MARCEL JOSÉ PALMIERI

LACTUCA SATIVA: BIOINDICADOR PARA ANÁLISE DO EFEITO GENOTÓXICO DE PEÇONHAS

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-graduação em Genética e Melhoramento de Plantas, área de concentração em Citogenética, para a obtenção do título de Doutor.

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Dedico este trabalho ao meu irmão Breno e aos meus pais José Antônio e Maria José que me apoiam, me ensinam, me incentivam e me fazem ser uma pessoa melhor. Dedico também à minha Orientadora de longuíssima data e inspiração profissional Profª Lisete e à minha mentora, amiga e companheira de trabalho Profª Larissa

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RESUMO

Estudos de citogenotoxidade permitem avaliar, estabelecer e compreender mecanismos de ação de diversos agentes tóxicos, sejam eles físico, químicos ou biológicos; bem como a extenção de seus efeitos. O efeito citogenotóxico da maioria das peçonhas ainda é pouco explorado. Toxinas de peçonhas tem uma ampla gama de atuação, inclusive na fabricação de fármacos, quando se conhece seus efeitos detalhadamente e é possível manipular suas concentrações. Os modelos vegetais se apresentam como bons organismos para testes alternativos às cobaias animais por serem de fácil proliferação e manejo, além de não esbarrarem em empecilhos legais e/ou éticos quanto a sua aplicação. Neste sentido, este trabalho objetivou mostrar os efeitos citogenotóxicos da peçonha de uma espécie de escorpião (*Tityus serrulatus*), duas espécies de vespas (*Polybia ocidentalis* e *P. fastidiosa*) e seis espécies de serpentes (*Bothrops atrox, B. jararacussu, B. moojeni e B alternatus*, *Lachesis muta* e *Crotalus durissus terrificus*) sobre células meristemáticas de ponta de raiz de *Lactuca sativa* (alface) – um modelo vegetal amplamente empregado em testes de toxidade, com eficiência comprovada. Foram observados efeitos tóxicos significativos para todas as nove espécies estudadas. Os resultados mostraram que o modelo vegetal foi eficiente para prospecção da atividade citogenotóxica das peçonhas, com resultados em concordância com efeitos em células humanas. Os mecanismos inerentes à toxidade foram atribuídos a uma série de metabólitos, proteases, enzimas e outras substâncias, como o mastoporam e também a ações neurotóxicas das peçonhas, interrompendo o funcionamento de canais iônicos e a indução e ação direta no mecanismo de sinalização e regulação do stress oxidativo.

Palavras-chave: *Tityus serrulatus*, *Polybias* sp., *Bothrops* sp., *Crotalus durissusterrificus* , *Lachesis muta,* TUNEL, morte celular, alterações no ciclo celular, fragmentação do DNA.

ABSTRACT

Cyto and genotoxicity studies allow the evaluation, understanding and establishment of action mechanisms and the extension of the effects of various toxicants, whether they are physical, chemical or biological. The cytogenotoxic effects of most of the venoms are still largely unexplored. Venom toxins have many uses, including the manufacture of pharmaceuticals, when their effects are well known and it is possible to manipulate their concentrations. Plant models are a good alternative to experiments that use animals as test subjects. Plants germinate easily, are easy to manage and maintain and are not hampered by legal and/or ethical impediments regarding their application. Therefore, this study aim was to show the citogenotoxic effects of the venoms of a scorpion species (*Tityus serrulatus*), two wasps species (*Polybia ocidentalis* and *P. tedious*) and six snakes species (*Bothrops atrox*, *B. jararacussu*, *B. moojeni*, *B alternatus*, *Lachesis muta* and *Crotalus durissus terrificus*) on *Lactuca sativa* (lettuce) meristematic root tip cells - a model widely used in toxicity with proven efficiency. Significant cito and genotoxic effects were observed for all nine species studied. The results showed that the plant model was efficient in the prospecting of the cytogenotoxic activity of venoms, the results were in accordance to effects observed for human cells. The mechanisms leading to the venoms toxicity was attributed to a series of metabolites, proteases, enzymes and other substances such as mastoporams as well as the neurotoxic actions of the venom interrupting the functioning of ionic channels and inducting and acting directly on the signaling and regulation of oxidative stress.

Keywords: *Tityus serrulatus*, *Polybias* sp., *Bothrops* sp., *Crotalus durissus terrificus*, *Lachesis muta* , TUNEL, cell death, cell cycle alterations, DNA fragmentation.

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PRIMEIRA PARTE

1 INTRODUÇÃO

A citogenética combina duas ciências separadas – citologia e genética. Ela estuda a estrutura, o número, os movimentos, a função e as variações dos componentes fisio e morfológicos dos cromossomos e moléculas de DNA, relacionando estes fatores tanto entre si quanto com a transmição, recombinação e expressão dos genes. Também estão no escopo da citogenética pesquisas sobre a biodiversidade, estudos de melhoramento genético de plantas e animais, e até estudos de fertilidade, tanto humana como anima e vegetal.

Desde seu estabelecimento esta ciência se aperfeiçoou agregando novas técnicas e tecnologias, sendo hoje de grande importância para diversas outras áreas dentro da genética. Uma das áreas da citogenética é a análise de efeitos genéticos de substâncias tóxicas através da avaliação de seus efeitos sobre os cromossomos e material genético de maneira geral, permitindo quantificar o seu potencial tóxico bem como elucidar seus mecanismos de ação.

Estudos de citogenotoxidade têm contribuído para o entendimento da ação de toxinas sobre o organismo humano, e do surgimento de doenças associadas a estas toxinas como, por exemplo, diferentes tipos de câncer e doenças degenerativas. Este tipo de pesquisa é de suma importância para o entendimento de alterações dos mecanismos de controle do ciclo celular, principalmente no caso de agentes biológicos, como as peçonhas, e podem levar também ao desenvolvimento de fármacos, uma vez que vários medicamentos têm como princípio ativo concentrações controladas de toxinas isoladas de organismos vivos. Entretanto, em relação às peçonhas de serpentes e artrópodes, esta área é ainda pouco explorada. Torna-se imperativo, portanto, estudos deste nível, para melhor elucidar os mecanismos de indução de danos citológicos e

sobre a molécula de DNA exercidos por estas peçonhas, visando gerar informações relevantes que podem vir, dentre outras coisas, a ser empregadas no desenvolvimento de novos fármacos.

Testes citognéticos com substâncias biológicas, como é o caso das peçonhas, costumam ser realizados utilizando-se de modelos animais. Estes modelos, frequentemente, são de manutenção complicada e dispendiosa, além de apresentarem empecilhos legais e éticos quanto a sua aplicação para fins científicos. Com isso em mente, um modelo de teste seguro que sirva para promover testes iniciais que possam ser realizados com alta eficiência, segurança, baixo custo e sem impedimentos legais e éticos, se faz necessário. Neste contexto, a utilização de sistemas vegetais pode ser uma alternativa confiável para aferir o potencial citogenotóxico de muitos compostos e servir para screening inicial da ação das toxinas presentes em peçonhas. Os testes de citogenotoxidade utilizando modelos vegetais, como, por exemplo, *Lactuca sativa*, foram validados por vários pesquisadores que realizaram de forma conjunta modelos testes animais e vegetais (BELCAVELLO et al., 2012; PALMIERI et al., 2016; STANGE et al., 2009; TEIXEIRA et al., 2003)..

Desta forma, o conhecimento sobre danos na molécula de DNA ou indução de morte celular, advindas da ação de peçonhas em células vegetais poderá contribuir com a predição, em nível comparativo, de possíveis danos também em células animais, tendo em vista que testes com modelos vegetais são de simples execução, baratos e fornecem resultados altamente confiáveis (FISKESJÖ, 1985; GRANT, 1994). A partir de dados inicias obtidos em modelos vegetais, podem ser realizados testes mais detalhados e mais amplos, com modelos animais *in vitro* e *in vivo*, direcionados apenas àquelas áreas onde se tenham encontrado efeitos de interesse. Isso resultará em menores custos e tempo despendido nos experimentos.

Tendo em vista o exposto acima, esta tese objetivou estudar o efeito da peçonha de uma espécie de escorpião (*Tityus serrulatus*), duas espécies de vespas (*Polybia occidentalis* e *Polybia fastidiosa*) e seis espécies de serpentes (*Lachesis muta muta*, *Crotalus durissus terrificus*, *Bothrops moojeni*, *Bothrops atrox*, *Bothrops jararacuçu* e *Bothrops alternatus*) sobre a organização cromossômica, ciclo celular e o DNA de células de *Lactuca sativa* (alface) a fim de não só entender os mecanismos de ação citogenotóxico destas peçonhas como também determinar a possibilidade da aplicação de um modelo vegetal para testes de toxidade com peçonhas.

2 REFERENCIAL TEÓRICO

2.1 Peçonhas

As peçonhas chamam àtenção quanto às suas inúmeras propriedades bioquímicas, funcionais e estruturais, sendo compostas principalmente por moléculas protéicas, as quais incluem: neurotoxinas, citotoxinas, proteínas que agem na hemostasia (proteases e fosfolipases A_2) e peptídeos, L-aminoácido oxidases, hialuronidases e lectinas (MATSUI et al., 2000), justificando assim, os inúmeros estudos funcionais e estruturais realizados nas últimas décadas com as mesmas.

Proteases de peçonhas de serpentes, por exemplo, classificadas em serinoproteases e metaloproteases, estão entres os principais grupos de enzimas que podem levar ao desequilíbrio hemostático, podendo atuar sobre a hemoglobina, colágeno, fibrina, elastina, fibrinogênio, insulina e glucagon (IWANAGA; SUZUKI, 1979). Algumas desintegrinas têm sido estudadas com vistas à aplicação na terapia de cânceres, angiogênese e doenças trombogênicas (MATSUI; FUJIMURA; TITANI, 2000; McLANE; JOERGER; MAHMOUND, 2008; MAZZI et al., 2004).

Muitas peçonhas são ricas em fosfolipases A_2 (PLA_2s) dos grupos I e II. As PLA_2s representam uma classe de enzimas versáteis, considerando sua função, localização, regulação, mecanismo de ação, sequência e estrutura. Elas se destacam em serpentes e abelhas, estando envolvidas em diversas doenças inflamatórias humanas, assim como em vários processos biológicos como adesão, migração e sinalização celulares (RODRIGUES et al., 2009), uma vez que apresentam atividade catalítica sobre membranas celulares. Independentemente da sua função catalítica primária, as PLA_2 de peçonhas de serpentes podem induzir diversos efeitos farmacológicos adicionais como

neurotoxidade pré e/ou pós-sináptica, cardiotoxidade, miotoxidade, iniciação e/ou inibição de agregação plaquetária, edema, hemólise, anticoagulação, convulsão e hipotensão (SOARES et al., 2004).

Gupta et al. (2007), ao investigarem os efeitos da peçonha do escorpião *Heterometrus bengalensis* sobre uma linhagem de células leucêmicas mostraram o potencial antiproliferativo, pro-apoptótico e citotóxico desta peçonha, sendo apontada como possível responsável pelos efeitos observados, a classe de toxinas PLA_2. Em outro trabalho, a peçonha do escorpião *Androctonus crassicauda* mostrou ação sobre o crescimento de neuroblastos e células cancerosas de mama por citotoxidade e limitação do ciclo celular (ZARGAN et al., 2011), havendo também sugestão, por parte dos autores, da ação de PLA_2 como indutoras dos efeitos observados. Esta classe de fosfolipases amplamente distribuída em animais, vegetais e microorganismos, podem também estar associadas ao potencial genotóxico dos venenos sobre linfócitos humanos relatado por Marcussi et al. (2011), quando analisaram a ação da peçonha de C*rotalus. durissus terrificus* (*C.d.t.*) e algumas toxinas isoladas.

Na peçonha de *Apis melífera* também podemos encontrar PLA_2, e estudos mostraram os efeitos genotóxicos (GAJSKI; GARAJ-VRHOVAC, 2008; GARAJ-VRHOVAC; GAJSKI, 2009; HOSHINA; MARIN-MORALES 2014) desta peçonha, assim como seu potencial antiproliferativo sobre células cancerosas. Pode-se sugerir então, a participação de PLA_2 também na indução de danos no DNA, uma vez que, outros efeitos como a indução de morte celular e citotoxidade já haviam sido descritos para estas moléculas (MARCUSSI et al., 2013; ZARGAN et al., 2011).

Outra classe importante de moléculas presentes em peçonhas é a das lectinas, proteínas capazes de reconhecer, de modo específico, carboidratos, associados (glicoconjugados) ou não a outras estruturas (ASHWELL; MORELL, 1974; LIS; SHARON, 1986). As lectinas são expressas em vários

organismos, desde vírus e vegetais a seres humanos (KARLSSON, 1995; SHARON; LIS, 1989; WILEY; WILSON; SKEHEL, 1981). Há uma grande diversidade estrutural de glicoconjugados nos seres vivos, a qual está associada a uma diversidade biológica muito significativa, pois estas glicoestruturas podem codificar várias informações biológicas, as quais podem ser decodificadas por lectinas (SHARON e LIS, 1989). Portanto, o reconhecimento de carboidratos por lectinas é um fenômeno bioquímico associado a vários processos fisiológicos e/ou patológicos como fertilização, embriogênese, migração celular, defesa imunitária, infecção por microorganismos e câncer (KARLSSON, 1995; LIS; SHARON, 1986; RABINOVICH, 2005; SHARON; LIS, 1989). Assim, a presença destas moléculas nas peçonhas pode também sugerir sua participação direta ou indireta nos mecanismos de degradação do DNA e indução de morte celular.

Alguns trabalhos da literatura ilustram a caracterização química e/ou biológica de lectinas derivadas de peçonhas de serpentes (ELÍFIO-ESPOSITO et al., 2007), aranhas (FITCHES et al., 2004; VASTA; COHEN, 1984a) e escorpião (VASTA; COHEN, 1984b), e algumas destas têm sido utilizadas como ferramentas experimentais para o estudo de eventos biológicos e como agentes terapêuticos ou biotecnológicos (FITCHES et al., 2004). De modo interessante, carboidratos, na forma de glicoconjugados, derivados de sementes *Mucuna pruriens* protegem camundongos do efeito tóxico de peçonha de serpente da espécie *Echis carinatus* (GUERRANTI et al., 2004). Esses achados sugerem que o reconhecimento de carboidratos pode participar dos eventos de proteção contra a ação tóxica de peçonhas.

A indução dos eventos locais e sistêmicos causados pelo envenenamento por serpentes e a velocidade pela qual os constituintes difundem na circulação sistêmica e atingem o sítio de ação dependem de inúmeras proteínas. Presente em várias peçonhas de serpentes e escorpiões, a hialuronidase é responsável por

intensificar a ação tóxica da peçonha, através da degradação do hialuronan, aumentando a difusão de outras toxinas através da matriz extracelular (EL-SAFORY; SAVARY; LEE, 2010; PESSINI et al., 2001). Apesar do efeito sinérgico na toxicidade da peçonha, essa classe de enzimas chama atenção para as aplicações terapêuticas em diversas áreas, como por exemplo, ortopedia, oftalmologia, oncologia, dermatologia e ginecologia, sendo de suma valia entender suas ações em diferentes organismos e a parte que lhe cabe durante o envenenamento.

Os diferentes mecanismos envolvidos na intoxicação resultam também dos efeitos indiretos de algumas toxinas sobre os sistemas biológicos. As L-aminoácido oxidases, por exemplo, possuem mecanismos complexos e pouco conhecidos. Porém, biologicamente, esse grupo de toxinas induz citotoxidade, provavelmente devido ao aumento na produção de peróxido de hidrogênio resultante de sua ação catalítica. Os estudos farmacológicos, ainda que preliminares, descrevem efeitos sobre processos de morte celular, agregação plaquetária, hemorragia, hemólise, indução de edema e efeitos bactericida e leishmanicida (DU; CLEMETSON, 2002).

Em relação à peçonha de escorpiões, a espécie de maior importância médica do Brasil, *Tityus serrulatus*, é também a que apresenta o maior número de trabalhos publicados abordando os efeitos locais e sistêmicos de sua peçonha e toxinas isoladas, destacando-se os peptídeos com atividade sobre canais iônicos (neurotoxinas) (QUINTERO-HERNÁNDEZ et al., 2013). Essas diversas neurotoxinas tem seu efeito potencializado, muito provavelmente, devido a ações de proteases e da hiarulonidase, que servem como agentes facilitadores da dispersão da peçonha (ALVARENGA et al., 2012; HORTA et al., 2014).

Já em relação às vespas, os trabalhos são escassos, inclusive os que se referem à composição de suas peçonhas, sendo um campo vasto a ser

cientificamente explorado. Entretanto sabe-se que peçonhas de Hymenopteras (vespas e abelhas) podem conter agentes antimicrobianos, enzimas, peptídeos citólicos e de baixo peso molecular e neurotoxinas (KUHN-NENTWIG, 2003). Especificamente nas peçonhas de vespas, podemos encontrar fosfolipases A e B, mastoporans, hiarulonidases, proteases e fosfatases ácidas (KING; VALENTINE, 1987; NAKAJIMA et al., 1985) na composição de sua peçonha. Essas peçonhas podem apresentar também agentes antigoagulantes (CZAIKOSKI et al., 2010).

Sabe-se que as toxinas animais são ferramentas importantes para se investigar vários mecanismos celulares e moleculares envolvidos em processos farmacológicos, inflamatórios e de intoxicação. Constituem modelos moleculares interessantes para o desenvolvimento de novos agentes terapêuticos, sendo alvos de indústrias farmacêuticas e de biotecnologia. Assim, enzimas isoladas de peçonhas, têm sido utilizadas para investigar os mecanismos de coagulação do sangue, formação de tumores, além de terem uso no estudo de diversas patologias e elaboração de fármacos (CZAIKOSKI et al., 2010; EBLE, 2010; HARVEY, 2014; KING, 2011; KOH; KINI, 2012; KUSMA et al., 2008; PRAZNIKAR; PETAN; PUNGERČAR, 2009).

Em vista da complexidade da mistura de toxinas de peçonhas, que podem se ligar a alvos relacionados com funções vitais, apresentar potencial para elaboração de fármacos e compreensão de mecanismos de desenvolvimento de patologias, torna-se de suma importância a realização de estudos que abordem as vias de interação destas toxinas nos organismos. Processos de morte celular e danos celulares são os mecanismos de ação propostos para a maioria das toxinas e peçonhas (BAPTISTA-SEINDBERG; SAIDEMBERG; PALMA, 2011; MARCUSSI et al., 2013; PATHAN et al., 2015). Sendo assim estudos que lancem mão de analogias entre células vegetais e animais poderão contribuir para o entendimento dos mecanismos de indução de morte celular e danos

citogenéticos das peçonhas bem como permitir uma alternativa barata, de simples manejo e sem empecilhos legais e éticos para avaliação dos efeitos das peçonhas e suas toxinas. Testes mais complexos, mais aprofundados e, consequentemente, mais onerosos podem ser focados apenas para aquelas toxinas e elementos que tenham apresentado atividade e mecanismos de ação que as caracterize como de potencial interesse nos screenings iniciais com modelos vegetais.

2.2 Avaliação de Toxidade

A avaliação do ciclo celular é uma ferramenta básica na citogenética que permite ao pesquisador ter acesso a uma série de informações importantes sobre a estrutura organizacional dos cromossomos de um organismo e como estes se comportam durante as diversas fases da divisão celular (GRANT, 1994).

Em vegeatis este teste é conduzido utilizando-se de pontas de raízes, as quais são compostas por tecido meristemático, apresentando células que passam por inúmeras divisões em rápida sucessão. Além disso, segundo Fiskejö (1988), a raiz é a parte do vegetal que, quase sempre, é a primeira a ser exposta aos agentes tóxicos dispersos no solo ou em água, portanto a análise das células radiculares constitue um método rápido para o monitoramento da toxidade do agente testado. Após a raiz ser exposta ao agente que se deseja testar, o meristema é separado da raiz e uma lâmina é preparada pela técnica de esmagamento. A lâmina é então avaliada sob um microscópio de luz e diversos parâmetros são analizados. A partir dos resultados obtidos é possível determinar qual o potencial genotóxico do agente testado (FISKEJÖ, 1984).

No âmbito dos bioensaios de toxidade essa avaliação pode detectar alterações no padrão do ciclo celular devido tanto à detecção de anormalidades no índice de divisão celular, quanto pela presença de aberrações cromossômicas

ou nucleares (ANDRADE; CAMPOS; DAVIDE, 2008; ANDRADE; DAVIDE; GEDRAITE, 2010; ANDRADE-VIEIRA et al. 2014; KLANCNIK et al. 2011; KUMARI et al. 2011; PALMIERI et al. 2014). O teste de ciclo celular permite ainda a análise de marcadores citológicos da morte celular pela observação da ocorrência de núcleos condensados, e também permite detectar a presença de micronúcleos e extravasamento da cromatina (ANDRADE-VIERA et al., 2011).

Alterações cromossômicas são caracterizadas como mudanças tanto na estrutura cromossômica quanto no número cromossômico, que podem ocorrer espontaneamente ou como resultado da exposição a agentes tóxicos, portanto, alterações cromossômicas são reconhecidas como importantes conseqüências de ações cito e genotóxicas induzidas por agentes químicos e biológicos (NATARAJAN, 2002). A Figura 1 mostra alguns exemplos de alterações cromossômicas (Fonte: acervo do grupo de pesquisa em toxidade do Laboratório de Citogenética da UFLA).

Existem vários mecanismos que podem levar ao surgimento destas alterações, eles estão relacionados a anormalidades na divisão mitótica e envolvem ações aneugênicas ou clastogênicas. Ações clastogênicas são caracterizadas pela indução de quebras cromossômicas, enquanto ações aneugênicas se baseiam na inativação ou desfunçao de uma estrutura celular, como o fuso mitótico por exemplo, ocasionando perdas cromossômicas (FENECH, 2000).

Os efeitos sobre o fuso mitótico podem causar alterações no número de cromossomos, pois afetarão a segregação correta durante a divisão celular. Essas alterações numéricas podem ser classificadas em dois tipos: (1)- Euploidias: alterações numéricas afetando todo o conjunto cromossômico de um organismo e (2)- Aneuploidias: alteração envolvendo um ou poucos elementos do conjunto cromossômico, sendo consequência da segregação anormal dos cromossomos. Ambos os tipos podem ocorrer tanto naturalmente como pela ação de agentes

aneugênicos (ALBERTINI et al, 2000). Muitas destas aberrações são irreversíveis e altamente prejudiciais às células fazendo com que estas entrem em um processo de morte celular. A inativação do fuso mitótico pelo agente tóxico pode estar relacionada ao bloqueio da sinalização para a ligação do fuso com o cinetócoro na região centromérica dos cromossomos, como mostrado por Freitas et al (2016).

