolated from the venom of *Crotalus durissus cascavella* showed ability to inhibit the growth of *Streptococcus mutans* and *Xanthomonas axonopodis pv passiflorae*. Analysing the results of this study, it was verified that H_2O_2 plays a key role in this action since the addition of catalase, a H_2O_2 scavenger, supressed the antimicrobial activity. Furthermore, it has been found membrane rupture [168]. Similarly, Salama *et al* (2018) isolated the Cv-LAAO from the venom of *Cerastes vipera*, which exhibited antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, as well as a selective cytotoxic activity against breast cancer cell lines (MCF-7). In addition to the influence of catalase on the effects promoted by CV-LAAO, they also verified a considerable increase of the lipid peroxidation in the MCF-7 treated cells [96]. Lipid peroxidation may

occur as a consequence of reactive oxygen species (ROS) presence and can compromise the assembly, composition, structure and dynamics of cell membrane [169]. Thus, the membrane damage promoted by the action of H_2O_2 can constitute one of the main mechanisms of action of SV-LAAOs. Furthermore, it has been found that some SV-LAAOs can activate extrinsic and intrinsic apoptosis pathways through processes related to H_2O_2 [170–172].

1.3.2 Phospholipases A2

Some PLA_2s isolated from Viperidae venoms display antimicrobial and antitumoral activities, however, mechanisms of action are yet unclear. Samy *et al* (2015) showed that the *Daboia russelli russelli* PLA_2, Viperatoxin-II, is capable of inhibiting growth of *S. aureus* and *Burkholderia pseudomallei* [173]. Daboiatoxin, other PLA_2 isolated from the venom of *Daboia russelli russelli* exhibits strong antibacterial activity against *Burkholderia pseudomallei* [174]. Membrane damage seems to be the main mechanism responsible for bactericidal effect shown by these toxins. Previous studies have demonstrated that PLA_2 catalytic activity may be related to inhibition of bacterial growth since it is involved in the lipid digestion [175–177]. However, a bactericidal effect independent of the enzymatic activity of PLA_2 has also been observed and appears in more recent studies as the most common bactericidal mechanism exhibited by snake venom PLA_2s [173,175,178,179]. This may be similar to the cationic peptides action. Thus, it is assumed that these toxins interact with the membrane leading to structural and permeability modifications such as the formation of pores [173,178,180]. Regarding the antitumoral activity, Azevedo *et al* (2016) verified that BnSP-6 isolated from the *Bothrops pauloensis* venom displayed a selective cytotoxic activity to human breast cancer cell line (MDA-MB31) when compared to non-tumorigenic breast epithelial (MCF10A) cells [181]. The anticancer of BthTX-I was reported on promyelocytic leukemia cells (HL-60), human hepatocellular carcinoma cells (HpG2) and murine melanoma cells (B16F10). It was shown that this toxin reduces the viability of these cancer cells [182]. In fact, some authors suggest that cytotoxicity of PLA_2s is not related with their enzymatic activity but to other mechanisms. This thesis is supported by the existence of cytotoxic effect promoted by Lys49 PLA_2s, which have low or no catalytic activity [182,183]. It was observed that these toxins interfere with gene regulation by promoting up-regulation of different pro-apoptotic genes such as TNF, TNFRSF10B, TNFRSF1A and CASP8 and down-regulation of anti-apoptotic genes. Genes involved in

cell cycle progression can also be modulated, leading to cell cycle arrestment. Furthermore, it was verified that PLA_2s may led to biosynthesis of tumor suppressor proteins, as well as, induce autophagy. These mechanisms can be triggered by the interaction of PLA_2s with specific membrane receptors [181,184,185].

1.3.3 Components that may interact with integrins

Integrins are bidirectional receptors found on the surface of cells. They interact with ECM and other cells (eg: endothelial cells), thereby being fundamental in metastasis and angiogenesis [39]. The $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_6\beta_4$, $\alpha_4\beta_1$ and $\alpha_v\beta_6$ are some of the integrins involved in cancer progression [48]. Since they are crucial in regulating tumor processes, integrins constitute a therapeutic target with potential. Taking into account their role as a venom component, disintegrins appear as the most obvious candidate for this task. Mojastin 1 and viridistatin 2 are two disintegrins isolated from *Crotalus scutulatus scutulatus* and *Crotalus viridis viridis* respectively, which inhibited the adhesion of pancreatic cells (BXPC-3) to laminin and vitronectin, as well as the migration of these cells after 24h of incubation. Interaction with $\alpha_v\beta_3$ and $\alpha_3\beta_1$ was suggested as the possible cause for these results [112]. Similarly, lebestatin interacts with the $\alpha_1\beta_1$ and leading to inhibition of adhesion of rat pheochromocytoma (PC12) and chinese hamster ovary cells to type I and IV collagen. Migration and angiogenesis were also affected [186]. Kerkkamp *et al* (2018) verified in *in vivo* assays that venom of *Echis carinatus* inhibited the migration of the pancreatic cancer cell line PaTu 8988t and resulted in partial inhibition of angiogenesis, which may be related with the presence of disintegrins in the venom [187].

Other components have the ability to recognize integrins too. For example, Bazaa *et al* (2009) reported a phospholipase A_2 (MVP-PLA_2) which inhibited adhesion and migration of several human tumor cells by interaction with $\alpha_5\beta_1$ and α_v-integrins [188]. Lectins are able to interact with different targets in the cell membrane. BJcuL, a lectin isolated from *Bothrops jararacussu* venom, can interact with specific glycans on the surface of gastric carcinoma cells MKN45 and AGS, resulting in decreased adhesion of these tumor cells to matrigel and cytotoxic effects [189]. Furthermore, C-type lectins were also described as modulators of integrin-ligand interactions. Sarray *et al* (2007) verified that lebectin and lebecetin from *Macrovipera lebetina* venom, inhibit adhesion, migration, invasion and proliferation of human cell lines: fibrosarcoma (HT1080), melanoma (IGR39, M21-L and M21-L4), colonic

adenocarcinoma (HT29-D4), leukemia (K562) and ovarian adenocarcinoma (IGROV1). In addition, it was observed that these interact with $\alpha_5\beta_1$ and α_v-integrins [190], however, it is not the only mechanisms responsible for the antitumoral activity of snaclecs. A recent study has shown that macrovipecetin can interfere with $\alpha_v\beta_3$ integrin, E-cadherin, vimentin, β-catenin, c-Src and RhoA expression leading to inhibition of cell adhesion, migration and invasion. The same study also verified that this toxin can activate extrinsic and intrinsic apoptosis pathways [191]. Finally, SVMPs may also promote inhibition of integrins, since some of them are constituted by a disintegrin-like domain. Maria *et al* [192] observed that jararhagin, a P-III class SVMP purified from *Bothrops jararaca* venom, decreased cell viability besides inhibitory effects on adhesion, proliferation of B16F10 murine melanoma cells. The effects were more severe after treatment with Jari, the same toxin with inactive, catalytic domain. In addition, in vivo studies showed that these toxins have an antimetastatic and antiproliferative effects in tumors. The authors suggest that these results are due to their disintegrin domain. Similar effects were observed in SK-Mel-28 human melanoma cells exposed to the same toxins [193]. Bothropoidin isolated from *Bothrops pauloensis* venom was able not only to inhibit the adhesion and migration of breast cancer cells (MDA-MB-231) in a dose-dependent manner but also has an antiangiogenic effect [165]. Nevertheless, SVMPs have a proteolytic activity that may lead to antitumoral effect. In the previous study, it was also verified that bothropoidin exhibited a cytotoxic effect of 30% on MDA-MB-231. Bonilla-Porras *et al* (2016) observed that nasulysin-1 induced apoptosis of Jurkat and K62 cells [194]. These authors suggest that catalytic activity of these enzymes may contribute to cytotoxicity. SVMPs are able to degrade ECM components, membrane proteins and interact with specific receptors [183,195], which might trigger anoikis or apoptosis through an extrinsic pathway. However, the exact mechanism still needs to be investigated.

Table II: Toxins isolated from several viper species, their activity and mechanisms of action.

Snake	Protein Family	Protein	Action	Major mechanism	Reference
Crotalus durissus cascavella	SV-LAAO	Casca LAO	Inhibits growth of gram-negative bacteria	Effects related with the production of H_2O_2	[168]
Crotalus mitchellii pyrrhus	SV-LAAO	Cmp-LAAO	Cytotoxic activity Induces apoptosis		[196]
Cerastes vipera	SV-LAAO	Cv-LAAO	Inhibits growth of *S. aureus* and *E. coli* Cytotoxic activity against tumoral cells		[96]
Crotalus scutulatus scutulatus *Crotalus viridis viridis*	Disintegrins	Mojastin 1 Viridistatin 2	Inhibits adhesion of tumor cells to matrix protein, cell migration Induce apoptosis	Interaction with integrins	[112]
Macrovipera lebetina	Disintegrins	Lebestatin	Inhibits adhesion, migration Anti-angiogenic effect		[186]
Calloselasma rhodostoma	Disintegrins	Rhodostomin	Inhibits adhesion, migration Anti-angiogenic effect		[197]
Cerastes cerastes	Disintegrins	CC5 CC8	Induce apoptosis of HMEC-1 Inhibits adhesion, migration, proliferation of tumor cells Anti-angiogenic effect	Activation of apoptosis pathways; Interaction with integrins	[198]

Cerastes cerastes	Disintegrins	-	Cytotoxic activity against SHSY5Y cells Disorganization and detachment Aggregate formation	-	[164]
Echis ocellatus *Echis pyramidum leakeyi* *Echis carinatus sochureki* *Echis coloratus*	PLA_2	$[Ser]^{49}PLA_2$	Inhibits growth of *E.coli* and *S.aureus* Cytotoxic activity	Membrane damage	[179]
Daboia russelli russelli	PLA_2	Daboiatoxin	Inhibits growth of *B. pseudomallei*		[174]
Crotalus durissus terrificus	PLA_2	Crotoxin B			
Daboia russelli russelli	PLA_2	Viperatoxin-II	Inhibits growth of *S.aureus* and *Burkholderia pseudomallei*		[173]
Cerastes cerastes	PLA_2	CC-PLA2-1 CC-PLA2-2	Inhibits adhesion, migration	Block ligand non-enzymatically	[199]
Bothrops pauloensis	PLA_2	BnSP-6	Cytotoxic activity		[181,184]
Bothrops jararacussu	PLA_2	BthTX-1			[185]
Crypteçytrops purpureomaculatus	PLA_2	-	Cytotoxic activity against SHSY5Y cells	-	[164]

Porthidium nasutum	SVMPs	Nasulysin-1	Induce apoptosis of tumor cells	Degrades membrane proteins and ECM Interact with specific receptors Activate extrinsic apoptosis	[194]
Bothrops pauloensis	SVMPs	Bothropoidin	Induce apoptosis of tumor cells Inhibits adhesion, migration, proliferation of tumor cells Anti-angiogenic effect	Degrades membrane proteins and ECM Interact with integrins	[165]
Bothrops jararaca	SVMPs	Jararhagin (Jara) Jari	Inhibits adhesion, migration, proliferation of SK-Mel-28 human melanoma cells Cytotoxic activity and inhibition of adhesion and proliferation of B16F10 murine melanoma cells Morphological changes Tumor volume and metastasis decrease in vivo	Interaction with integrins	[192,193]
Macrovipera lebetina transmediterranea	SVMPs	Leberagin-C	Inhibits adhesion, migration		[200]
Macrovipera lebetina transmediterranea	Kunitz-type serine protease inhibitor	PIVL	Inhibits adhesion, migration Anti-angiogenic effect		[201,202]
Echis multisquamatus	Snaclec	EMS16	Inhibits adhesion, migration	Inhibition of the $\alpha_2\beta_1$ integrin	[203]

