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GLÊZIA RENATA DA SILVA LACERDA

PAPEL BIOLÓGICO DA L-ASPARAGINASE PRODUZIDA POR *Streptomyces ansochromogenes* UFPEDA 3420: ATIVIDADE CITOTÓXICA, GENOTÓXICA E IMUNOMODULATÓRIA.

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Tese submetida à Coordenação do programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito para obtenção do título de Doutora em Ciências Biológicas.

Orientadora: Profª. Drª. Silene Carneiro do Nascimento
Co-orientadora: Profª. Drª. Gláucia Manoella de Souza Lima.

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RECIFE

2017

A Deus, aos meus pais, Antonio e Luiza, ao meu esposo Jefferson Lacerda, a minha amada irmã Graziela, meu cunhado Renan e a minha pequena sobrinha Maria Luiza, por todo apoio, incentivo, alegrias e por ser o alicerce da minha vida.

Dedico.

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“Aquele que habita no esconderijo do Altíssimo,
à sombra do Onipotente descansará.

Direi ao Senhor: Ele é o meu Deus, o meu
refúgio, a minha fortaleza, e nele confiarei.”

Salmos 91:1,2.

RESUMO

Streptomyces é produtor de diversos metabólitos secundários, incluindo L-asparaginase, uma enzima que hidrolisa a asparagina em ácido aspártico e amônia. Logo, tem sido utilizada como agente quimioterápico no tratamento da leucemia linfoblástica aguda, pois estas células não produzem asparagina e dependem do aminoácido livre para a síntese proteica. O objetivo deste estudo foi avaliar a atividade citotóxica, genotóxica e imunomodulatória da L-asparaginase produzida por *Streptomyces ansochromogenes* UFPEDA-3420. A enzima foi produzida a partir da determinação dos parâmetros ideais de cultivo do micro-organismo. Sua atividade foi determinada pela dosagem de amônia liberada por Nesslerização. O extrato bruto foi purificado por métodos cromatográficos. O peso molecular foi determinado por eletroforese SDS-PAGE. O efeito citotóxico da L-asparaginase foi observado frente às linhagens de células tumorais: HEp-2 (carcinoma de laringe), NCIH-292 (carcinoma mucoepidermoide de pulmão), MOLT-4 (Leucemia linfoblástica humana), K562 (leucemia mielóide), HL-60 (leucemia aguda promielocítica) e MCF-7 (adenocarcinoma de mama), utilizando o ensaio do MTT. Os efeitos genotóxicos foram determinados pelos Testes Cometa Alcalino e de Micronúcleo nas linhagens tumorais NCIH-292, MCF-7 e MOLT-4 e em células normais PBMC (Células Mononucleadas de Sangue Periférico), utilizando as concentrações 12,5, 25 e 50 µg/mL de L-asparaginase, como controle positivo a Doxorrubicina na concentração da sua IC_{50} para cada célula tumoral: 0,5 µg/mL (NICH-292), 0,04 µg/mL (MOLT-4) e 0,2 µg/mL (MCF-7) e como controle negativo as células sem tratamento. Os parâmetros ideais de cultivo foram fermentação em meio M9, 48 horas, pH=6, 37ºC e rendimento máximo de 743.29 UI/mL. A enzima foi purificada até homogeneidade com fator de purificação 6,06, rendimento 27,68% da atividade inicial e atividade específica final 35.205,76 UI/mg. A enzima purificada apresentou peso molecular de 63,99 kDa, com atividade catalítica ótima em pH=7 e 40ºC. L-asparaginase apresentou valores de Km de 3,224 mM e Vmax de 161,29 UI/min. Quanto aos efeitos citotóxicos, apresentou IC_{50} de 25 µg/mL frente à linhagem MOLT-4. Foi observado que esta substância possui efeitos genotóxicos sobre as linhagens estudadas. No parâmetro Índice de Danos, as três linhagens tumorais apresentaram resultados significativos para todas as concentrações testadas quando comparadas com células não tratadas, exceto na concentração de 12,5 µg/mL na linhagem MOLT-4. No Teste do micronúcleo, foi observado que em todas as células houve indução à formação de micronúcleos, coferindo efeito genotóxico a enzima. Isso demonstra que nas linhagens MOLT-4 e PBMC, tanto o parâmetro células micronucleadas quanto a frequência de micronúcleos aumentaram de acordo com o aumento da concentração de L-asparaginase, demonstrando um efeito dose/resposta positivo. L-asparaginase induziu a ativação e proliferação de linfócitos TCD8+ e produziu níveis elevados de TNF-α, IFN-γ, IL-2 e IL-10 em 24 horas. O presente estudo possui grande relevância científica, pois consiste no primeiro relato de produção de L-asparaginase por *Streptomyces ansochomogenes* UFPEDA-3420, sua ação genotóxica em células tumorais e normais e atividade imunomodulatória, de forma inédita. Esse fato é importante, pois L-asparaginase tem sido empregada nas terapias convencionais de controle e tratamento do câncer. Dessa forma, esta enzima configura-se como molécula promissora para utilização em modelos in vivo e para aprofundar testes pré-clínicos.

Palavras-chave: Enzima. Actinobactéria. Caracterização Molecular. Efeito Citotóxico. Efeitos Genotóxicos.

ABSTRACT

Streptomyces is a producer of several secondary metabolites, including L-asparaginase, an enzyme that hydrolyzes asparagine in aspartic acid and ammonia. Therefore, it has been used as chemotherapeutic agent in the treatment of acute lymphoblastic leukemia, since these cells doesn't produce asparagine and depend on free amino acid for protein synthesis. The objective of this study was to evaluate the cytotoxic, genotoxic and immunomodulatory activity of L-asparaginase produced by *Streptomyces ansochromogenes* UFPEDA-3420. The enzyme was produced from the determination of the ideal microorganism culture parameters. Its activity was determined by the dosage of ammonia released by Nesslerization. The crude extract was purified by chromatographic methods. The molecular weight was determined by SDS-PAGE electrophoresis. The cytotoxic effect of L-asparaginase was observed against tumor cell lines: HEp-2 (laryngeal carcinoma), NCIH-292 (mucoepidermoid carcinoma of the lung), MOLT-4 (human lymphoblastic leukemia), K562(myeloid leukemia), HL-60 (promyelocytic acute leukemia) and MCF-7 (breast adenocarcinoma) using the MTT assay. The genotoxic effects were determined by Alkaline Comet and Micronucleus Tests in NCIH-292, MCF-7 and MOLT-4 tumor cell lines and in PBMC (Mononuclear Peripheral Blood Cells), using the concentrations 12.5, 25 and 50 μg/mL of L-asparaginase, as a positive control to Doxorubicin at the concentration of its IC50 for each tumor cell: 0.5 μg/mL (NICH-292), 0.04 μg/mL (MOLT-4) and 0.2 μg/mL (MCF-7) and as a negative control the cells without treatment. The ideal culture parameters were fermentation in M9 medium, 48 hours, pH=6.37 and maximum yield of 743.29 IU/mL. The enzyme was purified to homogeneity with purification factor 6.06, yield 27.68% of the initial activity and final specific activity 35,205.76 IU/mg. The purified enzyme had a molecular weight of 63.99 kDa, with optimum catalytic activity at pH=7 and 40ºC. L-asparaginase had values of Km of 3.224 mM and Vmax of 161.29 IU/min. Regarding the cytotoxic effects, IC50 of 25 μg/mL was presented in relation to the MOLT-4 lineage. It was observed that this substance has genotoxic effects on the studied strains. In the parameter Damage Index, the three tumor lines presented significant results for all the concentrations tested when compared to untreated cells, except in the concentration of 12.5 μg/mL in the MOLT-4 lineage. In the Micronucleus test, was observed that in all the cells there was induction to the formation of micronuclei, cofering genotoxic effect to the enzyme. This demonstrates that in the MOLT-4 and PBMC lines, both the micronucleate parameters and micronucleus frequency increased as the L-asparaginase concentration increased, demonstrating a positive dose/response effect. L-asparaginase induced the activation and proliferation of TCD8+ lymphocytes and produced elevated levels of TNF-α, IFN-γ, IL-2 and IL-10, in 24 hours. The present study has great scientific relevance, since it consists of the first report of production of L-asparaginase by *Streptomyces ansochomogenes* UFPEDA-3420, its genotoxic action in normal and tumor cells and immunomodulatory activity, in an unprecedented way. This fact is important because L-asparaginase has been used in conventional cancer treatment and control therapies. Thus, this enzyme is configured as a promising molecule for use in vivo models and to deepen preclinical tests.

Key words: Enzyme. Actinobacteria. Molecular Characterization. Cytotoxic Effect. Genotoxic Effects.

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synthetize L-asparagine and require this amino acid from the blood serum for their survival. The L-asparaginase enzyme hydrolyzes asparagine into aspartic acid and ammonia and this process inhibits tumor growth. In addition, L-asparagine also hydrolyzes glutamine into glutamic acid and ammonia. Studies have shown that human and murine treatment using L-asparaginase is promising against Acute Lymphoblast Leukemia but can produce many side effect and compromise the immune system [15,16,18-21]…….

Fig. 2: Effects of L-asparaginase isolated from *Streptomyces ansochromogenes* UFPEDA 3420 actinobacteria against human lymphocytes. At all concentrations, L-asparaginase did not induce statistical and significant cell death in human lymphocytes. Apoptotic and necrotic values found in L-asparaginase-treated cells were similar to control cells (cells+medium). Horizontal bars represent the average of six independent experiments performed in duplicate…….. 117

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LISTA DE ABREVIATURAS

µm	Micrômetro
AML	Leucemia Mielóide Aguda
APC	Células Apresentadoras de antígenos
ASN	L-asparaginase
CC	Beta-quimiocinas
CCL/CXCL	Quimiocina
CD4	Linfócitos T CD4 Auxiliares
CD8	Linfócitos T CD8 Citotóxicos
CI_{50}/IC_{50}	Concentração Mínima Inibitória
CMN/MNC	Células Micronucleadas
CXC	Alfa-quimiocinas
DEAE	Dietilaminoetil
DMEN	Dulbecco's Modified Eagle's Medium
DMSO	Dimetilsulfóxido
DNA	Ácido Desoxirribonucleico
FD/DF	Frequência de Danos
FMN/MNF	Frequência de Micronúcleos
FMN/MNF	Frequência de Micronúcleos
GFP	Green Fluorescent Protein
HEp-2	Carcinoma de Laringe
HL-60	Leucemia Promielocítica Aguda
HTC-116	Células de Câncer de Cólon retal
ID/DI	Índice de Danos
IL	Interleucina
INCA	Instituto Nacional do Câncer
INF	Interferon
iNOS	Óxido nítrico sintetase induzida
IP/PI	Índice de Proporcionalidade
ISP	International *Streptomyces* Project
IU	International Unit
K562	Leucemia Mielóide Humana

Km	Constante de Michaelis-Menten
LA	L-asparaginase
LLA/ALL	Leucemia Linfoblástica Aguda
LM	Melting-point
MCF-7	Células de Câncer de Mama
MDA-MB-231	Células de Câncer de Mama
MDR	Resistência Multidroga
MHC	Major Histocompatibility Complex
MN	Micronúcleo
MOLT-4	Leucemia Linfoblástica Humana
MTT	(Brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio)
NADH	Dinucleótido de Nicotinamida e Adenina
NADPH	Fosfato de Dinucleótido de Nicotinamida e Adenina
NCIH-292	Carcinoma Mucoepidermiode de Pulmão
NK	Natural Killer Cells
OD	Densidade Ótica
OMS	Organização Mundial de Saúde
ON/NO	Óxido Nítrico
PBMC	Células Mononucleadas de Sangue Periférico
PBS	Tampão Fosfato Salina
PEG	Polietileno glicol
pH	Potencial Hidrogeniônico
RNA	Ácido ribonucleico
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TCD4+	Linfócito TCD4 ativado
TCD8+	Linfócito TCD8 ativado
TGF	Fator de crescimento Transformante
Th	Linfócitos auxiliares
TNF	Fator de Necrose Tumoral
T_{reg}	Células T reguladoras
UFPE	Universidade Federal de Pernambuco
UFPEDA	Departamento de antibióticos da Universidade Federal de Pernambuco
Vmax	Velocidade Máxima

SUMÁRIO

Introdução

1 INTRODUÇÃO

O Câncer é uma doença que se caracteriza pelo crescimento excessivo e descontrolado de células anormais. Este descontrole no crescimento celular pode ser causado pela inibição da apoptose ou da expressão de genes que promovem o crescimento celular (oncogenes) ou pelos supressores da transformação maligna (genes supressores de tumores). (POLLOCK et al., 2006; INCA, 2017).

Esta doença é uma das mais temida em todo o mundo devido à ausência de tratamentos efetivos para grande parte dos tumores metastáticos. A busca por fármacos antitumorais teve seu início em meados da década de 40 e desde então, a área de pesquisa de produtos naturais teve significativa expansão. Um elevado número destes produtos é oriundo de micro-organismos ou interações microbianas, o que indica que estes são produtores de moléculas bioativas que podem ser utilizadas como fármacos ou como estruturas-modelo para o planejamento e desenvolvimento de novas substâncias com aplicação farmacológica (NEWMAN & CRAGG, 2016).

No que diz respeito ao câncer, das 155 moléculas já identificadas e aprovadas para o tratamento, 75% são moléculas não sintéticas e destas, 47% são produtos naturais ou derivados diretamente destes. Os micro-organismos também são capazes de produzir enzimas, antibióticos, imunossupressores e agentes redutores do colesterol sanguíneo (NEWMAN & CRAGG, 2016).

Streptomyces, um gênero pertencente ao filo actinobactérias, vêm sendo estudado cada dia mais por ser caracterizado como grande produtor de uma diversidade de metabólitos secundários, sendo os antibióticos os metabólitos mais relatados. Além disso, os *Streptomyces* também são capazes de produzir enzimas, anti-inflamatórios e antitumorais (BIBB, 2005; TORTORA, 2012; MADIGAN, 2010).

O filo das actinobactérias é considerado como boa fonte para produção de L-asparaginase (ASN), enzima de alto valor terapêutico devido ao seu amplo espectro de atividade antitumoral. Esta enzima é utilizada no tratamento de leucemia linfoblástica aguda (LLA), doença de Hodgkin´s, tumores de NK, linfomas de célula T e alguns subtipos de leucemias mielóides (MULLER E BOOS, 1998).

Alguns tipos de células tumorais requerem grandes quantidades de L-asparagina para crescerem e desta forma captam este aminoácido do meio extracelular (AGHAIYPOUR et al., 2001). A asparaginase hidrolisa a asparagina liberando amônia e ácido aspártico. Diferente das células normais, a célula tumoral sofre silenciamento gênico e não consegue regular positivamente

o gene da asparagina sintase, obrigando a célula a captar o aminoácido na corrente sanguínea. Deste modo, a depleção de asparagina da corrente sangüínea conduz à destruição das células tumorais, por serem incapazes de completar a síntese proteica e, consequentemente, sintetizar suas próprias proteínas (NARTA et al., 2007).

Vários microrganismos são produtores desta enzima entre eles *Enterobacter cloacae, Serratia marcescens e Enterobacter aerogenes*. As enzimas isoladas de *E. coli* e *Erwinia carotovora* são as mais utilizadas no tratamento de leucemia linfoblástica aguda. Porém, a administração prolongadada L-asparaginase pode levar a produção de anticorpos provocando um choque anafilático ou neutralização do efeito do fármaco. Por isto, é necessária uma busca contínua por novas estirpes capazes de produzir e gerar alto rendimento de L-asparaginase. Entre as actinobactérias algumas espécies têm se destacado pela produção tais como *S.karnatakensis, S. venezualae, S. longsporusflavus*, *Streptomyces* sp. PDK2, *Streptomyces acrimycini*, *Streptomyces ginsengisoli*, *S. olivaceus* e *S. griseus* (NARAYANA et al., 2007; SELVAM e VISHNUPRIYA, 2013; DESHPANDE et al, 2014; EL-NAGGAR et al, 2015; MEENA et al, 2015a) e *Nocardiopisis alba* (MEENA et al., 2015b).

No mercado estão disponíveis as preparações de asparaginase derivadas de *E. coli* (Asparaginase®;Medac, Kidrolase®; EUSA Pharma, Elspar®) e de *Erwinia* chrysanthemi (Erwinase®) (ONCASPAR, 2006; AVRAMIS e TIWARI, 2006).

Pesquisadores do Departamento de Antibióticos da UFPE vêm estudando a diversidade de actinobactérias do bioma Caatinga do Nordeste brasileiro. Considerando que pouco se conhece sobre a diversidade dos micro-organismos neste habitat, acredita-se que condições ambientais extremas representem um local propício capaz de revelar a presença de actinobactérias fontes de novas moléculas bioativas. Além disso, o solo pouco fértil pode representar uma poderosa fonte de micro-organismos produtores de substâncias químicas com elevado potencial biotecnológico. A complexa interação ecológica e os mecanismos adaptativos destes micro-organimos ao semi-árido são de enorme interesse a biotecnologia, e configuram perspectivas para aplicação e geração de novos produtos.