Análises experimentais mostram que quebras na dupla fita de DNA são a principal lesão responsável pela formação de alterações cromossômicas (BRYANT, 1998; OBE; JOHANNES; SCHULTE-FROHLINDE, 1992). Essas quebras ocorrem espontaneamente em frequências significantes através de uma variedade de processos celulares, mas podem também ser diretamente induzidas por agentes tóxicos (OBE; JOHANNES; SCHULTE-FROHLINDE, 1992; PFEIFFER; GOEDECKE; OBE, 2000). As quebras no DNA podem gerar alterações estruturais cromossômicas. Alguns exemplos são: (1)- deficiências: perda de um segmento cromossômico, (2)- duplicações: segmento cromossômico duplicado devido a erros de pareamento gerado pela perda de fragmentos, (3)- inversões: envolve duas quebras cromossômicas e a reinserção do fragmento no mesmo cromossomo em sentido invertido e (4)- translocações: troca de partes entre cromossomos não homólogos (CARVALHO; RECCO-PIMENTEL, 2007).

Além disso, essas quebras na fita de DNA se relacionam com a ocorrência de morte celular. Em organismos multicelulares, as células se autodestroem quando sofrem danos ou como parte de seu desenvolvimento normal. Células podem tanto ser destruídas acidentalmente - “assassinadas” por meio de um processo conhecido como necrose celular – ou se autodestruir usando um mecanismo ativo, processo conhecido como morte celular programada (MCP), uma espécie de “suicídio” celular, a fim de eliminar células danificadas, servir como uma defesa do organismo contra patógenos e danos

genéticos e reaproveitar nutrientes e moléculas de células senis (DANON et al, 2000).

Gichner et al. (2005), diferenciam a MCP da necrose, afirmando que a MCP tem um importante papel na diferenciação de tecidos e envelhecimento, podendo ocorrer também em resposta a danos celulares, como a exposição a agentes tóxicos, ao passo que a necrose representa uma morte celular não programada.

Em animais, a MCP é muito bem estudada e dois tipos principais dela são descritos: autofagia e apoptose. Apoptose envolve o engolfamento de uma célula que esteja morrendo por uma célula viva e degradação da primeira no lisossoma da segunda. Já na autofagia a degradação celular se dá principalmente por lisossomos na própria célula. Existem ainda outros tipo de MCP que não involve degradação no lisossomo (VAN-DOORN;WOULTERING, 2005; 2010) ou não são apoptóticos como o caso da necroptose e a pyroptose (TAIT; ICHIM; GREEN, 2014).

Em vegetais, o processo de MCP tem semelhanças, mas não é idêntico daquele dos animais. As principais evidências citológicas da MCP são a condensação da cromatina e a fragmentação nuclear e celular (DANON et al, 2000). Em microscópio é pssível identificar a morte celular através da presença de núcleos condensados (ANDRADE-VIEIRA et al., 2011)). Já um critério bioquímico que evidencia a MCP é a fragmentação do DNA por endonucleases, o *DNA laddering*, que recebe este nome, pois a molécula forma um padrão de escada ao ser observada em eletroforese em gel de agarose. (GICHNER, 2005; VAN-DOORN; WOULTERING, 2005, 2010).

Outro parâmetro muito importante que pode ser avaliado é o índice mitótico, caracterizado pelo número total de células em divisão durante o ciclo celular. O aumento ou a redução deste índice pode ajudar a determinar o grau de toxidade de um agente (FERNANDES; MAZZEO; MARIN-MORALES, 2007).

Estudos de ciclo celular são amplamente empregados, não apenas para avaliar o efeito genotóxico de diversos agentes, mas também para avaliar seus mecanismos de ação (LEME; ANGELIS; MARIN-MORALES, 2008). Segundo Fernandes; Mazzeo; Marin-Morales (2007) um dos mais antigos e mais usados métodos para avaliar efeitos mutagênicos é o teste de ciclo celular. Este teste permite a identificação de quase todas as alterações estruturais e numéricas do cariótipo, escapando apenas aquelas não visíveis ao microscópio (MARCANO et al, 2004).

A análise de ciclo celular é um dos poucos métodos diretos para mensurar danos em sistemas expostos a mutagênicos ou carcinogênicos potenciais. Andrade; Davide e Gedraite (2010) e Palmieri et al. (2014) avaliaram o efeito tóxico do SPL (Spent Pot Liner) e de seus principais componentes em meristema de *Lactuca sativa* e observaram aumento na ocorrência de alterações cromossômicas e de núcleos condesados (o que é indicativo de morte celular programada – este tópico será discutido adiante) nas células expostas ao agente tóxico.

Com este bioensaio, é possível ainda a avaliação de anormalidades nucleares, conjuntamente com a avaliação de alterações cromossômicas, isso tem se mostrado como uma boa maneira de tornar os estudos da toxidade ainda mais precisos em relação aos efeitos de químicos no DNA dos organismos testes (LEME; MARIN-MORALES, 2009).

De acordo com Leme; Angelis e Marin-Morales (2008), anormalidades nucleares podem surgir como consequência de aberrações cromossômicas. Essas anormalidades são caracterizadas por alterações morfológicas no núcleo interfásico e são resultados da ação tóxica de algum agente (LEME; MARIN-MORALES, 2009).

Exemplos de tipos de aberrações nucleares são os brotamentos nucleares, que podem surgir devido à eliminação do excesso de material

genético decorrente de um processo de poliploidização celular; núcleos lobados e as células polinucleadas, ambos consequência de anáfases multipolares (FERNANDES; MAZZEO; MARIN-MORALES, 2007). Segundo Leme, Angelis e Marin-Morales (2008) a presença destas duas últimas anomalias pode ser um indicador da ocorrência de morte celular.

O micronúcleo é provavelmente a alteração nuclear mais bem estudada, sendo um excelente indicador de toxidade, como visto nos trabalhos de Andrade, Davide e Gedraite (2010), Corrêa et al. (2016), Liang et al. (2015), Palmieri et al. (2014) entre outros. Os micronúcleos podem ser definidos como estruturas no citoplasma contendo cromatina envolvida por uma membrana sem que haja nenhuma ligação aparente ou visível com o núcleo principal da célula (MA et al., 1995) (Figura 1A, 1B e 1C). Eles são formados pela exclusão de cromossomos inteiros ou fragmentos cromossômicos durante a divisão celular e, portanto, sua presença indica que o agente possui atividade aneugênica ou clastogênica, uma vez que micronúcleos derivados de fragmentos cromossômicos indicam ação clastogênica, enquanto aqueles provenientes de cromossomos que tenham sido perdidos inteiros indicam ação aneugênica (FENECH, 2000). Micronúcleos resultam de danos não reparados ou reparados erroneamente nas células parentais durante a divisão celular, sendo facilmente observados nas células filhas como uma estrutura similar ao núcleo principal, mas de tamanho reduzido. Podem ser identificados citogeneticamente através de uma variedade de corantes de DNA e sua frequência pode ser quantificada com o uso de microscópio (MA et al., 1995; MESCHINI et al., 2015).

Testes de micronúcleos são muitas vezes efetuados em paralelo ao teste do cometa (ou eletroforese de gel de célula única). Este teste permite estimar o grau de dano sofrido pela fita de DNA através da avaliação de imagens que são formadas ao se correr um gel de eletroforese contendo nucleóides, normalmente advindos de linfócitos humanos. Os nucleóides danificados formam um arraste

devido ao tempo de corrida diferenciado dos fragmentos de tamanhos distintos do DNA, dando a célula um aspecto semelhante a um cometa justificando o nome da técnica (SINGH et al. 1988). Este arraste é visível em microscópio de fluorescência (após marcação da molécula de DNA por um fluorocromo) e, baseado no tamanho da cauda do cometa, ele é classificado em uma categoria genérica que representa o grau de dano sofrido pela molécula de DNA apresentado pelo nucleóide. A Figura 2 mostra um exemplo de imagem de cometas de diferentes níveis observados em um microscópio (Fonte: acervo do grupo de pesquisa em toxidade do Laboratório de Citogenética da UFLA).

Existe uma grande quantidade de trabalhos realizados nos últimos anos que aliam os testes do cometa e do micronúcleo, a fim de obter informações a respeito do dano sofrido pela molécula de DNA induzido pela ação dos mais variados agentes, desde químicos até radiotivos (BENEDETTI et al. 2013; CIGERCI et al. 2016; MARCUSSI et al. 2013; ZAPATA et al. 2016).

Existe outro bioensaio muito útil, complementar aos demais testes citados na detecção de fragmentação da fita e avaliação de dano de DNA e que pode indicar a ocorrência de morte celular, trata-se do teste TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling). Descrito por Gavrieli, Sherman e Ben-Sasson (1992), esse é atualmente considerado um dos principais testes para se detectar morte celular e apontar a ocorrência de fragmentação no DNA (JUCHIMIUK; MALUSZYNSKA, 2005). Vários trabalhos já foram publicados utilizando o teste TUNEL (CHENLU et al. 2014; DESAI et al. 2014; LIU et al. 2007; ZHANG et al. 2012) .

O bioensaio consiste em marcar as extremidades 3'-OH (hidroxila) do DNA que são geradas quando ocorre fragmentação do material genético. Essa marcação ocorre por intermédio de uma enzina, a deoxinucleotídeo transferase terminal, através da ligação de dUTPs sinalizados com fluorocromos (PEDROSO; DURZAN, 1999; WOJCIECHOWSKA; OLSZEWSKA, 2003).

Para permitir a entrada de enzimas nas céluas a membrana plasmática deve ser permeabilizada antes da reação de TUNEL. Após o preparo da amostra monta-se uma lâmina e a observa em um microscópio de fluorescência. Atualmente este teste tem sido usado em associação com a citometria de fluxo oque dispensa a montagem de lâminas.

O TUNEL pode ser utilizado para avaliar a ação genotóxica de compostos como extratos de plantas, agrotóxicos, metais pesados, materiais orgânicos ou sintéticos em geral de interesse médico-científico, ou para averiguar a presença ou não de danos oxidativos indutores de processo apoptótico. O sinal positivo do TUNEL pode ser considero um importante marcador molecular da morte celular (BEHBOODI; SAMADI, 2004).

A análise de fragmentação do DNA pode ser feita também por meio de eletroforese em gel de agarose com o DNA total em um gel comum, uma vez que se espera que células em processo de morte celular apresentem fragmentação de DNA, o que confere ao gel uma aparência peculiar, semelhante a uma escada, num processo conhecido como DNA laddering (MENKE et al., 2001) .

2.3 Caracterização das Principais Alterações Cromossômicas

2.3.1 Cromossomos Aderentes

A ocorrência de cromossomos aderentes se dá após alterações na estrutura cromossômica que promove a perda das características normais de condensação causando a formação de aglomerados. Esta alteração está normalmente associada a um forte efeito citotóxico e pode levar a morte celular (AMIN, 2011; LEME; MARIN-MORALES, 2009)

O mecanismo de formação da aderência cromossômica foi atribuído ao efeito sobre a estrutura físico-química do DNA, das proteínas a ele ligado, ou de ambos; a formação de complexos com grupos fosfato no DNA; a condensação do DNA, ao efeito de agentes químicos ou quebras nas cromátides que levam a formação de ligações inter e intra cromatídicas. O cromossomo aderente pode ser interpretado como sendo um emaranhado de fibras cromatídicas que leva a conecções subcromatídicas entre cromossomos (EL-GHAMERY; EL-KHOLY; ABOU EL-YOUSSER, 2003) (Figura 1H).

2.3.2 C-metáfase e alterações correlacionadas

C-metáfases são alterações aneugênicas e resultantes de distúrbios no aparato do fuso. Como consequência, os cromossomos não se ligam apropriadamente ao fuso, não formando a placa equatorial típica e são, portanto, vistos espalhados no interior da célula durante a metáfase. (FISKESJÖ, 1985, 1988) (Figura 1D). Problema na formação do do fuso mitótico pode ocorrer devido à ação direta de agentes tóxicos sobre as fibras do fuso causando disturbios na polimerização ou depolimerização dos microtúbulos. Freitas et al. (2016) também verificaram que agentes tóxicos podem inibir a fosforilação da serina 10 na histona H3 promovendo a inativação de regiões pericentroméricas e afetando a coesão entre cromátides irmãs. Esse mecanismo epigenético influenciaria o arranjo dos cromossomos na placa metafásica e a segregação de cromátides na anáfase.

A indução de c-metáfases através do uso de substâncias bloqueadoras das fibras do fuso, como a Colchicina, 8 hidroxiquinoleina, ciclohexamida, conhecidas como antimitóticos, é comum em laboratórios de citogenética para estudos de cariótipo, já que os cromossomos são facilmente visualizados e distinguidos nas células c-metafásicas. Caso o ciclo celular de uma célula c-

metafásica não seja interrompido pode haver poliploidização da célula, pois a célula entraria em um novo ciclo celular sem ter dividido seu material genético na divisão anterior (FERNANDES; MAZZEO; MARIN-MORALES, 2007; PALMIERI et al., 2014).

Alterações como anáfase multipolar e cromossomos não orientados (Figura 1E e 1G) também estão relacionadas com distúrbios na dinâmica das fibras do fuso (LEME; MARIN-MORALES, 2009). Cromossomos não orientados que não sejam reintegrados ao conjunto cromossômico podem ser perdidos (Figura 1F) e formar micronúcleos (FENECH, 2000), enquanto que as anáfases multipolares podem formar microcélulas, cujo conteúdo nuclear reduzido leva à inviabilidade.

2.3.3 Pontes Cromossômicas e Fragmentos Cromossômicos

Pontes cromossômicas são alterações clastogênicas observadas em anáfases e telófases e que surgem devido a quebras terminais em ambas as cromátides de um cromossomo seguido pela união das mesmas, ou também devido a quebras em regiões ao longo dos braços cromossômicos, tornando suas extremidades reativas e propensas a ligações mesmo com outro cromossomo desprovido de telômero (MATSUMOTO er al. 2006) (Figura 1G e 1I). Esse evento ocorre devido à perda dos telômeros, região que tem como uma das funções garantir proteção e estabilidade ao cromossomo evitando fusões e pareamentos errôneos. Sua perda é decorrente de uma quebra, deixando as extremidades cromossômicas livres para a ocorrência de reações e ligações (LEME; MARIN-MORALES, 2009). Essas quebras podem levar também à formação de alterações estruturais como inversões, que por sua vez podem levar a formações de pontes e fragmentos cromossômicos, e a outras alterações estruturais como translocações, duplicações e deficiências (FISKESJÖ, 1985).

Esta fusão dos cromossomos na região telomérica resulta em cromossomos dicêntricos que irão caracterizar as pontes observadas em anáfase e em pequenos fragmentos acêntricos que podem vir a constituir micronúcleos. Devido a isto existe uma relação entre ocorrência de pontes, fragmentos cromossômicos e micronúcleos (CAMPOS et al., 2008; PALMIERI et al., 2014)

A aderência cromossômica também pode levar a ocorrência de pontes em anáfase, já que nessas condições os cromossomos tendem a se ligar uns aos outros devido à perda de sua estrutura organizacional padrão. Pontes cromossômicas formadas por aderência tendem a ser múltiplas (MARCANO et al., 2004).

2.4 Modelos vegetais para estudos de toxidade

Dentre os danos causados por agentes tóxicos, efeitos genotóxicos e mutagênicos são os mais problemáticos, devido a sua capacidade de induzir danos genéticos que podem ser prejudiciais à saúde. Esses efeitos são normalmente observados em longo prazo, o que pode tornar difícil a identificação da causa, quando esses efeitos aparecem. Esse tipo de dano se torna ainda mais preocupante quando se considera que ele pode ser transmitido, hereditariamente. Por isso é preciso avaliar a toxidade de diversos agentes para determinar os riscos que estes representam, e para tanto se fazem necessários teste eficientes e confiáveis.

De acordo com Grant (1994), testes biológicos para avaliar a toxidade e genotoxidade são indispensáveis para se conhecer as reações de organismos vivos à agentes tóxicos. Collins, Duthie, Dobson (1993) declaram que embora haja diferenças entre metabolismos de plantas e animais, há também similaridades, tais como o importante sistema de oxidase. Isto estabelece um

paralelo entre modelos animais e vegetais, permitindo afirmar, em muitos casos, que resultados obtidos no segundo podem ser extrapolados para o primeiro.

Alguns autores atribuem a indução de morte celular e danos ao DNA à geração de radicais livres, associados ao estresse oxidativo de células animais, resultantes da ação enzimática de L-aminoácido oxidases Proteases e fosfolipases A_2 presentes na maioria das peçonhas. Essas toxinas são responsáveis, respectivamente, pela geração de H_2O_2, pela quebra de proteínas em geral e diversos fosfolipídeos, além de auxiliarem a acessibilidade de outras toxinas às diferentes organelas e moléculas presentes nas células (NAWKAR et al., 2013; ZHANG et al., 2010). De forma análoga, pode-se inferir que modelos vegetais poderiam ser úteis em substituição aos modelos animais, visto que a ação de fosfolipases A_2 sobre fosfolipídios de membranas celulares é indiferente à espécie utilizada, assim como a ação de proteases sobre proteínas.

Leme E Marin-Morales (2009) definem as plantas superiores como ótimos modelos genéticos para avaliar substâncias tóxicas, pois, apresentam alta sensibilidade a agentes mutagênicos em diversos ambientes e permitem a análise simultânea de vários mecanismos de ação destes, englobando desde mutações pontuais até aberrações cromossômicas em células de diferentes órgãos e tecidos, como pólem, raiz e folhas. GRANT (1994) destaca também a alta sensibilidade dos bioensaios com vegetais superiores e adiciona que raramente dão resultados falsos e são, portanto, muito confiáveis e ótimos candidatos para programas de monitoramento de genotoxidade. Fiskejö (1985) também aponta vantagens para esse sistema de teste. Ele afirma que esses bioensaios são muito baratos e tem boa correlação com outros modelos, além disso os cromossomos de vegetais superiores são grandes e de fácil visualização sendo, portanto, muito bons para análise citológicas.

Vários vegetais superiores já foram ou ainda são utilizados como modelos para testes de toxidade. A lista é realmente muito extensa, entretanto

nem todos os casos apresentam resultados ideais ou são estudados afundo o suficiente e, por isso, os vegetais mais frequentemente utilisados são: *Lactuca sativa*, *Allium cepa, Vicia faba, Zea mays, Tradescantia, Crepis capillaris e Hordeum vulgare* (GRANT, 1994). Todas essas espécies listadas foram empregadas em diversos trabalhos e a confiabilidade dos tetes realizados com estes materiais é muito alta. Especificamente pontas de raízes de sementes de *Lactuca sativa* foram utilizadas como modelo inumeras vezes em trabalhos conduzidos pelo grupo de pesquisa do laboratório de citogenética da Universidade Federal de Lavras. Os resultados demonstram alta sensibilidade da mesma para detecção de efeitos cito e genotóxicos bem como sua aplicação como um determinante de alelopatia de variados agentes (ANDRADE; DAVIDE; GEDRAITE, 2010; ANDRADE-VIEIRA et al., 2014; LUBER et al., 2015; PALMIERI et al., 2014).

Çelik e Äslanturk (2007) destacam que os bioensaios vegetais possuem uma boa correlação com sistemas de testes em mamíferos, validando sua aplicação para ensaios de citogenotoxidade. Também, como foi mostrado anteriormente, existem trabalhos que aplicam modelos vegetais e animais simultaneamente (TEIXEIRA et al., 2003; STANGE et al., 2009; BELCAVELO et al., 2012; PALMIERI et al., 2016) que demonstram resultados similares ou complementares de um modelo para o outro. Por fim, o Programa Ambiental das Nações Unidas (UNEP), Organização Mundial de Saúde (OMS) e a Agência de Proteção Ambiental dos Estados Unidos (US- EPA) consideram bioensaios vegetais como sistemas de teste adequado para avaliação de toxidade (GRANT 1982; GRANT 1999). Todos estes dados validam e consolidam o uso dos modelos vegetais para testes de toxidade e atestam quanto a sua eficiência.

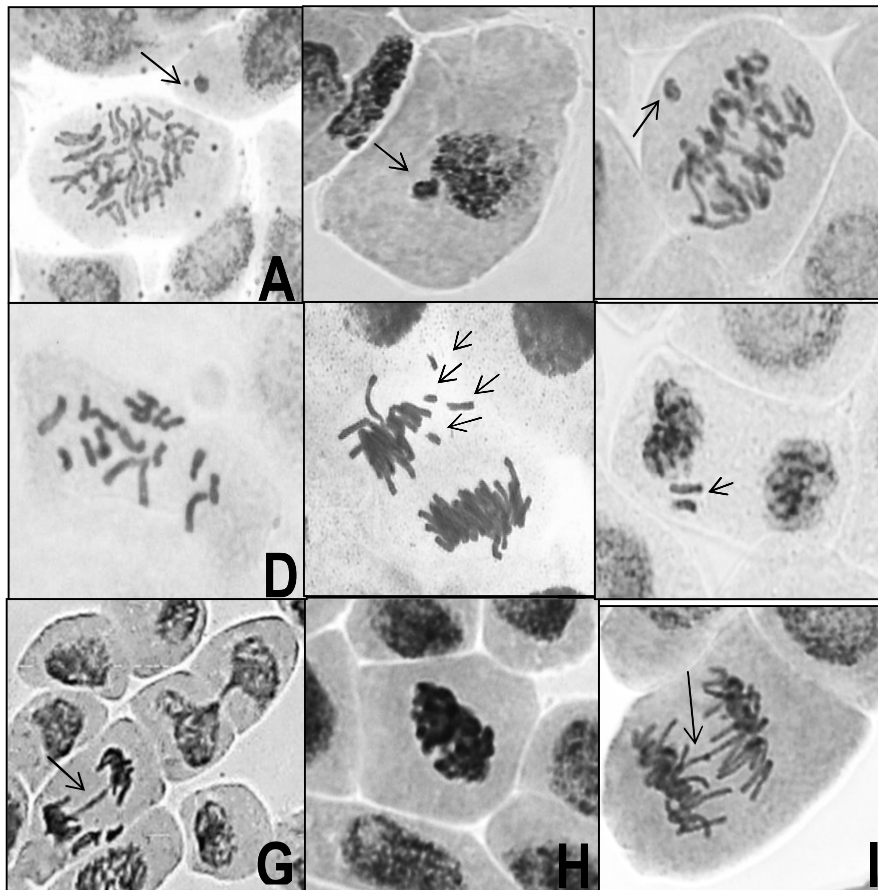

Figura 1 Exemplos de alterações cromossômicas em células de *L. sativa.* A. Micronúcleo (seta); B. Micronúcleo (seta); C. Micronúcleo (seta); D. C-metáfase; E. Fragmentos cromossômicos e cromossomos não orientados na anáfase; F. Cromossomos perdidos na telófase (seta); G. Telófase com ponte (seta) e cromossomos não orientados (seta sem cauda); H. Cromossomos aderentes; I. Ponte na anáfase (seta).

Fonte: Acervo do grupo de pesquisa em toxidade do Laboratório de Citogenética da Universidade Federal de Lavras (UFLA).