Macrovipera lebetina	Snaclec	Macrovipecetin	Cytotoxic activity Inhibits adhesion, migration and invasion	Activation of apoptosis pathways Interact with integrins Modulation of the expression of integrins α_v e β_3, E-cadherin, β-catenin, c-Src, RhoA	[191]
Macrovipera lebetina	Snaclec	Lebectin Lebecetin	Inhibits adhesion, migration and invasion	Inhibition of the integrins ($\alpha_5\beta_1$, α_v)	[190]
Bothrops jararacussu	C-Type lectin	BJcuL	Cytotoxic activity against gastric carcinoma cells (MKN45 and AGS) and human colon adenocarcinoma cells (HT29) Decreased adherence Morphological changes (Actin filament disorganization and disassembly) Induce apoptosis of tumor cells	Interact with glycans Activation of apoptosis pathways (Increased expression of TRAIL and cytochrome c release)	[189,204]
Bothrops leucurus	C-Type lectin	BIL	Inhibits growth of *S. aureus*, *Enterococcus faecalis* e *Bacillus subtilis*	Membrane damage	[205]

1.4 Objectives and Structure of the dissertation

Viperidae represents one of the most successful and deadly reptile families in the world. Its venom is characterized by its hemotoxic effect, presenting components that act essentially in the haemostatic system, in the coagulation cascade and in related tissues. The study of its application as therapeutic agents is relatively recent and the innovative scientific approaches (eg: in proteomics, genetic, among others), have allowed to increase the knowledge about the venom components. Thus, venom has increasingly emerged as a new source of therapeutic agents for the treatment of several diseases. Nowadays, multi-resistant microorganisms and cancer represent real threats to human life and therefore there is an urgent need for solutions. Several proteins isolated from the venom of vipers have revealed quite interesting effects in this scope and they mainly belong to five families: SV-LAAO, disintegrins, PLA_2, SVMPs and C-type lectin. Although some of these mechanisms are still unclear, they prove that the venom of vipers and their components can have an antimicrobial and/or antitumoral activities, leading to the death of microorganisms and/or tumor cells and the inhibition of some metastasis processes. However, it is important to understand that although a venom may have cytotoxic capacity this is not synonymous with antitumoral activity, so it is important to verify if there is also cytotoxic activity in non-tumor cells. Further investigations of these toxins as well as the venom of several species of vipers not yet studied may clarify many doubts and reveal new compounds with the potential to become new therapeutic agents.

Therefore, the main aim of this dissertation was to evaluate the antitumor and antibacterial potential of *Vipera latastei* crude venom providing thus the first study about the bioactivity of the venom of this species. In order to achieve this objective, the present dissertation was organized in four chapters. The present chapter (chapter 1) is an overview of the venom and toxins produced by species belonging to the Viperidae family, exploring its pharmacological properties, in order to demonstrate its antimicrobial and antitumor potential.

Chapters 2 and 3 consist of two studies about the antitumor and antibacterial potential of *V. latastei* crude venom. Both chapters are in preparation to be submitted for publication. Chapter 2 evaluates the antitumor potential of this venom on human melanoma cells, reporting its effects on cell viability and cell cycle dynamics of human melanoma and non-tumor human keratinocyte cell lines. Chapter 3 focuses on the antibacterial potential of *V.*

latastei crude venom, testing it against several Gram-positive and Gram-negative pathogenic bacteria.

The last chapter (chapter 4) consists of a general discussion of the most relevant conclusions, as well as, some suggestions to consider in future studies.

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Chapter 2- Antitumor potential of *Vipera latastei* crude venom against melanoma cells

2.1 Abstract

Nowadays, cancer is one of the main threats to human health, being responsible for many deaths globally. Among the various types of cancer, skin cancer represents one of the most common forms with an increasing incidence in recent years. Melanoma is the deadliest form of skin cancer and current treatments present some obstacles that may compromise prognosis of patients. Therefore, it is essential to develop novel treatment approaches and new therapeutic agents. Snake venoms are a rich source of compounds with potential application in various therapeutic areas, including cancer therapies. Many studies have reported antitumor activities expressed by several snake venoms and their toxins. Based on this, and in the lack of knowledge concerning the venom of vipers inhabiting Portugal, this study aimed to screen the antitumor potential of *Vipera latastei* crude venom by evaluating its effects on cell viability and cell cycle dynamics. Human Keratinocyte HaCaT and human melanoma MNT-1 cell lines were treated with different concentrations of crude venom (MNT-1: 25, 12.5, 6.25, 3.12, 2.5, 2, 1.56, 0.78, 0.39 μg protein/mL; HaCaT: 3.12, 1.56, 0.78, 0.39, 0.19, 0.09 and 0.04 μg protein/mL) for 24, 48 and 72h and their viability assessed by MTT assay. Flow cytometry was used to evaluate interferences in cell cycle dynamics. Additionally, we determined ROS formation after crude venom treatment. Our results demonstrated that the cell viability of both cell lines significantly decreases after venom exposure. Additionally, crude venom exerted a more potent action against HaCaT cells. Regarding cell cycle analyses, venom affected cell cycle progression of MNT-1 cells leading to a S-phase delay. No significant changes were noted in HaCaT cells. We also noted in MNT-1 cells an increase in ROS generation in a dose-dependent manner. A non-significant increase in IC50 was found in HaCaT cells, compared to the control.

These findings suggest that *V. latastei* crude venom presents a potent cytotoxic effect against both cell lines. However, we cannot demonstrate that it exhibits an antitumor selective activity since the viability of HaCaT cells were more affected than melanoma cells. Nevertheless, more advanced studies should be performed in order to clarify their potential and assess to the bioactivity of their constituents.

Keywords: Cancer; Skin cancer; Melanoma; Snake venom; *Vipera latastei*; MNT-1 cells; HaCaT cells; Cytotoxicity; Cell cycle

2.2 Introduction

Cancer represents a serious health problem in current society, being one of the leading causes of death worldwide [1]. According to WHO, in 2018 18,078,957 new cancer cases and approximately 9,555,027 deaths were identified globally, occurring mainly in Asia and Europe, the two most affected continents [2]. It defines a multi-cellular and multi-genic disease which is characterized by uncontrolled growth and proliferation of abnormal cells. Cancer can present a diverse etiology and develop in several types of tissues [3,4]. Skin cancer figures as one of the most prevalent forms of cancer in the world, and nowadays its incidence continues to increase as possible result of deterioration of the ozone layer and consequent increase in sun exposure [5,6]. Among skin tumours, melanoma appears as the deadliest type, especially in stages that present metastatic capacity. Statistically, 287 723 cases of melanoma skin cancer and about 60 712 deaths were observed in 2018, constituting an alarming public health concern that has been increasing annually [2,7]. Diagnosis of early-stage melanoma is essential since it is associated with high survival rates, however more severe cases still represent a poor prognosis [8]. Currently, several therapies are available for patients with skin cancer [9]. Surgical procedures are the usual approach in most cases, especially as primary treatment for early-stage melanoma [5,7]. Despite presenting excellent results at an early stage or in situations with limited metastatic sites, surgical intervention may not be as effective at advanced stages of disease [10]. Furthermore, tumor location may limit surgery use [5]. Non-surgical therapies may also be used such as radiotherapy, cryotherapy, immunotherapy, 5-fluorouracil (5-FU), photodynamic therapy (PDT), however exacerbated side effects, high costs and limited availability represent some disadvantages of these approaches [5,9]. In addition, melanoma tumors may develop chemoresistance mechanisms [11]. Thus, new therapeutic agents should be discovered in order to develop innovative strategies to overcome these obstacles and improve patient prognosis.

Biological compounds have a high scientific and medical interest since they may exhibit great therapeutic potential [12]. Snake venoms due to their complex composition, the particular characteristics of some of their constituents, and the intra- and inter- species

variations that may occur in the venom content, represent an example of a biological source with therapeutic interest [13–17]. Currently, snake venoms are considered one of the most promising sources of new compounds that could be used as antitumor drugs [18]. Several studies have already shown the cytotoxic and antitumoral potential of various snake venoms, as well as their components. *Montivipera bornmuelleri* crude venom exhibited a differential cytotoxic effect on non-tumoral HaCaT, benign A5 and malignant II4 keratinocytes in a concentration-dependent manner, being the first cell line the least affected [19]. A selective cytotoxic activity was also observed in MDA-MB31 human breast cancer cells and MCF10A non-tumorigenic breast epithelial exposed to BnSP-6, a phospholipase A2 isolated from the *Bothrops pauloensis* venom, suggesting an antitumoral effect [20,21]. Different disintegrins were tested for this purpose and revealed ability to inhibit migration, and proliferation of tumor cells, as well as, anti-angiogenic properties [22–25]. Nowadays, the comprehension of their mechanisms of action has been a major focus of study [26]. Although not always fully understood, mechanisms associated with cell death and interference with metastasis and angiogenesis processes are often observed [15,27]. These may include activation of apoptosis pathways, cell cycle interference, DNA damage, and interaction with different receptor types and other membrane components [28–30].

In Portugal there are two known viper species, being *Vipera latastei* the most abundant. *V. latastei* is a venomous serpent that belongs to the *Viperidae* family, easily distinguished due to a small nasal appendage at the end of the snout and the dorsal pattern that forms a dark zig-zag band [31]. It is distributed throughout the Iberian Peninsula as well as in regions of Northern Africa [32], and according to IUCN Red List of Threatened Species, is registered as vulnerable [33]. As seen in other viper species, *V. latastei* venom is characterized by exhibiting a hemotoxic effect, for which no antivenom has been reported so far [34,35]. Besides the reasons already mentioned that make the snake venoms a promising source, the distribution of the species and the lack of studies related to the therapeutic potential of its venom motivated the choice of *V. latastei* for the present work. This is the first study that explores the cytotoxic effects of *V. latastei* venom. Therefore, the overall aim of the present study was to evaluate the antitumor potential of *Vipera latastei* crude venom on human melanoma cells.

2.3 Material and Methods

2.3.1 Collection and preparation of venom

Individuals of *V. latastei* were collected in their natural habitat in Castro de São Paio, Portugal. *V. latastei* venom was extracted from several adult animals of both genders using parafilm coated microtubes, and stimulating bite, without exerting any pressure on the venom glands. These procedures were done in collaboration with Dr Fernando Martínez-Freiría (CIBIO-InBIO). Crude venoms collected were frozen at -80ºC, lyophilized and stored at -80ºC for later use. To prepare stock solution, the lyophilized crude venom was resuspended in cell culture medium (DMEM), filtered with a sterile 0.22 μm pore filter (VWR) and diluted to obtain the concentrations set for the assays.

2.3.2 Determination of protein content

Before antitumor tests, 1 mg of each freeze-dried venom sample was diluted in 1 mL of ultra-pure water, preparing a concentration of 1 mg crude venom/mL. Protein concentrations of these samples were then determined by Bradford assay [36] using a plate reader at a wavelength of 595 nm. Bovine γ – globulin was used as a standard. After that, the concentrations set for the assays (μg protein/mL) was prepared considering the protein concentration obtained for each sample in use.

2.3.3 Cell culture

Human Keratinocyte HaCaT cell line was purchased from Cell Lines Services (Eppelheim, Germany) and human melanoma MNT-1 cell line was supplied by Dr Manuela Gaspar from Faculdade de Farmácia da Universidade de Lisboa (Portugal). Cells were maintained in culture flasks (Corning, EUA) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L- glutamine, 100 μg/mL streptomycin, 100 μg/mL penicillin and 250 μg/mL fungizone at 37ºC in 5% CO2 humidified atmosphere. Confluence, morphology and viability of cells were routinely observed using an inverted microscope Nikon Eclipse 80i (Nikon, Tokyo, Japan). Subcultures were routinely performed every 2-3 days and procedures were adapted for each cell line.