Neste sentido, foi investigada a produção de L-asparaginase de *Streptomyces ansochromogenes* UFPEDA 3420, bem como os efeitos citotóxicos, genotóxicos em células normais e cancerígenas e sua atividade imunomodulatória.

Objetivos

2 OBJETIVOS

2.1 Objetivo geral:

Avaliar o papel biológico da L-asparaginase produzida por *Streptomyces ansochromogenes* UFPEDA 3420 na atividade citotóxica e genotoxidade em células normais e cancerígenas e atividade imunomodulatória em linfócitos humanos.

2.2 Objetivos específicos:

- Avaliar a influência de pH, temperatura e tempo na produção de L-sparaginase por *S. ansochromogenes* UFPEDA 3420;
- Definir as melhores condições de cultivo do *S. ansochromogenes* UFPEDA 3420 para produção de L-asparaginase;
- Produzir, purificar e caracterizar a enzima L-asparaginase a partir de *S. ansochromogenes* UFPEDA 3420;
- Observar o efeito citotóxico da L-asparaginase em células sadias e tumorais;
- Analisar o efeito genotóxico da L-asparaginase provocado nas células sadias e tumorais.
- Investigar o estímulo imunológico promovidos pela L-asparaginase, in vitro, contra linfócitos humanos.

Revisão de Literatura

3 REVISÃO DE LITERATURA

3.1 Gênero *Streptomyces*.

As Actinobactérias correspondem a um importante grupo de bactérias Gram-positivas, caracterizadas por um alto conteúdo de guanina e citosina. São capazes de formar micélio, que se ramificam em longos filamentos denominados hifas e que podem se diferenciar e formar cadeia de esporos, como o gênero *Streptomyces*. (KONEMAN et al, 2008; MADIGAN et al, 2010; BERGEY et al, 2012). É possível que estes organismos tenham desenvolvido estas estruturas para obter suprimentos nutricionais de vegetais rígidos. As actinobactérias produzem micélio aéreo e utilizam esta estrutura para fixação e penetração em tecidos vegetais, podendo liberar enzimas que degradam compostos essenciais para sua alimentação (CHATER, 2006). Elas são caracterizadas pela ampla produção de metabólitos secundários, dentre eles enzimas, inibidores enzimáticos, antitumorais e principalmente antibióticos, que são utilizados com frequência na Agricultura, Medicina e Veterinária (INBAR et al., 2005).

Ferdinando Cohn, em 1875, foi quem primeiro descreveu o micro-organismo que atualemente nós conhecemos como *Streptomyces*. O pesquisador observou células filamentosas que inicialmente denominou de *Streptotrix*, o que significa “cabelo enrolado”, e em 1916 o grupo de Actinomycetes foi reconhecido oficialmente como *Actinomycetales* por Buchaman (1916). Entretanto, foi Waksman e Henrice em 1943 quem primeiro classificou os *Actinomycetales* em três grupos principais: 1) *Mycobacterium*; 2) *Actinomyces* anaeróbio; *Nocardia* aeróbia e 3) *Streptomyce*s e *Micromonospora* os quais são aeróbios oxidativos de grande importância para a biotecnologia. Waksman, em 1943, sugeriu o nome *Streptomyces* que significa “fungo enrolado” e favorecia a idéia de actinobactéria como um grupo intermediário entre fungo e bactéria, sendo atualmente o gênero de maior importância para a biotecnologia que é responsável por 70% dos antibióticos de uso clínico.

De acordo com o banco de dados *National Center for Biotechnology Information* (NCBI, 2016), o gênero *Streptomyces* pertence à família *Streptomycetaceae*, à subordem *Streptomycineae*, à ordem *Actinomicetales*, à subclasse *Actinobacteridae*.

O gênero *Streptomyces* é o mais conhecido dentre as actinobactérias, pois abriga mais de 3000 espécies já identificadas. Eles são comumente encontrados no solo, embora também possam ocorrer em ambientes aquáticos. São organismos aeróbios obrigatórios, que sintetizam compostos

voláteis como a geosmina, que confere ao solo o odor característico de terra ou terra molhada (MADIGAN et al., 2010; TORTORA et al., 2012; BERGEY et al, 2012). Seus filamentos possuem geralmente 0,5 – 1,2 µm de diâmetro, mais estreitas que as hifas de fungos, com comprimento indefinido e frequentemente sem septos. O crescimento ocorre nas extremidades da hifas, acompanhado de ramificações (KONEMAN et al., 2008; MADIGAN et al., 2010).

INBAR et al.(2005), relatam que o gênero *Streptomyces* é um dos mais estudados devido a diversidade de metabólitos secundários produzidos por eles e por sua capacidade competitiva por substratos. Eles são potentes produtores de moléculas bioativas como enzimas, anti-inflamatórios, antibióticos e antitumorais. No entanto, outros gêneros também são significativos, pois diversas outras espécies foram isoladas confirmando que não só os *Streptomyces* são capazes de produzir novos metabólitos secundários (BIBB, 2005).

Diversos estudos com *Streptomyces* vêm mostrando que este gênero é responsável pela produção de uma variedade de compostos com a atividade antitumoral. Estudos recentes que avaliaram solo marinho e sedimentos oceânicos verificaram a atividade antitumoral contra uma série de cânceres, como o de cólon retal, adenocarcinoma cervical humano, hepatocarcinoma humano, epidermóide oral humano e câncer de mama (LOMBÓ et al, 2006; SHAABAN et al, 2011; ESPINOSA et al, 2012).

O *Streptomyces ansochromogenes* UFPEDA 3420 tem sido relatado como produtor de um metabólito com atividade antifúngica. Estudos realizados por Vasconcelos et al (2015) mostram esta atividade frente a diferentes gêneros como *Candida*, *Fusarium*, *Trichophyton* e *Aspergillus*.

3.2 Novos fármacos

Registros da década de 60 mostram que os pesquisadores daquela época já buscavam compostos com potencial atividade antitumoral. Várias linhagens de *Streptomyces* foram isoladas pelo Departamento de Antibióticos da Universidade Federal de Pernambuco, dentre estas o *S. olindensis* (UFPEDA 5622) um grande produtor de retamicina (complexo policetônico de ampla atividade antimicrobiana e antitumoral) (LIMA et al, 1969).

Ao longo do tempo, vários estudos vêm sendo realizados em busca de compostos com a atividade antitumoral a partir de actinobactérias. Espinosa et al, (2012) avaliaram a atividade antitumoral de actinobactérias isoladas de sedimento marinho no México e observaram que 19% dos extratos brutos de *Streptomyces* apresentaram atividade antitumoral contra células de Câncer de

Cólon retal HTC-116. YE et al, (2009) constataram que o *S. griseorubens* proveniente de solo marinho chinês também apresentou atividade antitumoral contra outros tipos de cânceres como adenocarcinoma cervical humano, hepatocarcinoma humano e epidermóide oral humano.

Shaaban e colaboradores (2011) relataram que o *S. cyanogenus* S-136 foi capaz de produzir oito compostos denominados Landomicina P-W, que em associação com 10 anguciclinonas tem atividade contra as células MCF-7 e MDA-MB-231 de câncer de mama. Outros compostos como a mitramicina e cromomicina, isolados respectivamente de *S. argillaceus* e *S. griseus* também tem demonstrado grande potencial como antitumorais (LOMBÓ et al, 2006).

Actinobactérias, principalmente aquelas pertencentes ao gênero *Streptomyces*, têm sido alvo de pesquisas quanto à produção de L-asparaginase (MEENA et al, 2015a). A L-asparaginase é uma enzima de grande importância terapêutica para o tratamento de Leucemia Linfoblástica Aguda (SALZER et al., 2014).

Deshpande et al. (2014) relatam a produção de L-asparaginase por *S. ginsengisoli* em diferentes condições de produção, como tempo, temperatura, fonte de carbono e pH. Outros estudos, como o realizado por El-Sabbagh et al (2013), mostram a atividade antitumoral da L-asparaginase produzida por *S. halstedii*, cuja enzima exibiu um efeito citotóxico contra a linhagem celular de Carcinoma de ascite de Ehrlich (carcinoma mamário de camundongos fêmeas) no ensaio in vitro e além disso reduziu o crescimento do tumor sólido induzida em ratos albinos.

3.3 L-asparaginase

De acordo com Clementi (1922), a L-asparaginase começou a ser estudada por volta do século XIX, por Caventon Bacon et al, Plisson et al e Pelouze et al, que relataram a degradação da asparagina em ácido aspártico e amônia. Este mesmo pesquisador, em 1922, verificou que a L-asparaginase ativa em soro de porco da guiné tinha atividade citotóxica e também atuava como inibidor de crescimento.

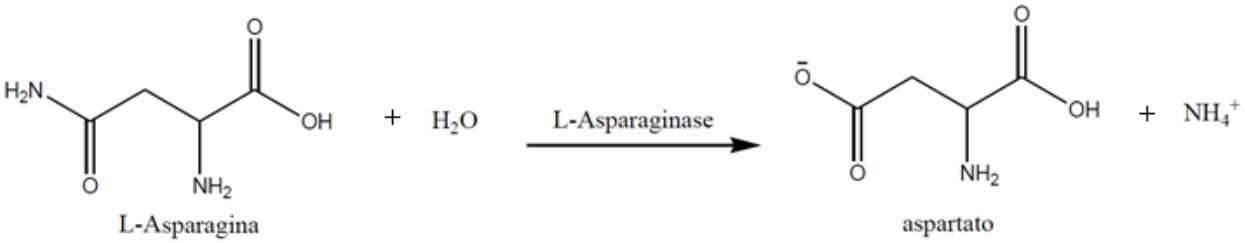
Em 1953, Kidd observou que ratos portadores de linfoma tinham regressão da doença quando tratados com o soro de porco da guiné quando comparados ao grupo controle não tratado, sem causar danos à cobaia.

Dez anos depois, Broome (1963) mostrou que a L-asparaginase possuía ação anti-linfoma. Este pesquisador realizou um processo de purificação da enzima por precipitação por sal, eletroforese e cromatrografia DEAE celulose e verificou que a atividade antilinfoma foi encontrada

apenas nas frações que continham a L-asparaginase. A partir daí, diversos estudos foram realizados e comprovaram a ação desta enzima. Entretanto, só em 1967 a L-asparaginase (L-Asnase) foi isolada em quantidade suficiente para ser submetida a testes clínicos e ter sua atividade antineoplásica comprovada (CAPIZZI et al., 1971)

A L-asparaginase é uma enzima de grande importância medicinal, devido a sua ação quimioterapêutica pela extensa capacidade de hidrolisar o aminoácido L-asparagina em ácido aspártico e amônia (figura 2). A L-asparagina é relatada como um aminoácido essencial tanto para as células sadias quanto para as células tumorais, pois participam das sínteses de proteínas, DNA e RNA (MCCREDIE, HO e FREIREICH, 1973; NARTA, KANWAR e WAMIK, 2007; JAIN et al., 2012; KAWEDIA e RYTTING, 2014).

Figura 1: Representação esquemática da hidrólise do aminoácido pela enzima L-asparaginase.



Fonte: Narta, Kanwar e Wamik (2007), modificado.

A enzima L-asparaginase tem sido bastante utilizada para abrandar os sintomas da Leucemia aguda desde meados dos anos de 1960, quando foi implementada para o tratamento desta doença. As células tumorais utilizam tanto a asparagina oriunda da dieta (livres no soro) como a quantidade limitada de asparagina produzidas por elas mesmas, para sua proliferação e sobrevivência. No entanto, como a L-asparaginase é capaz de hidrolisar a L-asparagina, ocorre redução nos níveis extracelulares de asparagina, privando a célula tumoral do consumo do aminoácido essencial. Células leucêmicas e outras linhagens tumorais não possuem a capacidade de sintetizar a L-asparagina devido a um silenciamento genético que culmina na não produção da enzima L-asparagina sintetase, resultando na redução da síntese de proteínas dependente de asparagina e morte celular. As células sadias não são afetadas diante a ação da L-asparaginase, uma vez que estas possuem a L-asparagina sintetase íntegra, possibilitando a produção da L-asparagina no citoplasma celular para seu próprio consumo (NARTA, KANWAR e WAMIK, 2007; JAIN et al., 2012; CHEN, 2014).

Uma ampla diversidade de micro-organismos é capaz de produzir a L-asparaginase. Dentre tais micro-organismos incluem os fungos (GURUNATHAN e SAHADEVAN, 2012; CHOW e

TING, 2014), leveduras (KIL, KIM e PARK, 1995), bactérias (SALZER et al., 2014) e actinobactérias (HATANAKA et al., 2011; KIRANMAYI, PODA e VIJAYALAKSHMI, 2014), sendo que a L-asparaginase mais relatada pelos pesquisadores são aquelas produzidas por *Escherichia coli* e *Erwinia chrysanthemi*, pois são relatadas como as principais enzimas utilizadas como agentes terapêuticos no tratamento da leucemia linfoblástica aguda infantil (SALZER et al., 2014).

Algumas bactérias, como *Escherichia coli*, apresentam duas proteínas independentes com atividades de asparaginase. Uma delas está localizada no citoplasma, a asparaginase I, e a outra no espaço periplasmático, a asparaginase II, e somente a do tipo II apresenta atividade antineoplásica (CAMPBELL et al., 1967).

Três tipos principais de asparaginase têm sido utilizados até agora: 1) a asparaginase nativa derivada de *Escherichia coli* (*E. coli* asparaginase: Kidrolase, EUSA Pharma, Oxford, Reino Unido; Elspar, Ovation Pharmaceuticals, Deerfield, Illinois; Crasnitin, Bayer AG, Leverkusen, Alemanha; Leunase, Sanofi-Aventis, Paris, França; Asparaginase Medac, a Kyowa Hakko, Tóquio, Japão), 2) uma forma da asparaginase de *E. coli* nativa (polietileno glicol [PEG]-asparaginase: Oncaspar, Sigma-Tau Pharmaceuticals, Inc., Gaithersburg, MD), (ONCOSPAR, 2006) e 3) uma enzima isolada a partir de *Erwinia chrysanthemi*, referido como *Erwinia asparaginase* (Erwinase, EUSA Pharma, Oxford, Reino Unido) (AVRAMIS e TIWARI, 2006).

3.4 Câncer

Câncer é o nome dado a um conjunto de mais de 100 doenças caracterizado pelo crescimento excessivo e descontrolado de células anormais, que podem invadir e destruir outros tecidos e espalhar-se para outras regiões do corpo em um processo denominado metástase. A rápida divisão celular permite que estas células tornem-se muito agressivas e incontroláveis, determinando a formação de tumores ou neoplasias malignas. É possível que apenas um pequeno número de células tumorais seja capaz de iniciar e sustentar o crescimento do tumor (O'BRIEN et al, 2009; ABBAS et al, 2010; INCA, 2017).

Apesar da origem incerta, o termo câncer tem sido descrito por alguns autores como uma palavra derivada do latim caranguejo, que faz analogia à maneira que o tumor maligno se comporta no tecido, preso, entranhado e infiltrado nas células sadias assemelhando-se aos tentáculos de um caranguejo. Além disso, as projeções podem parecer em forma de pinça, lembrando a pata do

crustáceo. No entanto, outros designam que a origem do termo parece estar mais relacionada ao termo Cancro (CUNHA, OLIVEIRA E LIMA, 2010).

O termo Carcinogênese é utilizado para descrever a cascata de eventos que transforma uma célula normal em uma célula cancerígena. Consiste em um processo de múltiplos estágios decorrentes de danos genéticos e alterações epigenéticas, que pode ser descrita por três principais etapas: iniciação, promoção e progressão. Na iniciação, ocorrem mutações nas células-tronco pela exposição à carcinógenos de modo irreversível, afetando a composição ou estrutura básica do componente nuclear de DNA, dando partida ao desenvolvimento do câncer. A etapa da promoção envolve a alteração da expressão do gene, a expansão clonal seletiva e a proliferação de células que sofrem iniciação, sendo de caráter reversível com a descontinuação do agente de promoção. A última etapa denominada progressão, é caracterizada por alterações moleculares adicionais, aumento da massa tumoral primária, desprendimento dessa massa e locomoção para outras áreas e tecidos. A fixação em outros lugares seguido de contínuo crescimento é chamado de metástase (POLLOCK et al, 2006).

O termo neoplasia significa “novo crescimento” e o termo tumor foi designado ao edema causado por inflamação. Os tumores podem ser classificados como benignos ou malignos. Os tumores benignos são automilitantes, não se disseminam em tecidos próximos e não formam metástases, porém podem causar algum comprometimento devido à pressão mecânica. Estes tumores geralmente recebem o sufixo –oma nas células de origem, como fibroma, papilomas, entre outros. Já os tumores malignos tem crescimento ilimitado e podem disseminar-se pelos tecidos adjacentes ou por metástase. Estes tumores possuem nomenclatura semelhante aos tumores benignos, porém acrescido de algumas expressões dependendo do seu local de origem (no caso de sarcomas, carcinomas, adenocarcinomas, entre outros) (ALBERTS et al, 2009; ABBAS et al, 2010; BORGES-OSÓRIO e ROBINSON, 2013).