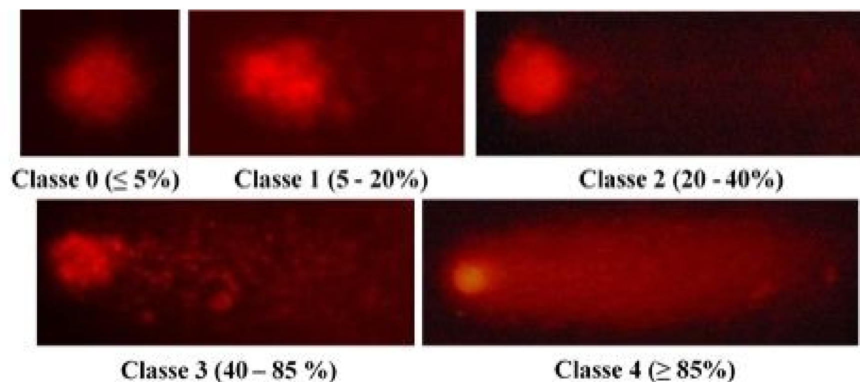

Figura 2 *Scores* de dano baseados no tamanho da cauda do cometa observado em leucócitos humanos. Valores entre parênteses se referem ao percentual de fragmentação da molécula de DNA (adaptados de COLLINS et al., 2004).

Fonte: Acervo do grupo de pesquisa em toxidade do laboratório de citogenética da Universidade Federal de Lavras (UFLA)

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Tityus serrulatus venom affects the cell cycle and DNA of plant cells and human leukocytes

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Tityus serrulatus venom affects the cell cycle and DNA of plant cells and human leukocytes

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ABSTRACT: The present work had the aim to validate plant bioassays for the screening of toxic effects of animal venom. For this end, the effects of *Tityus serrulatus* (Brazilian yellow scorpion) venom was accessed on the cell cycle and genetic material of the plant model *Lactuca sativa* L. (lettuce) and compared with the damages to human leukocytes. Thus, seeds of lettuce were exposed to venom solution and emitted roots were collected. The occurrence of cell cycle alterations and DNA fragmentation was evaluated by agarose gel electrophoresis and TUNEL assay. Incubations of venom with human peripheral blood cells were also held with subsequent evaluation of cell proliferation index, micronucleus formation and fragmented DNA molecules. The results demonstrated that the venom of *T. serrulatus* is mitodepressive and cytogenotoxic, reducing mitotic index and inducing alterations in the cell cycle (CCA) and micronuclei formation, considered an excellent marker of cytotoxicity. The occurrence of cell death (CD) was evidenced by the detection of condensed nuclei, positive TUNEL signals, and presence of DNA fragmentation in agarose gel on lettuce cells. The scorpion venom induced low levels of DNA fragmentation in the human leukocytes and a reduced number of micronuclei. Further, the role of peptides and proteases found in the venom in inducing the observed damage was discussed.

Keywords: Cell cycle alterations, TUNEL, DNA fragmentation, Comet assay, Micronuclei.

1 INTRODUCTION

Scorpionism is considered a public health problem all over the world. It is estimated that more than one million people are stung annually (CHIPPAUX; GOYFFON, 2008). In many cases there is development of sequelae, such as arthritis, as well as death (CHIPPAUX, 2012).

The species *Tityus serrulatus* (Brazilian yellow scorpion), is considered the most dangerous in South America (FREIRE-MAIA; CAMPOS; AMARAL, 1994). It presents wide distribution in Brazil, including the whole southeast and parts of the central and southern regions of the country, being responsible for the majority of the accidents related to venomous animals (COLOGNA et al., 2009).

The venom of *T. serrulatus* is composed by a complex mixture of substances. Among these are enzymes (hyaluronidases and proteases), peptides, mucus, amino acids, nucleic acids and salts. Neurotoxins (peptides) are the main components of the venom, and act on different ionic channels, being responsible for physiological alterations that may result in death. Moreover, the proteases and hyaluronidases may leverage the effects of the neurotoxins, since they act as spreading factors of the venom (ALVARENGA et al., 2012; HORTA et al., 2014). Also, antimicrobial components and other peptides are present, however the biological functions of these have not been completely established thus far (PUCCA et al., 2014).

Considering that the components of animal venoms may act on the hemostatic, immunological and neural systems, in addition to induce cell death, and that the occurrence of the *T. serrulatus* in Brazil is prolific, studies that allow understanding the mechanisms of action of its venom are of great value in the medical-scientific scope.

Active agents found in venoms have been used as model for the development of drugs. The works in this area have intensified over the last

decades, resulting in the discovery of new medicines with wide therapeutic uses, like anticoagulants (e.g. Ancrod, Batroxobin and Echistatin), antihypertensive (e.g. Captopril and Ranatensin) (KOH; KINI, 2012), as well as analgesics and pharmaceuticals that treat Diabetes Mellitus (HARVEY, 2014; KING, 2011; KOH; KINI, 2012).

However, both the characterization of pharmacologically active molecules and the development of new pharmaceuticals require tests that use animals as guinea pigs. This is a practice which often faces logistic and economic problems related to the maintenance of the animals, in addition to legal and ethical barriers. Researches with animals are subject to prior evaluation and supervision by ethics and animal rights committees, which makes the process slow and often unviable. An alternative for animal tests could thus be the use of plant organisms as models to investigate mechanisms of action, especially for pilot assays and initial screenings with different samples and dosages.

The use of plant models in bioassays that prospect toxicity (cyto- and genotoxicity) of environmental pollutants and plant extracts or oils has been of great value in ecotoxicology and allelopathy researches (ANDRADE-VIEIRA et al., 2011; CAMPOS et al., 2008; FISKESJÖ, 1985, 1988; LEME; MARIN-MORALES, 2008, 2009). These models are accepted as efficient methods to evaluate cytological and genetic material damage (ANDRADE-VIERA et al., 2014; LEME; MARIN-MORALES, 2009; PALMIERI et al., 2014). The assays using higher plants as models are advantageous and justifiable due to presenting low cost, good correlation with other test models and systems, such as animals, apart from being highly reliable (DONG; ZHANG, 2010; GRANT; OWENS, 2006). Besides, they are considered adequate test systems to evaluate toxicity by the United Nations Environment Programme (UNEP), the World Health

Organization (WHO) and the United States Environmental Protection Agency (US- EPA) (GRANT, 1982, 1999)

Therefore, the main objective in this work was to determine the validity of plant bioassays for the screening of toxic effects of animal venom. In order to achieve this tag, the effects of *T. serrulatus* venom were evaluated on chromosomes during the cell cycle, on the DNA and on the process of cell death of *Lactuca sativa* meristematic cells. According to Campos et al. (2008a), this plant model is considered suitable to evaluate toxic compounds, since it presents high sensitivity to toxic agents and have easily visible chromosomes. For this, we applied classic cytogenetics by means of cell cycle evaluation; molecular genetics, evaluating the fragmentation of the genomic DNA in agarose gel; and molecular cytogenetics, using the TUNEL test (Terminal deoxynucleotidyl transferase dUTP nick end labeling).

To further study the genotoxic/mutagenic potential of *T. serrulatus* venom the comet assay (single cell gel electrophoresis) and micronucleus assay on human leukocytes were also performed, allowing comparative analysis of results obtained with plant and animal cells. Both tests are considered of high value when studying ecotoxicology (GAUTHIER et al., 1999; TICE et al., 2000), nutrition (FENECH; FERGUSON, 2001; FENECH; RINALDI, 1995), risk assessment for cancer, biomonitoring of human populations (FENECH et al., 1999), molecular epidemiology (FALCK et al., 1999; NORPPA, 1997) and to identify the mutagenic potential of new pharmaceuticals, agrochemicals and chemicals or physical agents in general (KIRSCH-VOLDERS, 1997).

Finally studies on cytogenetic toxicology of venoms are still very scarce in the literature, which increases the importance of the present work extending the *T. serrulatus* venom characterization.

2 MATERIAL AND METHODS

2.1 Obtainment of the Biological Material

2.1.1 *Tityus serrulatus* Venom

The venom was collected at the serpentarium of the University of São Paulo (USP), campus of Ribeirão Preto, SP, by one of the authors (Marcussi, S.). After it was collected, the venom was immediately frozen and lyophilized, which allowed its weighing.

2.1.2 Root Tips Treatment

Commercial seeds of *L. sativa*, were pre-germinated in Petri dish, covered by filter paper moistened with distilled water, for 24 h. After this period, five seeds with emitted roots of about 0.5 mm length were transferred to a second Petri dish, covered with filter paper, containing 500 µL of *T. serrulatus* venom solution (0.5 mg/mL), in total the seeds were exposed to 0.25 mg of venom. These concentrations were based on pilot studies. The negative control was achieved with distilled water. The Petri dishes were sealed with *Parafilm*® and remained in germination chamber at 20 ºC for 24 h.

2.1.3 Human cell preparation

Human blood was obtained from 20 healthy volunteers (10 for the Micronucleus and 10 for Comet assay) after obtaining formal consent. The donors were between the ages of 18-30 years old and no distinction regarding gender were made. Volunteers had not made use of any medication in a

minimum period of one month before the blood collection. Venous blood was collected in heparinized tubes and distributed in fractions of 500 µL per flask for cultivation. Three cell culture flasks were used for each treatment/experiment/volunteer, and the culture period was of 72 h at 37 ºC for the micronucleus and 7 h at the same temperature for the comet assay. Peripheral blood mononuclear cells (PBMCs) were cultivated in total blood RPMI 1640 medium (5 mL) supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 100 U/mL penicillin and streptomycin and 1% phytohemagglutinin (Gibco BRL) in 5% CO_2 at 37°C. Experiments were approved by the Research Ethics Committee of FCFRP-USP (nº 102).

2.2 Bioassays in plant cells

2.2.1 Cell Cycle Evaluation

Exposed roots (2.1.2) were collected and fixed for 24 h in ethanol:acetic acid solution (3:1). Then, fixed roots were washed in distilled water and hydrolyzed in 5 N HCl at room temperature (20 to 25ºC) for 12 min. The slides were prepared by squashing technique and the material was stained with 2% acetic orcein. Ten slides were evaluated per treatment, and 500 cells were counted per slide (totalizing 5,000 cells analyzed per treatment).

The evaluated parameters were: (1) mitotic index (MI), given by the ratio between the total of dividing cells and the total of evaluated cells, (2) cell cycle alterations (CCA): the sum of sticky chromosomes, c-metaphases, chromosome fragments, multipolar anaphases, chromosome bridges and non-oriented chromosomes, micronuclei (MCN) and condensed nuclei (CN), expressed in frequency per one thousand cells. The percentage rate of each alteration within the total of observed abnormalities (CCA) was also measured.

2.2.2 DNA Fragmentation Evaluated in Agarose Gel

The total DNA of 1 g of freshly collected *L. sativa* roots exposed to venom solution was extracted according to DOYLE; DOYLE (1987). The roots were homogenized in liquid nitrogen and incubated in buffer containing 2% hexadecyltrimethylammonium bromide (CTAB) at 65 ºC. The DNA was extracted with chloroform-isoamyl alcohol (24:1) and precipitated in cold isopropanol. The isolated DNA was maintained in TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). The sample was subjected to 1.2% agarose gel electrophoresis in TBE 0.5 X containing 0.5 µg/mL of ethidium bromide. DNA fragmentation was observed under UV light, and the gel image was captured with a Kodak camera. As reference for evaluation of the fragmentation, a standard containing fragments of 100 bp to 1500 bp (Promega) was applied. DNA molecules extracted from the roots used as control were used as comparator. The data presented here is based on three repetitions of the assay.

2.2.3 TUNEL Assay

The TUNEL reaction was carried out according to the manufacturer's instructions for the kit DeadEnd™ Fluorometric TUNEL System (Promega®). For preparation of the slides, freshly exposed root meristems were hydrolysed with 1 N HCl at 60°C for 15 min., and next fixed in 4% paraformaldehyde for 25 min. at 4°C before performing the TUNEL reaction. The positive reaction was marked with fluorescein, and the countermarking was accomplished with use of propidium iodide. The slides were evaluated in epifluorescence microscope (Olympus BX60) using the filters with excitation interval of 460-

490 nm to evidence the fluorescein, and of 530-550 nm for propidium iodide. The test was repeated three times and 100 cells were analyzed per slide.

2.3 Bioassays in human cells

2.3.1 Cytokinesis-block Micronucleus (CBMN) test

This test allows the observation of human lymphocytes after one nuclear-division phase in the presence of treatments and the number of micronuclei observed can be determinant of mutagenic potential. The samples (*T. serrulatus* venom; 1 to 120 µg/mL) were added 24 h after the initiation of the cultures. After 44 h, cytochalasin-B (4 µg/mL, Sigma) was added to the cultures. The CBMN test preparations were performed according to FENECH; MORLEY (1985). The analyses were carried out after 72 h. Scores were taken according to the criteria of FENECH (2000). All slides were coded and scored blindly. Three slides were made for each flask/treatment/experiment-volunteer, and 1,000 binuclear cells were counted considering the presence or not of micronuclei, this way making it possible to determine the genotoxic effect of venom. The antineoplastic drug, Cisplatin (PLATINIL®, Quiral Química do Brasil S.A.) (6 µg/mL) was used as positive control.

2.3.2 Cell proliferation index

Values recorded of cytokinesis-block proliferation index (CBPI) are very important to identifying cytotoxic doses. The CBPI was calculated by counting 500 cells, considering the number of nuclei mono, bi, tri or tetranucleated. The CBPI defines whether the cultures are multiplying normally

after the addition of venom. The following formula was used according to KIRSCH-VOLDERS (1997): CBPI = 1 (mono) + 2 (bi) + 3 (tri + tetra) / 500.

2.3.3 Comet assay

This test was performed according to the methodology described by SINGH et al. (1988). The cells were incubated with different treatments for 4 h at 37ºC, and were then utilized to prepare the slides before the first cellular division. A cellular suspension containing approximately 10^5 cells/mL was used to work with 5 to 8 million cells per slide. Three slides were made for each flask of each treatment/experiment-volunteer. 100 nucleoids were evaluated per slide, being three slides per flask and three flasks per treatment/experiment, totalizing 900 nucleoids. Approximately 60 µL of each cell culture were transferred to microtubes containing 300 µL of LMP (low melting point) agarose, for the slides preparation in triplicate. The mixture was homogenized and dropped on the slides (100 µL/slide) with NMP (normal melting point) agarose and covered with the coverslips. The cells were submitted to 4 °C in the refrigerator for 10 min, after that, the coverslips were removed and the slides immersed in lyses solution (containing 0.25 M NaCl, 100 mM EDTA, 10 mM Trizma base, pH 10 adjusted with 10 N NaOH, 5% DMSO and 1% Triton X-100), remaining there for 2 h, being this procedure responsible for the achievement of the nucleoid. Doxorubicin (DRX) (Bergamo Ltda) (6 µg/mL) was used as a positive control. All the procedures described above and the electrophoretic running, were carried out in the dark.

2.3.4 Electrophoresis

Before the electrophoretic run, the slides were kept in electrophoresis solution (300 mM sodium hydroxide and 1 mM EDTA, pH 13) for 20 min at 4 ºC. The electrophoretic run was programmed at 25 V and 300 mA, and the run time was fixed as 35 min. After the run, the slides were immersed in neutralization solution (0.4 M Tris-HCl, pH 7.4) for 20 min., dried at room temperature and fixed with 100% ethanol for 5 min.

2.3.5 Staining and analysis

The stainig was performed with ethidium bromide solution at 2 µg/mL. To that end, 50 µL of this solution was placed over each slide, protected from light, covered with a coverslip and immediately analyzed by fluorescence microscopy at 200 and 400X magnificence. Comet standards were analyzed by visual scores according to COLLINS; DUTHIE; DOBSON (1993), with minimal modifications. The cells analyzed were classified by DNA injury extent in 5 classes: class 0, without damage (damage < 5%); class 1, low level of damage (5-20 %); class 2, medium level of damage (20-40 %); class 3, high level of damage (40-85 %) and class 4, totally damaged (damage > 85%). The average frequency of damage was calculated from the sum of the percentages of nucleoids with damage 1, 2, 3 and 4. In order to perform comparative analysis, data were calculated with arbitrary units (AU) as described by COLLINS (2004). The arbitrary units (0-400; where 0 = no damage and 400 = 100% damage) were calculated by the equation (1 x number of nucleoids grouped in class 1) + (2 x number of nucleoids in class 2) + (3 x number of nucleoids in class 3) + (4 x number of nucleoids in class 4).

2.4 Statistical Tests

Statistical analysis was performed at the free Software "R" (R DEVELOPMENT CORE TEAM 2015). The parameters evaluated in the cell cycle and TUNEL test assessments were subjected to analysis of variance (ANOVA) followed by Tukey's test of means at 5% significance. For the comet and micronucleus assays the AU values of each treatment were compared by Duncan ($P<0.05$) test.

3 RESULTS AND DISCUSSION

The present work describes for the first time the utilization of plant cells as model systems to evaluate the action of animal venom. The data obtained here show that *T. serrulatus* venom has a toxic effect on plant cells and DNA, affecting the chromosome structure, causing breaks directly in the DNA molecule, and leading to cell cycle alterations.

In the cell cycle analyses of lettuce, significant statistical differences were noticed between the two evaluated parameters (MI and CCA), compared to the control. An increase of about 15 times was detected in the CCAs of the cells exposed to *T. serrulatus* venom in relation to the control (Table 1).

MCN, sticky chromosomes, c-metaphases, bridges, chromosome fragments, non-oriented chromosomes, multipolar anaphases and condensed nuclei were observed. Figure 1 brings examples of the CCA most commonly (Figure 2) found in the slides evaluated for this work.

The most abundant alterations were MCN (Figures 1A and 1B), representing 32% of the total of observed alterations (Figure 2). The presence of MCN in large proportion serves as an excellent indicator for the genotoxic potential of the venom (HOSHINA; MARIN-MORALES, 2009). They can be seen as a subproduct of the various alterations of the cell cycle, since they are only visualized when initial damage suffered by the DNA molecule is not correctly repaired (GARCÍA-QUISPES et al., 2013). MCN are formed by exclusion of whole chromosomes or chromosome fragments during the cell division; thus, their presence indicates that the agent has aneugenic and/or clastogenic activity (FENECH; CROTT, 2002). Further, they reflect genomic instabilities that point to degradation of somatic DNA, which may have been inherited from previous cell generations or acquired *de novo* (BONASSI et al., 2006; CASELLA et al., 2003; KIRSCH-VOLDERS et al., 1997).

The second highest observed alteration was sticky chromosomes (Figure 1C), which represent 20% of the total alterations (Figure 2). The venom of *T. serrulatus* is composed by a variety of proteases (HORTA et al., 2014), which allows to infer their action on the protein scaffold of the chromosome, destabilizing it, affecting its normal complexion and resulting in the appearance of sticky chromosomes (EL-GHAMERY; EL-KHOLY; ABOU EL-YOUSSER, 2003). This occurs after alterations in the basic chromosome structure, which promotes loss of the normal condensation characteristics of the chromosomes, forming agglomerates. The mechanism that leads to the appearance of sticky chromosomes is attributed to damage on the physical-chemical structure of the DNA, its protein skeleton, or both (AMIN, 2011).

The various proteases found in *T. serrulatus* venom could also be acting over the proteins that form the mitotic spindle. Damage to the mitotic spindle affect the chromosome movements during division, possibly leading to the occurrence of non-oriented chromosomes, multipolar anaphases and c-metaphases (FISKESJÖ, 1985), all detected in the present work (observed in 5.33%, 1.33% and 12% of the total of CCAs, respectively – Figure 2).

The proteases might also be acting on proteins associated directly to the DNA, destabilizing the molecule and favoring the occurrence of breaks, which can be evidenced by the formation of bridges and fragments (each of them having been observed in the proportion of 5.33% of the total CCAs – Figure 2). The bridges result from breaks that lead to loss of the telomeres, a region located at the extremities of the chromosome arms. One of the functions of the telomeres is to grant protection and stability to the chromosome, avoiding fusions (LEME; MARIN-MORALES, 2009). The extremities without telomeres tend to fuse, leading the formation of a bi-centric chromosome. During the mitosis, in anaphases, the bi-centric chromosome is observed as a bridge between the two poles. Meanwhile, the acentric segments excised from the chromosomes

constitute fragments that are eliminated by way of micronuclei (MCN) (CAMPOS et al., 2008).

This instability of the DNA structure and the damage suffered by the molecule may also be determined by the occurrence of condensed nuclei (CN). CNs are considered cytological markers of cell death (CD) (ANDRADE-VIEIRA et al., 2011), and are related to DNA fragmentation induction by the toxic agent, observed in agaroses gel as DNA laddering pattern (DANON et al., 2000). CD is part of the normal life cycle of a plant, and may be activated under stress when the mechanisms of DNA repair are not capable of compensating the suffered damage. Thus, the occurrence of CD, evidenced cytologically by the presence of CN, is considered a great measurer of toxicity (VAN-DOORN; WOLTERING, 2010).

The high index of CN found, 18.67% (Figure 1C), suggests that *T. serrulatus* venom presents genotoxic potential, responsible for the induction of damage that is not repaired. As consequence, plant aborts the damaged cells. The DNA laddering effect becomes evident by the fragmentation of the genetic material observed in the agarose gel electrophoresis, which showed fragments of 100 to 500 bp (Figure 3). These data are corroborated by the positive result of the TUNEL assay, which detected damage in the DNA molecules in 23% of the evaluated cells (Table 1 and Figure 4).

The induction of DNA damage followed by CD may also be attributed to the presence of free radicals (reactive oxygen species – ROS), associated to oxidative stress (NAWKAR et al., 2013; ZHANG et al., 2010). It is thus possible that the occurrence of CD is induced as a defense and response mechanism to ROS, formed by stress caused by exposure of the root cells to the venom of *T. serrulatus*.

In addition, the identification and signaling of the ROS and CD are related to ionic channels. These processes are linked to the transport of various

ions, including K^+ (DEMIDCHIK, 2015; DEMIDCHIK et al., 2010). Among the peptides present in the venom of *T. serrulatus* are various neurotoxins, capable of acting by blocking or deregulating the functioning of ionic channels (COLOGNA et al., 2009). These toxins are the object of scientific interest due to the importance of these channels in the physiology of both animals and plants (QUINTERO-HERNÁNDEZ et al., 2013). Some peptides of the *T. serrulatus* venom may be acting on ionic channels transporters of K^+, so as to activate the CD mechanism, resulting in the great amount of CN observed in the present study. Moreover, the action of the neurotoxins on channels related to the recognition and signaling of ROS might interfere with the neutralization of the ROS, resulting in damage to the DNA molecules.