2.3.4 MTT assay

Cell viability was determined by the colorimetric MTT assay, as described by Carrola et al (2016). Briefly, cells were seeded on 96-well plates at 0.35 x 10^5 cells/mL (MNT-1) or 0.4 x 10^5 cells/mL (HaCaT), 0.25 x 10^5 cells/mL and 0.15 x 10^5 cells/mL for 24, 48 and 72h exposure periods, respectively, in a final volume of 100 µL/well. After 24h, cells were incubated with fresh medium as control and medium containing the stock solution of *V. latastei* venom with different concentrations (concentration range 25, 12.5, 6.25, 3.12, 2.5, 2, 1.56, 0.78, 0.39 µg protein/mL for MNT-1 and 3.12, 1.56, 0.78, 0.39, 0.19, 0.09 and 0.04 µg protein/mL for HaCaT) at 37ºC in 5% CO2 humidified atmosphere. After each exposure period, 50 µL of MTT (1 mg/mL in PBS) were added to each well, and incubated for 4h at 37ºC, 5% CO2. Then, MTT solution was removed and the medium was replaced with 150 µL dimethyl sulfoxide (DMSO). The plate was shaken under the dark for 2h at room temperature to dissolve the formazan crystals. The optical density was measured at 570 nm using a microplate reader and the percentage of viable cells was calculated with [Formula 1]. The concentration of crude venom that leads to a 20% (IC20) and 50% (IC50) decrease in cell viability were determined. Four independent assays, with three replicates, were performed.

$$\%\ \text{Cell viability} = \frac{(\text{absorbance of sample} - \text{absorbance of DMSO})}{(\text{absorbance of Control} - \text{absorbance of DMSO})} \times 100$$

Formula 1 – Formula used to calculate % cell viability.

2.3.5 Cell cycle analysis

Cell cycle analysis was performed according to the method described by Oliveira *et al*. [37]. Cells were seeded in 6-well plates with an initial density of 0.59375 x 10^5 and 0.51953 x 10^5 cells/mL for HaCaT and MNT-1, respectively, at 24h assay and 0.22265 x 10^5 cells/mL for 72h exposure. Then, they were incubated at 37ºC for 24h and 72h with IC20 (HaCaT,24h: 0.47 µg protein/mL; MNT-1, 24h: 2.87 µg protein/mL. HaCaT, 72h: 0.19 µg protein/mL; MNT-1, 72h: 0.64 µg protein/mL) and IC50 (HaCaT,24h: 0.56 µg protein/mL; MNT-1, 24h: 3.01 µg protein/mL. HaCaT, 72h: 0.24 µg protein/mL; MNT-1, 72h: 0.73 µg protein/mL). After incubation, cells were trypsinized and centrifuged at 700 g for 5 min.

Then, cells were washed with PBS pH 7.2, centrifuged, and resuspended in 85% cold ethanol until analysis.

For cell cycle analysis, cells were centrifuged (700 g for 5 min) to remove ethanol and the pellets were resuspended with PBS. Then, for each sample 50 μg/mL RNase and 50 μg/mL propidium iodide (PI) were added and incubated for 20 min, in the dark and at room temperature. Relative fluorescence intensity was measured in a flow cytometer and the percentage of cells in G0/G1, S and G2 phases was determined using the FlowJo software, applying the Dean – Jett – Fox model.

2.3.6 Analysis of ROS

The measurement of intracellular ROS was determined following the method described by Deng *et al* [38], with few modifications. This procedure was performed using the probe 2',7'-dichlorofluorescein diacetate (DCFH-DA), which in the presence of ROS is converted to the fluorescent compound 2',7' – dichlorofluorescein (DCF). Briefly, cells were seeded in 96-well black plates and incubated with complete growth culture medium for 24h. Next, medium was removed and cells were washed twice with PBS and treated with 10 μM DCFH-DA in medium without FBS. After 30 min incubation, excess was removed and the cells were washed again twice with PBS. They were then exposed to crude venom (IC20 and IC50 24h prepared in FBS-free medium) for 3h. Fresh medium was used as control. The exposure medium was replaced by culture medium without FBS and fluorescence was analysed in a plate reader with excitation and emission wavelengths of 475 and 528 nm, respectively.

2.3.7 Statistical analysis

For all experiments, at least 3 replicates, and four independent assays (for MTT) and three independent assays (for cell cycle analysis and analysis of ROS) were performed. All data are expressed as mean ± standard deviation (SD). SigmaPlot version 12.5 for Windows were used for the statistical analysis. Data were analysed by one-way ANOVA, followed by the Holm-Sidak's test ($p<0.05$). In the absence of assumptions of parametric ANOVA, one-way ANOVA on ranks followed by Dunnett's test ($p<0.05$) was performed. Mann-Whitney Rank Sum test was also performed to evaluate the differences between the two cell lines viability.

2.4 Results

2.4.1 Cell viability

MTT assay was performed to measure crude venom induced cytotoxicity on MNT-1 melanoma cells and non-tumorigenic HaCaT after 24, 48 and 72 h. The results are expressed by the percentage of viable cells after treatment and median growth inhibitory concentration (IC50).

2.4.1.1 Viability of MNT-1 melanoma cells after administration of *V. latastei* crude venom

The results obtained are shown in [Figure 7]. For 24h assay, the viability of MNT-1 decreased significantly in a dose-dependent manner ($p < 0.05$). However, it is important to note that there was a non-significant proliferative tendency for concentrations of 0.39-2 μg protein /mL. At concentrations of 2.5 and 3.12 μg protein/mL, a decrease in cell viability was found, becoming significant compared to the control only with the subsequent increase in the concentrations of venom to 6.25, 12.5 and 25 μg protein /mL ($p < 0.05$). According to the results, the IC50 value for melanoma cells exposed 24h to crude venom of *V. latastei* was 3.01 ± 0.09 μg protein /mL [Table III].

After 48h exposure, a significant decrease in cell viability was also noted with increasing venom concentration [Figure 7]. For the first two concentrations (0.39 and 0.78 μg protein /mL), a similar response to the previous assay was verified, with 119.00 and 102.60 % cell viability, respectively. Further increase in the concentrations led to a significant decrease in cell viability when compared to the control. An IC50 of 1.22 ± 0.13 μg protein /mL was obtained for this assay [Table III].

Similarly, a significant dose-dependent decrease in cell viability was observed in MNT-1 melanoma cells exposed 72h to crude venom concentrations. MNT-1 showed a significant decrease in cell viability above 1.56 μg protein /mL, when compared to the control. The IC50 value obtained in this treatment was approximately 0.73 ± 0.02 μg protein /mL [Table III]. Furthermore, comparing the variation of cell viability over time, it was observed that MNT-1 cell viability tends to decrease in a time-dependent manner ($p < 0.05$).

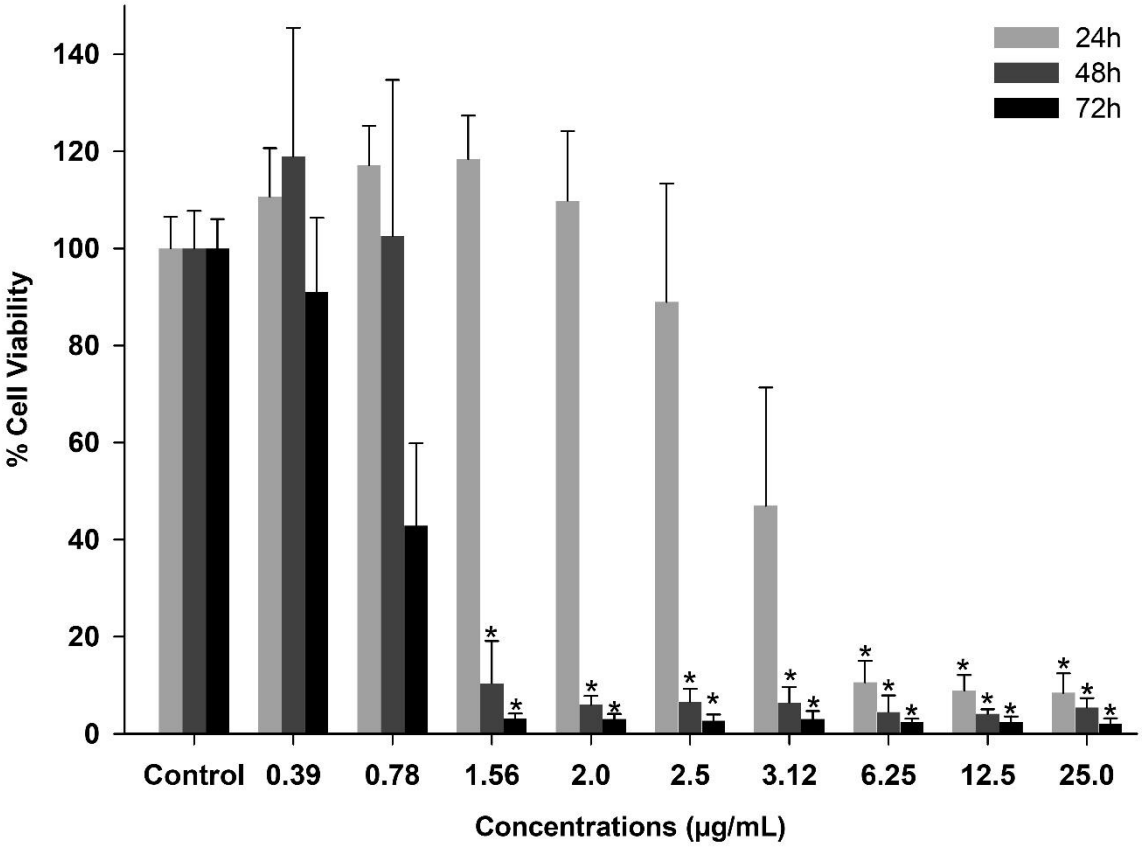

Figure 7 – Effect of *V. latastei* crude venom on the viability of MNT-1 cells at 24h, 48h and 72h. Results are expressed as mean ± SD of four independent experiments. Statistical analysis: * $p < 0.05$ compared to the control.

2.4.1.2 Viability of HaCaT after administration of *V. latastei* crude venom

The investigated concentrations led to a significant dose-dependent decrease in HaCaT cell viability ($p < 0.05$). It was also verified a tendency for the toxicity to increase overtime ($p < 0.05$) [Figure 8]. After 24h exposure, an increase in the concentrations of venom from control to 0.04, 0.09, 0.19 and 0.39 µg protein /mL induced a non-significant decrease in the percentage of viable cells. The subsequent increase in concentrations to 0.78, 1.56 and 3.12 µg protein /mL promoted a significant decrease in cell viability, reaching values of 18.45, 5.36 and 5.25 % respectively. An IC50 of 0.56 ± 0.03 µg protein /mL was calculated for this assay [Table III]. For 48h exposure, a similar response was found, with the three lower concentrations showing a non-significant decrease in cell viability relative to the control. However, from the concentration of 0.39 µg protein /mL, the cell viability decreased significantly, with values of 59.74, 6.29, 4.75 and 4.61%. The IC50 obtained for

this exposure time was 0.42 ± 0.03 µg protein /mL [Table III]. For 72h assay, although the reduction in cell viability was not significant at concentrations 0.04 and 0.09 µg protein /mL, the increase in venom concentrations led to a significant decrease in HaCat viability. The IC50 value obtained in this treatment was approximately 0.24 ± 0.01 µg protein /mL [Table III].