O câncer é uma doença genética e a falha no controle genético celular é o principal ponto de partida para o desencadeamento desta. Duas classes de genes específicos estão envolvidos no controle gênico celular: os proto-oncogenes e os genes supressores de tumor. Os proto-oncogenes são genes celulares normais que controlam o crescimento celular e a diferenciação do organismo. Em determinadas ocasiões, os proto-oncogenes podem diferenciar-se em oncogenes, que são genes dominantes em nível celular e codificam proteínas estimuladoras de crescimento, levando ao descontrole da divisão celular. Os genes supressores de tumor atuam reprimindo a divisão celular em um processo celular normal. Caso ocorra mutação nestes genes, sua função pode ser perdida ou alterada (ALBERTS et al, 2009; BORGES-OSÓRIO e ROBINSON, 2013).

A história natural de grande parte dos tumores malignos pode ser dividida em quatro importantes fases: alteração maligna da célula alvo (transformação), crescimento das células transformadas, invasão local e metástase à distância. Para que se inicie a alteração maligna é necessário que ocorra o mau funcionamento de algum processo regulatório, como a auto-suficiência nos sinais de crescimento, insensibilidade aos sinais inibidores de crescimento, invasão da apoptose, defeitos no reparo do DNA, potencial infinito de replicação, angiogênese mantida e a capacidade de evadir e metatastizar (ABBAS et al, 2010).

3.4.1 Epidemiologia do câncer

O câncer tem sido considerado uma doença de importância pública não só no Brasil, mas em todo o mundo. A Organização Mundial de Saúde (OMS) tem como um dos seus principais programas, o Programa de Controle do Câncer, que tem um importante papel de promover planos nacionais de controle do câncer, políticas e programas integrados para doenças não transmissíveis e outros problemas relacionados.

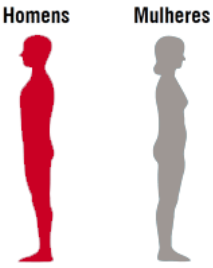
Os levantamentos epidemiológicos realizados pela Organização Mundial de Saúde e pelo Instituto Nacional do Câncer (INCA) são realizados a cada dois anos. De acordo com a OMS (2017), o câncer é uma das principais causas de morte no mundo, sendo responsável por 8,2 milhões de mortes em 2012. Através do projeto Globocan, realizado em 2012, as causas mais comuns de morte por câncer são; Câncer de pulmão (1,59 milhão de mortes); fígado (745 000 mortes); estômago (723 000 mortes); colorretal (694 000 mortes); de mama (521 000 mortes) e câncer esofágico (400 000 mortes). O número de novos casos no mundo todo deverá aumentar em cerca de 70% ao longo das próximas duas décadas. Entre os homens, os cinco sítios mais comuns de câncer diagnosticados em 2012 foram de pulmão, próstata, colorretal, estômago e câncer de fígado. Entre as mulheres dos cinco sítios mais comuns diagnosticados eram de mama, colorretal, pulmão, colo do útero e câncer de estômago (OMS, 2017).

No Brasil, as estimativas quanto ao número de casos de Câncer em 2016, de acordo com o INCA, eram que ocorressem cerca de 600 mil novos casos (dados também válidos para 2017), sendo os tipos mais incidentes, com exceção do câncer de pele do tipo não melanoma (o mais incidente na população brasileira), o câncer de próstata, pulmão, intestino, estômago e cavidade oral. Para as mulheres, a maior incidência estimada seria para os cânceres de mama, intestino, colo do útero, pulmão e estômago (figura 2). Ainda de acordo com esse órgão, a previsão é que em 2030, a carga global seja de 21,4 milhões de casos novos de câncer e 13,2 milhões de mortes por câncer,

em consequência do crescimento e do envelhecimento da população, bem como da redução na mortalidade infantil e nas mortes por doenças infecciosas em países em desenvolvimento.

Figura 2: Tipos de Câncer mais incidentes, estimados para 2016-2017, na população brasileira.

Localização primária	casos novos	%
Próstata	61.200	28,6%
Traqueia, Brônquio e Pulmão	17.330	8,1%
Cólon e Reto	16.660	7,8%
Estômago	12.920	6,0%
Cavidade Oral	11.140	5,2%
Esôfago	7.950	3,7%
Bexiga	7.200	3,4%
Laringe	6.360	3,0%
Leucemias	5.540	2,6%
Sistema Nervoso Central	5.440	2,5%



Localização primária	casos novos	%
Mama Feminina	57.960	28,1%
Cólon e Reto	17.620	8,6%
Colo do Útero	16.340	7,9%
Traqueia, Brônquio e Pulmão	10.890	5,3%
Estômago	7.600	3,7%
Corpo do Útero	6.950	3,4%
Ovário	6.150	3,0%
Glândula Tireoide	5.870	2,9%
Linfoma não Hodgkin	5.030	2,4%
Sistema Nervoso Central	4.830	2,3%

*** Números arredondados para múltiplos de 10**

Fonte: INCA, 2017.

Em Pernambuco, estimou-se que em 2016-2017 pudesse surgir mais de 20.000 novos casos de câncer no estado, sendo o maior índice, o câncer de próstata para homens (2.750 casos) e o câncer de mama feminina para mulheres (2.550 casos) (tabela 1).

Não há como contestar que o câncer é hoje, no Brasil, um problema de saúde pública. É evidente que o controle e prevenção desta doença sejam prioridade em todas as regiões, desde as mais desenvolvidas até às mais desiguais. É fundamental a implementação de medidas socioeducativas em todos os níveis da sociedade; prevenção, apoio e monitoramento continuado dos programas de prevenção e controle para combater o câncer e seus fatores de risco.

Tabela 1: Estimativas para o ano de 2016-2017 em Pernambuco das taxas brutas de incidência por 100 mil habitantes e do número de casos novos de câncer, segundo sexo e localização primária*.

Localização Primária da Neoplasia Maligna	Estimativa dos Casos Novos							
	Homens				Mulheres			
	Estado		Capital		Estado		Capital	
	Casos	Taxa Bruta	Casos	Taxa Bruta	Casos	Taxa Bruta	Casos	Taxa Bruta
Próstata	2.750	61,73	550	73,19	-	-	-	-
Mama Feminina	-	-	-	-	2.550	53,18	740	84,89
Colo do Útero	-	-	-	-	970	20,23	150	17,74
Traqueia, Brônquio e Pulmão	550	12,46	170	22,41	420	8,70	120	14,16
Cólon e Reto	370	8,35	120	15,75	570	11,98	190	22,27
Estômago	500	11,36	90	11,73	350	7,34	70	7,55
Cavidade Oral	380	8,62	90	12,57	220	4,50	40	5,02
Laringe	300	6,65	80	10,23	40	0,82	**	1,21
Bexiga	230	5,18	70	9,05	90	1,88	20	2,00
Esôfago	230	5,27	40	5,95	110	2,22	20	1,81
Ovário	-	-	-	-	320	6,69	90	10,21
Linfoma de Hodgkin	50	1,21	**	1,68	30	0,66	**	0,95
Linfoma não Hodgkin	200	4,44	50	6,19	180	3,75	50	6,36
Glândula Tireoide	70	1,52	**	2,23	370	7,82	100	11,48
Sistema Nervoso Central	220	4,86	50	7,10	190	4,03	50	5,84
Leucemias	200	4,40	40	5,87	180	3,76	40	5,03
Corpo do Útero	-	-	-	-	380	7,88	110	12,82
Pele Melanoma	100	2,34	30	4,64	80	1,71	20	2,22
Outras Localizações	1.750	39,29	380	50,53	1.770	36,94	370	42,31
Subtotal	**7.900**	**177,62**	**1.790**	**239,88**	**8.820**	**183,75**	**2.200**	**252,77**
Pele não Melanoma	2.340	52,65	540	72,74	2.790	58,20	440	51,06
Todas as Neoplasias	**10.240**	**230,23**	**2.330**	**312,24**	**11.610**	**241,88**	**2.640**	**303,33**

* Números arredondados para 10 ou múltiplos de 10
** Menores que 15 casos
(-) Não determinado.
Fonte: INCA, 2017.

3.5 Citotoxicidade e genotoxicidade

3.5.1 Ensaios de citotoxicidade

3.5.1.1 Teste de viabilidade celular

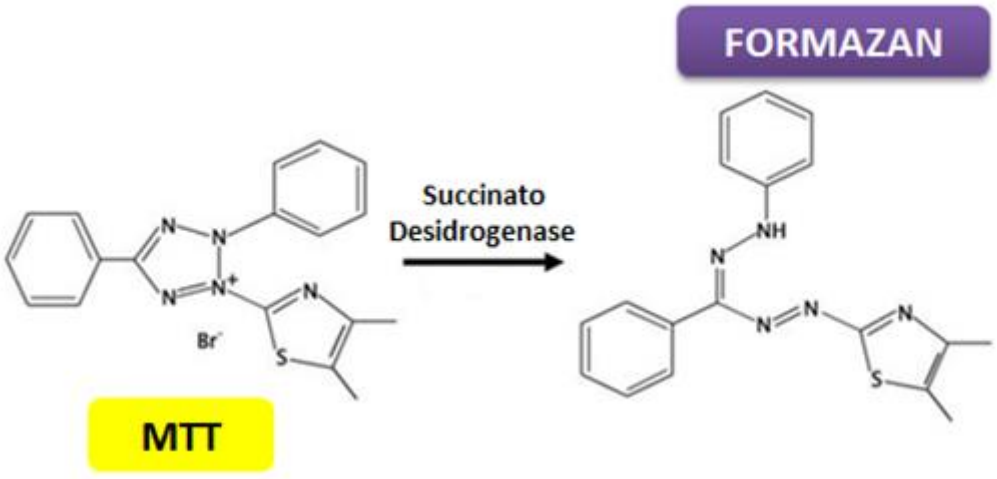
As terapias contra cânceres sofrem a cada dia mais limitações, devido à insatisfatória eficácia ou elevados efeitos colaterais que afetam a vida do paciente. A busca por novos compostos com potencial atividade anticâncer tem crescido continuamente, a fim de atenuar tais adversidades (NEWMAN E CRAGG, 2016; ŚLIWKA et al, 2016). Para o rastreio de potenciais compostos anticancerígenos e combinações de compostos, diversos ensaios que aferem o efeito do composto sobre o câncer através de ensaios em cultura de células tem sido muito utilizado (EDWARDS et al, 2008; RISS et al, 2013) .

Um dos principais testes utilizados é o teste de viabilidade celular, como MTT (brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio) que utiliza a capacidade das células vivas de catalisar reações, produzindo produto mensurável, o Formazan ((2E,4Z)-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan) (EBADA et al, 2008; CHAN et al, 2013). O teste do MTT foi o primeiro ensaio de viabilidade celular homogéneo desenvolvido para um formato de 96 poços que era adequado para rastreio de alto rendimento (MOSMANN, 1983).

O reagente MTT tem como cor característica o amarelo e quando em contato com as células viáveis é reduzido a cristais de formazan de cor púrpura, devido à ação de enzimas redutases de origem mitocondrial (Figura 3) (MOSMANN, 1983; EBADA et al, 2008). A reação de redução utiliza succinato e os co-factores de nucleótidos de piridina, NADH e o NADPH como substratos (BERRIDGE E TAN, 1993). A concentração de células viáveis em cultura é diretamente proporcional à concentração de cristais formados. É necessária a utilização de um solvente aprótico, geralamente o DMSO, com o intuito de solubilizar os cristais de formazan formados internamente nas células. Desse modo, a viabilidade celular é medida através de espectrofotômetro com absorbância próxima a 540 nm (MOSMANN, 1983). A leitura de cada concentração testada é então comparada com controle negativo de células (100%), onde é possível distinguir o percentual de sobrevida em cada ensaio. A partir da análise do percentual de sobrevida das células x diluição das amostras em teste é possível calcular a máxima Concentração Inibitória 50% (CI50%) que mata 50% das células (DAGUANO et al, 2007).

A análise dos danos citotóxicos através do teste do MTT é uma boa escolha por este ser um método rápido e sensível, que apresenta a capacidade de analisar parâmetros importantes, como a viabilidade e o estado metabólico da célula (CRAGG; NEWMAN, 2000).

Figura 3: Redução do MTT (brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio) em Formazan ((2E,4Z)-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan).



Fonte: MOSMANN, 1983; EBADA et al, 2008.

3.5.1.2 Citometria de fluxo

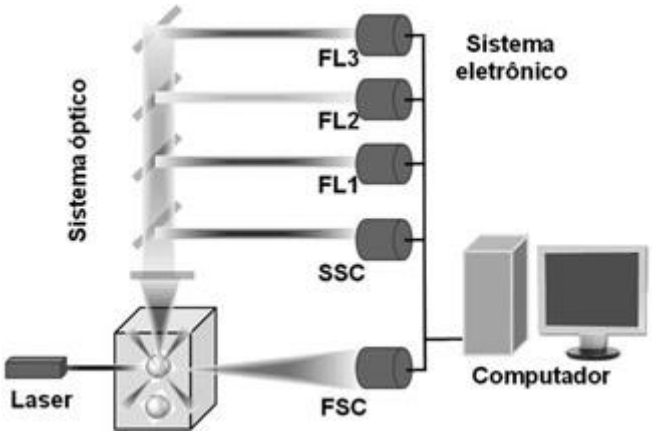
A citometria de fluxo é um instrumento muito importante para diferenciação de múltiplas características de células, quando as estas percorrem rapidamente em um fluxo através de um sistema de detectores fotônicos. Por ser um método eficiente para análise e classificação de células individualizadas, tem sido de grande relevância para as pesquisas científicas, pois agrega diversos benefícios e configura-se como uma importante ferramenta para o estudo dos eventos celulares envolvidos na resposta imunológica e permite uma ampla gama de aplicações biológicas e médicas. (MACEY, 1994; SAEYS et al, 2016).

O primeiro citômetro de fluxo surgiu entre os anos de 1967 e 1969, idealizado por Van Dilla, com configuração ortogonal usando a câmara descrita por Crosland-Taylor. A partir deste citômetro foi possível demonstrar a relação existente entre ploidia e intensidade de fluorescência na quantificação de DNA, possibilitando identificar definidamente as fases do ciclo celular por meio de histogramas (BERTHO, 2017). Uma característica bastante vantajosa da citometria de fluxo é que os ensaios realizados através desse instrumento permitem que as condições vitais da célula após

sua manipulação sejam mantidas, propiciando estudos mais apurados sobre avaliação funcional da população em estudo, bem como o seu completo comportamento biológico (MACHADO-JUNIOR, 2006).

O citômetro é resumidamente composto por um sistema fluídico, laser, sistema óptico, sistema de detectores (FSC, SSC, FL1, FL2 e FL3), sistema eletrônico (figura 4) (CARVALHO et al, 2011). As células são conduzidas uma a uma pelo fluxo e ao interromper o feixe luminoso dispersam a luz em diversas direções. A direção para onde o feixe de luz é desviado depende de alguns fatores consideráveis, como tamanho da célula, sua estrutura interna, características topográficas celulares e até mesmo da densidade óptica de cada célula (MACEY, 1994). Todas as características das células que passam pelo citômetro são determinadas através da dispersão da luz ocasionada pelo feixe de laser são detectadas e medidas por detectores (SCHACHNIK, 2008).

Figura 4:Principais componentes de um citômetro com cinco parâmetros: sistema fluídico, laser, sistema óptico, sistema de detectores (FSC, SSC, FL1, FL2 e FL3), sistema eletrônico.



Fonte: CARVALHO et al, 2011.

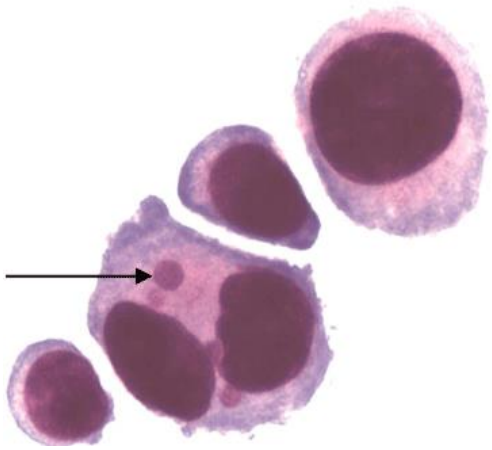
A utilização da citometria de fluxo proporciona a avaliação de diversas características particulares de cada célula em uma população pura ou mista. O amplo espectro de aplicações que esta ferramenta traz, permite análises de imunofenotipagem, avaliação imunológica de paciente transplantado, contagem de células da subpopulação linfocítica, diagnóstico e acompanhamento de leucemias e linfomas, incluindo todas as análises hematológicas, análises de ploidias, ciclo celular, apoptose, expressão gênica, metabolismo celular e análise de expressão de GFP (Green Fluorescent Protein), entre outras (BACAL E FAULHABER, 2003; SHAPIRO, 2003).