The presence of DNA damage due to the formation of ROS induced by the different proteases and classes of snake toxins such as LAAOs, metalloproteases and PLA_2s is also reported on literature (IWANAGA; SUZUKI, 1979; KANG et al., 2011). Specifically on *T. serrulatus* it is known that Ts2 and Ts6 toxins are capable of affecting the immune system causing leukocytosis and trigger the increase of inflammation via a mechanism dependent on lipid mediators and cytokine production in human cells (ZOCCAL et al., 2013). A dysfunctional immune system coupled with inflammation could result in the formation of ROS that would not trigger proper regulatory responses by the organism resulting in high levels of oxidative stress and causing widespread DNA damage.

Both the DNA fragmentation and the detected alterations have an impact on the MI, which decreased approximately 33% in cells exposed to venom in relation to the control (Table 1). Cells in CD process or that present alterations, and thus have their standard physiology hampered, do not enter the process of cell division. This harms the organism as a whole, since its development and growth are affected. Another factor that may be acting on the reduction of MI is

the inadequate transport of K^+ ions, which in plants are associated to cell elongation and proliferation (LEBAUDY; VÉRY; SENTENAC, 2007).

Thus, the results obtained in the present work demonstrate a clear genotoxic potential of *T. serrulatus* venom on lettuce cells. However, it is important to point that the effects of this venom were also evaluated over leukocytes of human peripheral blood (assessed by comet and micronucleus tests) and its genotoxic potential was once again noticeable.

For the comet assay, the venom was evaluated at concentrations between 1.0 and 120 µg/mL. Significant differences for the higher comet classes, especially on the higher concentrations, in relation to the negative control were observed (Table 2). The AU count and the total percentage of damaged cells demonstrates in a clear way the magnitude of such damage. Figure 5 shows the percentage of damage and the AU count on human leukocytes exposed to concentrations of 1, 2.5, 15, 30, 60 and 120 µg/mL of *T. serrulatus* venom. In addition, at the highest concentration applied, the total percentage of induced damage (approximately 40%) and AU count (approximately 75) were very similar to those observed for the positive control (doxorubicin 6µg/mL). This data is in accordance with what was detected by the TUNEL test and by the fragmentation on DNA observed in the agarose gel electrophoresis on *L. sativa* cells. It can also be relate to data found for the effects of snake venom or its isolated toxins on the DNA molecule when utilizing the comet and the micronucleus assay (MARCUSSI et al., 2011, 2013).

Nevertheless, for the MCN test in human leukocytes, the venom of *T. serrulatus,* on the concentrations between 1 and 30 µg/mL, did not induce micronucleus formation significantly different from the negative control (1.1 ± 0.1 MCN) (Table 3). It is suggested that the low frequency of MCN found may be the result of effective action of the cell repair mechanisms. This way, the venom is inducing damage that is liable to correction, and thus not observed by

the mentioned test. However, the data obtained here show that at least part of this damage is not repaired effectively, since the occurrence of CN and MCN was observed in high frequency on the tests using *L. sativa* as model and the two highest concentrations tested (60 and 120 µg/mL) showed a significant increase on the MCN formation in human leukocytes when compared to the negative control.

Citogenotoxic researches studying the venom of different species can be found on the literature. These studies relate to the present work, showing similar results. ZARGAN et al. (2011) demonstrated that *Androctonus crassicauda* venom has toxins that are able to limit the growth of SH-SY5Y and MCF-7 cancer cell lines by a mechanism of apoptosis induction and DNA synthesis blocking. Another work showed that *Apis mellifera* venom induced the formation of a significant number of micronuclei on HL-60 cancer cells and also DNA fragmentation when evaluated by micronucleus and comet test, respectively (LEE et al., 2007). GAJSKI; GARAJ-VRHOVAC (2008) observed DNA instability in human lymphocytes after prolonged exposure (up to 24 h) to high concentrations (100 µg/mL) of *Apis mellifera* venom. Lastly, DAS GUPTA et al. (2007) observed the antiproliferative, cytotoxic and pro-apoptotic effects induced by the venom of *Heterometrus bengalensis* on a line of leukemic human cells.

All these studies cited used either animals or human cells as test models. It is thus demonstrated that the data available in the literature stablish a direct relationship to the results found in this work, which used both cells of humans and of a plant model. Furthermore, the importance of toxicological screenings that allow the broadening of the functional characterization of venoms and generate mechanistic discussions about the toxins by which these venoms are composed cannot be underestimated. With this in mind, venoms of different species are currently under evaluation by the authors of the present work with

the aim of complementing the data obtained thus far and reaffirming the usefulness of plant cells as a model for the screening of cytogenotoxic effects induced by natural compounds, including venom.

However it is worth noting that the amount of venom used in the tests with plant cells (0.25 mg or 250µg) was more than twice the highest concentration evaluated on leukocytes (0.12 mg, or 120 µg). The choice for the elevated dose resulted from the consideration of two aspects: 1) five *L. sativa* seeds were exposed to venom solutions applied on germination paper over a petri dish, therefore the actual amount of venom per seed was of 0.05 mg or 50 µg; and 2) the presence of cell wall could impose an additional barrier for the penetration of the venom, when comparing the morphology of plant and animal cells. Nevertheless, the observed results demonstrate that the toxins present in the venom were able to cross the cell wall and induce toxicity in plant cells. Moreover, in plant models the exposure occurs in the meristematic tissue of root tips, which undergoes many rapid cell divisions, thus increasing its sensitivity to external agents. The mentioned characteristic makes plant test models very responsive, and leverages their use in screening for toxic substances (FISKESJÖ, 1988).

4 CONCLUSION

The venom of *T. serrulatus* presented high cytotoxicity in the meristematic cells of *L. sativa*, evidenced by the reduction in the mitotic index, and high genotoxicity, demonstrated by the high frequency of abnormalities in the cell cycles.

Under the evaluated conditions, the venom induced cell death, manifested by damage to the DNA molecule, positive TUNEL assay, fragmentation of the genomic DNA subjected to agarose gel electrophoresis, and by the presence of micronuclei, condensed nuclei, and chromosome bridges and fragments.

The results revealed relevant information for better understanding the mechanisms of action of *T. serrulatus* venom. The proteases present in the venom probably act on the protein scaffold structure of the chromosomes, spindle fibers and proteins associated to the DNA molecules, resulting in its fragmentation. The neurotoxins may cause damage to the DNA molecules, deregulating or hampering the functioning of ionic channels related to the activation of cell death processes and ROS identification and signaling.

Similarly the comet assay showed that the *T. serrulatus* venom induced DNA damage in human leukocytes. The results suggest that smaller concentrations of the venom are dealing damage but this damage is being effectively repaired by the cells natural repair mechanisms and cell cycle checkpoints. However the damage is not repaired effectively on the highest concentration tested suggesting a dose dependent effect.

Moreover, the employed plant model responded well to the toxic effects of this venom on the genetic material and the cell cycle, thus being a viable test system to evaluate the toxicity of animal venoms, representing a rapid, low-cost, easy-to-use alternative to complement or even substitute animal models during

early screenings. In addition, the observation of cytogenotoxic effects induced by the venom on the morphology and development of plant cells, as well as on their genetic material, uncovers a broad perspective for utilization of plant cytogenetic tests for investigation of natural, chemical or synthetic compounds of pharmaceutical interest, aiming to reduce research time and costs.

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Table 1. Effect of *T. serrulatus* venom on the mitotic index (MI), cell cycle alterations (CCA) and TUNEL test after exposure of *L. sativa* roots to 0.25 mg of venom.

Treatments	MI	CCA	TUNEL
Control	9.29 ± 2.40	0.99 ± 1.03	0.20 ± 1.49
T. serrulatus	6.23 ± 2.19*	14.9 ± 5.80*	23 ± 7.74*

Observed results followed by standard deviation in the two evaluated parameters and in the TUNEL test. MI is expressed as percentage, and CCA as frequency per thousand cells. The TUNEL result is given by the percentage of cells with the positive mark. Data followed by * differ significantly from the control according to Tukey test (α=5%).

Table 2. Distribution of comet classes observed in nucleoids from leukocytes treated with *Tityus serrulatus* venom in different concentrations, or the antitumor agent doxorubicin.

Treatment [µg/mL]		**Comet classes (%)**				
		0 (damage ≤ 5%)	**1 (5-20%)**	**2 (20-40%)**	**3 (40-85%)**	**4 (damage ≥85%)**
Negative control	-	78±0.02	22±0.03	0±0.01	0±0.02	0±0.02
Positive control (DXR)	6	33.5±0.04a	25.75±0.05	2.5±0.02	1±0.05	10.5±0.04a
T. serrulatus	1	70.2±2.1ab	23.5±1.3	4.1±0.8a	2.2±1.7a	0±0.03
	2.5	69.1±5.4ab	24.6±2.5	5.2±1ab	1.1±0.3	0±0.2
	5	72.9±7b	21.8±3.1	4.8±0.5a	0.5±0.1	0±0.1
	7.5	74±6.4b	22.5±1.1	2.5±0.6	1±0.1	0±0.4
	10	75±4b	20.4±1.4	3.6±0.8	1±0.4	0±0.8
	15	64.5±3.2ab	31±3.5ab	4.5±1.2a	0±0.2	0±0.5
	30	61.5±5.4ab	26±2.8	9±1.3ab	3.5±0.2ab	0±0.5
	60	75±3.8b	19±1.6	11.5±1.1ab	3±0.7ab	1.5±0.2
	120	60.5±2.6ab	15.5±2.2ab	14.5±1.6ab	6.5±1.1ab	3±0.3ab

Data represents the mean of each treatment ± S.D. for 10 individual experiments, one for each volunteer. Three flasks were prepared for each treatment and for each volunteer's blood, with cells remaining in culture for 7 h. 100 nucleoids were observed by flask, with three flasks per treatment/volunteer resulting in a total of 300 nucleoids. The classification was made according to Collins et al. (1993). DXR = doxorubicin.
a Significantly different from the negative control (p<0.05).
b Significantly different from the positive control DXR (p<0.05).

Table 3. Distribution of micronuclei and cytokinesis-blocking proliferating index (CBPI) in leukocytes treated with *Tityus serrulatus* venom in different concentrations or with the antitumor agent cisplatin.

Treatment [µg/mL]		**% cells/500cells**				**MN/1000BN cells, mean ± S.D.**	**CBPI ± S.D.**
		Mono	**Bi**	**Tri**	**Multi**		
Negative control	--	50.3	39	7.3	3.4	1.1±0.1	1.604±0.6
Positive control	6	48.5	38.7	7.6	5.2	12±0.5	1.643±0.5
T. serrulatus	1	48.9	42.5	6.2	2.4	1.0±0.1b	1.686±0.3
	2.5	47.8	36.2	11	5	0.8±0.2b	1.652±0.4
	5	46.9	41.3	8.9	2.9	1.2±0.2b	1.649±0.7
	7.5	35.3ab	48.6ab	8.6	7.5a	1.2±0.1b	1.808±0.2
	10	46.9	37.6	9.3	6.2	1.4±0.3b	1.686±0.6
	15	52.4	37.9	6.4	3.3	1.5±0.3b	1.572±0.9
	30	51.4	38.3	5.8	4.5	1.8±0.4b	1.598±0.9
	60	37.4ab	41.2	13.3ab	8.1a	5.4±0.2ab	1.840±0.5
	120	45.3	38.2	14.1ab	2.4	9.7±0.5a	1.712±0.4

BN: binucleated cells; MN: micronuclei; CBPI: cytokinesis-blocking proliferating index. 1000 binuclear cells were analyzed per volunteer for each treatment, with ten volunteers for counting micronuclei. Data are represented as means ± S.D. The values of CBPI were not statistically different compared to the control ($p<0.05$). Negative control = PBS. Positive control = cisplatin.

a Significantly different from the negative control ($p<0.05$).

b Significantly different from the positive control, Cisplatin ($p<0.05$).

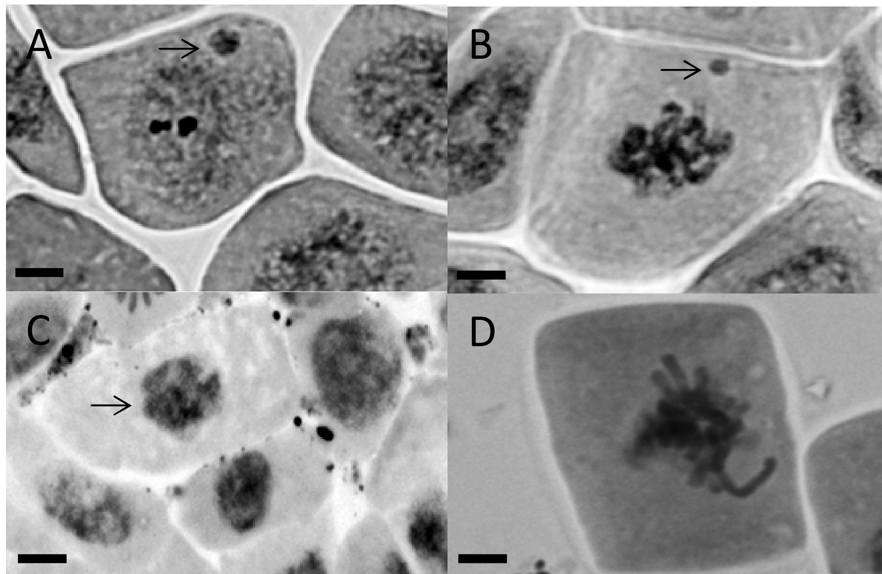

Figure 1. Most frequent cell cycle alterations found in root tip cells of *Lactuca sativa* exposed to *Tityus serrulatus* venom.

A and B – Micronuclei (arrow); C – Condensed nuclei (arrow); D – Metaphase with sticky chromosomes. Bar = 5 μm.

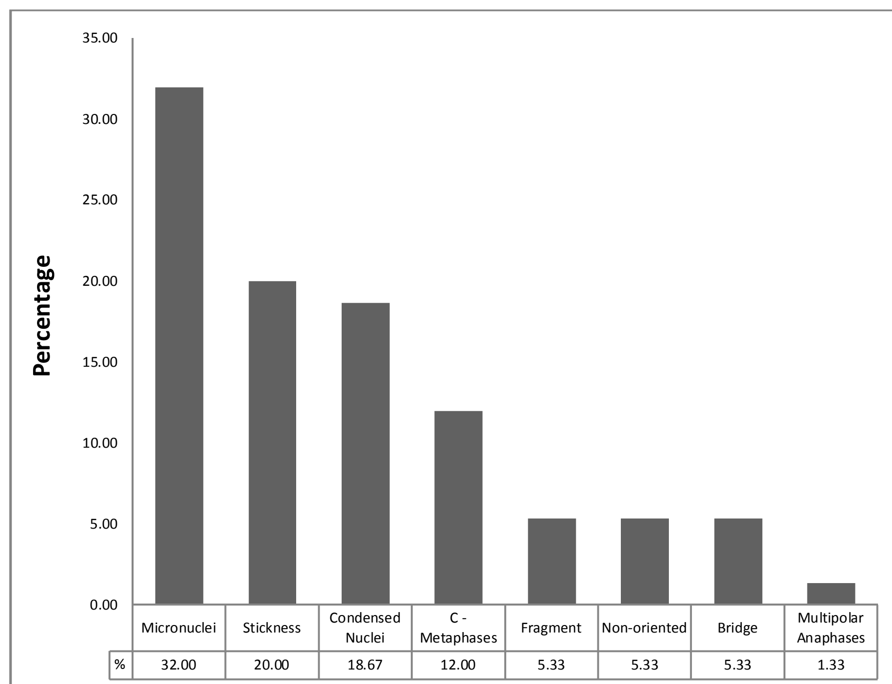

Figure 2. Percentage of CCAs observed on root tips of *L. sativa* exposed to *T. serrulatus* venom

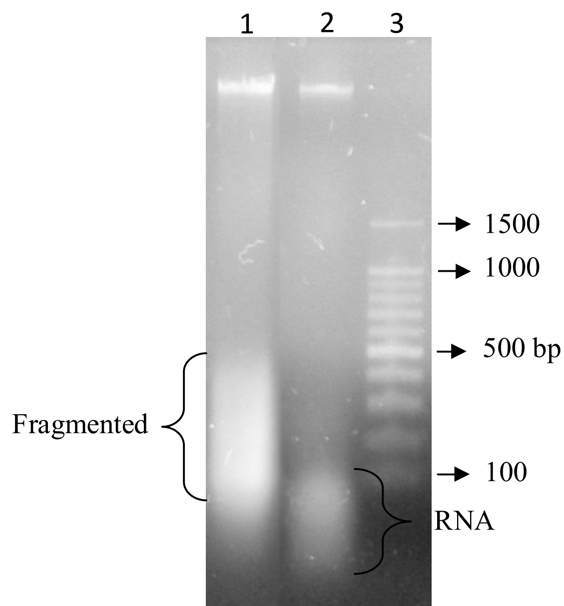

Figure 3. Gel demonstrating the occurrence of DNA-laddering in the genetic material of *Lactuca sativa* cells exposed to *Tityus serrulatus* venom.
1 – *Tityus serrulatus venom;* 2 – Comparator (distilled water); 3 – Fragmentation pattern (100 bp).

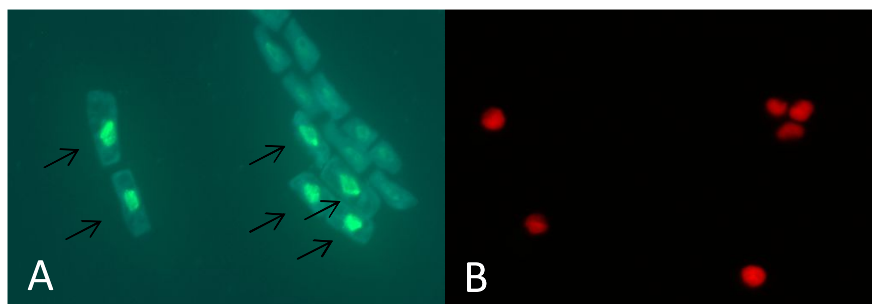

Figure 4. TUNEL test. Results observed after exposure of *L. sativa* roots to 0.25 of *Tityus serrulatus* venom.
A – Positive signal of the TUNEL test on cells exposed to the *Tityus* venom (seen on the fluorescein filter). B – Control cells showing no positive markings (image is composed of the juxtaposition of the fields observed on the fluorescein and iodide filters)

(A)

(B)

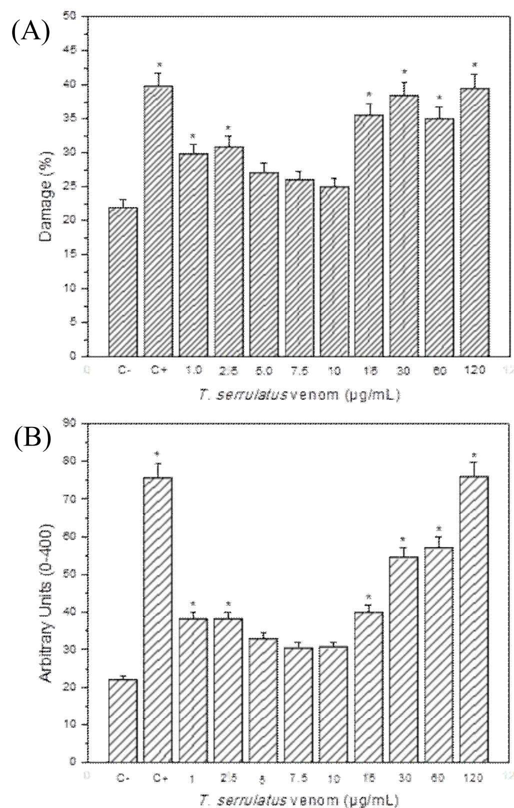

Figure 5. Comet test. (A) Frequency of nucleoids from leukocytes with comet and (B) Values of arbitrary units calculated to nucleoids with damage exposed for 4 hours at *Tityus serrulatus* venom in different concentrations, or the antitumor agent doxorubicin.

Data represent the mean of each treatment ± S.D. for 10 individual experiments, one for each volunteer. Three flasks were prepared for each treatment and for each volunteer's blood, with cells remaining in culture for 7 h. 100 nucleoids were observed by slide, with three slides by flask, three flasks per treatment/volunteer resulting in a total of 900 nucleoids.

The percentage of nucleoids with damage was calculated on the sum of nucleoids classified with damage 1, 2, 3 and 4. Arbitrary units (0–400) calculated according to Collins (2004).

* Significantly different from the negative control ($p<0.05$).

ARTIGO 2

Effect of *Polybia occidentalis* and *Polybia fastidiosa* venom on the cell cycle and the DNA integrity of *Lactuca sativa* L.

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Effect of *Polybia occidentalis* and *Polybia fastidiosa* venom on the cell cycle and the DNA integrity of *Lactuca sativa* L.

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ABSTRACT: Plant bioassays are efficient tools to detect toxicity by chemicals on cells and DNA. Therefore, it could be an alternative approach for screening the citogenoetixicity of venoms. Thus, the aim of the study was to evaluate the effects of two wasps venom, *Polybia occidentalis* and *Polybia fastidiosa*, on the cell cycle and genetic material of the plant model *Lactuca sativa* L. (lettuce). For this, seeds were exposed to venom solution; emitted roots were collected and the occurrence of cell cycle alterations and DNA fragmentation was evaluated by agarose gel electrophoresis and TUNEL assay. The results demonstrated that the venom of both wasps induce several cell cycle alterations (CCA) and reduces the mitotic index (MI) on treated cells. High frequencies of fragments were observed in cell exposed to *P. occidentalis* venom, while those exposed to *P. fastidiosa* showed a high frequency of non-oriented chromosome. Both species venom induced the occurrence of various condensed nuclei (CN). This alteration is considered an excellent cytological mark to cell death (CD). Additionally, CD was evidenced by positive signals in TUNEL assay, and DNA fragmentation in agarose gel electrophoresis. Further, the role of mastoporam, hiarulonidases, phospolipasesand, proteases and small pepitides of the venoms in inducing the observed damage was discussed.

Keywords: Cell cycle alterations, TUNEL, DNA fragmentation, Cell Death

1 INTRODUCTION

Hymenoptera (bees and wasps) venom is a complex mixture of different substances. It can have antimicrobial compounds, enzymes, neurotoxins, low molecular weight peptides and citolic peptides (Kuhn-Nentwig, 2003). There are a great variety of wasp species on Brazil but not much is known about the biochemical, pharmacological and immunological composition of their venoms. The little information existent show us that they contain, A and B phospholipases, hiarulonidases, proteases, mastoporan and acid phosphatases (Nakajima et al., 1985; King and Valentine, 1987).

Some components of venoms can be benefic if isolated and administered in the proper dose, as there are many medicines fabricated from isolated fractions of venoms, i.e. antihipertensives (e.g. Captopril and Ranatensin), anticoagulants (e.g. Ancrod, Batroxobin and Echistatin) (Koh and Kini, 2012), pharmaceuticals against Diabetes Mellitus and lastly analgesics (Harvey, 2014; Koh and Kini, 2012; King, 2011).