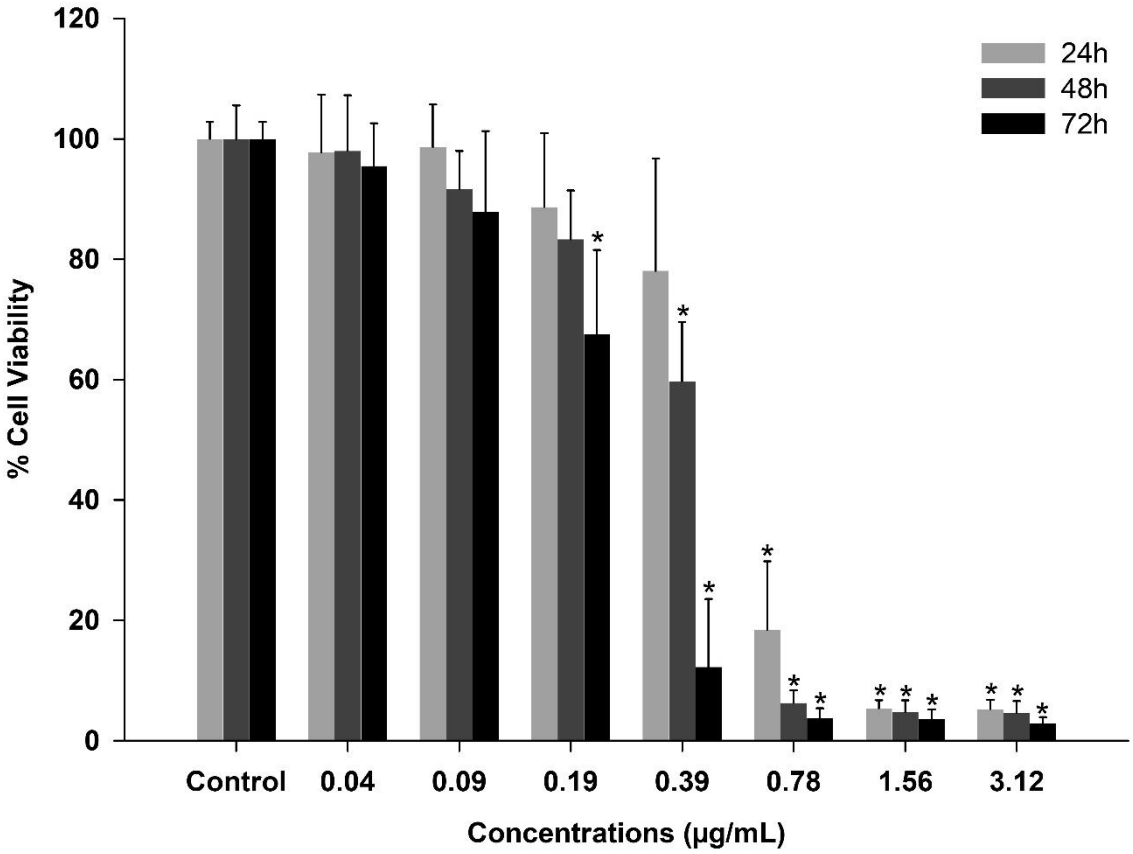

Figure 8 – Effect of *V. latastei* crude venom on the viability of HaCaT cells at 24h, 48h and 72h. Results are expressed as mean ± SD of four independent experiments. Statistical analysis: * $p < 0.05$ compared to the control.

Table III – IC50 values (μg protein/mL) of *V. latastei* crude venom treated cell lines.

Cell lines	Time		
	24h	48h	72h
MNT-1	3.01 ± 0.09	1.22 ± 0.13	0.73 ± 0.02
HaCaT	0.56 ± 0.03	0.42 ± 0.03	0.24 ± 0.01

2.4.1.3 Comparison of *V. latastei* venom cytotoxicity in the two cell lines

HaCaT Keratinocytes proved to be more sensitive to the venom and there was a need to readjust the range of concentrations tested. For the common treatments, differences were observed between cell lines at all exposure times ($p < 0.05$) [Figure 9]. Concentrations exhibited higher toxicity on HaCaT compared to MNT-1. For example, for the three exposure times, at the lowest concentration (0.39 μg protein /mL) HaCaT cell viability was 78.07, 59.74 and 12.24% for 24,48 and 72h, while for the same treatment, the viability of MNT-1 was 110.67, 119.00 and 91.09%.

Differences in the effect of snake venom are also observed in the calculated IC50. Based on the IC50 values, the crude venom has the most potent activity on HaCaT cells, as observed in table III. IC50 obtained for MNT-1 cells was approximately 5 times higher than IC50 calculated for HaCaT at 24h exposure and 3 times higher at 48 and 72h treatments.

2.4.2 Effect of crude venom on cell cycle dynamics

To investigate possible causes for the reduction in cell viability, the effect of crude venom on cell cycle was also performed. Briefly, both cell lines were treated for 24h and 72h with their respective IC20 and IC50 and cell cycle profiles were analysed by flow cytometry. The results are presented in Figures 10 and 11.

2.4.2.1 Effect of *V. latastei* crude venom on cell cycle profile in MNT-1 cells after 24h exposure

Crude venom treatment resulted in a significant decrease of cells in the G0/G1 phase from 50.5% to 45.9% (IC20) and 47.2% (IC50) [Figure 10A]. However, only cells exposed

to IC20 showed a significant reduction compared to control. Furthermore, it was also observed a significant accumulation of cells in the S-phase, with a significant increase in cells exposed to IC20 (31.9%) and IC50 (32.6%) compared to untreated cells (25.4%) [Figure 10A]. The proportion of cells in the G2/M phase tended to decrease, however no significant differences were observed [Figure 10A].

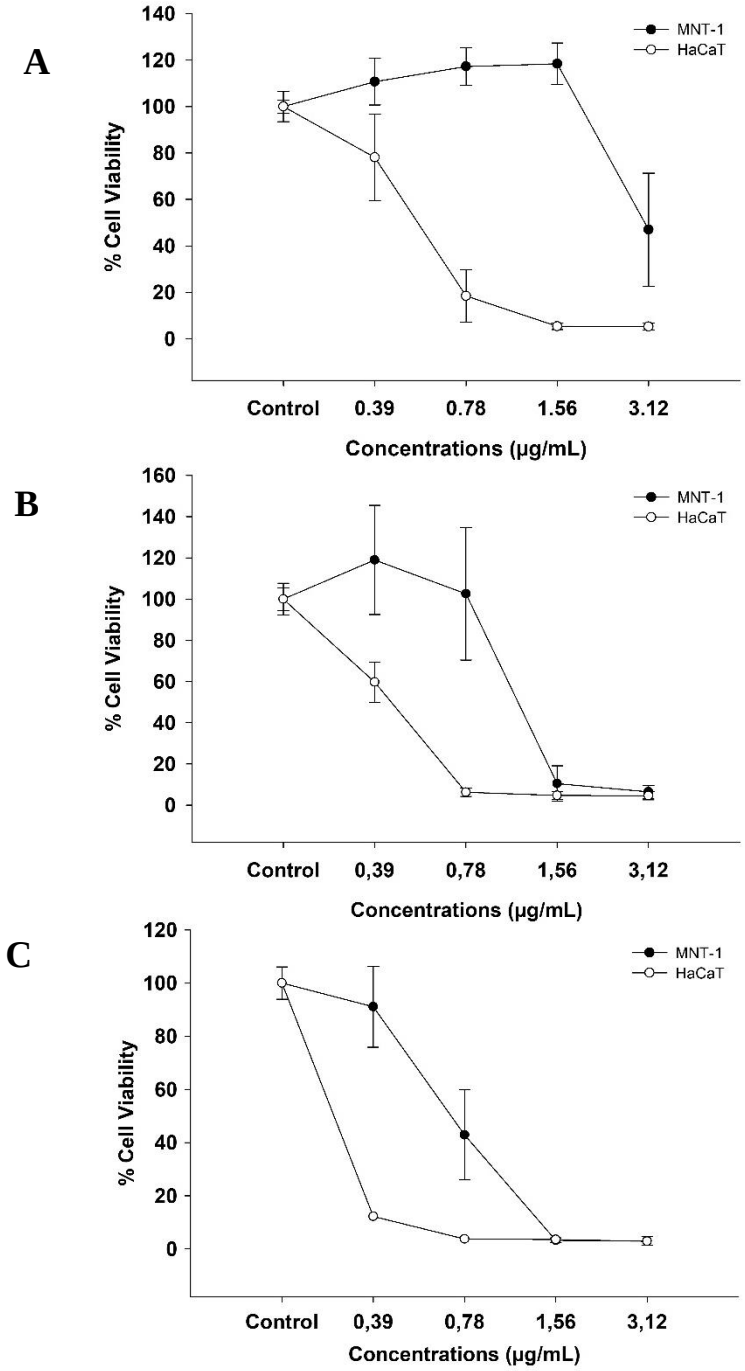

Figure 9 – Effect of *V. latastei* crude venom on the viability of MNT-1 and HaCaT cells at 24h (A), 48h (B) and 72h (C). Results are expressed as mean ± SD of four independent experiments.

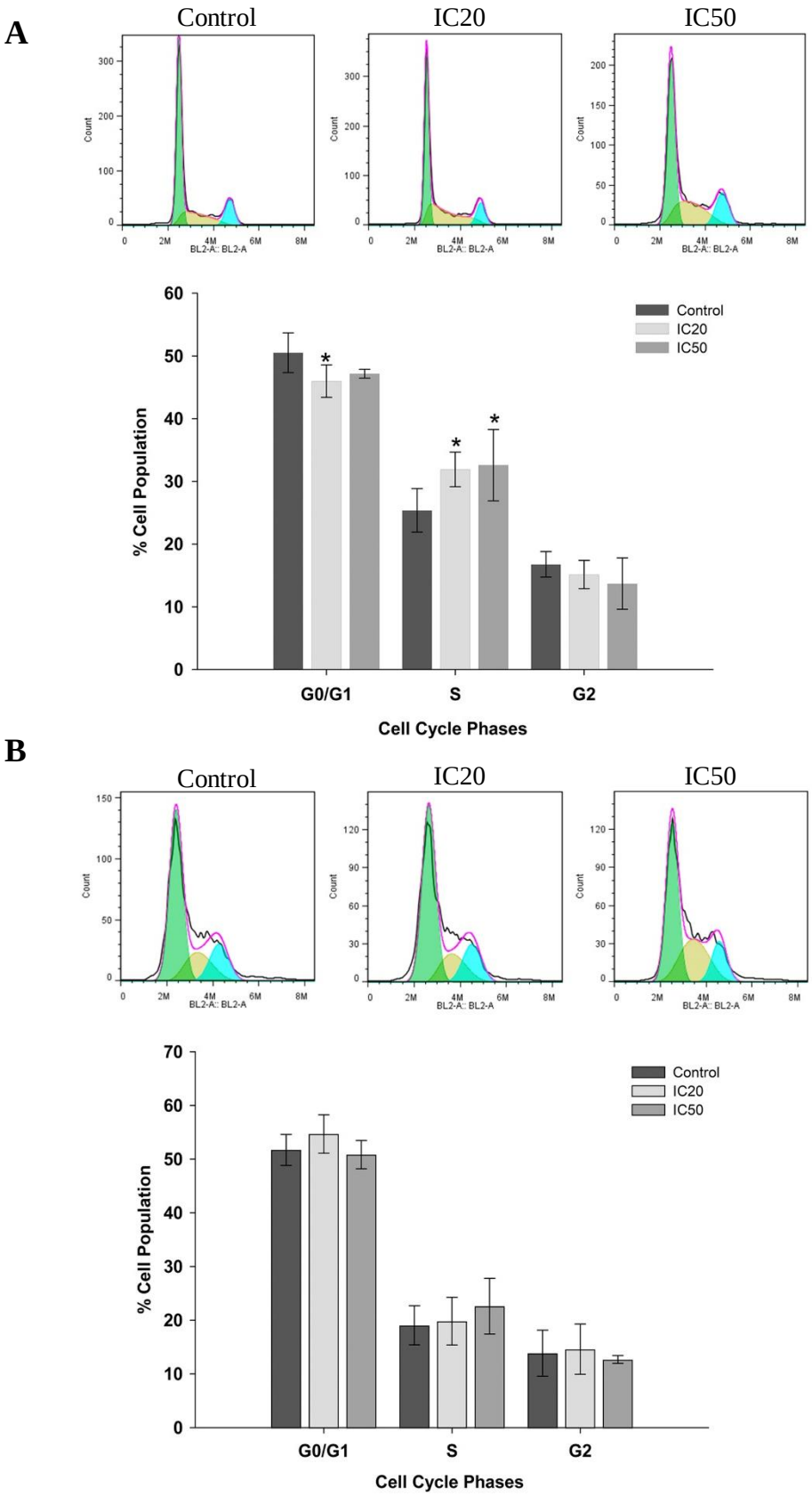

Figure 10– Cell cycle analysis of crude venom treated MNT-1 cells at 24h (A) and 72h (B). Green – G0/G1; Yellow – S; Blue – G2 Results are expressed as mean ± SD of three independent experiments. * $p < 0.05$ compared to the control.