3.5.2 Ensaios de genotoxicidade

3.5.2.1 Micronúcleo

Micronúcleos são pequenas estruturas compostas de cromatina resultante de mitoses aberrantes, delimitada por membrana e separada do núcleo principal. Estas estruturas se formam devido à lise na molécula de DNA, dias ou semanas após a ação de carcinógenos, a partir da extrusão de cromossomos inteiros ou fragmentos destes durante o processo de divisão celular das células da camada basal. Eles se apresentam em forma ovalada ou redonda, localizados em proximidade ao núcleo da célula (figura 5) (STICH E ROSIN, 1983; STICH et al, 1984; JOKSIÉ, 2004; REIS et al., 2004).

Figura 5: Células de origem epitelial de cólon que apresenta múltiplos micronúcleos.



Fonte: BERNSTEIN et al, 2011.

Na maioria das vezes, os micronúcleos constituem-se de cromossomos inteiros ou fragmentos cromossômicos acêntricos que se desligam e perdem-se durante a mitose, mais precisamente na anáfase, e consequentemente acabam não compondo o núcleo principal das células filhas. No entanto, a formação dos micronúcleos está totalmente associada à perda de material genético como resposta a um dano cromossômico ou no aparelho mitótico (D'AGOSTINI, 1998; BONASSI et al., 2003; SALVADORI et al, 2003).

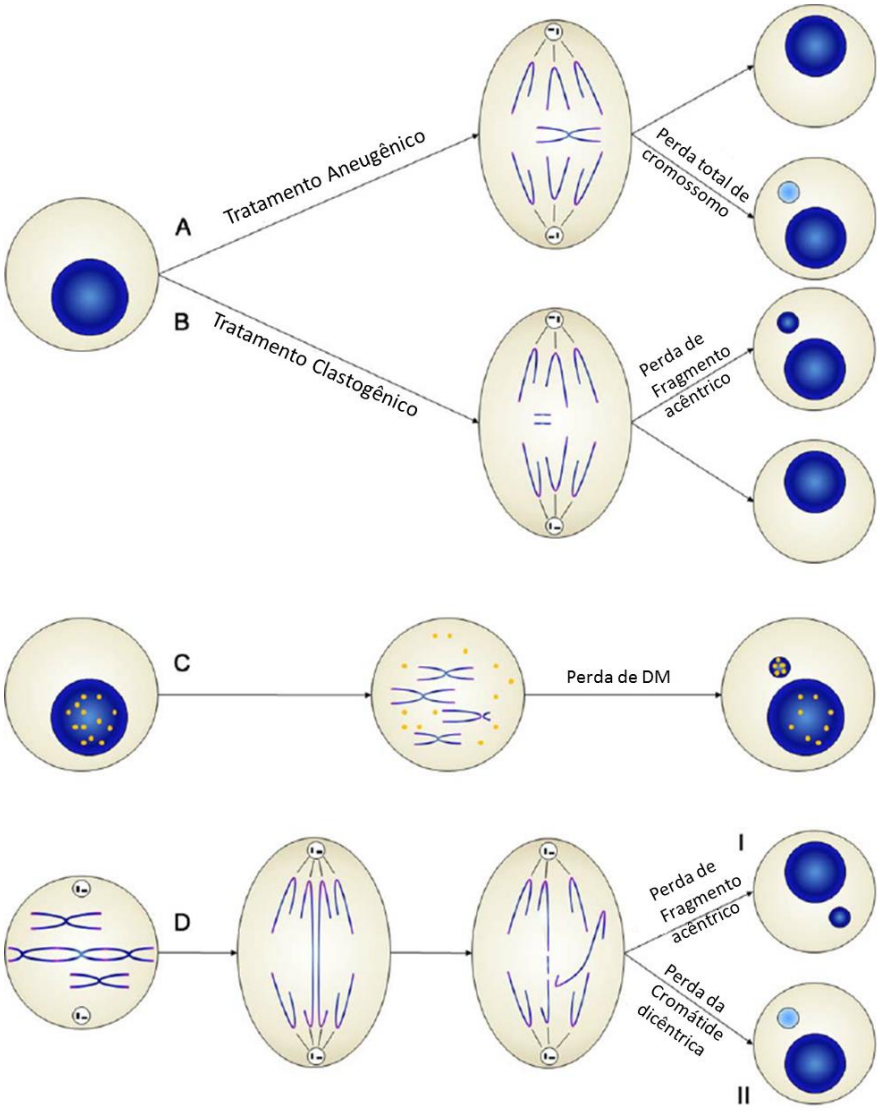
O processo de formação dos micronúcleos pode decorrer de cinco principais mecanismos (figura 6). O primeiro mecanismo seria a ação de agentes aneugênicos que interrompem a formação

do aparelho mitótico, causando problemas durante a anáfase. Isso resulta em micronúcleos constituídos de cromossomos inteiros até o final da mitose, passando essa característica para as células filhas. O segundo mecanismo corresponde à ação de agentes clastogênicos que atuam diretamente na dupla fita de DNA, formando fragmentos acêntricos que não se ligam às fibras do fuso, nem permitem a integração com o núcleo das células filhas. Tais fragmentos são deixados para trás durante a mitose. O terceiro mecanismo envolve sequências genéticas altamente amplificadas, derivadas de "double minuts" extracromossômicos que podem estar contidos dentro dos micronúcleos. O quarto mecanismo corresponde a uma torção que ocorre entre dois centrômeros de um cromossomo dicêntrico que dá origem a ponte de anáfase que se rompe e resulta na formação de fragmentos acêntricos que são excluídos dos núcleos das células filhas e formam um ou mais micronúcleos no final da mitose. O último mecanismo ainda pode ser decorrente da ponte de anáfase, onde ao invés de se romper, os cromossomos dicêntricos são separados dos dois centrossômos, esquecidos durante a anáfase e retido em micronúcleos (TERRADAS et al, 2010).

Os primeiros relatos sobre a utilização da metodologia baseada na análise de micronúcelos sugiram em meados da década de 1950. Evans et al. (1959) utilizaram a freqüência de micronúcleos para mensurar as alterações cromossômicas em plantas irradiadas (EVANS et al., 1959).

O "Teste do micronúceo" é realizado tanto *in vivo* quanto *in vitro*, par a determinação da capacidade de mutagenicidade de substâncias que possam ser capazes de causar danos nos cromossomos, chamadas de substâncias clastrogênicas ou daquelas substâncias que possam interferir na formação e funcionamento do aparelho mitótico, resultando em uma distribuição desigual dos cromossomos no processo de divisão celular (REIS et al, 2004). Diante a grande capacidade de o Teste de micronúcleo identificar células com alterações em nível cromossomal ele configura-se como um importante marcador biológico da exposição à carcinógenos (MAJER et al, 2001). Para a execução deste teste, diferentes tipos de células podem ser empregadas, como células animais de modo geral e vegetais, desde que possuam a capacidade de se dividir ou que possa ter esse processo induzido, que este processo seja conhecido e de possível manipulação (FENECH, 2000).

Figura 6: Mecanismos de formação de micronúcleos (MNs). (A) Formação de MNs por exposição a agente aneugênicos; (B) Formação de MNs por exposição a agente clastogênicos; (C) Formação de MNs derivadas de "double minuts" extracromossômicos; (D(I)) Formação de MNs pela quebra da ponte de anáfase, que torce os cetrômeros de um cromossômo; (D(II)) Formação de MNs pela separação dos dois centrosomos.



Fonte: TERRADAS et al, 2010.

O método "teste do micronúcleo" ganhou muito reconhecimento na esfera científica, sendo bastante recomendado para estudos de biomonitoramento ambiental, principalmente por permitir a detecção de alterações como quebra de cromossomos, causadas por agentes clastogênicos, e segregação cromossômica anormal (agentes aneugênicos) (FENECH, 2000; RIBEIRO et al., 2003). O método também garante várias vantagens. Os micronúcleos podem ser detectados em células em intérfase, são facilmente distinguíveis, rapidez (pois, para detectar os micronúcleos é necessário que a célula passe por algumas divisões), é um procedimento simples e sua interpretação é menos

trabalhosa quando comparado a outros testes de avaliação a nível cromossomal e permite a detecção da ação de agentes clastogênicos e aneugênicos (HEDDLE et al, 1983). Além disso, o baixo custo e a alta confiabilidade desta técnica são fatores que contribuíram para o sucesso internacional e a sua adesão como biomarcador em estudos *in vitro* e *in vivo* do dano genômico (BONASSI et al., 2003).

3.5.2.2 Ensaio cometa

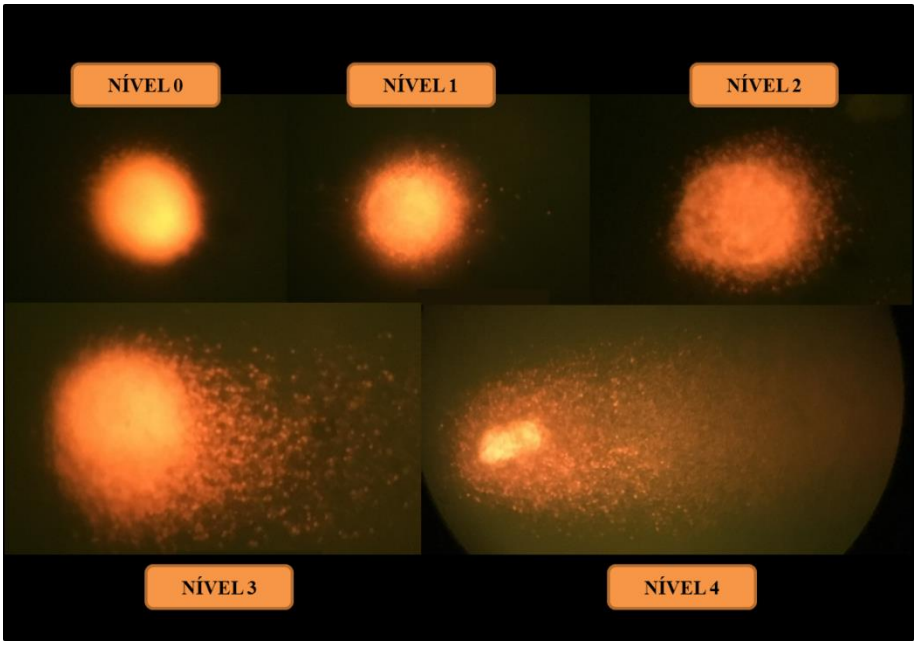
O ensaio cometa é um teste que permite a identificação de compostos genotóxicos, ou seja, aqueles que causam danos ao DNA, através da visualização de células danificadas por microscopia de fluorescência. Os primeiros ensaios foram relatados por Östling e Johanson, em 1984, quando estes pesquisadores desenvolveram o teste de avaliação genotóxica com células individualizadas, tratadas, aplicadas em um gel de agarose de baixo ponto de fusão sobre lâminas de microscopia, previamente preparadas, submetidas a um processo de ruptura de membrana e a um campo elétrico utilizando um tampão alcalino (CANTALICE, 2014; GONTIJO E TICE, 2003).

A utilização de substâncias alcalinas neste método garante a observação de rupturas duplas e simples na estrutura helicoidal do DNA. Em sítios lábeis alcalinos, a cadeia polinucleotídica pode sofrer rupturas quando o DNA encontra-se incubado em pH elevado, e as ligações cruzadas em posições diferentes no DNA (crosslinks), resultantes da ação de compostos genotóxicos, podem alterar a estrutura do DNA das células (COOK E BRAZELL, 1976; RAZIN et al, 1995; ERIKSSON et al, 2002). Essa alteração inclui o relaxamento em partes da molécula do DNA, que no compo elétrico, migram em direção ao cátion. O uso de corantes de ácidos nucleicos fluorescentes específicos no ensaio cometa permite a visualização de células com característica diferenciada, devido à migração do DNA que semelhante a um cometa, que pode ser visualizado por microscópio de fluorescência (SINGH et al, 1988; TICE et al., 2000).

O ensaio cometa segundo Mota et al., (2011) correlaciona a epifluorescência das células analisadas em 5 possíveis níveis de danificação genômica (Figura 7): nenhuma lesão ou dano visível no DNA, com ausência de fragmentação nuclear ou que esta seja menor que 5% (dano 0); leve danificação no DNA e leve fragmentação nuclear com discreta formação de halo, com níveis de dano correspondendo de 5 a 20% (dano 1); danificação de leve a moderada no DNA, com fragmentação nuclear visível e formação de um halo mediano. Níveis de dano correspondendo de 20 a 40% (dano 2); danificação de moderada a forte no DNA, com fragmentação nuclear visível e formação de um halo longo. Níveis de dano correspondendo de 40 a 95% (dano 3) e danificação

intensa no DNA e observação de um halo bastante extenso, ao redor, do pouco material nuclear e a formação de uma cauda semelhante a de um cometa, sendo este último, a forma mais grave de danificação, com níveis de dano superior a 95% (dano 4).

Figura 7: Padrão de escores utilizados para determinação do índice de dano no teste do cometa segundo Mota et al (2011).



Fonte: Imagens do autor.

Ensaio do Cometa traz consigo inúmeras vantagens que incluem o seu rápido desempenho, a sua simplicidade e sua alta sensibilidade para vários tipos de danos no DNA, além do baixo custo e por grande aplicabilidade em estudos tanto *in vivo* como *in vitro* ou no monitoramento da exposição humana a agentes tóxicos, podendo ser utilizado em qualquer tipo de tecido sem a limitação da proliferação celular para a análise (DA SILVA et al., 2002; GONÇALVES et al., 2003; DEHON et al., 2008). Nas últimas décadas, esta metodologia tem sido bastante empregada no estudo de células sanguíneas humanas desde o conhecimento da ação de substâncias químicas mutagênicas até avaliação de suscetibilidade à radiação (CORONAS et al., 2008; FAUST et al., 2004).

O Ensaio Cometa não requer como condição, que as células utilizadas para o estudo estejam em constante divisão para observação da viabilidade celular em cultura, ao contrário de outros tipos de ensaios de genotoxicidade. Contudo, o teste pode ser realizado com células normais, como os linfócitos que são obtidos facilmente por meio de coleta de sangue e até mesmo em células

provenientes de diversos tecidos, como o fígado, mama, pulmão e os rins, também têm sido testados. A avaliação de diversos tipos celulares permite determinar os efeitos de genotoxicidade das substâncias tóxicas que são muitas vezes peculiares para cada tipo de tecido do organismo (MITCHELMORE E CHIPMAN, 1998).

3.6 O sistema imunitário e a resposta imune

O Sistema Imunológico atua no organismo de todos os indivíduos na defesa contra antígenos através de um mecanismo conhecido como resposta imune. A resposta a um patógeno inicialmente ocorre em três fases. A primeira delas denomina-se resposta imune inata, a segunda resposta imune induzida precocemente e por fim, a resposta imune adaptativa. A resposta imune inata atua através do reconhecimento por efetores inespecíficos, pré-formados e amplamente específicos. A resposta imune induzida precocemente atua através de um processo de inflamação, recrutamento e ativação de células efetoras, enquanto que a imunidade adaptativa ocorre posteriormente, através do reconhecimento do antígeno por células T e B virgens, sofrendo expansão clonal antes de se diferenciarem em células efetoras capazes de eliminar a infecção (MURPHY et al, 2010).

As barreiras físicas, químicas e biológicas, células especializadas e moléculas solúveis, são os principais compontentes da imunidade inata, existindo em todos os indivíduos sem a necessidade de contato anterior com antígenos. As células mais atuantes da imunidade inata são principalmente os macrófagos, os neutrófilos, as células dendríticas e as células Natural Killer (NK) (MEDZHITOV et al, 1997; MEDZHITOV E JANEWAY, 2000). Em contrapartida, a imunidade adquirida necessita que ocorra a ativação de células especializadas para desenvolver a resposta. A resposta adquirida possui algumas características peculiares como: possui uma ampla especificidade no processo de reconhecimento, executam uma resposta rápida e especializada, são autolimitantes e tolerantes à componentes do próprio organismo e proporcionam a memória imunológica. As principais células envolvidas na resposta adaptativa são os linfócitos. Porém, existem células que desempenham uma importante função na ativação destes linfócitos, as chamadas células apresentadoras de antígenos (APCs). Estas células associam os antígenos a moléculas MHC (Major Histocompatibility Complex) e apresentam-os para os linfócitos T (DELVES E ROITT, 2000).