For wasps venom, specifically, substances with pharmacological potential as anticonvulsant (Cunha et al., 2005), anticoagulant (Han et al., 2008) and antimicrobial (Cerovský et al., 2008) capacity have been already described. Therefore, Hymenoptera venom is a very promising area of study for the discovering of medical useful molecules leading to the development of new biopharmaceuticals.

Both the characterization of pharmacologically active molecules and the development of new pharmaceuticals require various tests. The first goal to achieve this is to know the effects of venoms on target organisms. It is documented that venoms can cause cell death, acting on the immunological, neural and hemostatic systems, disrupting the normal physiology of living organism which may lead to death (Czaikoski et al., 2010). Thus far, the data

available about their capacity of inducing chromosome and cell cycle alterations, DNA breaks, or cell death is scarce, not many works in this field exist (Kirsch-Volders et al., 1997; Gupta et al., 2007; Gajski and Garaj-Vrhovac, 2008; Marcussi et al., 2011; Marcussi et al., 2013, Hoshina et al. 2013).

Therefore understanding how these venoms act on the cell cycle and DNA and their possible applications stands out as a promising field of research, establishing the groundwork for further studies that can use the data obtained in these more basic screenings to single out and/or determine potential toxins of medicinal interest.

The most common models used for studying the mechanism of action of a venom in an organism are mammals, often rats. This is, however, a practice looked down by many, since it puts the animals through a lot of suffering, being considered cruel by many researchers. Also, caring and handling these animals is expensive, requires lots of room and faces legal barriers, as the practice requires the approval of ethics and animal rights committees. This can sometimes make the tests unviable; therefore an alternative test model would be helpful. The use of plants to determine the cito and genotoxicitiy potential of natural products could be this alternative (Andrade-Vieira et al, 2014). Plant models have none of the legal and ethical problems that are common on animal tests and are especially interesting for pilot assays and screening when a great number of different samples and dosages are tested.

Plants have been used as models in allelopathy and ecotoxicology for toxicity prospection bioassays to evaluate environmental pollutants, oils or plant extracts for many years and to great avail (Fiskejö 1985; 1988; Leme and Marin-Morales,2008; 2009; Campos et al., 2008; Andrade-Vieira, 2011). Plants are considered adequate test models to evaluate toxicity by the United Nations Environment Programme (UNEP), the World Health Organization (WHO) and the United States Environmental Protection Agency (US- EPA) (Grant, 1985;

Grant, 1999). Grant and Owens (2006), Ferreti et al. (2007) and Dong and Zang (2010) consider test systems using higher plants as models advantageous for being easy to handle, having low maintenance costs, showing reliable results and presenting good correlation with other models, including human cells (Palmieri et al., 2016).

One work testing the efficiency of using plant as models for citogenotoxic screening of venoms has already been conducted. It tests the effects of *Tityus serrulatus* (Brazilian yellow scorpion) venom on the DNA molecule and the cell cycle of *Lactuca sativa* L (Palmieri et al., 2016 – submitted). The results proved the usefulness of the higher plant systems once more and showed that *L. sativa* is an efficient model for evaluating genetic and cytological effects of venoms. The present work aims to corroborate or deny such claim while also testing the application of the model for screening of different species venoms.

Therefore, the objective of the present work was to evaluate the effects of two wasps venom, *Polybia occidentalis* and *P. fastidiosa*, on cell cycle and the DNA of *L. sativa* cells. For this goal classic cytogenetics was applied to observe the mitotic chromosomes during cell cycle; as well as molecular genetics, evaluating the fragmentation of the genomic DNA in agarose gel; and also molecular cytogenetics, using the TUNEL test (Terminal deoxynucleotidyl transferase dUTP nick end labeling) to confirm the occurrence of cell death.

The observation of theses cytogenotoxic effects induced by the venoms on the morphology of chromosomes and the cell cycle of plant cells, as well as on their genetic material, uncovers a broad perspective for utilization of plant tests for investigation of natural, chemical or synthetic compounds of pharmaceutical interest, aiming to reduce research time and costs.

2 MATERIAL AND METHODS

2.1 Obtainment of the Biological Material

2.1.1 *Polybia* Venom

The venoms of *P. occidentalis* and *P. fastidiosa* was kindly provided by Doctor Marta Chagas (Universidade Federal do Paraná – UFPA). Wasps were euthanized on a gas chamber. Their venom glands were surgically extracted with the aid of a magnifying glass, tweezers and a scalpel, and then, frozen. For the venom extraction, glands were thawed, macerated after adding PBS and subjected to centrifugation for removal of cellular debris. The supernatant was then frozen and submitted to lyophilization, making it possible for the material to be weighted.

2.1.2 Root Tips Treatment

Commercial seeds of *L. sativa,* variety loose leaf Grand Rapids, were pre-germinated in Petri dish covered by paper moistened with distilled water for 24 h. After this period, the seeds that emitted roots of about 0.5 mm length were transferred to a second Petri dish, covered with moistened filter paper, containing 500 µL of *P. occidentalis* or *P. fastidiosa* venom solution (0.5 mg/mL), for a final dose of 0.25 mg. These concentrations were based on pilot studies. The negative control was achieved with distilled water. The Petri dishes were sealed with *Parafilm*® and remained in germination chamber at 20º C for 24 h. Next, some roots were collected and fixed for 24 h in ethanol:acetic acid solution (3:1) to be used in the cell cycle evaluation. For the TUNEL test and the

analysis of DNA fragmentation in agarose gel, the exposed roots were collected and immediately used on tests.

2.2 Bioassays

2.2.1 Cell Cycle Evaluation

The fixed roots were washed in distilled water and hydrolyzed in 5 N HCl at room temperature (20 to 25ºC) for 12 min. The slides were prepared by squashing technique and the material was stained with 2% acetic orcein. Ten slides were evaluated per treatment, and 500 cells were counted per slide (totalizing 5,000 cells analyzed per treatment).

The evaluated parameters were: (1) mitotic index (MI), given by the ratio between the total of dividing cells and the total of evaluated cells, (2) cell cycle alterations (CCA): the sum of sticky chromosomes, c-metaphases, chromosome fragments, multipolar anaphases, chromosome bridges and non-oriented chromosomes, micronuclei (MCN) and condensed nuclei (CN), expressed in frequency per one thousand cells. The percentage rate of each alteration within the total of observed abnormalities (CCA) was also measured.

2.2.2 DNA Fragmentation Evaluated in Agarose Gel

The total DNA of 1 g of freshly collected *L. sativa* roots exposed to venom solution (*P. occidentalis* or *P. fastidiosa*) was extracted according to Doyle and Doyle (1987). The roots were homogenized in liquid nitrogen and incubated in buffer containing 2% hexadecyltrimethylammonium bromide (CTAB) at 65 ºC. The DNA was extracted with chloroform-isoamyl alcohol (24:1) and precipitated in cold isopropanol. The isolated DNA was maintained in TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). The sample was subjected

to 1.2% agarose gel electrophoresis in TBE 0.5X containing 0.5 µg/mL of ethidium bromide. DNA fragmentation was observed under UV light, and the gel image was captured with a Kodak camera. As reference for evaluation of the fragmentation, a standard containing fragments of 100 bp to 1,500 bp (Promega) was applied. DNA molecules extracted from the roots used as control were used as comparator. The data presented here is based on three repetitions of the assay.

2.2.3 TUNEL Assay

The TUNEL reaction was carried out according to the manufacturer's instructions for the kit DeadEnd™ Fluorometric TUNEL System (Promega®). For preparation of the slides, freshly exposed root meristems were hydrolysed with 1 N HCl at 60°C for 15 min., and next fixed in 4% paraformaldehyde for 25 min. at 4°C before performing the TUNEL reaction. The positive reaction was marked with fluorescein, and the countermarking was accomplished with use of propidium iodide. The slides were evaluated in epifluorescence microscope (Olympus BX60) using the filters with excitation interval of 460-490 nm to evidence the fluorescein, and of 530-550 nm for propidium iodide. The test was repeated three times and 100 cells were analyzed per slide.

2.3 Statistical Tests

The parameters evaluated in the cell cycle and TUNEL assay assessments were subjected to analysis of variance (ANOVA) followed by Tukey's test of means at 5% significance using the free software R (R DEVELOPMENT CORE TEAM, 2015).

3 RESULTS AND DISCUSSION

Studies regarding the pharmacological applications of arthropods venom are relatively plentifully and have been in vogue for a long time (Habermann 1972; Nakajima,1986; Belboni et al., 2004; Monteiro et al., 2009), one of such studies focused exclusively on *P.ocidentalis* showing that the venom presents anticoagulant effects (Czaikoski et al., 2010). However, data concearning the citogenotoxic effects of *P.occidentalis* and *P.fastidiosa* venom are very scarce. Regarding other *Polybias* species, some cytogenetic studies are available. For instance, *Polybia paulista* (social wasp), is the most well researched one; but most of the papers that exist deal with molecular characterization of the venom or with isolated substances from the venom (Dias et al., 2014; Gomes et al., 2014; Hoshina et al, 2013; Wang et al.; 2011). Therefore more studies concerning the cytogenetic effects of arthropods venom are necessary.

Cell cycle analyses detected significant statistical differences between the two evaluated parameters (MI and CCA) and the control for both tested venoms. Cells exposed to *P. fastidiosa* venom showed an increase of about 15 times the frequency of CCAs in relation to the control treatment; this increase was approximately 16 times on the cells that were exposed to *P. occidentalis* venom (Table 1).

MCN, sticky chromosomes, c-metaphases, bridges, chromosome fragments, non-oriented chromosomes, multipolar anaphases and condensed nuclei were detected. For *P. occidentalis* venom no c-metaphases was registered during metaphasis, while *P. fastidiosa* venom induced neither fragments, nor multipolar anaphases (Figure 1 and Figure 2).

Non-oriented chromosomes (Figure 1A) were very frequent on cells exposed to *P.fastidiosa* venom (36% of the total CCAs) (Figure 2). This type of alteration is generally the result of a defective mitotic spindle, or due to the lack

or faulty signaling for the mitotic spindle on the centromere (Freitas et al., 2016). This affects chromosome movements during the cell division which may lead to the miss orientation of some (Fiskejö, 1985). C-metaphases (Figure 1B) are associated with spindle malfunction as well, this CCA was also abundant on cells exposed to *P.fastidiosa* venom (10.24% of the total CCAs) (Figure 2). Therefore, the spindle is most likely being damaged by proteases contained in the venom or its singnaling in the centromere is being hampered by neurotoxins.

On the cells exposed to *P. occidentalis* venom a high frequency of fragments (Figure 1A). was observed (17.50% of the total CCAs) (Figure 2). Fragments can originate from chromosome breakages resulting from bridges, but they can also occur due to damage caused directly on the DNA molecule (Leme and Marin-Morales, 2009).This results in acentric segments being excluded from chromosomes, constituting the fragments. These may be eliminated by way of MCN (Figure 1C) (13.75% of the total CCAs) (Figure 2) (Campos et al. 2008a). It is likely that the proteases contained in the venom are also damaging proteins associated directly with the DNA, which would disrupt the molecules normal organization and favor the occurrence of breaks.

There are plenty of other molecules that could interact with DNA associated proteins, the spindle and directly with the DNA on *Polybia* venom, like biogenic amines, biologically active peptides and a diversity of proteins and enzymes (Lorenzi, 2002; Nakajima et al., 1986). These molecules need to penetrate the cell in order to interact with the DNA. This process is most likely facilitated by three key components of the venom: (1) mastoporans: mastoporan: this molecule can act on ion channels changing the flow of ions through lipid membranes, which may lead to cell lysis (Li et al., 2000), as well as forming pores that can increase the membrane permeability to small molecules and ions (Gusovski et al. 1991); (2) hyaluronidase: this protein is capable of hydrolyzing the hyaluronic acid contained in the extra cellular matrix, disrupting it and

increasing cell permeability to other components of the venom (El-Safory et al., 2010; Hoffman, 2006); (3) phospholipases: they act on the lipid bilayer of membranes, affecting the phospholipids of the biological membranes, leading to the formation of pores, facilitating the entrance of other substances in the cell, as well as possibly causing cell lysis, tissue damage (Santos et al., 2007; Dotimas and Hider, 1987).

However plant cells have cell walls and this could present a barrier for the passage of the various molecules acting as a barrier for the venom spread. The primary plant cell walls are composed of cellulose microfibrils embedded in a highly hydrated matrix composed of pectins and hemicellulose. Structural proteins are added to the cellulose/hemicellulose scaffolding to help stabilize the cell wall. The primary cell wall is composed of approximately 25% hemicellulose, 35% pectins and 25% cellulose, with structural proteins varying in frequency up to a total of 8% (on a dry weight basis). These values may vary according to the species and environmental influences (Taiz and Zeiger, 2002). This complex structure is likely being disrupted by the action of the many proteases and peptides that compose the *Polybia* venom; they could be acting on the structural proteins or directly on the cell wall matrix reducing its cohesion and therefore increasing its permeability. Furthermore cell walls are naturally permeable to small molecules (Knox and Benitez-Alfonso, 2014).

The increase in cell permeability incurs in a direct exposition of the internal cell structures, including the DNA, to a diversity of substances that are potentially damaging. If the damage accumulates it could lead to cell death (CD). CD can work as a defense mechanism in plants, and may be activated under stress when the DNA repair mechanisms are not capable of properly fixing the molecule. A cytological marker of CD is the condensed nuclei (CNs) (Figure 1F) (Andrade-Vieira et al., 2011). They occur when there is fragmentation of the genetic material, resulting in a phenomenon known as DNA

laddering (Danon et al., 2000). Thus, the occurrence of CD, evidenced cytologically by the presence of CNs, is considered a great measurer of toxicity (Doorn and Woultering, 2010).

Both venoms tested induced an expressive amount of CNs (32.50% and 18.67% of the total CCAs for *P. occidentalis* and *P. fastidiosa,* respectively) (Figure 1D and Figure 2). Also 12.6% of the cells treated with *P. occidentalis* and 10.6% treated with *P. fastidiosa* were positive for the TUNEL signal (Table 1) which indicates DNA damage and fragmentation. The occurrence of DNA laddering, was also visible on the agarose gel electrophoresis (Figure 3). As discussed before, mastoporans, hyrulonidases and phospholipases can all disrupt a series of cell structures and membranes and result in cell lyses causing CD.

Furthermore a reduction in the mitotic index (MI) was noticed for the cells exposed to the venom of both species (15.5% reduction for *P. occidentalis* and 16.5% reduction for *P. fastidiosa* when comparing to the control cells) (Table 1). MI reduces as a result of alterations and CD, the normal mitotic cycle can be hampered by DNA damage and chromosomal abnormalities and cells undergoing CD do not divide. With the decreased on MI, a reduction in cell growth is also expected as cell division and cell growth are parameters that are closely related in plants (Harashima and Schnittger, 2010). Reduced MI may indicate a noxious toxic effect as the organisms slows down its normal development due to the cytological and genetically damage sustained.

It is possible to assume, based on a prior study (Palmieri et al. 2016 – submitted) and on the results showed here that *L. sativa* is efficient when screening the toxicity of the venom of at least three species of arthropods (*T. serrulatus*, *P. occidentalis* and *P. fastidiosa*). Therefore cytogenetic test systems using higher plants as models show great potential as a cheaper, easier maintenance, devoid of ethical contrivances, substitute for the tests using animals on the preliminary stages of venom cytogenotoxicity evaluation.

Lastly, while the aim of this study was to evaluate the application of *L. sativa* as a model for screening possible effects and mechanisms of action of venoms it also showed the cytotoxic and genotoxic effects of *P. occidentalis* and *P. fastidiosa*'s venom. This information coupled with a previous study by Hoshina et al. (2014) - the authors showed that even in very small concentrations *Apis mellifera* (bees, another Hymenopthera) venom can induce genotoxicity and mutagenicity on human cells - indicate that venom from some arthropods represent a high risk and should be studied carefully when used for pharmacology in order to avoid undesirable side-effects.

4 CONCLUSION

The results presented here show that both *Polybia occidentalis* and *Polybia fastidiosa* venoms have cytotoxic and genotoxic effects, causing DNA damage, leading to the occurrence of CCAs and reduction of MI. The mechanism of action of these venoms are likely related to an increase in cell permeability by the action of mastoporans, hyarulonidases and phospholipases followed by direct action on cell structures of various small peptides and different proteases.

Else more, *Lactuca sativa* has proven to be an effective test model for venom cytogenotoxicity screening as proposed by Palmieri et al. (2016, submitted).

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Table 1 –Effect of *Polybia occidentalis* and *Polybia fastidiosa* venom on the mitotic index (MI), cell cycle alterations (CCA) and TUNEL test after exposure of *Lactuca sativa* roots to 0.25mg of venom.

Treatments	MI	CCA	TUNEL
Control	11.29 ± 1.21	0.5 ± 0.103	0 ± 1.49
P. occidentalis	7.26 ± 0.82*	8.00 ± 3.65*	12.6 ± 3.24*
P. fastidiosa	6.82 ± 1.37*	7.53 ± 2.22*	10.6 ± 3.24*

Observed results followed by standard deviation in the two evaluated parameters and in the TUNEL test. MI is expressed as percentage, and CCA as frequency per thousand cells. The TUNEL result is given by the percentage of cells with the positive mark. Data followed by * differ significantly from the control according to Tukey test (α=5%).

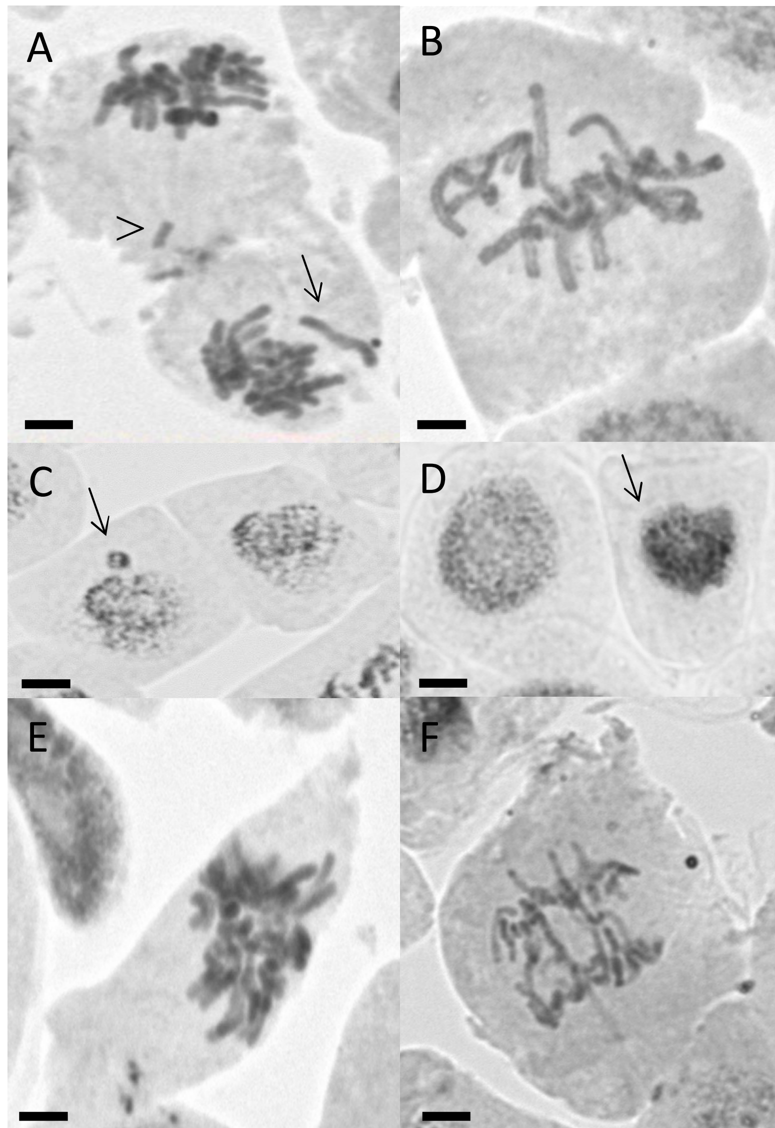
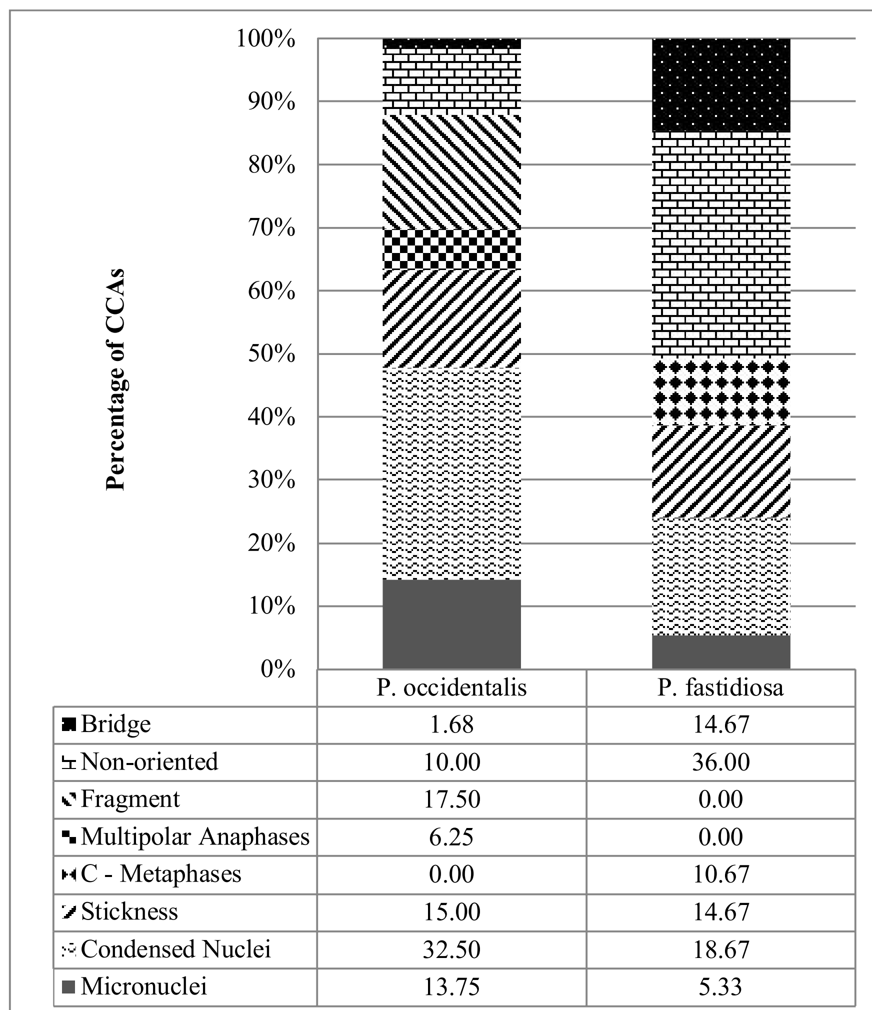


Figure 1 - Some of the CCAs found in root tip cells of *Lactuca sativa* exposed to *Polybia occidentalis* and *Polybia fastidiosa* venom.