2.4.2.2 Effect of *V. latastei* crude venom on cell cycle profile in MNT-1 cells after 72h exposure

After 72h exposure, the percentage of MNT-1 cells in the G0/G1 phase tended to decreased from 51.7% to 50.8% (IC50). A subtle increase was also found in IC20 exposure (54.7%) [Figure 10B]. However, in both cases, differences did not reach statistical significance. The proportion of cells in the G2/M phase followed a similar trend. In addition, for both IC20 (19.8%) and IC50 (22.6%), it was verified a non-significant accumulation of cells on S phase, compared to control (19.0%) [Figure 10B].

2.4.2.3 Effect of *V. latastei* crude venom on cell cycle profile in HaCaT cells after 24h exposure

Analysis of HaCaT cells exposed for 24h to *V. latastei* crude venom suggest a decrease in the percentage of G0/G1 cells, with a slight increase from 42.9% to 43.4% (IC20) followed by a decrease to 39.9% (IC50) [Figure 11A]. The percentage of cells population in the S-phase tended to increase from 23.5% in the control group to 26.2% at IC50 [Figure 11A]. A subtle decrease was observed in IC20 exposure (22.5%). Similarly, and accumulation of cells in G2/M has also been verified, from 21.4% in the untreated group to 21.3% (IC20) and 22.1% (IC50) [Figure 11A]. Nevertheless, in all cases no significant differences were observed.

2.4.2.4 Effect of *V. latastei* crude venom on cell cycle profile in HaCaT cells after 72h exposure

After 72h exposure, the results showed minimal changes in the proportion of cells in each phase with increasing concentration [Figure 11B]. As seen at 24h exposure, a non-significant decrease in G0/G1 cells was observed, with 47.1% (IC20) and 47.2% (IC50) compared to 48.1% in the control. Also, for the S-phase a slight increase on cell percentage was verified, from 21.6% in the untreated group to 22.9% (IC50). The values obtained on IC20 exposure were similar to those in the control (21.1%). In addition, statistical differences were not verified. Similarly, the proportion of cells in G2/M increased in a non-significant way with concentration.

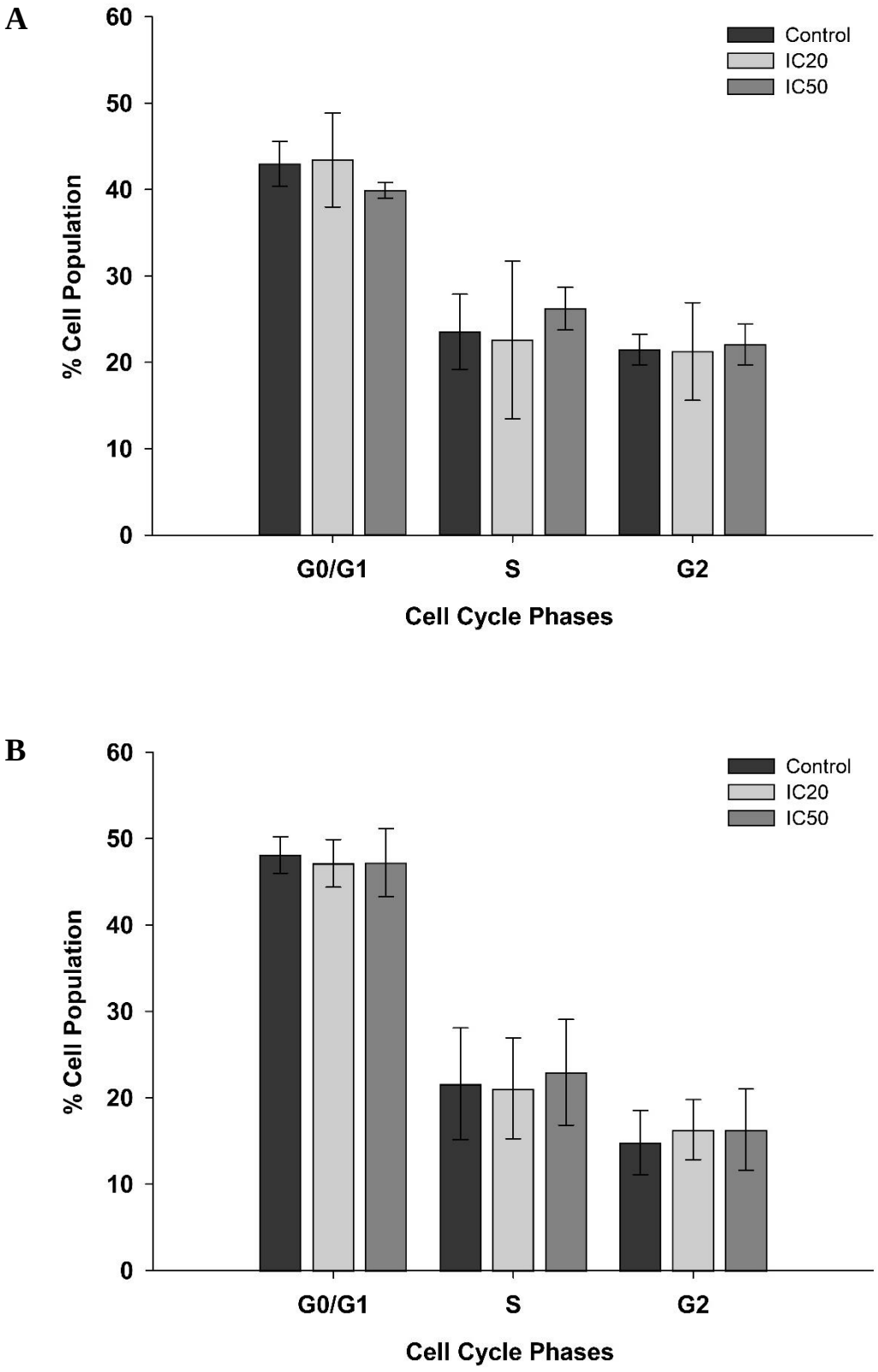

Figure 11 – Cell cycle analysis of crude venom treated HaCaT cells at 24h (A) and 72h (B). Results are expressed as mean ± SD of three independent experiments.

2.4.3 Effect of crude venom on the intracellular ROS levels

Intracellular ROS generation in HaCaT and MNT-1 cells exposed to crude venom was assessed by DCFH-DA fluorescence.

As presented in Figure 12, the levels of ROS of MNT-1 cells increased in a dose-dependent manner (IC20: 118.2%; IC50: 188.9%). Cells exposed to IC50 revealed that ROS generation was significantly higher compared to control levels ($p<0.05$).

Regarding HaCaT cell line, it was observed a slight decrease in ROS formation in IC20 treated cells (79.6%), while exposure to IC50 led to an increase in intracellular ROS levels (125.2%) [Figure 13]. Nonetheless, no significant differences were recorded compared to the control.

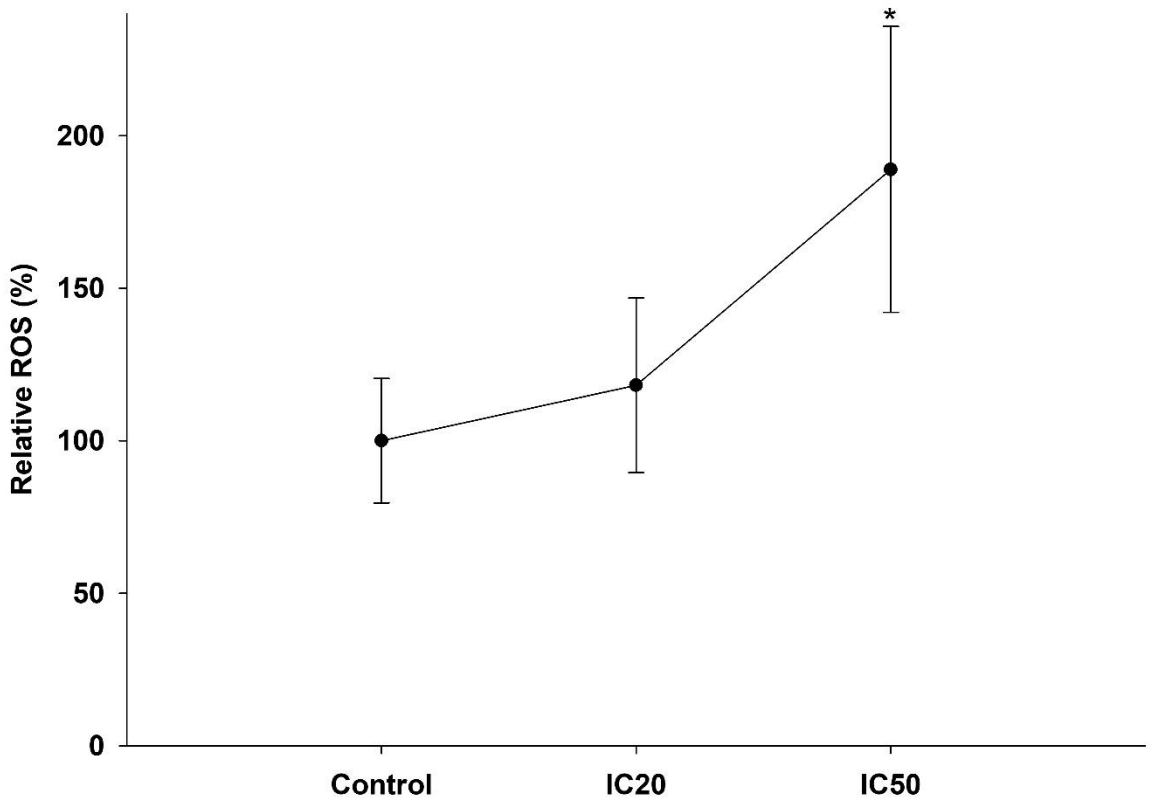

Figure 12 – Relative abundance of intracellular ROS in MNT-1 cells exposed to *V. latastei* crude venom for 3h. Results are expressed as mean ± SD of three independent experiments. * $p < 0.05$ compared to the control.