As respostas imunes e as respostas proliferativas são complexas mediadas por diversos componentes plasmáticos, como fatores de crescimento, citocinas, receptores celulares e células

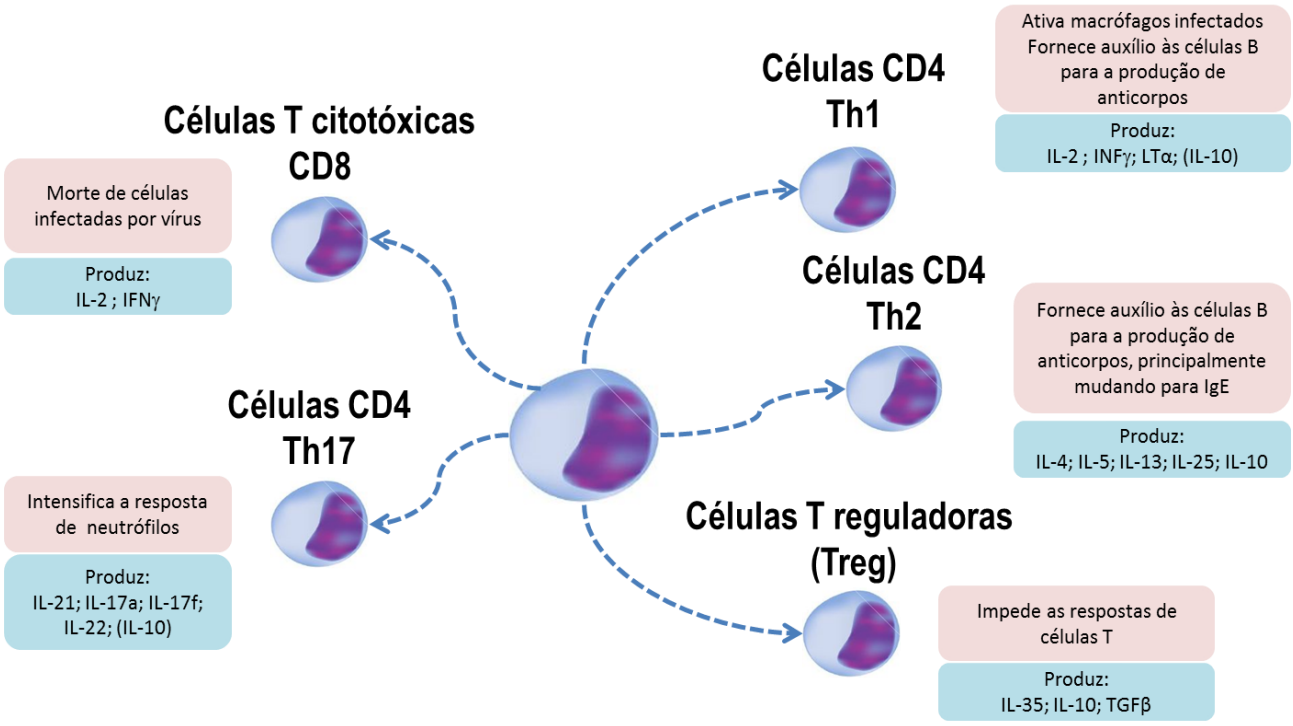
específicas que permitem a eficácia do sistema imunológico (KOURILSKY E TRUFFA-BACHI, 2001; SIKKELAND et al, 2007).

Após uma lesão, as citocinas são secretadas em poucos minutos, uma vez que se encontram armazenadas intracelularmente como moléculas preformadas e com o estímulo, tenderm a se tornar moléculas ativas (MORGANTI-KOSSMANN et al., 2002). As mesmas citocinas podem ser liberadas por diferentes tipos de células e atuar através de um fenômeno denominado pleiotropia, onde uma determinada citocina pode exercer uma ação em diferentes tipos de células. Além disso, a mesma atividade desencadeada pode ser semelhante para diferentes citocinas. As citocinas podem desenvolver uma resposta em forma de cascata com frequência, de modo que uma citocina estimula uma célula específica a produzir outras citocinas (ZHANG E AN, 2007).

Várias citocinas estão relacionadas à resposta imunológica, principalmente no que diz respeito à mediação da inflamação. Em geral, estas moléculas estão associadas com a ativação de linfócitos T e linfócitos B pela expressão de agentes imunomoduladores, sendo classificadas como citocinas pró-inflamatórias (como, TNF-α, IFN-γ IL-1, IL-2, IL-12), anti-inflamatórias (como, IL-10) e quimiocinas (VAN DEN BRINK E BURAKOFF, 2002, ABBAS et al, 2008). Linfócitos também são capazes de produzir espécies reativas de oxigênio, denominadas EROs, como ânion superóxido, radical hidroxila e peróxido de hidrogênio (H_2O_2), e outros intermediários reativos do nitrogênio, sendo o óxido nítrico (ON) seu principal representante (ABBAS et al, 2008).

Os linfócitos podem atuar de diversas formas, sendo que os principais subtipos correspondem aos linfócitos auxiliares (Th) e os citotóxicos, denominados CD4 ou CD8, respectivamente (PARKIN E COHEN, 2001). Os Linfócitos T CD4 Auxiliares são classificados pelo tipo de citocinas que produzem e por sua funcionalidade. Quando um linfócito precursor Th0 é estimulado por uma APC, ele pode ser diferenciado em um linfócito Th1, Th2 ou Th17 (figura 8), de acordo com a citocina envolvida no estímulo. Mesmo quando ocorre a diferenciação dos linfócitos, estas células acabam não apresentando diferenças morfologicamente distinguíveis, porém apresentam resposta diferenciada de acordo com a citocinas que secretam (BRADLEY, 2003). Outra forma de diferenciação dos linfócitos é no tipo de células T reguladoras (T_{reg}) que atuam na inibição de respostas adaptativas através da produção de citocinas que possuem grande afinidade com receptores de superfícies para diferentes tipos de células, sendo importantes na prevenção das respostas imunes descontroladas e na prevenção da autoimunidade (ESTAQUIER E AMEISEN, 1997; MURPHY et al, 2010).

Figura 8: Esquematização das diferentes vias de diferenciação das células TH0 em Th1, Th2, Th17 e T_{REG}, algumas características pertinentes a cada tipo e as principais citocinas secretadas.



Fonte: Adaptado de MURPHY, et al. (2010) e ZHU E WE (2008).

Abbas et al (2008) descreve a produção de citocinas por diversos tipos de células para cada tipo de resposta, que pode ser observado detalhadamente na tabela 2.

A interleucina 1 (IL-1) é uma citocina primariamente produzida por macrófagos e monócitos, células endoteliais e algumas células epiteliais e tem como células-alvo as células endoteliais e fibroblastos ativados durante lesão celular, infecção, processos inflamatórios e de coagulação (ZHANG E AN, 2007; ABBAS et al, 2008).

Uma importante citocina que atua no controle da proliferação e diferenciação de células do sistema imunitário é a interleucina 2 (IL-2). São produzidas principalmente por células TCD4+ e em menor quantidade pelas células TCD8+. A IL-2 é também considerada forte indutora na produção de outras citocinas, como IFN-γ e IFN-β, ocasionando ativação de monócitos, neutrófilos e células NK, resultado em uma resposta imunológica antígeno-específica (CURFS et al, 1997; HOYER et al, 2008).

O interfon gama (IFN-γ) é produzido pelas células Th1 e são moléculas essenciais para a eliminação de patógenos intracelulares. Em contrapartida, as células Th2 secretam IL-4, IL-5, IL-6,

IL-10 e IL-13, que são fundamentais para a retirada de organismos extracelulares principalmente no que diz respeito a manutenção de reações alérgicas (FASANMADE E JUSKO, 1995; BARTEN et al, 2001). A IL-10 é uma citocina Th2 imunorreguladora com efeitos específicos pars diversos tipos de células, que influenciam as respostas imunes inatas e adaptativas (MOORE et al., 2001).

Tabela 2: Principais citocinas do sistema imunológico, relacionadas com o tipo de resposta e as respectivas células produtoras.

Citocina	**Tipo de resposta**	**Importantes Células Produtoras**
Fator de crescimento Transformante (TGFβ)	Adaptativa	Macrófagos, Células T e outros tipos celulares
Fator de Necrose Tumoral (TNF)	Inata	Macrófagos, Células T
IFN-α	Inata	Macrófagos
IFN-β	Inata	Fibroblastos
IFN-γ	Adaptativa	Células T $CD8_+$ (Th1) e Células NK
IL-1	Inata	Macrófagos, células endoteliais e algumas células epiteliais
IL-2	Adaptativa	Células T
IL-4	Adaptativa	Células T $CD4_+$ (Th2)
IL-5	Adaptativa	Células T $CD4_+$ (Th2)
IL-6	Inata	Células endoteliais, células T e Macrófagos
IL-10	Inata	Macrófagos, células T (manutenção de Th2)
IL-12	Inata	Células dendríticas e Macrófagos
IL-13	Adaptativa	Células T $CD4_+$ (Th2)
IL-15	Inata	Macrófagos
IL-18	Inata	Macrófagos
Quimiocinas	Inata	Células T, macrófagos Fibroblastos, plaquetas e células Endoteliais

Um efetor de grande importância na mediação da resposta imune é o óxido nítrico (ON). O óxido nítrico é produzido principalmente por macrófagos na forma da enzima óxido nítrico sintetase

induzida (iNOS), e a sua produção é estimulada por citocinas, como IFN-γ. Quando a produção de óxido nítrico pela iNOS é iniciada, ela permanece ativa por horas e em concentrações suficiente para apresentar ação citotóxica contra microorganismos, parasitas e células tumorais, exercendo uma significativa função na modulação do sistema imunológico (MONCADA et al, 1991; LAURENT et al, 1996).

Uma grande família de pequenas citocinas, denominadas quimiocinas, também é de grande importância na resposta imunológica. As quimiocinas são classificadas em duas famílias baseada no primeiro resíduo de cisteína que as compõe. A primeira família é chamada quimiocinas CC ou beta-quimiocinas e são capazes de estimular monócitos, basófilos, eosinófilos, linfócitos T e células NK. A segunda família é a das quimiocinas CXC ou alfa-quimiocinas, e são capazes de estimular a quimiotaxia de neutrófilos, através de uma sequência Glu-Leu-Arg no seu N-terminal que é essencial para a ligação de receptores (MURPHY et al, 2000).

Geralmente, as quimiocinas CCL2, CCL3, CCL4, CCL5, CCL11 e CCL13 são classificadas como inflamatórias, enquanto as quimiocinas CCL18 CCL19, CCL21, CCL25 e CCL27 são homeostáticas. Já as quimiocinas CXCL9 e CXCL10 têm dupla função inflamatória e homeostática (LE et al, 2004). Em geral, CXCL9 e CXCL10 tem função na esposta Th1, tráfego de Th1, CD8 e NK enquanto que a CXCL8 atua no tráfego de neutrófilos (MURPHY et al, 2000).

Artigo 1

4 ARTIGO 1

4.1 L-asparaginase from *Streptomyces ansochromogenes* UFPEDA 3420: purification, characterization and in vitro evaluation of cytotoxic potential.

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Artigo submetido ao periódico **SEPARATION AND PURIFICATION TECHNOLOGY**

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L-ASPARAGINASE FROM *Streptomyces ansochromogenes* UFPEDA 3420: PURIFICATION, CHARACTERIZATION AND IN VITRO EVALUATION OF CYTOTOXIC POTENTIAL.

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Abstract

L-asparaginase is considered an important anti-neoplasic enzyme used as a drug of choice for treatment of lymphoblastic leukemia in association with others chemotherapeutic agents. This study aimed to produce, purify and characterize the L-asparaginase from *S. ansochromogenes* UFPEDA 3420 and cytotoxic activity. An extracellular asparaginase from *S. ansochromogenes* UFPEDA 3420 achieved high production with M9 medium for 48 hours at pH 7 and temperature of 35 °C and was purified to homogeneity with a record of 35.205,76 U/mg, 6.06 fold purification and 27.68% of yield. The purified L-asparaginase has a molecular mass of 63.99 kDa with an catalytic activity at pH and temperature 7.0 and 40 °C, respectively, Km of 3.224 mM and Vmax 161.29 U/min. L-asparaginase from *S. ansochromogenes* UFPEDA 3420 showed cytotoxic effect against cancer cell line *in vitro*, including human lymphoblastic leukemia (MOLT-4) with IC_{50} 25 µg/mL. This study reports for the first time an important productivity of L-asparaginase by the species *Streptomyces ansochomogenes* UFPEDA 3420, making appropriate studies on the applicability of this molecule in *in vitro* and *in vivo* models.

Key words: Enzyme; cancer; Actinomycetes; Streptomicetaceae; human lymphoblastic leukemia

1. Introduction

L-Asparaginase (E.C.3.5.1.1) is an enzyme responsible for catalyzing the hydrolysis reaction of the amino acid L-asparagine resulting in ammonia and aspartic acid, widely used in the treatment of acute lymphoblastic leukemia. The administration is performed intravenously in order to decrease the plasma levels of L-asparagine and disturbing the development of neoplastic cells. The lymphatic cancer cells require large amounts of L-asparagine to quickly perform malignant growth. However, malignant cells, because of gene silencing, have not L-asparagine synthetase and requires to capture extracellular L-asparagine [1-3].

Escherichia coli and *Erwinia* (*E. chrysanthemi* e *E. carotovora*) are considered the most important producers of L-asparaginase commercially available, however, other bacterial asparaginases of *Erwinia* have been characterized by heterologous expression of the enzyme in cells from *E. coli*. The mechanisms of action and toxicity are identical in the two drugs, however, the pharmacokinetic properties differ from one another [4,5].

Besides bacteria, L-asparaginase could be isolated from diverse other sources as from filamentous fungi and yeast, actinobacteria, plants, among others. Several studies report that some genera of fungi such as *Aspergillus*, *Fusarium* and *Penicillium*, can be an excellent producers of L-asparaginase [6-8]. In recent years, the study of actinobacteria producers of L-asparaginase increased significantly, mainly due to the large search for microorganisms that produce an enzyme with less adverse effects. The actinobacteria represent a potential source for production of L-asparaginase. The production of this enzyme has been reported in *Streptomyces acrimycini, Streptomyces ginsengisoli, S. olivaceus, S. griseus* [9-12], and *Nocardiopisis alba* [13].

However, even the scientific literature providing a range of information about the various microorganisms producers of L-asparaginase, were not found reports of *Streptomyces ansochromogenes* as a producer of this enzyme, being this the first report of asparaginase production by this species. The microorganism of this study, *S. ansochromogenes* UFPEDA 3420,

was recently reported by Vasconcelos et al. [14] as a producer of a metabolite with antifungal activity, which was isolated from the rhizosphere of a plant from biome Caatinga, exclusively Brazilian biome, characterized by dry weather and a soil little fertile. The microbial diversity Caatinga Biome represents a powerful source of metabolites due to the extensive metabolic activity that exerts even in extreme conditions of cultivation, such as high temperatures and low moisture content. The complex ecological interaction with this biome and the adaptive mechanisms of these microorganisms to the semi-arid region are of enormous interest to biotechnology and represent prospects for application and generation of new products.

This study aimed to produce, purify and characterize the L-asparaginase from *S. ansochromogenes* UFPEDA 3420 and cytotoxic activity.

2. Materials and Methods

2.1. *Streptomyces* strain and culture conditions

Streptomyces ansochromogenes UFPEDA 3420 is deposited into the Collection of Microorganisms of Antibiotics Department (UFPEDA) with the identification number UFPEDA 3420. The strain was preserved in mineral oil and was reactivated in ISP-2 broth (International *Streptomyces* Project) [15] at 37 °C for 48 hours. Then, the *Streptomyces* culture was transferred to ISP-4 agar plates at 37 °C for 48 to 120 h, to obtain fresh cultures for realization of the experiments.

2.2. Production of L-asparaginase

Six circular 8 mm diameter agarose blocks containing *S. ansochromogenes* UFPEDA 3420 was inoculated in 100 mL of liquid medium M9 [$Na_2HPO_4.2H_2O$, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 0,5 g/L; L-asparagine, 5 g/L; $MgSO_4.7H_2O$, 0,5 g/L; $CaCl_2.2H_2O$, 0,014 g/L; Glucose, 2 g/L; Distilled water, 1 L; pH 7.0] and the enzimatic activity was measured after 120 hours of fermentation at 37ºC. Subsequently was evaluated the effect of pH (range from 5 to 9), time (from 24 hours to 120

range) and temperature (ranging from 25 °C to 50 °C) in the production of the enzyme. All experiments were performed in triplicate and the mean values with standard deviation (SD) were calculated. Then, fermentation was performed in a bioreactor New Brunswick Bioflo 110, using 4 liters of culture medium under the optimal conditions for production of L-asparaginase.

2.3. Quantification of L-asparaginase

The activity of L-asparaginase was determined by quantifying ammonia formed by Nesslerization [16]. A mixture of 0.5 mL of enzyme extracts (cell free supernatant), 0.5 mL of 0.05 M Tris-HCl (pH 8.0) containing L-asparagine 16 mM was incubated for 30 min at 37 °C. The reaction was interrupted by adding 0.5 mL of 1.5 M trichloroacetic acid, centrifuged at 6,160 g for 3 minutes and 0.5 mL of the supernatant was diluted with 2.25 mL of distilled water and added to 0.25 mL of the Nessler reagent. The reading was performed using a spectrophotometer at wavelength of 450 nm. An ammonium sulfate solution was used to prepare the standard curve. One international unit (IU) of L-asparaginase was defined as the amount of enzyme required to release 1 µM of ammonia per minute at 37 °C.