A– Non-oriented chromosomes (arrow) and lost chromosome and fragment (head arrow); B– C-Metaphases (*P. fastidiosa*); C- Micronuclei (arrow) (*P. fastidiosa*); D– Condesed Nuclei (arrow) (*P. occidentalis*); E- Stickiness (*P. occidentalis*); F- Multiple Bridges (*P. fastidiosa*) (Bars represent 10µm).



	P. occidentalis	P. fastidiosa
Bridge	1.68	14.67
Non-oriented	10.00	36.00
Fragment	17.50	0.00
Multipolar Anaphases	6.25	0.00
C - Metaphases	0.00	10.67
Stickness	15.00	14.67
Condensed Nuclei	32.50	18.67
Micronuclei	13.75	5.33

Figure 2. Percentage of CCAs observed on root tips of *L. sativa* exposed to *Polybia occidentalis* and *Polybia fastidiosa* venom

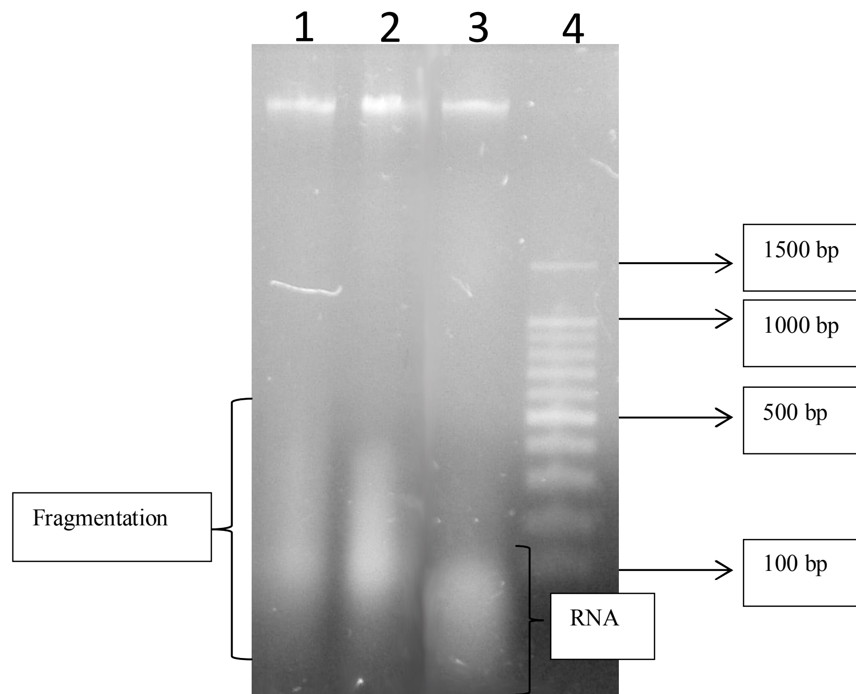


Figure 3 – Gel demonstrating the occurrence of DNA-laddering in the genetic material of *Lactuca sativa* cells exposed to *Polybia occidentalis* and *Polybia fastidiosa* venom.

1- *P. Fastidiosa venom;* 2- *P. Occidentalis venom;* 3 - Comparator (distilled water); 4 – Fragmentation pattern (100 bp).

ARTIGO 3

Cell cycle alterations and DNA fragmentation on *Lactuca sativa* L. cells exposed to *Crotalus durissus terrificus* (CDT) and *Lachesis muta muta* venoms

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Cell cycle alterations and DNA fragmentation on *Lactuca sativa* L. cells exposed to *Crotalus durissus terrificus* (*C.d.t.*) and *Lachesis muta muta* venom

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ABSTRACT: Plant models are efficient on detecting toxicity of a variety of substance on cells and DNA. They are also easy to handle, cheap to maintain and quick to show results. Plant bioassays could present a valid alternative to animal models when screening effects of biologic substances, like venoms. Animal models usually have to deal with ethical and legal matters, problems which plant models are unaffected by. Therefore this work objective was to study the effects of the venom from two snake species, *Crotalus durissus terrificus* (*C.d.t.*) and *Lachesis muta muta* on the cell cycle and DNA of *Lactuca sativa* (lettuce) meristematic (root tip) cells. Seeds were exposed to venom solution; emitted roots were collected and the occurrence of cell cycle alterations (CCA) and the the mitotic index (MI) was evaluated trough the cell cycle test, DNA fragmentation was evaluated by agarose gel electrophoresis and TUNEL assay. Amongst the CCAs found, condensed nuclei were the most common for both snakes venom, followed by sticky chromosomes. The TUNEL assay had a significant positive result and the agarose gel electrophoresis showed a "drag" pattern characteristic of DNA fragmentation also for the venom of both species. Phospholipases A_2, LAAO, Crotoxin and Zn^{2+} metaloproteinases were all considered to be related in some way to the citogenotoxic activity of the venoms tested. *L. sativa* proved to be an effective model for testing the cytogenotoxicity of biological substances, such as snake venoms.

Keywords: venomous snakes, TUNEL, DNA fragmentation, cell death, cytogenotoxicity

1 INTRODUCTION

Snake venoms are highly effective biological substances composed of a mixture of molecules, many of which are toxins, designed to immobilize or kill the prey (DU et al., 2016). There are more than 250 different snake species in Brazil, amongst which over 70 are considered venomous. It is estimated that approximately twenty thousand accidents involving snake bites happen every year in this country (BOCHNER; STRUCHINER, 2003; PINHO; PEREIRA, 2001).

One genus of snake endemic Brazil is the *Lachesis*. This genus inhabits tropical forest areas in Central and South America. These snakes are known popularly as “bushmasters” and in Brazil they are called “surucucu”. They average 2 to 2.5 meters in length but can reach up 3 meters being the longest venomous snake species in America (MADRIGAL et al., 2012; SANZ et al., 2008). Human envenoming by these snakes is not very common, mostly because they tend to avoid urban areas and are easy to spot, but when it happens it can be extremely dangerous as the “bushmaster” can inoculate up to 400mg of venom in one bite (MÁLAQUE; FRANÇA, 2003). The effects of the venom are various, presenting neurotoxic and myotoxic action, including hemorrhage, necrosis on the bite site, intense pain, edema, renal failure, nausea, shock, bradycardia, hypotension and coagulopathies (DAMICO et al., 2005).

The *Lachesis* genus is composed of four especies, one of wich is the *L. muta* , a species commonly found on Brazil that subdivides into two subspecies, *L. muta rhombeata* (endemic of the Brazilian Atlantic Forest) and *L. muta muta* (endemic of the Brazilian Amazonic Forest). Their venom contains a series of pharmacological active substances including metaloproteases, serine proteases, L-amino oxidases, phospholipases A_2 and a series of other enzymes (BREGGE-SILVA et al., 2012; DA SILVA CUNHA; FULY; GIESTAL DE ARAUJO,

2011; FERREIRA et al., 2009). However, *L. muta* venom is not very well studied because the venom is hard to extract and the snakes are hard to handle (DAMICO et al., 2005). Therefore this work can shed light on the cyto and genotoxicity of the *L.muta* venom and lead to interesting findings regarding its mechanisms of action.

Another snake species endemic to Brazilian territory is the *Crotalus durissus terifficus* (*C.d.t.*), popularly known as the Brazilian rattlesnake (JONATHAN A. CAMPBELL, 2003). As most venomous snake, their venom is composed by a mixture of substances and it is know that it has a neurotoxic, hemorrhagic, myotoxic and hypotensive effect on its victims (GUTIÉRREZ, 2009; VIALA et al., 2015). Recently it has come to knowledge that *C.d.t.* venomics is even more complex than initially imagined when a series of previously unidentified molecules were detected in its venom using a novel proteomics approach (MELANI et al., 2015).

Cd.t. venom is amongst the most well studied venoms there is, , however studies on cytoxicity of this venom are scarce. Therefore more studies are necessary to better understand the effects of this venom on the chromosomes structure and organization, which could lead to interesting discoveries regarding the mechanism of action of the various toxins that compose *Cd.t.*. venom.

Even though venoms are designed to be harmful on their natural dosage and serve as "weapons" for the animals that produce and inoculate these substances, isolated toxins in controlled concentrations can have beneficial effects. In fact venoms constitute a vast wealth of molecules with pharmaceutical properties that still has not been very well tapped on (GEORGIEVA; ARNI; BETZEL, 2008). Therefore studying the venom's mechanisms of action and composition are extremely important since the findings can lead to the development of a variety of medicines (BHUNIA et al., 2015; CZAIKOSKI et al., 2010; HARVEY, 2014; KING, 2011; KOH; KINI,

2012). Some examples of medicine produced via controlled usage of venoms toxins are: pharmaceuticals against Diabetes Mellitus, analgesics, antihypertensive (e.g. Captopril and Ranatensin) and also anticoagulants (e.g. Ancrod, Batroxobin and Echistatin) (HARVEY, 2014; KING, 2011; KOH; KINI, 2012).

To achieve this level of understanding, however, groundwork needs to be established first. This is done through broad screening tests of effects and response to the venom of interest. These tests usually use animals as test subject and this have a series of drawbacks, maintaining the test subject on adequate health and fitness condition is expensive and there are legal and ethical contrivances that comes with working with animals in a laboratory. An alternative to screening tests using animals would be the usage of higher plants. Plant as models for cytogenotoxicity screening are applied since the 1900s being well established and effective models. They are of low maintenance cost, easy to handle and devoid of the legal and ethical problems associated with animal models (ANDRADE-VIERA et al., 2014; FISKESJÖ, 1985, 1988; LEME; MARIN-MORALES, 2009b). In addition plants are considered adequate bioassays to evaluate toxicity by the United Nations Environment Program (UNEP), the World Health Organization (WHO) and the United States Environmental Protection Agency (US- EPA) (GRANT, 1982, 1999). Furthermore the results found on plant and animal model are in agreement, and can present similar results through different assays that complement or are equivalent to each other (PALMIERI et al., 2016; DONG; ZHANG, 2010; GRANT; OWENS, 2006).

With this in mind the current work objective was to make an initial and broad screening of the effects and mechanisms of action of the venom from two snake species, *Lachesis muta muta* and *C.d.t.* using *Lactuca sativa* L. root meristem cells as a model. In order to achieve this the cell cycle test was

performed and the results found were compared and complemented with data from the TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and genomic DNA fragmentation in agarose gel.

2 MATERIAL AND METHODS

2.1 Obtainment of the Biological Material

2.1.1 Venom Samples

The venom, from both species (*L. muta muta* and CDT), was acquired commercial from the Bioagents (Batatais-SP) serpentarium. The samples were collected and immediately underwent crystallization on a vacuous chamber.

2.1.2 Root Tips Treatment

Commercial seeds of *L. sativa* were pre-germinated in Petri dish covered by paper moistened with distilled water for 24 hours. After this period, the seeds that emitted roots of about 0.5 mm length were transferred to a second Petri dish, covered with moistened filter paper, containing 500 μL of venom solution (0.5 mg/mL), for a final dose of 0.25 mg of venom. These concentrations were based on pilot studies. The negative control was achieved with distilled water. The Petri dishes were sealed with *Parafilm*® and remained in germination chamber for 24 hours. Next, some roots were collected and fixed for 24 hours in ethanol:acetic acid solution (3:1) to be used in the cell cycle evaluation. For the TUNEL test and the analysis of genomic DNA fragmentation in agarose gel, the roots were used immediately after collection.

2.2 Bioassays

2.2.1 Cell Cycle Evaluation

The fixed roots were washed in distilled water and hydrolyzed in 5 N HCl at room temperature (20 to 25ºC) for 12 minutes. The slides were prepared by squashing technique and the material was stained with 2% acetic orcein. Ten slides were evaluated per treatment, and 500 cells were counted per slide (totalizing 5,000 cells analyzed per treatment).

The evaluated parameters were: (1) mitotic index (MI), given by the ratio between the total of dividing cells and the total of evaluated cells, (2) cell cycle alterations (CCA): the sum of sticky chromosomes, c-metaphases, chromosome fragments, multipolar anaphases, chromosome bridges and non-oriented chromosomes, micronuclei (MCN) and condensed nuclei (CN), expressed in frequency per one thousand cells. The percentage rate of each alteration within the total of observed abnormalities (CCA) was also measured.

2.2.2 DNA Fragmentation Evaluated in Agarose Gel

The total DNA of 1 g of freshly collected *L. sativa* roots exposed to venom solution (*L.muta* or CDT) was extracted according to Doyle and Doyle (1987). The roots were homogenized in liquid nitrogen and incubated in buffer containing 2% hexadecyltrimethylammonium bromide (CTAB) at 65 ºC. The DNA was extracted with chloroform-isoamyl alcohol (24:1) and precipitated in cold isopropanol. The isolated DNA was maintained in TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). The sample was subjected to 1.2% agarose gel electrophoresis in TBE 0.5X containing 0.5 µg/mL of ethidium bromide. DNA fragmentation was observed under UV light, and the gel image was captured

with a Kodak camera. As reference for evaluation of the fragmentation, a standard containing fragments of 100 bp to 1500 bp (Promega) was applied. DNA molecules extracted from the roots used as control were used as comparator. The data presented here is based on three repetitions of the assay.

2.2.3 TUNEL Assay

The TUNEL reaction was carried out according to the manufacturer's instructions for the kit DeadEnd™ Fluorometric TUNEL System (Promega). For preparation of the slides, freshly exposed root meristems were hydrolysed with 1 N HCl at 60°C for 15 minutes, and next fixed in 4% paraformaldehyde for 25 minutes at 4°C before performing the TUNEL reaction. The positive reaction was marked with fluorescein, and the countermarking was accomplished with use of propidium iodide. The slides were evaluated in epifluorescence microscope (Olympus BX60) using the filters with excitation interval of 460-490 nm to evidence the fluorescein, and of 530-550 nm for propidium iodide. The test was repeated three times and 100 cells were analyzed per slide.

2.3 Statistical Tests

The parameters evaluated in the cell cycle and TUNEL test assessments were subjected to analysis of variance (ANOVA) followed by Tukey's test of means at 5% significance using the free software R (R DEVELOPMENT CORE TEAM, 2015).

3 RESULTS AND DISCUSSION

Significant statistical differences between the two evaluated parameters (MI and CCA) and the control were detected on the cell cycle analyses for both tested snakes' venoms (Table 1). *L sativa* root meristem treated with *C.d.t.* venom had a CCA occurrences increase of approximately 6.6 times when compared to the control. For *L.muta muta* the increase was much more drastic being as high as 10.72 times the amount of CCA found in the control.

The CCAs detected for both venoms were: micronuclei (MCN), sticky chromosomes, c-metaphases, bridges, chromosome fragments, non-oriented chromosomes and condensed nuclei (CN). (Figure 1 and Figure 2). This shows clear cytogenotoxic effect for the venom of both species studied.

For both venoms the most common CCA found was CNs (Figure 1A). CNs encompassed 33.9% and 31.11% of the total amount of CCAs for *C.d.t.* and *L.muta* respectively (Figure 2). This nuclear alteration is considered to be very good cytological marker for cell death (ANDRADE-VIEIRA et al., 2011). However basing the occurrence of cell death on just the observation of CNs can be a mistake as visual analysis of nucleus size can be deceiving and also a cell presenting a CN may recover from this condition trough the action of the natural repair mechanism of DNA and checkpoints of the cell division (GARCÍA-QUISPES et al., 2013).

One of the first steps of a cell's mechanism of programmed cell death is the fragmentation of the DNA molecule, known as DNA laddering. This name is due to the DNA strands looking similar to a ladder when observed in agarose gel due to all the breakages sustained by the molecule (DANON et al., 2000; VAN-DOORN; WOLTERING, 2010). There are tests capable of detecting these molecular markers of cell death like the TUNEL assay and the genome DNA

pattern agarose gel electrophoresis (ANDRADE-VIEIRA et al., 2011). The first test binds fluorescent marked dUTPs to the 3'OH breakage sites on the DNA molecules. The second test visually shows the occurrence of DNA laddering through the observation of a "drag" pattern on the image.

For the *C.d.t.* venom 16% of the cells showed positive TUNEL assay signals (Table 1 and Figure 3A), the result was slightly more pronounced for the *L. muta muta* venom, where 20.3% of the *L. sativa* meristematic cells exposed to the venom showed positive TUNEL signals (Table 1 and Figure 3B). The agarose gel electrophoresis showed a "drag" pattern initiating on 1000 pb going all the way to 100pb denoting the presence of various differently sized DNA fragments in this range (Figure 4). These results, alongside the cell cycle observations reported before in this paper, indicate the induction of CD by the tested venoms.

CD induction could be related to oxidative stress caused by the venoms of both species. The venoms could trigger the formation of reactive oxygen species (ROS) which can be highly damaging due to their strong capacity to react with a variety of molecules and destabilize cellular structures. CD could be happening both as a defense mechanism to the ROS, aborting cells to try to prevent the spread of the damage or as a direct result of the damage caused by ROS (NAWKAR et al., 2013; ZHANG et al., 2010). It is also known that both *C.d.t* and *L.muta* have L-aminoacid oxidase (LAAO) amongst the components of their venoms (BREGGE-SILVA et al., 2012; MELANI et al., 2015). These enzymes are capable of catalyzing the deamination of L-amino-acids, generating as a result, amongst other things, hydrogen peroxide, causing widespread damage (DU; CLEMETSON, 2002). In fact they are considered to be multifunctional enzymes, displaying a vast range of biological effects like stimulation of edema formation, inhibition or induction of platelet aggregation,

antibacterial and antiviral functions and induction of apoptosis via inflammatory pathways and oxidative stress (TAN; FUNG, 2009).

Furthermore the main component of *C.d.t.* venom is the Crotoxin. This was the first venom toxin to ever be crystalized (SLOTTA; FRAENKEL-CONRAT, 1938) and is a non-covalent heterodimer of two subunits, CrotoxinA (CA), wich is acidic, and CrotoxinB (CB), a phospholipase A_2. CB depends on CA to be able to bond to cell membranes. CA/CB Crotoxin complex may differ greatly from species to species and even within individuals of the same species resulting in different pharmacological and biological properties for the toxin itself (FAURE; BON, 1987; FAURE et al., 1993). The multiple isoforms of this toxin made studying its structure very difficult and only recently its tridimensional structure was better elucidated (FAURE; XU; SAUL, 2011; PEREAÑEZ; GÓMEZ; PATIÑO, 2012). It is known that Crotoxin has cytotoxic activity in animals, promoting mitochondria uncoupling (YAN et al., 2006), the same could be happening here and that would trigger the CD mechanisms on the cells resulting in CNs and DNA fragmentation. Crotoxin also has genotoxic activity causing DNA damage that was detected by the comet assay and micronucleus test on human lymphocytes (MARCUSSI et al., 2011).

Another common CCA found for both venoms was the presence of micronuclei (MCN) which was observed with a frequency of 19.65% for *C.d.t.* and 11.11% for *L.muta* (Figures 1B and 2). MCN is amongst the most well studied CCAs, being an excellent indicator of toxicity, as seen in a number of recent studies (CORRÊA et al., 2016; LIANG et al., 2015; PALMIERI et al., 2014). It only occurs if the initial damage suffered by the DNA molecule and/or chromosome is not repaired. MCNs are secondary cytoplasmatic nuclei that have no visible connection to the main cellular nucleus. In some cases MCNs can even be expelled from the cell (FERNANDES; MAZZEO; MARIN-

MORALES, 2007). They can either be formed by chromosomes fragments or entire chromosomes lost during a defective cell division (MA et al., 1995).

Bridges (7.14% of the CCAs for *C.d.t.* and 10% of the CCAs for *L.muta* – Figure 1C and Figure 2) were also detected amongst the CCAs. Bridges might also be related to the presence of chromosome fragments (3.57% of the CCAs for *C.d.t.* and 2.22% of the CCAs for *L.muta* – Figure 1D and Figure 2) and in turn contribute to the MCN formation. Bridges form when the chromosome suffers damage on its telomeric region or when this region is lost completely. This affects the chromosome stability causing it to become very likely to form erroneous bonds during cell division. These bonds result in dicentric chromosomes, and constitute the bridges observed in anaphases. (LEME; MARIN-MORALES, 2009b). The segments excised from the chromosomes will form acentric chromosomes and may be enveloped by a nuclear membrane and form a MCN (CAMPOS et al., 2008).

Two other CCAs, C-metaphases and non-oriented chromosomes (3.57% and 7.14% for *C.d.t.* and 13.33 and 10% for *L .muta* - Figures 1E, and Figure 2) denote failure on the normal functioning of the mitotic spindle. It is known that failures on the spindle functions are related either to damage sustained by the spindle structure (LEME; MARIN-MORALES, 2009b) or to lack of proper signaling for the spindle to attach with the kinetochores on the centromeres (FREITAS et al., 2016). Toxic agents and molecules can disrupt this signaling and/or damage the spindle resulting in CCAs. *L. muta muta* venom have a variety of phospholipases A_2 in its composition, it is known that this category of phospholipases act on membranes cleaving the sn-2 bonds on phospholips releasing fatty acids (DU et al., 2016). They can also disrupt signaling and have post and pre-synaptic neurotoxic activity (SOARES et al., 2004). Therefore it is safe to assume that these enzymes can be “jamming” the

spindle bond signaling resulting in non-oriented chromosomes and also C-metaphases.

Non oriented chromosomes might “detach from the group” and be lost in its entirety, these chromosomes will later be involved by a nuclear membrane and form a MCN (FENECH, 2000). Therefore the MCNs found here are also originated from whole chromosomes; this is more prominent on *L. muta muta* venom since the rate of non-oriented chromosomes is higher for this species venom.

Also found in high frequency on cells exposed to both venoms were sticky chromosomes (25% of all CCAs for *C.d.t.* and 22.22% of all CCAs for *L .muta* – Figure 1F and Figure 2). Stickiness is usually characterized by the formation of chromosomal agglomerates. These arise from the loss of normal condensation of the chromosomes structure. Damage to the protein scaffold responsible for maintaining the correct chromosomal form and organization, direct damage to the structure of the DNA strands or both are considered to be the mechanism behind stickiness formation (AMIN, 2011).

Many toxins common to snake venoms can possibly cause damage to the chromosome proteins scaffold. Amongst them are the Zn^{2+} metaloproteases, this enzyme is particularly common on *L. muta muta*, it is the most common toxin protein of the species consisting over 31% of the total toxin proteins found in their venom (SANZ et al., 2008). This class of enzymes can cleave several proteins, including cellular receptors and plasma membrane proteins (KIRKIN et al., 2007; WHITE, 2003), leading to cell death (TRUMMAL et al., 2005). There are no evidences that these metaloproteases have direct action on the DNA, however considering their wide range of action it is possible that they are affecting the chromosome protein skeleton and indirectly they are affecting DNA integrity and chromosome structure by triggering CD. They can also

unbind plasma membrane proteins (WHITE, 2003) which would make the whole cell, including the chromosomes vulnerable to the action of other toxins.