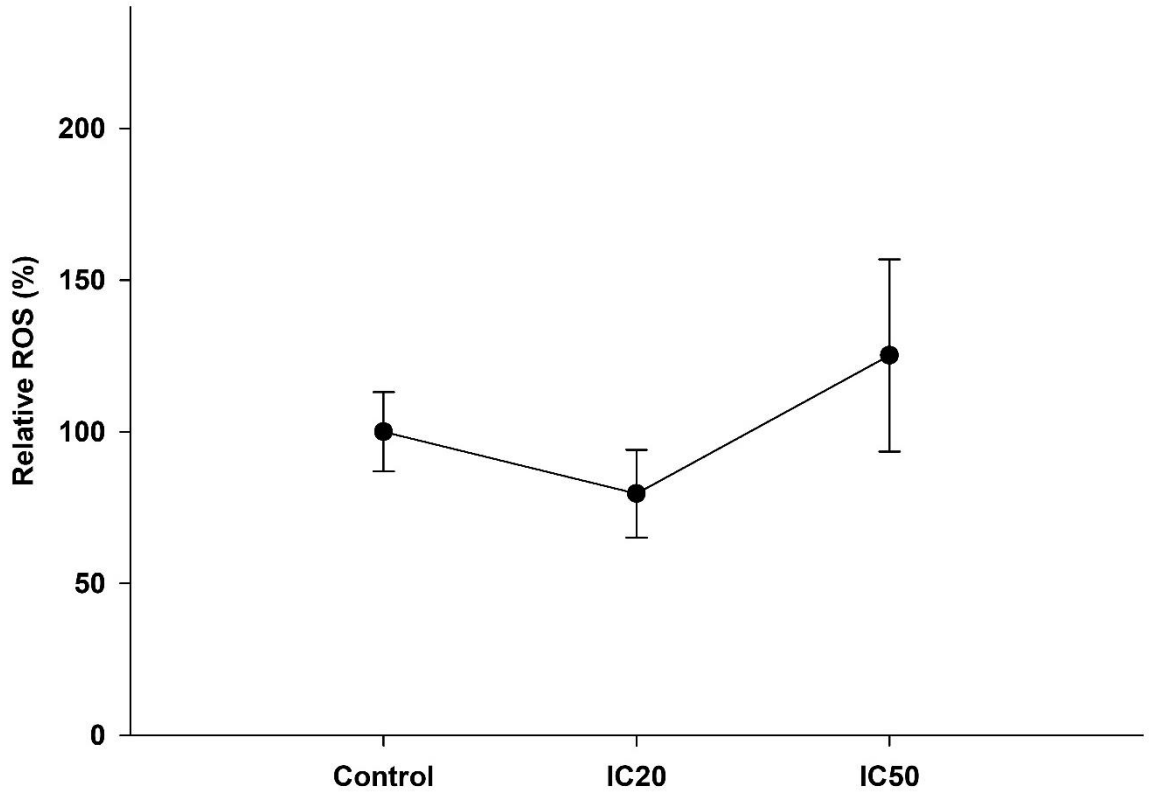

Figure 13 – Relative abundance of intracellular ROS in HaCaT cells exposed to *V. latastei* crude venom for 3h. Results are expressed as mean ± SD of three independent experiments.

2.5 Discussion

Snake venoms are currently recognized as a truly gold mine, containing new compounds of therapeutic interest that can be applied for different clinical purposes [26,30]. As far as the cancer is concerned, several studies have focused the antitumoral potential of snake venoms and some of their constituents in order to develop new cancer therapies. Nalbantsoy *et al* (2016) [39] previously demonstrated the cytotoxic activity of *Montivipera wagneri* venom against different cancerous and noncancerous cell lines. More potent activity was observed against human lung adenocarcinoma (A549) and human colorectal adenocarcinoma (CaCo-2) cells treated with venom, exhibiting a lower IC50 than that obtained for the control positive drug agent parthenolide. In the opposite direction, the IC50 of non-tumor cells was higher than positive control. In another study, *Cerastes cerastes* venom presented a relevant cytotoxic effect against human glioblastoma carcinoma cells (SHY5Y) compared to other cell lines tested. Further venom purification revealed that fraction of heterodimeric disintegrins was the most active [17]. Maria *et al* (2014) [40] have shown that cellular viability of B16F10 murine melanoma decreased after treatment with jararhagin, a toxin isolated from *Bothrops jararaca* venom, and jari (jararhagin with catalytic

domain inactivated). These in vitro treatments also led to cell cycle alterations, apoptosis induction (jararhagin) and necrosis (jari), as well as, in vivo experiments revealed antiproliferative and antimetastatic effects. Nevertheless, there is no study in the literature exploring the antitumor potential of *Vipera latastei* crude venom. Therefore, in a first phase it is essential to obtain a screening study in order to characterize the bioactivity of this venom.

In the present work, we explored the cytotoxic effects of *V. latastei* crude venom on human melanoma MNT-1 and human Keratinocyte HaCaT cells. Crude venom presented a significative cytotoxic activity in a concentration-dependent manner for both cell lines at all exposure times. Comparison of these results with others in the literature cannot truly be made since this is the first study that addresses the antitumor potential of *V. latastei* crude venom in skin cancer cell lines. However, they are consistent with the cytotoxic effects observed in several nontumor and tumor cells treated with different snake venoms belonging to *Viperidae* family [19,41–43]. It should be noted that at 24h and 48h exposures, a non-significant increase in cell viability of MNT-1 cells treated with lowest doses of crude venom was observed, followed by a decrease with increasing concentration. It has also been found that stimulation at lower doses tends to disappear over time of exposure. These results suggest that it may be a biphasic-dose response named hormesis, which is characterized by a stimulation in lower doses and an inhibition in higher doses [44]. Stimulatory responses may represent an adaptive action or a compensatory effect of cells exposed to a moderate and intermittent stress [45,46]. Furthermore, low dose stimulation may no longer be noted in case of chronic stress [47]. Similar effect was observed by Yalcin *et al* (2014) [48].

A different cytotoxicity of crude venom was found between the two cell lines, indicating a possible non-selective antitumor effect. In fact, HaCaT cells were the most affected by exposure to *V. latastei* venom. For example, at concentration 3.12 µg protein /mL, HaCaT cell viability was 5.25% while for MNT-1 a value of 46.99% was registered. This is also supported by the IC50 values shown in table III. In contrast, Sawan *et al* (2017) [19] reported that *Montivipera bornmuelleri* crude venom presented a more potent cytotoxic activity for skin tumor cell lines (benign A5 and malignant II4 keratinocytes) than for nontumor cell line (human Keratinocyte HaCaT). In a similar study, *Vipera ammodytes* crude venom reduced significantly the viability of human colorectal adenocarcinoma (CaCo-2), human breast adenocarcinoma (MCF-7), human glioblastoma-astrocytoma (U-87 MG)

and human prostate carcinoma cells, exhibiting less activity against African green monkey kidney epithelial noncancerous cells (Vero) [49]. On the other hand, Ozen *et al* (2015) [50] showed a higher cytotoxic effect against Vero cells treated with *Macrovipera lebetina obtuse* venom. One of the major problems of conventional therapies used in cancer treatment (eg: chemotherapy) is related to the non-selective action of therapeutic agents, affecting both tumor and nontumor cells, which may result in severe side effects. Although in the present study the crude venom of *V. latastei* does not exhibit a selective activity against MNT-1 cell line, it is not pertinent to rule out its therapeutic potential in this area without purifying the active components.

Further experiments were performed to investigate the mechanisms responsible for the cytotoxic activity of crude venom. The cell cycle is a basic cellular process and essential for cell proliferation. It is a continuous and repetitive event, that is divided into two major phases - interphase (G0, G1, S and G2) and mitosis - that result in cell division [51]. Cell cycle is usually regulated by specific molecular mechanisms that ensure genome integrity and prevent the transmission of DNA errors to future generation cells [52–54]. In response to DNA damage, these mechanisms lead to cell cycle arrest to allow DNA repair, and in more severe cases, can result in cell death. These responses depend on the type of damage and the phase of the cell cycle in which it is detected [55]. However, tumor cells are characterized by a unregulated cell cycle that results in uncontrolled cell proliferation [56]. Cell cycle disruption is one of the preferred strategies for cancer treatment using therapeutic agents that target components of these pathways [53,57]. Cell cycle analysis were performed by flow cytometry to evaluate the effects of *V. latastei* crude venom on the MNT-1 and HaCaT cell cycle. It was shown that crude venom affected the progression of MNT-1 cell cycle after a 24h exposure, causing a delay in the S phase and consequent reduction in the percentage of cells in G2. Toxins present in venom can promote a genotoxic stress during DNA replication leading to cell cycle arrest during the S phase [58]. This action may be related to mechanisms involving ATM (ataxia telangiectasia) and ATR (ataxia telangiectasia – related protein) and checkpoint proteins such as CKI's, CDK's and CHK [54,58]. In contrast, no significant change in HaCaT cell cycle was observed for the same exposure time. At 72h also no significant change was verified for both tumor and nontumor cells. Few studies have focused on the effects of snake crude venom on the cell cycle, and none of which refer to skin cancer cell lines. Bernardes-Oliveira *et al* (2016) [59] reported that

Bothrops jararaca and *Bothrops erythromelas* venoms led to cell cycle arrest of SiHa human squamous cell carcinoma in G0/G1 phase. Similar results were obtained by Son *et al* (2007) [60]. It was observed that *Vipera lebetina turanica* crude venom induced cell cycle blockade of PC-3 prostate cancer cells in G0/G1 and G2/M phases. Despite the differences with our study, these data suggest that interference with tumor cell cycle may be one of the mechanisms of cytotoxicity exhibited by snake venoms. It should be noted that these differences may be related to the specific venom composition of each snake species and the cell type tested. The results obtained with HaCaT cells may indicate that *V. latastei* crude venom has no significant influence on the cell cycle of these cells. On the other hand, we hypothesize that the complexity of the venom and the quantitative differences in its components may mask these effects. The presence of proteins such as phospholipases A_2 and snake venom metalloproteases may act on cells, promoting membrane damage, which may preclude the action of other compounds in cell cycle dynamics [26,61–64]. In any case, purified fractions of the venom should be tested. Some studies focus on these experiments revealing remarkable results [26,40,57].

Tumor cells have been associated with increased oxidative stress related to a high abundance of ROS. Despite contributing to the molecular and biochemical changes characteristic of cancer cells, ROS appears as an important therapeutic target, which is capable of causing severe cell damage and leading to cell death [65]. Some chemotherapeutic drugs promote ROS increase and consequent tumor cell apoptosis [66,67]. Here, we verified that MNT-1 cells treated with *V. latastei* crude venom exhibited an increase in ROS formation in a dose-dependent manner, which was significant after exposure to the IC50. Regarding HaCaT cells, there was a non-significant decrease in ROS production on IC20 exposure, which may be associated with antioxidant enzymes. As observed in tumor cells, the ROS ratio increased in IC50, however, no significant differences were found compared to the control. Similar results were noted in colorectal and breast cell lines (HCT-8 and MDA-MB-231) exposed to 4 different snake venoms (*Bitis arietans, Cerastes gasperettii*, *Echis coloratus* and *Echis pyramidum*) [68]. These may be related to the activity of L-amino acid oxidase that may be present in the venom composition of this species. L-amino acid oxidase catalyse the oxidative deamination of an L-amino acid leading to the generation of ROS [69]. CV-LAAO isolated from *Cerastes vipera* venom promoted a cytotoxic activity against MCF-7 breast cancer cell lines, which may be a consequence of the production of

H_2O_2 since in the presence of catalase this action was inhibited [70]. Furthermore, some studies propose that the activity of Sv-LAAO and consequent increase in ROS production lead to the activation of apoptosis [71,72]. Park *et al* (2012) [73] observed in colon cancer cell lines (HCT116 and HT29) that *Vipera lebetina turanica* venom induced an increase on ROS generation that may mediate the activation of JNK pathway and lead to the upregulation of TRAIL receptors (DR4 and DR5) which may result in cell death. Remarkably, in the presence of an antioxidant (N-acetylcysteine) these effects were not verified, including snake venom cytotoxicity. In addition, the increase of ROS levels in MNT-1 cells may have contributed to cell cycle arrest, since the oxidative stress is related to DNA damage.

Snake venom consists of a complex mixture of proteins which may exhibit a set of mechanisms associated with cytotoxic activity. Besides the above mentioned Sv-LAAO, other proteins may perform specific effects that contribute to the results observed here. Several studies reported the antitumor potential of PLA_2 proteins isolated from different snake venoms, describing not only their cytotoxic activity, but also antimetastatic and antiangiogenic capabilities [20, 26]. In addition to the membrane damage associated with the activity of PLA_2s, these results may also result from their involvement in apoptosis activation by interference with pro- and anti-apoptotic gene regulation, as well as, due to a possible ROS production and genotoxic effect [20,26,61,63,74]. Furthermore, some PLA_2 are able to interact with specific receptors related to cell growth and proliferation [21,75]. Other proteins such as snake venom C-type lectins, snake venom metalloproteases and disintegrins may also act leading to similar results. Although most often associated with antimetastatic and antiangiogenic activities, there are some studies that have noted that these toxins are also capable of promoting decreased cell viability [25,27,64,76].