2.4. Enzyme purification

The ammonium sulfate was added to the supernatant to a concentration of 60% with constant stirring and cooling on ice bath (± 4 °C) to precipitate the proteins. The sample was centrifuged, the supernatant discarded and the pellet was resuspended and dialyzed into 50 mM Tris-HCl (pH 8.5) to remove salt. The enzyme was applied to the column of diethylaminoethyl cellulose (DEAE cellulose) and equilibrated with 50 mM Tris-HCl (pH 8.5). The column was washed with two volumes of starting buffer and the protein was eluted with a linear gradient of NaCl (0.05-0.5 M) prepared in 50mM Tris-HCl buffer (pH 8.5) at 50 mL/h flow rate. The active

fractions were added to the Sephadex G-75 (1x50 cm) equilibrated with 50 mM Tris-HCl (pH 8.5) and eluted with the same buffer at 48 mL/h flow rate. During each purification step, the proteins presents at both fractions of the DEAE column and at fractions of the Sephadex G-75 column were detected by reading absorbance at 280 nm. L-asparaginase activity was quantified by nesselerization and the protein profile and homogeneity of the protein were evaluated through SDS-PAGE gel. Fractions with similar characteristics were pooled and concentrated in a Speed Vac Concentrator.

2.4.4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS–PAGE was performed to analyze the proteins of the samples according to the method of Laemmli [17] using stacking gel at 5% and 12% of polyacrylamide separating gel. Electrophoresis was carried out at a constant current of 150 W. After separation, the gel was subjected to rapid staining with silver nitrate for detection of proteins. Broad Range Protein molecular markers (Ref. V849A, Promega®) was used protein marker.

2.5. Dosage of total proteins

The dosage of total protein was determined by the method of Bradford [18] using bovine serum albumin as the standard (BSA). During the steps of chromatography, the proteins were detected by reading absorbance in a optical density of 595 nm. Assays were performed in triplicate and the averages were used for the calculations. The specific activity was calculated and expressed in units per milligram of protein (U/mg protein).

2.6. Characterization of the purified L-asparaginase

2.6.1. Effect of pH and temperature on activity and stability of L-asparaginase

The pH optimum for the purified L-asparaginase activity was determined by enzymatic reactions in phosphate-citrate buffer (5.0 to 7.0), Tris-HCl (8.0) and Glycine-NaOH (9.0 and 10.0). Stability at different pH was determined by checking the remaining activity after incubation of the purified enzyme v/v (1:1) for 24 h at room temperature and the residual activity was determined under the prescribed conditions (0.05 M Tris-HCl, pH 8.0). For optimum temperature, the enzymatic reactions were performed at different temperatures (5 to 80 °C) and thermal stability of the enzyme was performed on 30, 40 and 50 °C for up to 1 h [19].

2.6.2. Effect of various effectors on L-asparaginase activity

The effect of ions was verified by the assay of L-asparaginase activity with a variety of compounds dissolved in distilled water. The following substances were evaluated in 10 mM: $MnCl_2$, $CaCl_2$, $MgCl_2$, KCl, $FeCl_3$, NaCl, $HgCl_2$, $BaCl_2$, $CuCl_2$, $ZnCl_2$, $CoCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, KOH, $NaNO_3$, urea, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF) and β-mercaptoethanol [20]. All assays were performed in triplicate.

2.6.3. Kinetic parameters

The Michaelis-Menten constant (Km) and maximum velocity (Vmax) were determined at 40 °C using L-asparagine as a substrate in concentrations ranging from 15 to 55 mM. The Michaelis-Menten parameters were determined from Lineaweaver-Burk graphics [21], using the equation derived from a linear regression analysis curve (Microsoft Office Excel version 12.0). Three experiments were performed for each substrate concentration in triplicate and the line was plotted by using mean values from each experimental point.

2.7. Cytotoxic evaluation

The cytotoxic activity of L-asparaginase was evaluated using the colorimetric method. These assay is based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) into an insoluble. After 3 hours the culture medium was removed and 100 µL of DMSO were added in each well for quantitation of formazan, which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells [22, 23].

The human cell lines: laryngeal carcinoma (HEp-2), human lung cancer (NCIH-292) and human breast adenocarcinoma (MCF-7) were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco); the human cell lines myelogenous leukemia (K562), acute promyelocytic leukemia (HL-60) and human lymphoblastic leukemia (MOLT-4) were maintained in RPMI-1640 medium (Himedia). All media were supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% antibiotics solution (penicillin 1000 UI/mL + streptomycim 250 mg/mL) and 1% glutamine (200 µM). (Gibco) and maintained at 37 °C and 5% of CO2.

An initial screening was conducted to determine the cytotoxic potential of the enzyme at a concentration of 25 µg/ml. The cells HEp-2, NCIH-292 and MCF-7, (10^5 cells/mL) and HL-60, MOLT-4 and K562 (0.3×10^6 cells/mL) were plated in 96-well plates and incubated for 24 h. Subsequently, the enzyme were dissolved in 1% dimethyl sulfoxide (DMSO) (Himedia) and culture medium added to the wells, and the plates were incubated for an additional 72 h. Doxorubicin (5 µg/mL) (Eurofarma) was used as positive control. As negative control cells were used without any treatment. Then, 25 µl of MTT (5 mg/mL in phosphate buffered saline - PBS) (Himedia) were added to the wells, and after 3 hours of incubation, the culture medium with MTT solution was aspirated and 100 µl of DMSO was added to each well. The absorbance was read on an automatic

microplate reader at 560 nm after dissolution of the formazan crystals [22]. The mean optical density (OD) of the samples was compared with the mean OD of the control DMSO.

These experiments were performed in triplicate, and the percentage of inhibition was calculated using the program GraphPad Prism 5.0. The percentage growth inhibition was calculated using the following formula:

Cell inhibition (%) = 100-{(At-Ab)/(Ac-Ab)} × 100

Where, At = Absorbance value of test compound; Ab = Absorbance value of blank, Ac = Absorbance value of control.

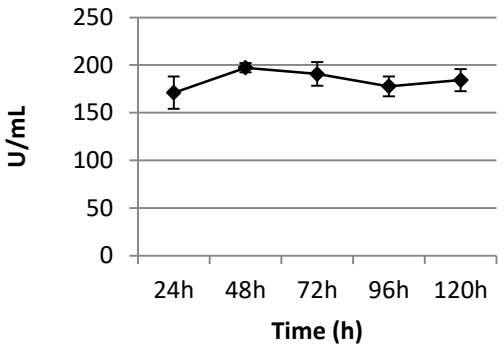
3. Results and Discussion

3.1. Determination of Kinetic parameters for enzyme production

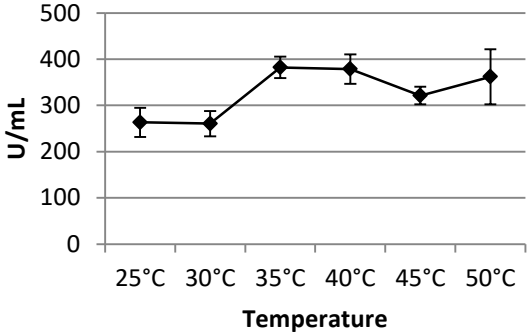
To determine the best conditions for production of L-asparaginase enzyme, some parameters such as temperature, time and pH were evaluated. *S. ansochromogenes* presented an enzymatic L-asparaginase activity of 133.22 U/mL when grown in M9 medium, under initial growing conditions. The optimum time was 48 h of fermentation, with a peak of 197.28 U/mL (Figure 1a). Among the measured temperatures (25 °C to 50 °C), the temperature of 35 °C showed the best activity with values that reached 382.19 U/mL (Figure 1b). The last parameter to be evaluated was the pH ranging between 5 and 9, and the pH 7 which stood out as the best condition for the production of the enzyme, with enzymatic activity of 743.29 U/mL (Figure 1c).

Figure 1: Parameters evaluated to determine the best conditions for the production of L-asparaginase enzyme. (a) fermentation time, (b) incubation temperature and (c) pH.

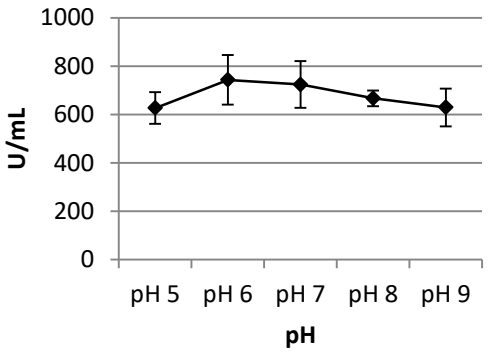
(a)



(b)



(c)



The influence of nutritional and metabolic parameters for cultivation is an important strategy for protein production [24]. Mahajan et al [25] report that the production of L-asparaginase from *Bacillus licheniformis* is viable within the pH range of 5.5-7.5 with a maximum production at pH 6.0 and 37 °C. A similar result was also evidenced by Meena et al [12] that report the optimum temperature for enzyme production in *Streptomyces griseus* as variable between 30-35 °C in the medium of production optimized in yield of 5.97 IU mL^{-1}. Narayana et al [26] highlight the maximum production of L-asparaginase by *Streptomyces albidoflavus* at approximately 35 °C.

3.2.Purification of L-asparaginase enzyme and Determination of Molecular Weight

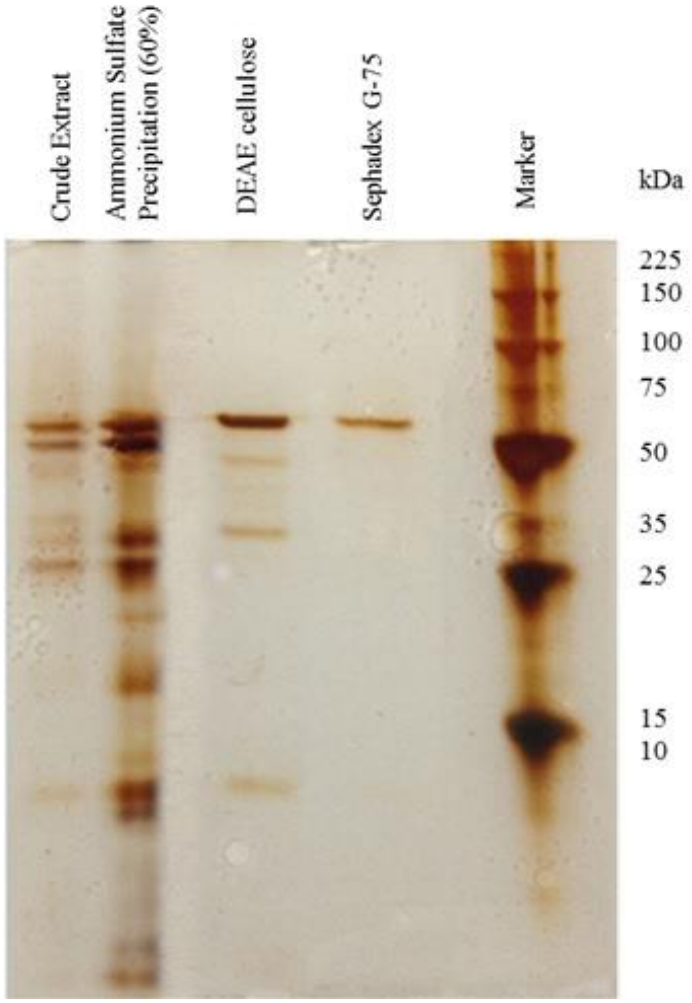
L-asparaginase produced by *S. ansochromogenes* UFPEDA 3420 was purified to their homogeneity with purification factor of 6.06, yield of 27.68% of the initial activity and final specific activity of 35205.76 U/mg (Table 1). Several studies describe the use of ion-exchange chromatography followed by molecular exclusion chromatography as an effective method for purification of L-asparaginase enzyme [27, 28]. Although the purification factor has presented a low value, it was enough to present a specific activity in a level very appreciable and significant to L-asparaginase. A study of the purification and characterization of *Salinicoccus* sp. M KJ997975 reveals a final enzyme yield of 27.3% [29]. Amena et al [30] reported that the production yield of L-asparaginase from *Streptomyces gulbargensis* was 32% after the steps of purification by chromatography. Thus, the data from this study showed a yield of 27.68% of L-asparaginase corroborating with literature reports.

Table 1: Purification steps of L-asparaginase from *Streptomyces ansochromogenes* UFPEDA 3420.

Purification steps	Total Activity (U)	Total Protein (mg)	Especific Activity (U/mg)	Yield (%)	Purification Fold
Crude Extract	11220.12	1.93	5802.94	100	1
Ammonium Sulfate Precipitation (0-60%)	393.89	0.095	4124.15	3.51	0.71
Ion-exchange (DEAE cellulose)	2026.60	0.21	9696.21	18.06	1.67
Gel filtration (Sephadex G-75)	3105.50	0.09	35205.76	27.678	6.06

In SDS-PAGE analysis was revealed a single homogenous band after the steps of purification with a molecular mass of approximately 63.99 kDa (Figure 2). The molecular weight of asparaginases produced by microorganisms, and also from other sources such as plants, may have different variations. The molecular weights of L-asparaginase from *Streptomyces albidoflavus*, *Penicillium cyclopium*, *Bacillus licheniformis* and *Capsicum annuum* were determined to 112 kDa, 55 kDa, 134.8 kDa and 70 kDa, respectively, by SDS-PAGE [26,8,28,20].

Figure 2: Determination of molecular weight of enzyme purified through a polyacrylamide gel SDS PAGE for steps of L-asparaginase purification.



3.3. Physicochemical properties of L-asparaginase from *S. ansochromogenes* UFPEDA 3420

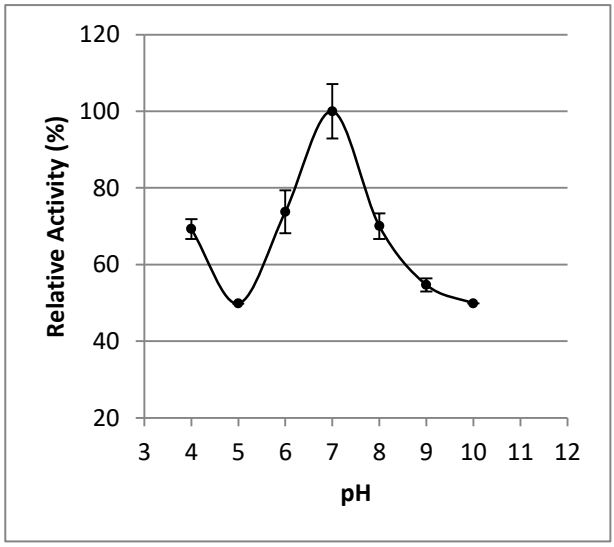
3.3.1. Effect of pH and temperature on activity and stability of L-asparaginase

The enzyme assay at different pH showed a peak of enzyme activity in the neutral region with maximum activity at pH 7.0 (Figure 3a). The enzyme had an activity above 50% at pH 6.0 and 8.0 and sharply reduced in pH 9.0 and 10.0. Much of the asparaginases maintains its activity in physiological pH. Similar results to this study were reported by Sinha et al [31] and Bhat and Marar [29], which reported the pH 7 as great in the activity of L-asparaginase from *Pseudomonas fluorescens* and *Salinicoccus* sp. KJ997975 M, respectively.

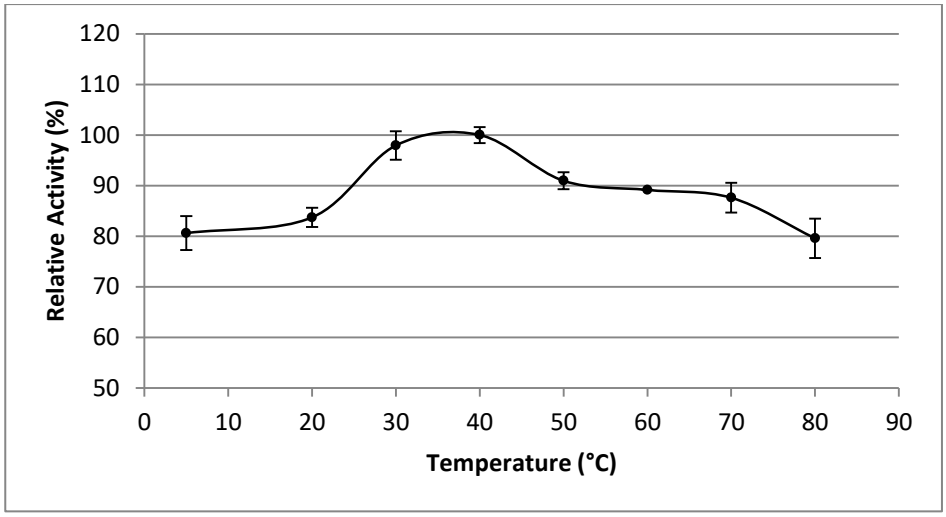
The study of stability of L-asparaginase in different pH revealed a maximal relative activity at pH 7.0 (Figure 3b). Regarding to temperature, was observed a maximum activity (Figure 3c) and stability at temperature of 40 °C, decreasing when subjected to higher temperatures. The stability declined sharply in temperature to 50 °C after 1 hour exposure, with a final activity of 50%. The enzyme retained about 90% of the initial activity at 40 °C in the first 20 minutes of exposure, following a slight decrease until 60 minutes of exposure. However, the stability of temperature was maintained in over 70% activity (Figure 3d). In temperatures above 50 °C, the enzyme may become unstable and begin to decrease activity. The low activity of L-asparaginases observed at higher temperatures can be attributed to the partial denaturation of the enzyme under the conditions of the tests [32]. The stability of an enzyme at a pH and physiologic temperature is considered a desirable feature for medical application of enzymes, since the maximum activity owned by L-asparaginase near 37 ° C may be more suitable for the complete elimination of asparagine by the body when the patient with the tumor is treated with L-asparaginase [33,8].