CCAs and DNA damage can negatively influence the mitotic index (MI). This is the case here, as MI decreased approximately 46% and 39% on cells exposed to *C.d.t.* and *L.muta* venoms respectively (Table 1). Alterations may lead to CD or disrupt the cell cycle, which in turn depletes the cell proliferation rates, this will show in the cell cycle test as a reduction in the MI (FERNANDES; MAZZEO; MARIN-MORALES, 2007). In addition the multiple neurotoxic toxins present in snake venom may also be having an impact on the MI reduction, since these toxins can disrupt the proper functioning of ionic channels, such as those that transport K^+ ions. K^+ ions, in plants, are associated with cell proliferation and elongation (LEBAUDY; VÉRY; SENTENAC, 2007).

The results presented here show that both *L. muta muta* and *C.d.t.* venom have cytogenotoxic effects, inducing CDs, disrupting the chromosomes normal structure, affecting the signaling for the kinetochore/spindle connection and causing DNA fragmentation, which leads to the occurrence of CCAs and reduction of MI. Finaly, *Lactuca sativa* has effectively detected the cytogenotoxicity of both snake venoms tested and could consist in an alternative or complementary model for animal models.

4 CONCLUSION

The results found here prove that *L. sativa* is an effective model for the screening of cytogenotoxicity of biologic molecules. In fact the present work show the effectiveness of the model for screening the cytotoxic activity of the venoms of two different genus of snakes, therefore its probable that it would be equally adequate for evaluating venoms from snakes in general and not just the two species boarded here. Since higher plant models are cheap, low maintenance and devoid of ethical and legal contrivances, they constitute suitable alternative to animal models for initial cytogenotoxicity assessment of venoms.

Lastly, it became evident that *C.d.t.* and *L. muta* venom, through the action of phospholipases A_2, LAAO, Crotoxin, proteases and many other biologically active molecules are highly cytogenotoxic, a plethora of alterations to the cell cycle, cell death and damage to the DNA molecule.

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Table 1 – Effects of *Lachesis muta* and *Crotalus durissus terrificus* venom on the mitotic index (MI), cell cycle alterations (CCA) and TUNEL test after exposure of *Lactuca sativa* roots to 0.25mg of venom.

Treatments	MI	CCA	TUNEL
Control	11.41 ± 1.33	0.85 ± 0.127	0.1 ± 1.01
C.*d.t.*	6.21 ± 1.15*	5.60 ± 3.06*	16 ± 7.74*
L.muta muta	6.98 ± 1.48*	9.11 ± 2.89*	20.3 ± 5.82*

Results followed by standard deviation for the two evaluated parameters and in the TUNEL test. MI is expressed as percentage, and CCA as frequency per thousand cells. The TUNEL result is given by the percentage of cells with the positive mark. Data followed by * differ significantly from the control according to Tukey test (α=5%).

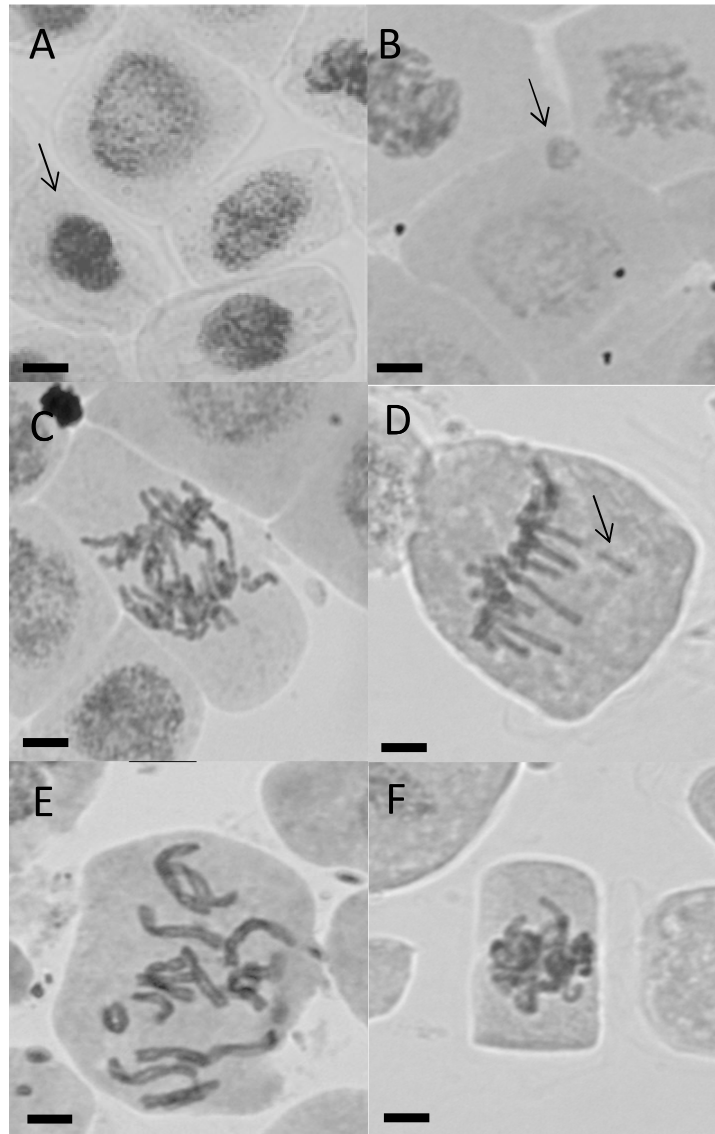
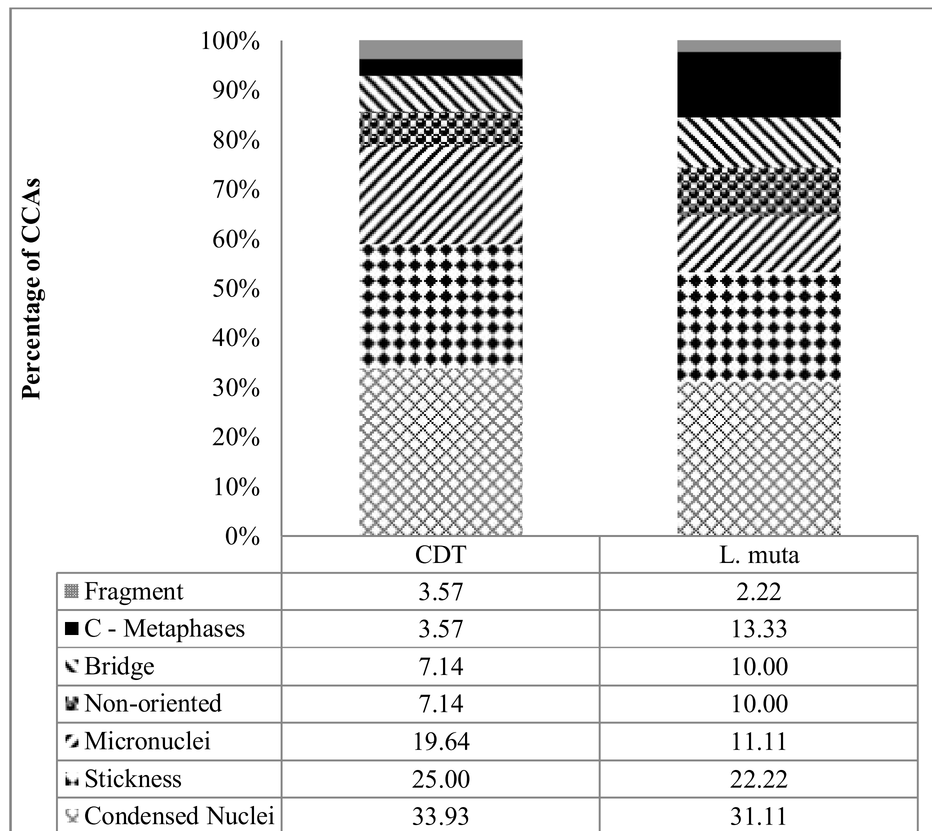


Figure 1 - Some of the CCAs found in root tip cells of *Lactuca sativa* exposed to *Crotalus durissus terrificus and L.muta muta* venom.

A – Condensed Nuclei (*C.d.t.*); B – Micronuclei (arrow) (*C.d.t.*); C – Multiple Bridges (*L. muta*); D – Chromosome Fragment (arrow) (*L. muta*); E - C-Metaphase (*L. muta*); F - Stickness (*C.d.t.*) (Bars represent 10µm).



	CDT	L. muta
Fragment	3.57	2.22
C - Metaphases	3.57	13.33
Bridge	7.14	10.00
Non-oriented	7.14	10.00
Micronuclei	19.64	11.11
Stickness	25.00	22.22
Condensed Nuclei	33.93	31.11

Figure 2 - Percentage of each CCA observed on root tips of *Lactuca sativa* exposed *Lachesis muta muta* and *Crotalus durissus terrificus* venom.

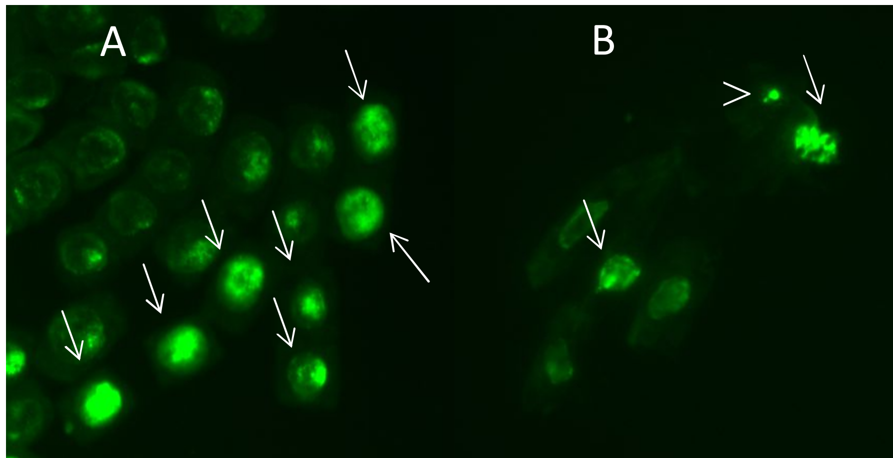


Figure 3. TUNEL test. Results observed after exposure of *Lactuca sativa* roots to 0.25 mg of *Crotalus durissus terrificus* and *Lachesis muta* venom.
A – Positive signal of the TUNEL test on cells exposed to the CDT venom (seen on the fluorescein filter). B – Positive signal of the TUNEL test on cells exposed to the *L.muta* venom (seen on the fluorescein filter), notice the presence of a positively marked micronuclei (head arrow).

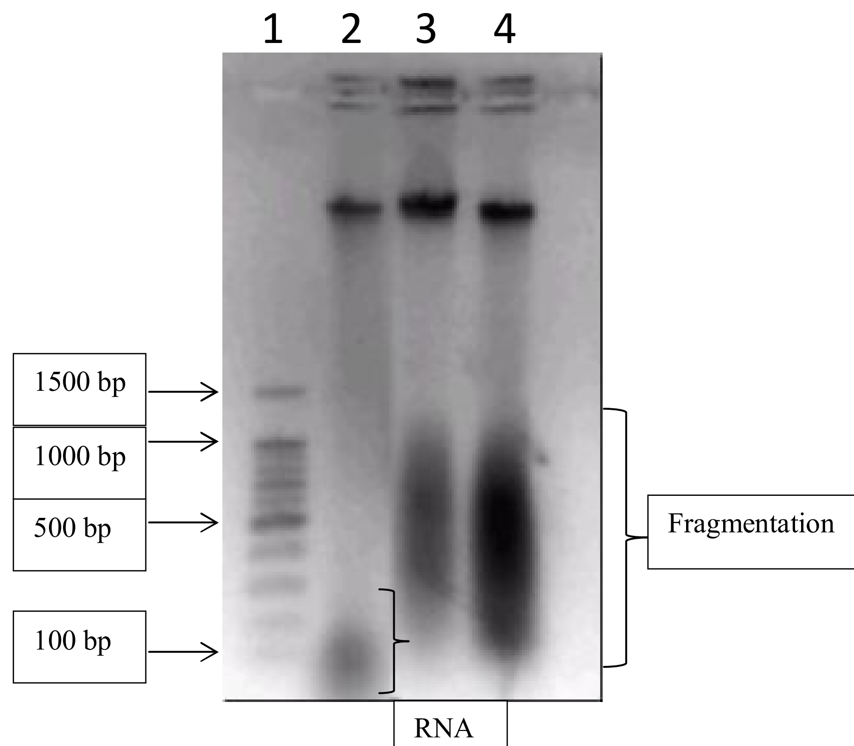


Figure 4 – Gel demonstrating the occurrence of DNA-laddering in the genetic material of *Lactuca sativa* cells exposed to *Crotalus durissus terrificus* and *Lachesis muta* venom.

1- Fragmentation pattern (100 bp), 2- Comparator (distilled water), 3- C.*d.t.* venom, 4- *L. muta venom*

ARTIGO 4

Screening of the effects of the *Bothrops* snakes venom on the cell cycle and the DNA of *Lactuca sativa* L.

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Screening of the effects of the *Bothrops* snakes venom on the cell cycle and the DNA of *Lactuca sativa* L.

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ABSTRACT: Higher plants are excellent models for cytogenotoxicity bioassays. They are cost effective, efficient and develop fast. When studying the toxic effects of biological substances, such as venom, plants could present and alternative or as a complementary bioassay to animal ones. The venom composition from the *Bothrops* genus is highly variable. This work objective was to study the effects of the venom of four snake species of the *Bothrops* genus, *Bothrops alternatus*, *Bothrops atrox*, *Bothrops moojeni* and *Bothrops jararacussu* on the cell cycle and DNA of *Lactuca sativa* (lettuce) meristematic (root tip) cells. Seeds were exposed to venom solution; emitted roots were collected and the cell cycle alterations (CCA) and the mitotic index (MI) were measured through the cell cycle test. DNA fragmentation was demonstrated by DNA pattern agarose gel electrophoresis and by the presence of positive signals on the TUNEL assay. Amongst the CCAs found, condensed nuclei were very prominent for all venoms evaluated. This alongside the results of the TUNEL, which was positive, and the "drag pattern" observed on the DNA agarose gel electrophoresis indicates that cell death (CD) is occurring. Phospholipases A_2, L-amino acid oxidases and metalloproteinases were all toxins considered to be related in some way to the cytogenotoxic activity of the venoms tested. *L. sativa* was an effective test model to evaluate initial cytogenotoxic effects of the four snake venoms tested.

Keywords: venomous snakes, TUNEL, DNA fragmentation, Cell Death, cytogenotoxicity

1 INTRODUCTION

Venomous snakes are highly efficient predators that utilize their venom as a weapon. These biological substances are composed of a mixture of molecules including proteins, resins and a wide variety of toxins, designed to weaken, paralyze and/or kill the prey (DU et al., 2016). In Brazil there are over 70 species of snakes classified as venomous and the number of accidents involving snake bites range in the twenty thousand yearly (BOCHNER; STRUCHINER, 2003; PINHO; PEREIRA, 2001).

The *Bothrops* genus, constitute some of the more common venomous snakes in Brazil and on the American territory and are responsible for most of the deadly cases of human envenomation on these regions (GARCIA DENEGRI et al., 2014). This is due to these snakes highly specialized buccal apparatus, suited for easy and effective venom injection (WÜSTER et al., 2008).

Generaly, *Bothrops* venom can cause severe renal and respiratory failure, cardiac arrest and coagulation disorders on humans (RIBEIRO et al., 1998) and is a rich source of phospholipase A_2 (PLA_2), calcium dependent enzymes that have post and pre-synaptic neurotoxic activity, disrupting signaling (SOARES et al., 2004). These enzymes also act on membranes, destabilizing them by releasing fatty acids via the cleaving of the sn-2 bounds (DU et al., 2016). Other components of the pit viper venom include metalloproteinases, serineproteinases, L-amino acid oxidases (LAAO), nucleotidases and hyarulonidases (BRAUD; BON; WISNER, 2000; KINI, 2006; TOYAMA et al., 2011).

The *Bothrops* genus is highly diverse, with approximately 30 species classified. Some authors do not even consider it to be monophyletic, subdividing it in three genus, *Bothrops*, *Bothrocophias* and *Botriopsis* (CARRASCO et al., 2012). Regardless of monophyly there is a consensus that these snakes are

divided in seven great groups named after the representative species *Bothrops alternatus*; *B. atrox*; *B. jararaca*; *B. jararacussu*; *B. neuwiedi*; and *B. microphthalmus; B. taeniatus* (the last two groups are classified as belonging to the *Bothrocophias* and *Botiopsis* genus respectively depending on the author) (CAMPOS et al., 2013; GUTBERLET, JR; CAMPBELL, 2001).

Commonly this diversity also reflects on the venom composition, fluctuations on the venoms proteomics and toxins concentrations from species to species; which may cause variations on the pharmacological effects and affect the efficiency of anti-ophitic serums. Even intraspecific venom composition variety can be noticed when comparing populations that are geographically distant from one another (BARLOW et al., 2009; CALVETE et al., 2011; MORA-OBANDO et al., 2014).

Considering this venom variability an interesting question arises. How would this affect the action of the venom on the chromosomes structure and organization during the cell cycle and on the DNA molecule? To answer this question cytogenetic and molecular genetics tests are necessary. Frequently the test subjects for these tests are animals. This can pose problems however, especially on initial tests and screening when the results are largely unpredictable and elevated numbers of repetitions are needed. Feeding and housing the animal subjects can be expensive and demand a lot of time. Also working with animals in a laboratory requires authorization from ethical and legal committees. An alternative test subjective would be higher plants. Plant models have been in use for toxicity assays since the early 1900s and therefore are very well established. They have proven efficiency, are highly cost effective, very easy to handle and are free of any legal or ethical contrivances (ANDRADE-VIERA et al., 2014; FISKESJÖ, 1985, 1988; LEME; MARIN-MORALES, 2009b). Higher plant models are also validated by the United Nations Environment Program (UNEP), the World Health Organization (WHO)

and the United States Environmental Protection Agency (US- EPA) as being appropriate test subjects for toxicity bioassays (GRANT, 1982, 1999). Finally, results found trough assays on plants are in agreement and can be equivalent or complement those obtained through animal assays (PALMIERI et al., 2016; DONG; ZHANG, 2010; GRANT; OWENS, 2006).

Furthermore venoms can be a source of a wide variety of medicines, since isolated toxins applied in small and controlled dosages can have beneficial targeted effects. Some examples of pharmaceuticals developed through controlled usage of venom toxins are: anticoagulants (e.g. Ancrod, Batroxobin and Echistatin), antihypertensive (e.g. Captopril and Ranatensin), Diabetes Mellitus combat drugs and analgesics (HARVEY, 2014; KING, 2011; KOH; KINI, 2012). The potential of venoms for pharmaceutical usage is still not fully explored (GEORGIEVA; ARNI; BETZEL, 2008). Comprehending how a venom acts in every level, even in a cellular and genetic one, can be of great importance in the development of new medicines in the future (BHUNIA et al., 2015; CZAIKOSKI et al., 2010; HARVEY, 2014; KING, 2011; KOH; KINI, 2012).

With this in mind the objective of the present work was to make an initial and broad evaluation of the effects and mechanisms of action of the venom from four *Bothrops* species: *B. atrox*, *B. jararacussu*, *B. alternatus* and *B. moojeni* (a species belonging to the *B. atrox* group) (FURTADO et al., 2010). This was achieved through the cell cycle test in *Lactuca sativa* L.. Data obtained were complemented and compared with data from the TUNEL assay (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and genomic DNA fragmentation in agarose gel.

2 MATERIAL AND METHODS

2.1 Obtainment of the Biological Material

2.1.1 Venom Samples

The venom, from all species, was acquired commercial from the Bioagents (Batatais-SP) serpentarium. The samples are collected and immediately undergo crystallization on a vacuous chamber.

2.1.2 Root Tips Treatment

Commercial seeds of *Lactuca sativa* were pre-germinated in Petri dish covered by paper moistened with distilled water for 24 hours. After this period, the seeds that emitted roots of about 0.5 mm length were transferred to a second Petri dish, covered with moistened filter paper, containing 500 µL of venom solution (0.5 mg/mL), for a final dose of 0.25 mg of venom. These concentrations were based on pilot studies. The negative control was achieved with distilled water. The Petri dishes were sealed with *Parafilm* and remained in germination chamber for 24 h. Next, some roots were collected and fixed for 24 hours in ethanol:acetic acid solution (3:1) to be used in the cell cycle evaluation. For the TUNEL test and the analysis of DNA fragmentation in agarose gel, the roots were used immediately after collection.

2.2 Bioassays

2.2.1 Cell Cycle Evaluation

The fixed roots were washed in distilled water and hydrolyzed in 5 N HCl at room temperature (20 to 25ºC) for 12 minutes. The slides were prepared by squashing technique and the material was stained with 2% acetic orcein. Ten slides were evaluated per treatment, and 500 cells were counted per slide (totalizing 5,000 cells analyzed per treatment).

The evaluated parameters were: (1) mitotic index (MI), given by the ratio between the total of dividing cells and the total of evaluated cells, (2) cell cycle alterations (CCA): the sum of sticky chromosomes, c-metaphases, chromosome fragments, multipolar anaphases, chromosome bridges and non-oriented chromosomes, micronuclei (MCN) and condensed nuclei (CN), expressed in frequency per one thousand cells. The percentage rate of each alteration within the total of observed abnormalities (CCA) was also measured.

2.2.2 DNA Fragmentation Evaluated in Agarose Gel

The total DNA of 1 g of freshly collected *L. sativa* roots exposed to venom solution (*B. atrox, B. alternatus, B. moojenis or B.jararacussu*) was extracted according to Doyle and Doyle (1987). The roots were homogenized in liquid nitrogen and incubated in buffer containing 2% hexadecyltrimethylammonium bromide (CTAB) at 65 ºC. The DNA was extracted with chloroform-isoamyl alcohol (24:1) and precipitated in cold isopropanol. The isolated DNA was maintained in TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA). The sample was subjected to 1.2% agarose gel electrophoresis in TBE 0.5X containing 0.5 µg/mL of ethidium bromide. DNA

fragmentation was observed under UV light, and the gel image was captured with a Kodak camera. As reference for evaluation of the fragmentation, a standard containing fragments of 100 bp to 1500 bp (Promega) was applied. DNA molecules extracted from the roots used as control were used as comparator. The data presented here is based on three repetitions of the assay.