Vipera latastei crude venom exhibited potent cytotoxic activity for both MNT-1 and HaCaT cells. However, our results suggest that the venom may not show a selective antitumor activity since it did not reveal greater selectivity for the tested tumor cell line. Additionally, crude venom led to a S-phase arrest of the MNT-1 cell cycle, having no significant effect on HaCaT. Furthermore, crude venom treatment resulted in an increase of ROS generation, especially in tumor cell line, which may be one of the possible mechanisms that may lead to cell death. Given the lack of knowledge about the antitumor potential of *V. latastei* venom, this work represents the first study in this regard. Our results demonstrate that this venom may be a relevant source of compounds that can be applied to treat tumors,

including melanomas. However, further research is required to determine the constituents responsible for cytotoxic activity and the mechanisms that result in this effect. It is also suggested to study other types of antitumor activity such as antimetastatic and antiangiogenic potential.

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Chapter 3- Antibacterial activity of *Vipera latastei* crude venom

3.1 Abstract

The emergence of multi-resistant bacteria is a burden in current society, being a serious threat to human health. The inefficiency of several known antibiotics and limited options for treating resistant bacterial infections, lead to a high mortality risk and jeopardize future life quality. Hence, there is an urgent need to develop new therapeutic agents that can represent an added value in the treatment of these infections. Despite the severe damage, snake venoms are a complex cocktail of proteins and other molecules that may provide an interesting source of new therapeutic elements, including compounds with antimicrobial activity. In this study, we aimed to assessing the antibacterial potential of *Vipera latastei* crude venom against pathogenic bacteria. To do so, Gram positive (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 14579) and negative bacteria (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853) were exposed, using the disc diffusion method, to several concentrations of *V. latastei* venom.

Our results showed that the growth of both Gram positive and negative bacteria was inhibited. For *E. faecalis*, *S. aureus*, *E. coli*, *K. pneumoniae* and *S. typhimurium* the inhibition was observed at a concentration of 800 μg protein/mL, while for *P. aeruginosa* and *B. cereus* the antibacterial effects of crude venom were only found at the concentration of 1600 μg protein/mL. However, the obtained inhibition zone diameters were significantly lower than positive controls (tetracycline and chloramphenicol).

The present findings suggest that *V. latastei* venom presents antibacterial activity, with potential to be explored in future studies. This is the first study focusing on the antimicrobial capacity of the venom of this species.

Keywords: Multi-resistant bacteria; Resistant bacterial infections; Snake venom; *Vipera latastei*; Antibacterial activity

3.2 Introduction

Antibiotic resistance is currently one of the most serious problems globally [1]. Despite their initial success and unquestionable contribution to improving the population's life quality, presently some antibiotics are completely ineffective in treating several bacterial infections, posing a dangerous threat to human health [2,3]. It is estimated that in the United States approximately 2 million cases of multi-resistant bacterial infections are reported annually, resulting in at least 23,000 deaths [4]. In Europe are observed each year about 25, 000 deaths associated with antibiotic resistant bacteria [5]. Organisms as *Staphylococcus aureus, Enterococcus faecium, Streptococcus pneumoniae, Acinetobacter baumannii, Klebsiella* and *Salmonella* species, *Escherichia coli* and *Pseudomonas aeruginosa* are among the most threatening [1]. Some strains of *P. aeruginosa* are resistant to most of the antibiotics used to treat infections, like carbapenem and cephalosporins [6,7]. According to WHO, *P. aeruginosa* resistant to carbapenem constituted 19% of isolates in Europe, presents a great risk of mortality with few treatment options [8]. Methicillin resistant *S. aureus* strains are other common example of this problem [9]. MRSA-*S. aureus* infections result in approximately 11, 000 deaths in the United States, representing one of the deadliest bacteria according to Centers for Disease Control and Prevention (CDC) [4]. In Europe, Carbapenem- resistant *Klebsiella pneumoniae* has been the leading cause of population morbidity and mortality related to antibiotic resistance [10]. Considering this present problem, much related to the overuse and misuse of antibiotics, and also with the decrease in the discovery of new antibiotics, the search for new therapeutic agents urges [11–14].

Snake venoms are a rich and complex source of compounds with therapeutic interest [15–17]. In current medicine, it is possible to find the presence of therapeutic agents from snake venoms for the treatment of several diseases [18]. Recent studies regarding their antibacterial potential have reported remarkable results, showing an inhibitory capacity of different venoms against various Gram positive and negative species. *Vipera latifii* crude venom exhibited the ability to inhibit the growth of *S. aureus* and *Bacillus subtilis* [19]. Ciscotto *et al* (2009) verified that venoms from *Bothrops jararaca* and *Bothrops jararacussu* inhibited the growth of *Eubacterium lentum, Peptostreptococcus anaerobius, Propionibacterium acnes, S. aureus, S. epidermidis, Porphyromonas gingivalis, Prevotella intermedia, Salmonella typhimurium* and *Pseudomonas aeruginosa* [20]. In addition, proteins isolated from the venom of different snakes also have antibacterial activity [21–25].

Taking into account the urgent need for new antibiotics, the excellent results found in the literature and the lack of knowledge about the therapeutic properties of *Vipera latastei* venom, this study aims to investigate the antimicrobial potential of the venom of this species. To attain this objective, in this study the crude venom of the Snub-nosed viper venom was extracted and tested against several Gram positive and negative pathogenic bacteria.

3.3 Material and Methods

3.3.1 Collection and preparation of venom

Adult animals from both genders of *Vipera latastei* were collected manually in Castro de São Paio, Portugal, between January and June 2019. Venom of these individuals was extracted using parafilm coated microtubes, and stimulating bite, without exerting any pressure on the venom glands, and stored at -80ºC. These procedures were done in collaboration with Dr Fernando Martínez-Freiría (CIBIO-InBIO). For later use, crude venoms were lyophilized and stored at -80ºC.

3.3.2 Determination of protein content

Before antibacterial tests, 1 mg of each freeze-dried venom sample was diluted in 1 mL of ultra-pure water, preparing a concentration of 1 mg crude venom/mL. Protein concentrations of these samples were then determined by Bradford assay [26] using a plate reader at a wavelength of 595 nm. Bovine γ – globulin was used as a standard. After that, the concentrations set for the assays (μg protein/mL) was prepared considering the protein concentration obtained for each sample in use.

3.3.3 Antibacterial activity

Freeze-dried crude venom was dissolved in 50mM Tris-HCl buffer (pH 7.4), filtered with a sterile 0.22 μm pore filter (Frilabo) and diluted to obtain the concentrations set for the assays (50 – 1600 μg protein/mL). Antibacterial activity of crude venom samples was tested against Gram-negative bacteria *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, and Gram-positive bacteria *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 14579 by disc diffusion susceptibility tests following the methods described by Lalitha (2004) [27]. Briefly, an isolated colony of each

bacteria was selected from a tryptone soy agar (HIMEDIA) plate, cultured in tryptic soy broth (Liofilchem) and incubated under continuous shaking at 37ºC overnight. The turbidity of bacterial suspensions was measured at 625 nm and compared to that of 0.5 McFarland standard. Then, suspensions were adjusted to obtain 1 x 10^8 colony forming units (CFU)/mL. Each bacterial inoculum was uniformly spread using a sterile cotton swab on a squared Petri dish (120 x 120 mm) (Corning) containing Muller Hinton (MH) Agar. Sterile paper discs (6 mm diameter) were applied to the MH agar surface and impregnated with 15 µL of each venom concentration. Tetracycline (10µg/disc, Oxoid) and Chloramphenicol (10µg/disc, Bioanalyse) were used as positive controls and 50mM Tris-HCl buffer (pH 7.4) was used as a negative control. The plates were incubated for 24h at 37ºC. After that, the diameter of inhibition zones was measured. All experiments were performed in triplicate.

3.3.4 Statistical analysis

Data are expressed as mean ± standard deviation (SD). SigmaPlot version 12.5 was used for the statistical analysis. One-way ANOVA followed by Holm-Sidak test was performed to analyse statistical differences between data. The differences were considered significant at $p<0.05$.

3.4 Results

3.4.1 Antibacterial activity

The antibacterial activity of *V. latastei* venom were evaluated using the disc diffusion method. The results obtained are listed in Table IV and represented in [Figure 14].

No inhibitory response was found for the Tris-HCl negative control. Overall, the results showed a similar pattern of inhibition between Gram-positive and Gram-negative bacteria. Only at crude venom concentrations of 800 and 1600 µg protein/mL was observed the presence of inhibition zones for *E. faecalis, S. aureus, E. coli, K. pneumoniae* and *S. typhimurium*. The growth of *P. aeruginosa* and *B. cereus* was not inhibited at 800 µg protein/mL, however these bacteria showed susceptibility to venom at 1600 µg protein/mL. Furthermore, at concentrations of 50, 100, 200 and 400 µg protein/mL no inhibition zones were visible for the mentioned microorganisms. In the tests with *K. pneumoniae* two distinct inhibition zones were observed, which became more evident at 800 and 1600 µg protein/mL.

Partial inhibition characterized by showing growth inhibition but with development of some resistant colonies can still be considered at concentrations of 100-400 μg protein/mL. Regarding Gram-positive bacteria, *E. faecalis* showed the highest inhibition diameter compared to *S. aureus* and *B. cereus*, at both concentrations. For Gram-negative bacteria, *K. pneumoniae* exhibited an inhibition diameter greater than those obtained for the other strains. Nevertheless, the inhibition zones visible after treatment with crude venom were always lower than those formed by tetracycline and chloramphenicol.

Table IV - Antibacterial activity of *V. latastei* crude venom accessed through the disc diffusion method. Diameter of the growth inhibition zone was measured in millimetre (mm).

Bacteria	**Tris-HCl**	**Crude Venom concentration (μg protein/mL)**						**Tetracycline**	**Chloramphenicol**
		50	100	200	400	800	1600		
E. faecalis	-	-	-	-	-	8.24 ± 4.22[a]	10.44 ± 1.75[b]	30.83 ± 4.62[c]	30.56 ± 5.89[c]
S. aureus	-	-	-	-	-	7.66 ± 0.75[a]	9.89 ± 0.60[b]	27.20 ± 1.91[c]	28.49 ± 2.25[d]
B. cereus	-	-	-	-	-	-	9.46 ± 1.54[a]	24.90 ± 6.37[b]	30.15 ± 4.41[c]
E. coli	-	-	-	-	-	7.55 ± 1.07[a]	8.33 ± 0.91[a]	30.24 ± 2.32[b]	31.50 ± 3.00[c]
S. typhimurium	-	-	-	-	-	8.10 ± 1.00[a]	9.59 ± 1.83[a]	28.03 ± 5.18[b]	31.13 ± 3.89[b]
K.pneumoniae	-	-	-	-	-	8.21 ± 1.17[a]	9.87 ± 1.37[b]	26.28 ± 2.75[c]	14.52 ± 3.32[d]
	-	-	8.05 ± 1.53*	10.49 ± 1.42*	11.87 ± 1.88*	12.94 ± 1.86*	15.82 ± 2.07*		
P. aeruginosa	-	-	-	-	-	-	8.73 ± 0.94[a]	36.18 ± 7.94[b]	30.65 ± 5.43[c]

Each diameter of the growth inhibition zone is represented as mean ± standard deviation of three independent assays.