Figure 3: Physical and chemical properties of L-asparaginase from *S. ansochromogenes* UFPEDA 3420. a) Effect of pH on enzyme activity; b) Effect of temperature on enzyme activity; c) Effect of pH on the stability of the enzyme; d) Effect of temperature on the stability of the enzyme.

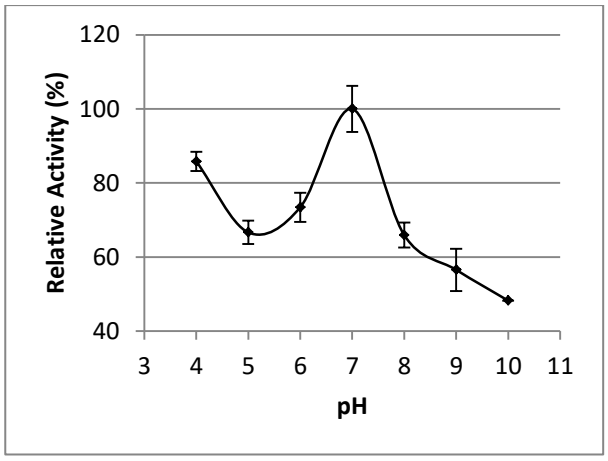
a)



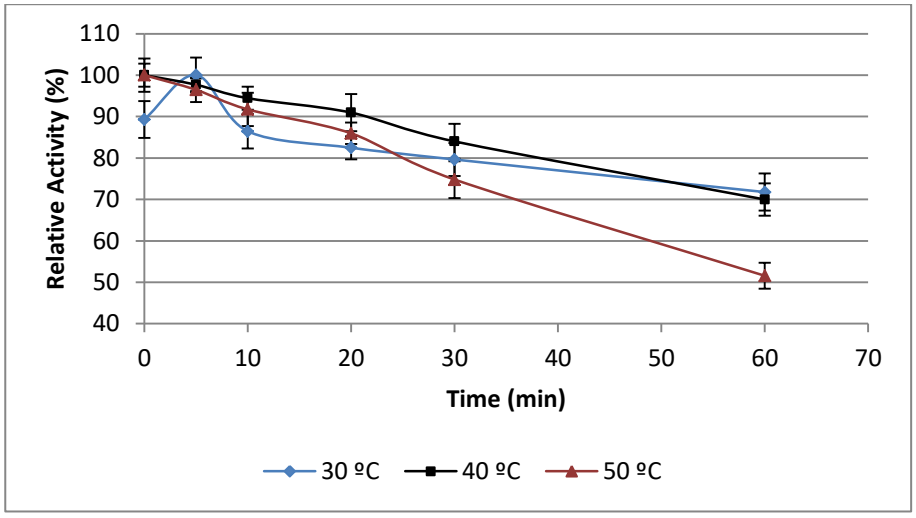
b)



c)



d)



3.3.2. Effect of different effectors on L-asparaginase activity

The L-asparaginase activity was investigated and the residual activities were calculated related to the control without effectors. Clearly, the enzyme activity was significantly enhanced in the presence of some compounds such as $HgCl_2$, $CoCl_2$ and β-mercaptoethanol and maintained above 70% in the presence of $MnCl_2$ and $CaCl_2$. The action of compounds such as $BaCl_2$, $CuCl_2$, $CuSO_4$ and $NaNO_3$, and denaturation caused by SDS detergent resulted in total loss of enzyme activity. The other compounds were considered enzyme inhibitors, because reduced L-asparaginase activity in more than 50%.

The increase of enzymatic activity of L-asparaginase in the presence of β-mercaptoethanol may be related to interaction of this compound with enzyme due to a higher affinity and thereby, prevent the formation of enzyme complexes with heavy metal. Warangkar and Khobragade [34] affirm that this behavior can be explained by a model that proposes the activation of non-essential enzymes of asparaginase in *Erwinia carotovora*. At the moment that substances such as β-mercaptoethanol are added, bind to the other site different to the site of the enzyme substrate. From there occurs a conformational change in the enzyme resulting in decreased binding affinity for heavy metal and the enzyme can recover their catalytic activity.

The significant decrease of more than 50% of L-asparaginases activity in the presence of sulfhydryl inhibitors may occur due to formation of complexes with the enzyme active sites [35]. Heavy metals ions can bind non-specifically to other regions than thiol group of cysteine of enzyme and induce inhibition [36].

3.3.3. Kinetic parameters

The analysis of Lineweaver Burk graphic infers that Km and Vmax of L-asparaginase purified from *Streptomyces ansochromogenes* UFPEDA 3420, under the conditions studied in this work and using L-asparagine as a substrate was 3.22 mM and 161.29 U/mL, respectively.

L-asparaginases enzymes produced by different microorganisms can have different affinities for the substrate and therefore play multiple physiological roles on enzyme activity [8]. Patro and Gupta [37] reported a Km value slightly higher for L-asparaginase from *Penicillium* sp. (4 mM) and higher values were observed for L-asparaginase from *Aspergillus aculeatus* (Km= 12.5 mM and Vmax= 104.06 U/mL) [38], higher values compared to the Km of *S. ansochromogenes* UFPEDA 3420.

3.4. Cytotoxic evaluation

The cytotoxicity effect of L-asparaginase was monitored against six different human tumor cell lines: HEp-2, NCIH-292, MOLT-4, K562, HL-60 and MCF-7, by using MTT cell viability assay. Analyses to determine the inhibition of cell lines exposed to the L-asparaginase from *S. ansochromogenes* UFPEDA 3420 demonstrated that this substance decreases the viability of the three cancerous cell lines. The L-asparaginase enzyme inhibited the growth of the MOLT-4, presenting IC_{50} value of 25 µg/mL. The IC_{50} was reported as greater than 25 µg/mL for others tumor cell lines.

Studies have shown that there may be increased asparagine synthetase expression in cells treated with L-asparaginase. However, the heightened activity can lead leukemia cells to become resistant to treatment. In addition, other adaptive processes may provide an alternative substrate for asparagine synthetase such as aspartate and glutamine, which are derived from extracellular and intracellular sources, hindering the action of the enzyme under study [39,40]. Studies by Mahajan et al (2014) show that L-asparaginase from *Bacillus licheniformis* RAM-8 showed activity against the K562 and MCF-7 cells. Beyond to these cells, L-asparaginase have also been reported to cytotoxic action against MOLT-4 cells and NCIH-292, but the activity for leukemic lines are very common due to its mechanism of action already established [1,41].

The literature reports the use of doses above 50 µg/mL for the inhibition of certain tumor cell lines. According Shanmugaprakash et al [20], L-asparaginase purified from *Capsicum annuum* L. is effective in decreasing tumor cells such Hela (human cervical carcinoma cell line), A549 (human lung carcinoma) and KB (oral squamous cell carcinoma human) with IC_{50} of 410 µg/mL, 535 µg/mL and 360 µg/mL, respectively. As L-asparaginase *S. ansochromogenes* UFPEDA 3420, the treatment using the enzyme from *Capsicum annuum* L. significantly decreased the number of cell lineages, but there was need higher doses of the compound to occur inhibition of tumor cell lines. Faced with this data, new research on L-asparaginase produced by *S. ansochromogenes* UFPEDA 3420 will still be made in order to improve the applicability of this compound.

Conclusions

The present study demonstrates for the first time the production of L-asparaginase by *Streptomyces ansochromogenes* and enhances the prospects of obtaining the enzyme from other genera of microorganisms as alternative sources. L-asparaginase from *S. ansochromogenes* UFPEDA 3420 showed Michaelian behavior and cytotoxicity activity against tumor cell lines, primarily for the MOLT-4 acute lymphoblastic leukemia. Such as L-asparaginase of this study was

obtained from a different bacterial source of the conventional, it may be in the future a new benchmark for the therapeutic enzyme industry as well as provide relevant information to enrich the literature. In addition, it is important to emphasize that *S. ansochromogenes* UFPEDA 3420 species that was isolated from the Brazilian biome Caatinga, represents a powerful source of metabolites of great potential for biotechnology.

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Artigo 2

5 ARTIGO 2

5.1 Genotox activity of l-asparaginase produced by *Streptomyces ansochromogenes* UFPEDA 3420 in normal and neoplastic cells

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GENOTOX ACTIVITY OF L-ASPARAGINASE PRODUCED BY *Streptomyces ansochromogenes* UFPEDA 3420 IN NORMAL AND NEOPLASTIC CELLS

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Abstract

L-asparaginase is an enzyme that hydrolyzes asparagine in aspartic acid and ammonia. Because this, it is widely used as an antineoplastic in the treatment of Acute Lymphoblastic Leukemia, since these cells use asparagine for their survival. This study aimed to evaluate the mutagenic effects and genomic damage in healthy and tumor cells exposed for 48 hours to different concentrations of L-asparaginase (12,5, 25 and 50 µg/mL) produced by *Streptomyces ansochromogenes* UFPEDA 3420, as a positive control the Doxorubicin at the concentration of its IC50 for each tumor cell line: 0,5 µg/mL (NICH-292), 0,04 µg/mL (MOLT-4) and 0,2 µg/mL (MCF-7) and as a negative control cells without any treatment. The genotoxic assay was performed on neoplastic cell lines NCIH-292 (mucoepidermoid carcinoma of the lung), MCF-7 (Breast adenocarcinoma), MOLT-4 (Lymphoblastic Leukemia) and normal cell lines PBMC (Peripheral Blood Mononuclear Cell). L-asparaginase used was produced by *S. ansochromogenes* UFPEDA 3420, isolated and purified by chromatographic methods. The evaluation of genomic damage was performed through the Alkaline Comet and Micronucleus Test. It was observed that in the parameter Damage Index, all tumor lines presented considerable genotoxic effects with levels of significance ($p < 0.05$) for all the concentrations tested when compared to the untreated cells, except for the concentration of 12.5 µg/mL to MOLT-4 lines. In mutagenic effects analysis, L-asparaginase was able to induce micronucleus formation in tumor cells as well as in healthy cells. The genotoxic activity of L-asparaginase equates with doxorubicin. It is very important to carry out in-depth studies on the toxicity of L-asparaginase, since the results show that there were also genotoxic effects for normal cells. Since L-asparaginase is already used in the treatment of leukemias, it is necessary to collect sufficient information so that the use of this drug is safely performed in patients.

Keywords: Micronucleus test; genomic damage; tumor lines; enzyme.

Introduction

The search for antitumor drugs started in the mid-1940s and since then, the area of natural products research has expanded significantly, aiming to obtain compounds with important therapeutic action and lower side effects. A high number of these products come from microorganisms or microbial interactions, which indicates that they are producers of bioactive molecules that can be used as drugs or as model structures for the planning and development of new substances with pharmacological application. Regarding cancer, of the 155 molecules already identified and approved for treatment, 75% are non-synthetic molecules and 47% are natural products or derived directly from these molecules. Microorganisms are also capable of producing antibiotics, immunosuppressants and blood cholesterol lowering agents (Newman e Cragg, 2016).

Actinobacteria belonging to the genus *Streptomyces* have been reported as an important source of natural products, being able to produce a wide variety of secondary metabolites such as antibiotics, antifungal, therapeutic enzymes, insecticides, biolarvicides, antitumorals, immunosuppressants, antiviral and for their competitive ability for substrates (Inbar, 2005; Barka et al., 2016).

The enzymes are biological catalysts produced by living cells, which present high catalytic activity and specific selectivity on the substrate (Nelson and Cox, 2014). They can come from a variety of natural sources, including animals, plants and microorganisms, the latter being the most used source for enzyme production for application in the pharmaceutical and biotechnology industries (Singh et al., 2010). Several enzymes have been characterized and identified, however, few of these enzymes have gained space in the scientific field and have become the target of major investigations (Batool et al., 2016).

L-asparaginase (Lasparagine amidohydrolase, EC 3.5.1.1) is a therapeutic enzyme, proven action, that has been extensively studied in the last decades. However, the relevance of the studies does not restrict the characteristics and performance as an enzyme, but because of its significant

capacity to combat neoplastic cells. The enzyme L-asparaginase belongs to the group of amidase enzymes, also known as aminohydrolase due to its ability to break down the amino acid L-asparagine in aspartic acid and ammonia, being this, its antileukemic mechanism (Narta et al., 2007; Kumar e Verma, 2012).

When administered, L-asparaginase induces nutritional stress in tumor cells by depletion of asparagine at its serum levels. The lack of the amino acid asparagine by the low regulation of asparagine synthetase leads to inhibition of the biosynthesis of DNA, RNA and proteins in acute lymphoblastic leukemia (ALL) cells and in other asparagine-dependent tumor cells induces cell cycle arrest and inhibits the proliferation of these cells (Yang et al, 2014). According to Narta et al. (2007), normal cells have the enzyme asparagine synthetase, which is absent in ALL cells, which allows these cells to perform the biosynthesis of their own asparagine. Thus, normal cells are not affected during treatment with L-asparaginase.

When L-asparaginase is used in the treatment of leukemias, it may present two important models of toxicity in individuals: such as the occurrence of hypersensitivity reactions, by the introduction of unusual compounds into the organism, or by the reaction of toxicity resulting from the inhibition of protein synthesis, result of the reduction of plasma L-Asparagine levels and also the accumulation of the products resulting from the enzymatic hydrolysis (Kurtzberg, 2000; Graham, 2003; Rizzari et al., 2013).

The promising therapeutic activity of the enzyme L-asparaginase makes evident the importance of further studies of its antineoplastic properties. However, the literature is scarce when referring to the genotoxic studies performed with L-asparaginase and, therefore, the mechanism by which this enzyme can act causing damage to the cellular genome is inconclusive, worrying and unknown.

Therefore, this study aimed to evaluate genomic damage in human normal and tumor cells exposed to different concentrations of L-asparaginase produced by *Streptomyces ansochromogenes* UFPEDA 3420.

Material and Methods

Purification and characterization of L-asparaginase

L-asparaginase used in this study was produced by the actinobacteria *Streptomyces ansochromogenes* UFPEDA 3420. The enzyme production process was carried out through a fermentative process under the ideal pre-established culture conditions: culture medium M9 [$Na_2HPO_4.2H_2O$, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 0,5 g/L; L-asparagine, 5 g/L; $MgSO_4.7H_2O$, 0,5 g/L; $CaCl_2.2H_2O$, 0,014 g/L; Glucose, 2 g/L; Distilled H_2O, 1 L], time of 48 hours, pH 7 and temperature of 35 °C. The activity of the enzyme was determined by the dosage of ammonia released by Nesslerization (Imada et al, 1973). The crude extract was precipitated with ammonium sulfate and enzyme purification was performed by chromatographic methods such as ion exchange interaction (DEAE (Diethylaminoethyl Cellulose) Chromatography) and gel filtration chromatography (Sephadex G75) (Silva et al., 2015). Protein homogeneity and molecular weight were determined by SDS-PAGE gel electrophoresis (Laemmli, 1970).

Tumor Cells and Culture Conditions

For the evaluation of the differential genomic damage induced by L-asparaginase, three tumoral lines were selected, NCIH-292 (mucoepidermoid carcinoma of the lung), MCF-7 (Breast adenocarcinoma) and MOLT-4 (lymphoblastic leukemia), as well as a normal lines of Peripheral Blood Mononuclear Cells (PBMC). The lines were made available by the Laboratory of Cell Cultures and Pharmacological Testing, of the Department of Antibiotics of the Federal University of Pernambuco (UFPE). The NCIH-292 and MCF-7 lines were cultured ($2x10^5$ cells/mL) in flasks (25 cm^2/60 mL) for cell culture, containing the Dulbecco's modified Eagle's minimal essential medium (DMEM, Sigma) and the lines PBMC ($2x10^5$ cells/mL) and MOLT-4 ($0.3x10^6$ cells/mL) were cultured in similar flasks in RPMI-1640 medium (Himedia). All media used were

supplemented with 10% fetal bovine serum (Gibco), 1% antibiotic solution (penicillin 1000 IU/mL + streptomycin 250 mg/mL) and 1% of L-glutamine 200 mM and incubated at 37 °C for 24 h in a humidified atmosphere containing 5% CO_2 (Mosmann, 1983).