2.2.3 TUNEL Assay

The TUNEL reaction was carried out according to the manufacturer's instructions for the kit DeadEnd^{TM} Fluorometric TUNEL System (Promega). For preparation of the slides, freshly exposed root meristems were hydrolysed with 1 N HCl at 60°C for 15 minutes, and next fixed in 4% paraformaldehyde for 25 minutes at 4°C before performing the TUNEL reaction. The positive reaction was marked with fluorescein, and the countermarking was accomplished with use of propidium iodide. The slides were evaluated in epifluorescence microscope (Olympus BX60) using the filters with excitation interval of 460-490 nm to evidence the fluorescein, and of 530-550 nm for propidium iodide. The test was repeated three times and 100 cells were analyzed per slide.

2.3 Statistical Tests

The parameters evaluated in the cell cycle and TUNEL test assessments were subjected to analysis of variance (ANOVA) followed by Tukey's test of means at 5% significance using the free software R (R DEVELOPMENT CORE TEAM, 2015).

3 RESULTS AND DISCUSSION

The results show that the venom from all four species tested (*B. atrox*, *B. alternatus*, *B. jararacussu* and *B. moojeni*) have cytogenotoxic effect on the *L. sativa* meristematic cells. Both parameters evaluated for the cell cycle analyses, MI and CCAs, were statistically different from the control (Table 1). Also the TUNEL assay showed the presence of positive signals in highly significant amounts on the cells exposed to the venoms (Table 1 and Figure 1) and the DNA pattern agarose gel electrophoreses presented a “drag pattern” (Figure 2). Both this results characterize DNA fragmentation and points towards the induction of cell death (CD) by the venoms.

Regarding the CCAs the increase was the greatest for *B. alternatus*, which presented a rating of CCAs approximately 14 times bigger than the control (Table 1). The increase was also very high for *B. jararacussu*, which displayed approximately 13.4 times more CCAs than the control (Table 1). *B. atrox* and *B. moojeni* had a lower increase in CCAs induction, although it was still significant. The CCAs for these two species venom were approximately 7.8 and 9.0 times more abundant than those of the control. Amongst the CCAs observed there were chromosome bridges (Figure 4A and 4D), micronuclei (MCN) (Figure 4B), condensed nuclei (CN) (Figure 4B), non-oriented chromosomes (Figure 4C and 4D), multipolar anaphases, c-metaphases, polyploid cells and chromosome fragments (Figure 4D). To better comprehend this data a comparative/associative analysis of the results obtained here and the composition of the venom of these four snake species is necessary.

The ratings of each CCA vary from venom to venom tough (Figure 3). This is the case even for *B. atrox* and *B. moojeni* wich are species considered to be form the same group withim the *Bothrops* genus (FURTADO et al., 2010). This variability is likely associated with the differences in venom composition

inherent to this genus (CALVETE et al., 2011; MORA-OBANDO et al., 2014). However some patterns could be observed amongst all species tested, like the high frequency of CN and sticky chromosomes for all four venoms tested (Figure 3). As mentioned above, a highly common CCA for all four species was the CN (ranging from 21.01% on *B. atrox* to 50.79% on *B. alternatus* of all CCAs observed) (Figure 3). These are considered to be cytological markers to cell death (CD) (ANDRADE-VIEIRA et al., 2011). However the presence of a CN does not necessarily mean that a cell is going through a process of CD, the cells repair mechanism might be able to correct the damages sustained and recover the cell to normal activity again (GARCÍA-QUISPES et al., 2013). Therefore confirmation via the TUNEL assay and the DNA pattern agarose gel electrophoreses is necessary. These two tests can identify molecular markers of CD. One of the first processes that a cell undergo when it is going to CD is the fragmentation of its DNA, also known as DNA *laddering*, this name arises from the fact that the DNA molecule assumes an aspect similar to a ladder (due to multiple breakages on the double strand) when observed in an agarose gel electrophoreses (DANON et al., 2000; VAN-DOORN; WOLTERING, 2010).

The percentage of cells with positive TUNEL signals was the greatest for *B. jararacussu*, wich presented the signal on over 36% of the cells and the smallest for *B.moojeni*, where cells were positively marked 17% of the time. *L sativa* cells exposed to *B alternatus* and *B.atrox* venom displayed the TUNEL signal 29.6 and 20 % of the time respectively (Table 1). The DNA pattern agarose gel electrophoreses showed a “drag pattern” starting on 1500pb and going all the way to 100pb, which indicates that the DNA fragments are of multiple different sizes within this range (Figure 2). Correlating the CNs observed with the TUNEL and the agarose gel electrophoreses results it is safe to assume that cell death is occurring.

Bothrops venom, like most other snake venoms, have LAAOs on their composition. These toxins are pharmacologically active largely because they can cause widespread cell death, leading to internal bleeding and also platelet aggregation inhibition (ALVES et al., 2008; SAKURAI et al., 2003). These enzymes catalyze the deamination of specific L-amino acids producing the corresponding alpha-keto acid, ammonia and hydrogen peroxide (DU; CLEMETSON, 2002). Hydrogen peroxide is a reactive oxygen specie (ROS) and can cause oxidative stress. It is know that oxidative stress triggers CD, both as a direct result of the damage caused by the ROS and due to the triggering of programmed CD mechanism to try to contain the action of the ROS (NAWKAR et al., 2013; ZHANG et al., 2010).

Another class of toxin that is likely involved with the induction of CD is the PLA_2s. As mentioned before PLA_2s are abundant on *Bothrops* (SOARES et al., 2004), and their many isoforms constitute the majority of the toxins present on the genus venom (CAMPOS et al., 2009). These toxins have catalytic activity over cell membranes, wich would explain why the Bothrops venom showed such a high rate of positive TUNEL signals (36.6%) (Tale 1) as well as of CN (36.84% of all CCAs) (Figure 3). In animals this toxin effect would be even more severe, as PLA_2s are active pharmacologically and can lead to acute inflammation. It is known that inflammation is intimately related to CD, both as a result and as a cause of each other, resulting in an auto-amplification loop (LINKERMANN et al., 2014; ROCK, 2009).

Non oriented chromosomes was another CCA that was relatively common on the venom of all four snake species, ranging from 6.35% of all the CCAs on *B. alternatus* to 21.85% of all CCAs on *B atrox*. This CCA arises from dysfunction on the spindle, either due to damage sustained by its structure (LEME; MARIN-MORALES, 2009b) or because of defective signaling for the

attachment of the spindle to the kinetochores during cell division (FREITAS et al., 2016).

Proteases could be at work here, damaging the spindle. In fact Amazonian *B. atrox* are known for having a very high concentration of snake venom metalloproteinase (SVMPs), over 50% of the protein count of their venom is composed by SVMPs (FREITAS-DE-SOUSA et al., 2015). This could also be related to the induction of sticky chromosomes, a CCA resulting from damages to the chromosome protein scaffold, which causes loss of normal condensation and destabilize its structure causing it to fold within itself and bond with other chromosomes forming a dense and entangled agglomerate (AMIN, 2011). Sticky chromosomes was the most frequent CCA on *B. atrox* encompassing 42.02% of all CCAs observed (Figure 2).

Non-oriented chromosomes may lead to the loss of a chromosome in its entirety. This lost chromosome could in turn be enveloped by a nuclear membrane and form a MCN. This CCA is considered to be an excellent indicator of cytogenotoxic potential (HOSHINA; MARIN-MORALES, 2009). MCNs originate as a byproduct of the multiple cell cycle alterations and can only be seen when initial damage isn't properly repaired by the molecular repair machinery of the cells (GARCÍA-QUISPES et al., 2013). MCN was present on all four venom treatments, however it was very infrequent for *B. jararacussu* (only 0.88% of the observed CCAs) (Figure 3). This indicates that most of the CCAs are being properly repaired for this species venom and can also relate to the high ratting of CD observed on *B. jararacussu* as CD can happen as a defense mechanism against genetic damage and instability, aborting cells to prevent the inheriting of genetical imperfections (DANON et al., 2000). *B. atrox* also induced a low MCN count (3.36% of all CCAs) (Figure 3), this information indicates that most of the non-oriented chromosomes observed for this treatment are not being eliminated.

On the other hand MCN was abundant on *B. moojeni* and *B .alternatus* (11.79 and 12.70% of all CCAs observed respectively) (Figure 3) indicating that the damage caused by these venoms are not being repaired properly and are resulting in MCNs. However the presence of both fragments and bridges was low for the venom of all four species (ranging from 0.84 to 4.39 % and from 1.69 to 4.39% respectively) (Figure 2). These two alterations are also related to MCNs, bridges spur from erroneous chromosomal fusion due to the loss or damaging of chromosomes telomeres, which causes them to become unstable and highly reactive. These erroneous bounds result in dysenteric chromosomes that constitute the bridges and acentric fragments that can be eliminated as MCNs. Fragments can also be the result of damage sustained directly by the DNA molecule and chromosomal organizational structure (CAMPOS et al., 2008; LEME; MARIN-MORALES, 2009b). The fact that fragments and bridges are much more infrequent than non-oriented chromosomes suggests that *Bothrops* venom have a predominant aneugenic effect.

Also found on the venom of all four species tested is another class of toxins, the hyaluronidases. This toxin is ubiquitous to snake venoms (and to most other venoms on nature as well) (GONÇALVES-MACHADO et al., 2015). These toxins are proteins capable of disrupting hyaluronic acid depolymerizing proteoglycans and glycosaminoglycans, proteins that form an entwined net that constitutes the extra cellular matrix. Therefore the hyaluronidases lead to a loss of cohesion on the extra cellular matrix facilitating the intra tissue diffusion of the venom (STERN; JEDRZEJAS, 2006). Plant cell wall could potentially constitute a barrier for the spread of the venom and its toxins on the *L. sativa* cells but the results show otherwise. This is likely because, like the extra cellular matrix, the cell wall integrity is related to an interconnected net of glycans and proteins that are integral for its cohesion; the venom toxins are therefore having a similar effect on the plants as they do on the animals, facilitating the diffusion

of the venom, increasing its toxicity. It is also know that plant cell walls are naturally permeable to small molecules

Lastly a decrease on the Mitotic Index (MI) was evident for the cells exposed to all four species venoms. This decrease was very steep for *B. atrox* where the MI was almost 58% lower than on the control (Table 1). This decreased ranged from 32% to 37% for the other three species (Table1). The MI is considered to be an effective cytotoxicity marker as both CD and CCAs will negatively impact the cell cycle which will result in less entering division (FERNANDES; MAZZEO; MARIN-MORALES, 2007). Snakes venoms are also replete with neurotoxins which are capable of interfering with the proper functioning of ion channels such as the K+ channels. In plants K+ ions are responsible for a multitude of physiologic processes, one of such process is related to cell proliferation and elongation (LEBAUDY; VÉRY; SENTENAC, 2007).

The results presented here show that the venom from all four species tested induced CD, disrupted the normal cell cycle and chromosomal structure, caused damage to the DNA molecule and impaired the signaling for the kinetochore/spindle attachment, having both cytotoxic and genotoxic effects that lead to the reduction in MI and the induction of CCAs. Moreover, *Lactuca sativa* was an efficient model for testing the cytogenotoxicity of the 4 venoms tested and could be used on future researches for complementing or as an alternative to animal models.

4 CONCLUSION

The results found here prove that *L. sativa* is an effective model to evaluate the cytogenotoxic action of snake venoms. The results were both conclusive and rich in details allowing the analysis of the venoms mechanisms of action. This work suggests that *L. sativa* is an adequate model for evaluation of biologicaly active substances, such as venom, in general and not only for the species tested here. Considering the low costs and lack of any ethical and legal hindrances for the model it could constitute an interesting alternative or complementary model to animal ones.

Lastly *B. alternatus*, *B. atrox*, *B. jararacussu* and *B. moojeni* venoms were highly cytogenotoxic inducing damages to the DNA molecule, cell cycle alterations and reduction in the mitotic index. This toxicity was achieved through the action of several biologically active toxins like LAAOs, metaloproteases and $PLA_{2s.}$

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Table 1 – Effects of *Botrhops atrox, Botrhops alternatus, Botrhops moojenis and Botrhops jararacussu* venom on the mitotic index (MI), cell cycle alterations (CCA) and TUNEL test after exposure of *Latuca sativa* roots to 0.25mg of venom.

Treatments	MI	CCA	TUNEL
Control	11.41 ± 1.33	0.85 ± 0.127	0.1 ± 1.01
B. atrox	4.82 ± 1.83*	6.33 ± 1.45*	27 ± 7.18*
B. alternatus	8.98 ± 2.19*	11.91 ± 2.70*	29.6 ± 7.34*
B. moojeni	7.16 ± 1.53*	7.70 ± 2.26*	17 ± 4.47*
B. jararacussu	7.66 ± 1.13*	11.42 ± 2.26*	36.6 ± 6.62*

Results followed by standard deviation for the two evaluated parameters and in the TUNEL test. MI is expressed as percentage, and CCA as frequency per thousand cells. The TUNEL result is given by the percentage of cells with the positive mark. Data followed by * differ significantly from the control according to Tukey test (α=5%).

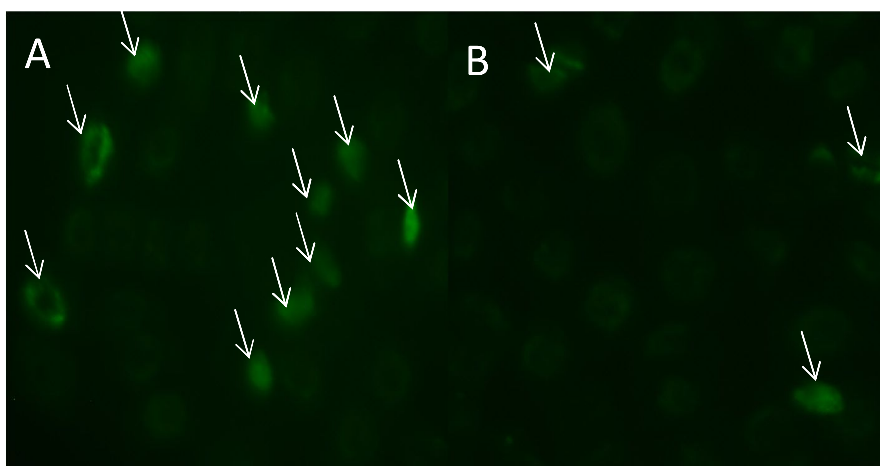


Figure 1. TUNEL test. Results observed after exposure of *Lactuca sativa* roots to 0.25 mg of *Bothrops* venom

A – Positive signals of the TUNEL test on cells exposed to the *B. jaracussu* venom (seen on the fluorescein filter). B – Positive signals of the TUNEL test on cells exposed to the *B. moojeni* venom (seen on the fluorescein filter).

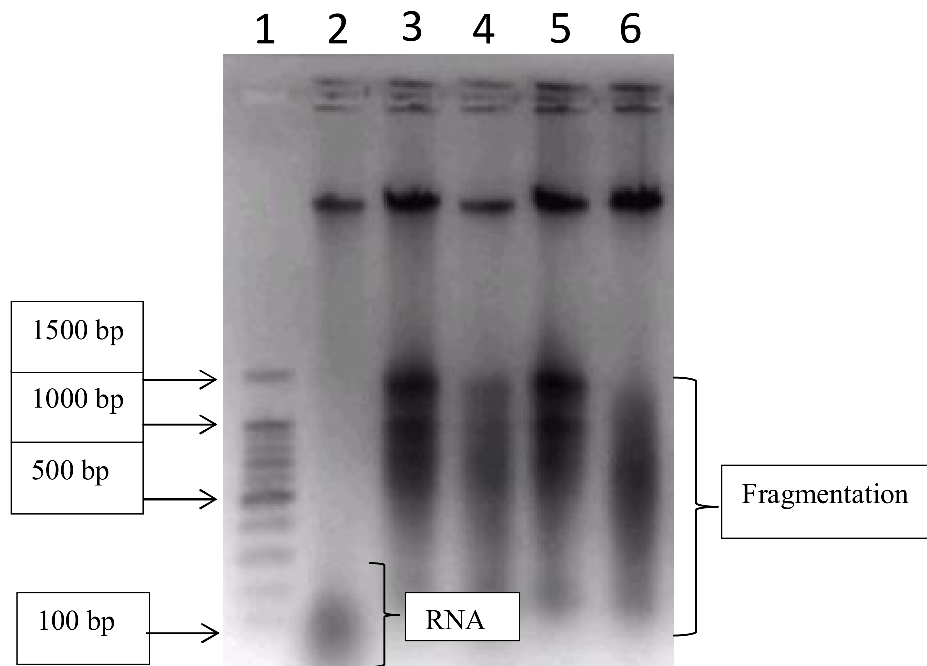
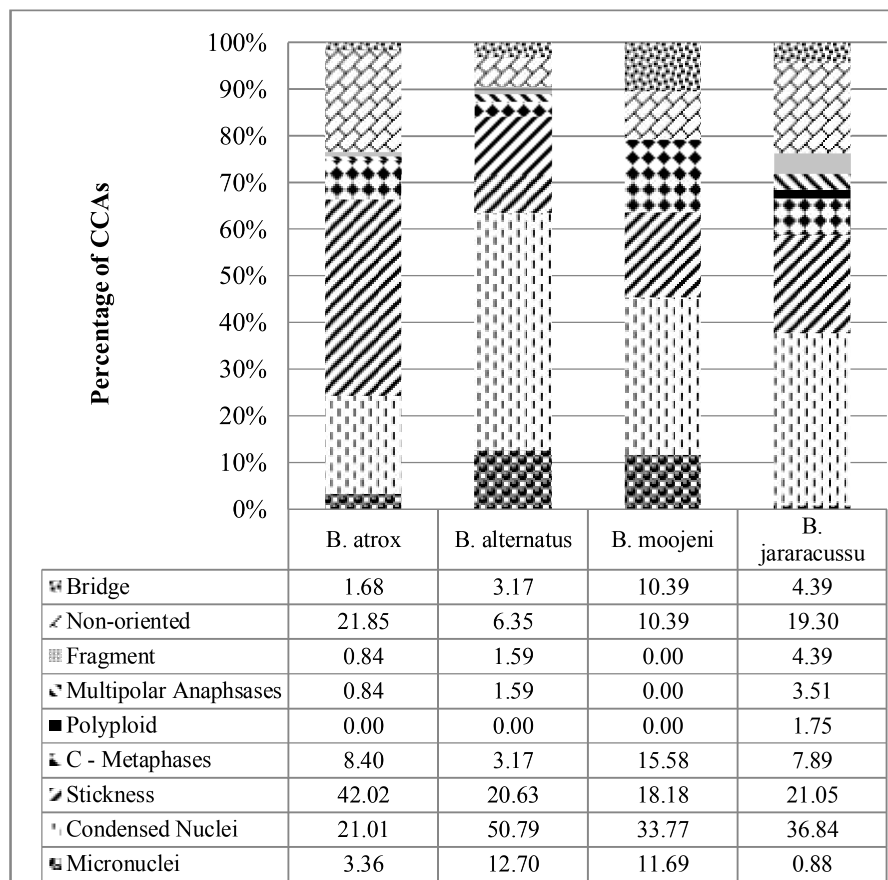


Figure 2 – Gel demonstrating the occurrence of DNA-laddering in the genetic material of *Lactuca sativa* cells exposed to *Bothrops* venom
1- Fragmentation pattern (100 bp); 2- Comparator (distilled water); 3- *B. atrox* venom; 4- *B.alternatus* venom; 5- *B. moojeni* venom; 6- *B. jararcussu* venom



	B. atrox	B. alternatus	B. moojeni	B. jararacussu
Bridge	1.68	3.17	10.39	4.39
Non-oriented	21.85	6.35	10.39	19.30
Fragment	0.84	1.59	0.00	4.39
Multipolar Anaphsases	0.84	1.59	0.00	3.51
Polyploid	0.00	0.00	0.00	1.75
C - Metaphases	8.40	3.17	15.58	7.89
Stickness	42.02	20.63	18.18	21.05
Condensed Nuclei	21.01	50.79	33.77	36.84
Micronuclei	3.36	12.70	11.69	0.88

Figure 3 - Percentage of CCAs observed on root tips of *Lactuca sativa* exposed to four species of the *Bothrops* genus venom.

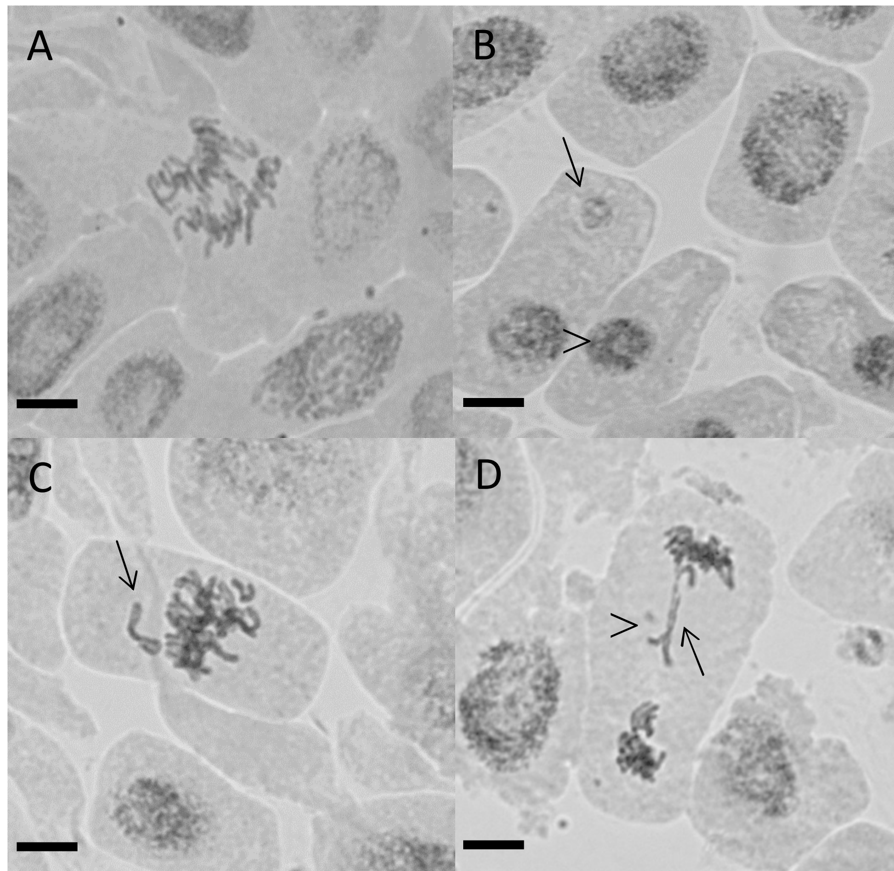


Figure 4 - Some of the CCAs found in root tip cells of *Lactuca sativa* exposed to venom from four *Bothops* species.
A – Bridges (*B. moojeni*); B – Micronuclei (arrow) and Condensed Nuclei (head arrow) (*B. alternatus*); C – Non-oriented (arrow) (*B. atrox*); D – Non-oriented (arrow) and Fragment (head arrow) (*B. jaracussu*). (Bars represent 10µm