Different letters in the same line represent significant difference ($p<0.05$)

- : Not detected

* Partial inhibition zone diameter

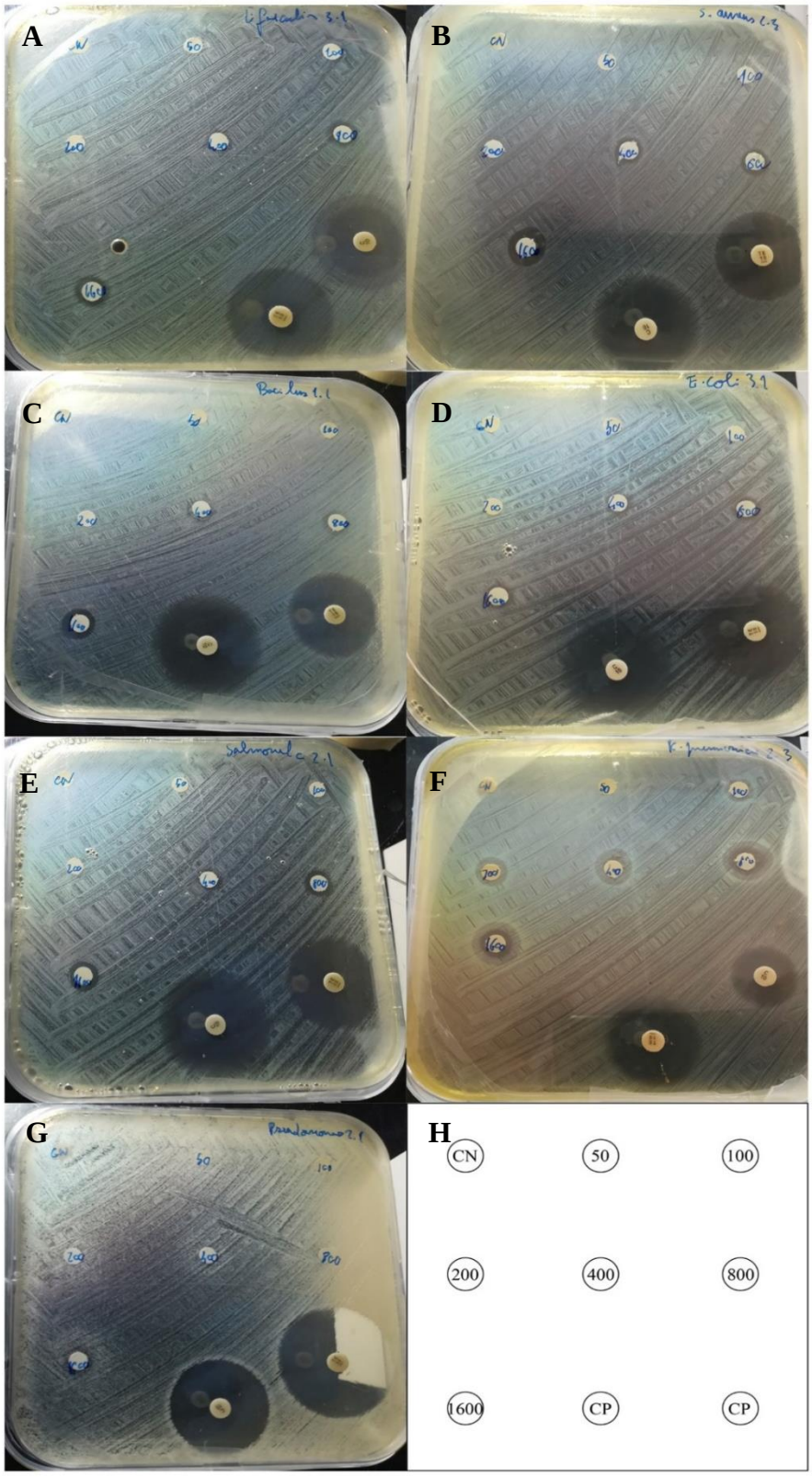

Figure 14 – Growth inhibition of different pathogenic bacteria by *V. latastei* venom. A - *E. faecalis*; B – *S. aureus*; C – *B. cereus*; D – *E. coli*; E – *S. typhimurium*; F - *K. pneumoniae*; G – *P. aeruginosa*; H – Schematic map: CN – negative control and CP – positive control.

3.5 Discussion

Antibiotic resistant bacteria infections pose a severe threat to human health, being one of the leading causes of death worldwide [28,29]. The lack of options to combat these microorganisms exacerbates this problem, which may result in an increase in infections rates and consequently higher mortality in the future [30,31]. Thus, there is an urgent need for solutions and a keen interest in the discovery and development of new agents that could be applied in the infections' treatment [32]. Due to the great diversity of proteins in its constitution and its biological and biochemical activities, snake venom represents a rich and promising source of bioactive molecules with high therapeutic interest that may constitute new drugs for various clinical purposes [33,34]. The results evidenced in the literature and the necessity to develop new antibiotics for the treatment of multi-resistant bacterial infections, validate the snake venom as appealing sources of antimicrobial agents.

In the present study, the antibacterial potential of *V. latastei* crude venom was evaluated, testing it against 7 pathogenic bacteria through disc diffusion method. The results obtained showed for the first time the antibacterial activity of the crude venom of this species, revealing an inhibitory action against all bacteria tested. This activity is in accordance with the antimicrobial effect observed in the venom of related species. Crude venoms of vipers such as *Bitis gabonica rhinoceros, Crotalus adamanteus, Daboia russelli russelli* and *Daboia russelli siamensis,* inhibited *Burkholderia pseudomallei* growth [23]. Ferreira *et al* (2011) [35] noted that venoms from *Agkistrodon rhodostoma* and *Bothrops atrox* exhibited an antibacterial effect against *E. faecalis* and *S. epidermidis*, while venom of *Bothrops jararaca* inhibited *S. aureus* growth. Furthermore, inhibition was also detected in *P. aeruginosa*, *S. enterica* serovar Typhimurium, *S. aureus*, *Micrococcus luteus* and *Corynebacterium glutamicum* treated with *Crotalus durissus terrificus* crude venom [36]. These results corroborate the conclusions reported by Charvat *et al* (2018) [37]. According to their study, venoms mainly from species belonging to the Viperidae family present the most notorious antimicrobial effect. Similar conclusions were also described by Stiles *et al* (1991) [38].

V. latastei crude venom exhibited a broad spectrum of activity and it should be noted that the susceptibility verified in this study is identical between Gram-positive and negative bacteria, which may indicate that the structural characteristics of these organisms do not influence the antibacterial mechanisms promoted by exposure to crude venom. Similar

results were observed for *B. jararaca* and *B. jararacussu* venoms, which equally inhibited the growth of several Gram-positive and negative bacteria, including *S. aureus* and *S. typhimurium*. However, no antimicrobial action against *E. coli* and *E. faecalis* was shown [20]. In contrast to what was verified in these works, crude venom of *Vipera latifii* had a differential antimicrobial effect [19]. Moreover, we observed that the inhibition zone diameters obtained at the exposure concentrations were significantly lower than those shown by positive controls. For example, the diameter of the inhibition zone measured for *E. coli* at concentration of 800 μg protein/mL was 7.55 ± 1.07 mm, while for chloramphenicol and tetracycline was 31.50 ± 3.00 and 30.24 ± 2.32 mm, respectively. These results may suggest that the venom may have presented a weaker activity against the bacteria tested than that exhibited by chloramphenicol and tetracycline. Nonetheless, the antibacterial activity of *V. latastei* venom should be considered and explored in further studies. An identical effect was also reported against *E. coli* exposed to the crude venom of Russell's viper (*Daboia russelli russelli*). It was observed that venom of Russell's viper inhibited *E. coli* growth with an inhibition zone diameter of 7.8 ± 0.83 mm, which is far below the value obtained for the Chloramphenicol (31.8 ± 1.64 mm). In contrast to our study, a strong activity against *S. aureus* was noted, presenting inhibition zones similar to those of positive controls (chloramphenicol and streptomycin) [39]. Regarding the *K. pneumoniae* tests, partial inhibition zones were observed in our study. We hypothesized that this partial inhibition may represent a heteroresistance phenomenon, which results from the existence of a subpopulation of resistant bacteria in a major susceptible population that can grow in the presence of antibiotic [40].

Venom composition may be associated with activity identified in various snake venoms. This suggest that the type of protein, its activity and even its relative amount in the venom profile may influence inhibitory capacity of snake venoms and consequently result in different patterns of antibacterial activity [23,35,38,39,41–43]. Antibacterial activity may be related to the multiple biological effects exhibited by some venom toxins such as PLA_2 and SV-LAAO. Previous studies have demonstrated that these proteins may promote membrane damage, resulting in bactericidal effects. PLA_2 toxins are very common among viper venoms and may act enzymatically leading to the digestion of membranes lipids, as well as, they may present a non-enzymatic activity that interferes with membrane structure and permeability through the formation of pores [22,39,44,45]. Furthermore, SV-LAAO

toxins may exhibit antibacterial activity, leading to membrane damage mediated by oxidative stress associated with H_2O_2 production [21,46,47].

In conclusion, the present results suggest that *V. latastei* crude venom exhibits promising antibacterial activity against both Gram positive and negative bacteria. Here, we provided the first study about the antimicrobial potential of *V. latastei* venom and demonstrated that it may be an important source of new molecules with antibacterial properties. Further studies may include the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by dilution methods and fractionation of the crude venom and characterization of the interest fractions. In addition, it is pertinent to assess to mechanisms responsible for antibacterial activity.

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Chapter 4 – Final remarks and future perspectives

Cancer and the emergence of multi-resistant bacteria represent a serious burden on human health and for which is urgent the development of new treatment strategies and therapeutic agents. Snake venoms have been studied for this purpose, seeking to identify constituents of relevant clinical interest. Due to their rich and complex composition, mechanisms of action of their toxins, and inter- and intra-specific diversity, these venomous cocktails appear as a promising source. Studies show that snake venoms and various compounds isolated from them present remarkable results, evidencing an antitumor action against different types of tumor cell lines in in vivo and in vitro experiments, as well as, an antibacterial effect against several pathogenic agents.

Throughout this dissertation, it was explored the antitumor and antibacterial potential of *Vipera latastei* crude venom, being so far the first study that evaluates the bioactivity of the venom of this species. The present study revealed that crude venom has a potent cytotoxic effect against human melanoma MNT-1 and human keratinocyte HaCaT cell lines. However, both tumoral or non-tumoral cell lines were affected and no selectivity effect for the tumor cell line tested in this work was observed. Crude venom treatment caused a cell cycle arrest of MNT-1 cells, resulting in a S-phase delay. On the other hand, it was not observed any interference with cell cycle dynamics of HaCaT cells. These results indicate that the cytotoxicity observed in crude venom treatments may be related to toxins that interfere with cell cycle. Furthermore, other compounds such as PLA_2 may promote cell death acting on cell membranes and causing cell disruption, which may preclude the action of other compounds in cell cycle dynamics. We also observed a significant increase in ROS generation in a dose-dependent manner in MNT-1 cells treated with crude venom, as well as, a non-significant increase compared to the control in IC50- treated HaCaT cells. We hypothesized that these results may be related to the activity of L-amino acid oxidase that is a possible component of *V. latastei* crude venom. Therefore, the increase of ROS and consequent oxidative stress may represent one of the cytotoxicity mechanisms exhibited by crude venom. In addition, this increase in ROS levels may be associated with cell cycle arrest observed in MNT-1 cells.

Regarding antibacterial potential, *V. latastei* crude venom exhibited antibacterial activity, inhibiting the growth of both Gram-Positive and Gram-Negative bacteria. Inhibition zones were observed in all screened test bacteria *Escherichia coli* ATCC 25922, *Salmonella*

typhimurium ATCC 14028, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, and *Bacillus cereus* ATCC 14579.

This dissertation is of great relevance since it provides the first analysis of the bioactivity of *V. latastei* venom, demonstrating for the first time its cytotoxic and antibacterial potential. Remarkably, the present findings revealed the potential of *V. latastei* venom as a reservoir of bioactive compounds, supporting that it can be a promising source of new therapeutic agents, particularly in the treatment of cancer and multi-resistant infections.

Further studies should test the venom on different tumoral cell lines, as well as, its antimetastatic and antiangiogenic potential. With respect to antibacterial potential, determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) is also essential. A key idea advocated throughout this study is that the fractionation and purification of crude venom should be considered in order to identify and characterize the compounds with the activity of interest. In addition, the assessment of the mechanisms that lead to the mentioned effects is also required.