Obtaining Peripheral Blood Mononuclear Cells (PBMC)

Peripheral Blood Mononuclear Cells (PBMC) were obtained by peripheral blood collection from a healthy patient. All the researches using the patient's material were approved by the Ethics Committee in Research involving human beings of the Federal University of Pernambuco, Center of the Sciences of the Health / UFPE, under the number 1.005.947 and the consent was provided by the participant of the study informed by written. The process of obtaining and culturing the cells was performed according to Yssel and Spits (2002). Initially, blood sample was diluted 1: 1 (v/v) in PBS buffer. Then, Ficoll-hypaque at the ratio of 1: 1.5 (v/v) was added to the suspension and centrifuged at 900 × g for 30 min, 20 ° C and without brake and acceleration. After centrifugation, the interface was collected, washed with PBS and again centrifuged at 400 ×g for 10 min, 20 °C and without brake and acceleration. Subsequently, the pellet was resuspended in RPMI 1640 and centrifuged under the same conditions as above. Finally, the pellet was resuspended in RPMI 1640 supplemented with 10% fetal bovine serum.

Genotoxicity assay of L-asparaginase in tumor cell lines

Treatment of cell cultures and exposure period

The L-asparaginase concentrations employed for analysis were calculated based on the previous determination of half the maximal inhibitory concentration (IC_{50}). The dose reduced to half the IC_{50} (12.5 μg/mL), the IC_{50} (25 μg/mL) and the duplicate dose (50 μg/mL) were tested. As

a negative control, the cell culture without any test substance was added and as a positive control, Doxorubicin at the concentration of its IC_{50} was used for each tumor cell lines: 0.5 μg/mL (NCIH-292), 0.04 μg/mL (MOLT-4) and 0.2 μg/mL (MCF-7). After culturing the cells, L-asparaginase at concentrations of 12.5, 25 and 50 μg/mL was added to the NCIH-292, MCF-7, MOLT and PBMC cell cultures. Cells were kept exposed to the test substance for 48 hours at 37 °C.

The adhered cells NCIH-292 and MCF-7 were initially subjected to a pre-treatment with trypsin. The medium was aspirated and the cells were washed with 300 μL buffer (PBS, pH 7.2) and then treated with 300 μL trypsin/EDTA. Subsequently, for inactivation of trypsin, 600 μL of DMEM medium was added and then the samples were aspirated and transferred to microtubes for processing. No pre-treatment with trypsin was required in the PBMC control and lines MOLT-4.

The samples were processed for application in the genotoxic assays. Initially, a wash was performed with 60 μL of buffer solution (PBS, pH 7.2) and centrifuged at 806 × g for 20 minutes. After centrifugation, the culture medium was removed and the cells were resuspended with 60 μL of the same buffer (Lins et al, 2015).

Micronucleus test

For the micronucleus test, the methodology of Fenech et al. (1993) was used. Microscopy slides were previously washed to remove interferents and rinsed with 70% ethanol. After the slides were dried, 15 μL of each sample was added onto the slides and spread over the smear. The slides were held horizontally until completely dried at room temperature. After drying, the slides were fixed with absolute ethanol (P.A) for 5 minutes and then washed in running water. For counting the micronuclei, the slides were stained with Giemsa (Merck). The dye was evenly deposited on the slides for about 1 minute and washed with running water. After drying at room temperature, the

reading was performed under an optical microscope (100x), where a total of 10^3 cells were counted with micronuclei.

DNA Damage Alkaline Comet Test

The comet assay was performed according to the methodology of Singh et al (1988) and Tice et al (2000) with modifications. For the comet assay, 15 μL of the homogenized cell suspension was used in 100 μL of low melting-point (LM) agarose preheated in a 37 °C waterbath. The homogenate was transferred to microscopy slides coated with standard agarose, covered with coverslips and incubated for 10 minutes under refrigeration at 4 °C. After this period, the coverslips were removed and the slides were immersed in a lysis solution for 2 hours under refrigeration at 4 °C. After the elapsed time, the slides were electrophoresed (40 V for 20 minutes and 300 mA). The slides were then immersed in a neutralization solution for 15 minutes, fixed in absolute ethanol solution for 5 minutes and dried at room temperature. The slides were kept under refrigeration until the time of staining. For staining, Gel Red dye (Biotarget GelRed®) was used, where 1 μl of the dye was homogenized in 1000 μL of sterile deionized water (1: 1000). The slides were analyzed by fluorescence microscopy (Olympus-BX Series) at a 100X amplification with an excitation filter at 535 ηm. Then, Cell counts were performed. Approximately 100 cells per type of cell lines treated at the different concentrations of L-asparaginase were analyzed and scored from 0 to 4 points, depending on the degree of damage in the nucleoid. The following criteria were used to classify comets based on epifluorescence of 4 levels of genomic damage: 0 damage, no visible lesion or damage to the DNA of the cell and absence of nucleoid fragments, damage 1, observation of slight DNA damage with slight fragmentation in DNA and formation of a discrete halo. Damage 2, moderate damage of DNA and visible nuclear fragmentation with formation of a halo to the extensive, damage 3, intense damage in the DNA and observation of a very extensive halo, around, of the little nucleoid material and formation of a tail similar to a comet , being the latter the most severe form of damage.

The paramters Damage Indices (DI) and Damage Frequency (DF) ware calculated using the following formula:

$$DI = \frac{(0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)}{100 \text{ cells}}$$

$$DF = \frac{(n1 + n2 + n3 + n4)}{100 \text{ cells}}$$

n= Number damaged cells

Statistical analyzes were performed through the comparisons between the Damage Indices (DI), Damage Frequency (DF) and Micronucleus Frequency (MNF) from the chi-square test (X^2), analyzed individually by the different treatments of each tumor lines and of such lines compared to the normal lines PBMC. The significance level adopted for the tests was 0.05. The data were evaluated in the program Excel 12.0 (Microsoft® Office).

The damage proportionality (PI) indices were established in each treatment in relation to its own control (without addition of L-asparaginase) and in relation to doxorubicin, as well as in relation to the coincident concentrations of the PBMC lines, for all methodological parameters used.

Results

L-Asparaginase showed a genotoxic effect against the NCIH-292 and MCF-7 tumor lines tested. From the statistical analysis of the parameter Damage Index (DI), acquired by the Alkaline Comet Test, it was observed significance for all the concentrations tested in relation to the negative control. In the MOLT-4 tumor line (fig. 1), significant concentrations of 25 and 50 μg/mL were observed, while in the PBMC healthy line, significant data, ie genotoxicity, were observed only at 25 μg/mL. Another parameter that was also evaluated from the Cometa Assay was the Damage Frequency (DF), which despite the damage index (DI) was statistically significant, the DF, however, did not present any significance for any of the studied strains (table 1).

Figure 1: Genomic damage at different levels observed in MOLT-4 tumor cells, when exposed to different concentrations of L-asparaginase in 48 hours. A) Damage 0, negative control; B) Damage 1, negative control; C) Damage 2, concentration of 12.5 μg/mL; D) Damage 3 and E) Damage 4, concentration of 50 μg/mL.

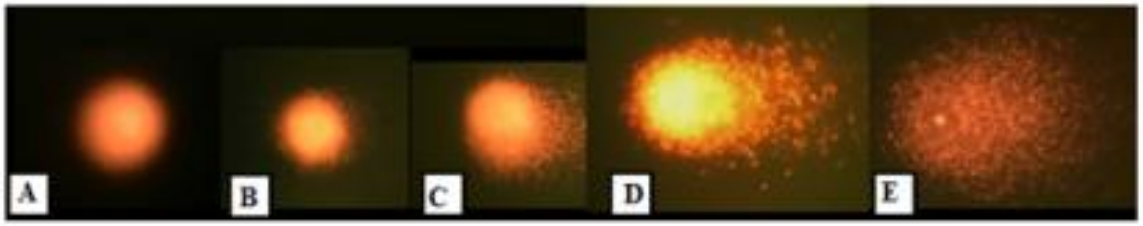


Table 1. Indices and frequency of genomic damage observed in normal and tumor cell lines (PBMC) after 48-hour exposure to different concentrations of L-asparaginase (12.5, 25 and 50 μg/mL), proportionality indexes and p-values relative to comparisons with the respective negative controls (cells without L-asparaginase treatment) of each lines, obtained by X^2.

μg/mL	Cell lines											
	NCIH-292						MCF-7					
	DI	PI	*p*	DF	PI	*p*	DI	PI	*p*	DF	PI	*p*
0,0	67,08	1,00		0,050	1,00		141,14	1,00		0,8	1,00	
LA 12,5	133,52	1,99	<0,001**	0,755	1,50	>0,5	164,82	1,17	<0,05*	0,955	1,19	>0,5
LA 25	133	1,98	<0,001**	0,79	1,56	>0,5	176,8	1,25	<0,01**	0,955	1,19	>0,5
LA 50	211,1	3,15	<0,001**	0,995	1,97	>0,5	175,08	1,24	<0,01**	0,965	1,21	>0,5
	MOLT-4						PBMC					
	DI	PI	p	DF	PI	p	DI	PI	p	DF	PI	p
0,0	110,54	1,00		0,7	1,00		146,12	1,00		1	1,00	
LA 12,5	116,54	1,05	>0,5	0,705	1,01	>0,5	143,58	1,02	>0,5	0,955	1,05	>0,5
LA 25	169,06	1,53	<0,001**	0,915	1,31	>0,5	186,54	1,28	<0,001**	0,995	1,01	>0,5
LA 50	221,06	2,00	<0,001**	1	1,43	>0,5	167,6	1,15	>0,5	0,99	1,01	>0,5

LA (L-asparaginase); DI (Genomic damage index); DF (Frequency of genomic damage); PI (Proportionality Index); Accepted level of significance p<0,05.

The evaluation of the DI in relation to the positive control Doxorubicin (Dox 0.5 μg/mL) presented significant results in the NCIH-292 and MOLT-4 tumor lines at all concentrations, except for the 50 μg/mL concentration in NCIH-292 and 25 μg/mL in MOLT-4. The DIs in the MCF-7 lines were not significant in the comparison with Doxorubicin. The comparative analysis of the DI between L-asparaginase and Doxorubicin in the PBMC line revealed that L-asparaginase presents a significant in the concentration of 12.5 μg/mL in relation to Doxorubicin at concentrations of 0.5 μg/mL and 0.2 μg/mL, but there is also a significant difference between L-asparaginase at the concentration of 25 μg/mL with Doxorubicin at 0.04 μg/mL. In the DF parameter, no statistical significance was found for any of the tested lines, either in the comparison of cells without treatment (negative control) or in those treated with Doxorubicin (positive control). Table 2 shows the DI and DF of the tumor lines compared to the DI of positive control (Doxorubicin), as well as the proportionality index for each parameter. The evaluation of damages by these parameters allows to observe that the damages induced by L-asparaginase in the concentration of 50 μg/mL were compared to the damages caused by Doxorubicin.

Table 2. Comparative analysis between indices and frequencies of genomic damage observed in normal and tumor cell lines (PBMC) after 48-hour exposure to different concentrations of L-asparaginase (12.5, 25 and 50 µg/mL), proportionality indices and the p-values relative to comparisons with the respective positive controls (Doxorubicin) for each lines obtained by X^2.

µg/mL	Cell Lines											
	NCIH-292						PBMC					
	DI	PI	*p*	DF	PI	*p*	DI	PI	*p*	DF	PI	*p*
Dox 0,5	191,62	1,00		0,915	1,00		181,36	1,00		1	1,00	
LA 12,5	133,52	1,44	<0,001*	0,755	1,21	>0,5	143,58	1,26	<0,01*	0,955	1,05	>0,5
LA 25	133	1,44	<0,001*	0,79	1,16	>0,5	186,54	1,03	>0,5	0,995	1,01	>0,5
LA 50	211,1	1,10	<0,3	0,995	1,09	>0,5	167,6	1,08	<0,5	0,99	1,01	>0,5
µg/mL	MCF-7						PBMC					
	DI	PI	P	DF	PI	p	DI	PI	p	DF	PI	p
Dox 0,2	163,18	1,00		0,98	1,00		141,14	1,00		1	1,00	
LA 12,5	164,82	1,01	>0,5	0,955	1,03	>0,5	143,58	1,17	<0,01*	0,955	1,05	>0,5
LA 25	176,8	1,08	>0,05	0,955	1,03	>0,5	186,54	1,11	>0,05	0,995	1,01	>0,5
LA 50	175,08	1,07	>0,05	0,965	1,02	>0,5	167,6	1,00	>0,5	0,99	1,01	>0,5
µg/mL	MOLT-4						PBMC					
	DI	PI	P	DF	PI	p	DI	PI	p	DF	PI	p
Dox 0,04	182,2	1,00		0,89	1,00		150,1	1,00		0,975	1,00	
LA 12,5	116,54	1,56	<0,001*	0,705	1,26	>0,5	143,58	1,05	>0,5	0,955	1,02	>0,5
LA 25	169,06	1,08	>0,5	0,915	1,03	>0,5	186,54	1,24	<0,01*	0,995	1,02	>0,5
LA 50	221,06	1,21	<0,01*	1	1,12	>0,5	167,6	1,12	>0,05	0,99	1,02	>0,5

Dox (Doxorubicin); LA (L-asparaginase); DI (Genomic damage index); DF (Frequency of genomic damage); Accepted level of significance p<0,05.
* Significative values.

The rates of genomic damage and the frequency of damage caused by L-asparaginase in tumor lines compared to DIs and DFs of PBMC are shown in Tables 3 and 4. In both parameters considered, none of the lines differ statistically from normal cells (PBMC) at all concentrations of L-asparaginase. The results of p observed for DI and DF were the same for all lines indicating that this enzyme causes genotoxic effects in both tumor cells and healthy cells. In table 3 it is possible to evaluate the proportionality index (PI) for the DI for each tumor line in relation to the PBMC. The three tumor lines NCIH-292, MFC-7 and MOLT-4 had a high DI and a higher proportionality index in relation to the PBMC, at the concentration of 50 μg/mL, with DI values of 211.1, 175.08 and 221 , 06 and PI of 1.26, 1.04 and 1.32, respectively, against the 167.6 DI of the PBMC healthy cells. The concentration of 25 μg/mL of L-asparaginase caused greater damage in healthy cells than in tumor cells, with a DI of 186.54, which is higher than in tumor cells. Thus, it is estimated that the reduction of PBMC lines damage when treated with the highest concentration (50 μg/mL) may be related to increased cell damage, which induces apoptosis in these cells and thus the levels of damage are not classified. These results demonstrate that at the concentration of 50 μg/mL the genotoxic damages in these tumor lines were greater than the damages caused in the healthy cells. Table 4 shows the frequencies of damage in all study lines, but these data were not significant.

Table 3. Genomic damage indices observed in normal and tumor cell lines (PBMC) after 48-hour exposure to different concentrations of L-asparaginase (12.5, 25 and 50 µg/mL), damage proportionality indices and p-values relative to comparisons to the control cell (PBMC) comparisons obtained by X^2.

µg/mL	Cell Lines									
	NCIH-292			MCF-7			MOLT-4			PBMC
	DI	PI	*p*	DI	PI	*P*	DI	PI	*p*	DI
Dox 0,04	-	-	-	-	-	-	182,2	1,21	>0,05	150,1
Dox 0,2	-	-	-	163,18	1,03	>0,05	-	-	-	167,82
Dox 0,5	191,62	1,06	>0,05	-	-	-	-	-	-	181,36
0,0	67,08	2,18	>0,05	141,14	1,04	>0,05	110,54	1,32	>0,05	146,12
LA 12,5	133,52	1,08	>0,05	164,82	1,15	>0,05	116,54	1,23	>0,05	143,58
LA 25	133	1,40	>0,05	176,8	1,06	>0,05	169,06	1,10	>0,05	186,54
LA 50	211,1	1,26	>0,05	175,08	1,04	>0,05	221,06	1,32	>0,05	167,6

LA (L-asparaginase); DI (Genomic damage index); PI (Proportionality Index); Accepted level of significance p<0,05.

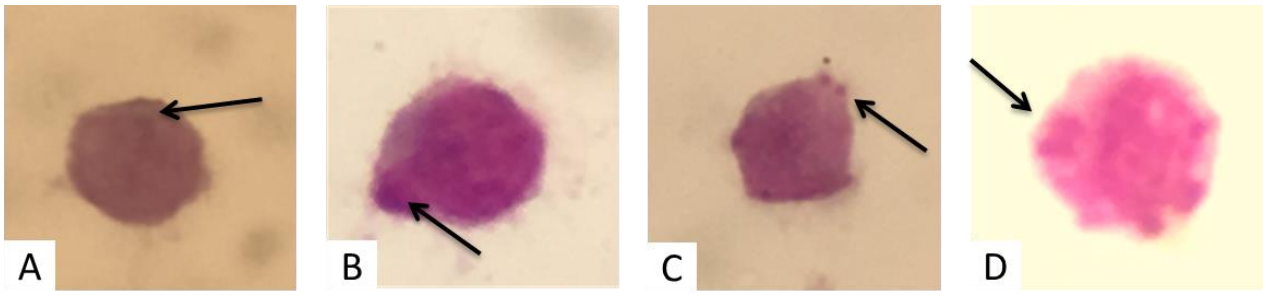
Table 4. Frequencies of genomic damage observed in normal and tumor cell lines (PBMC) after 48-hour exposure to different concentrations of L-asparaginase (12.5, 25 and 50 µg/mL), damage proportionality indices and p-values relative to comparisons to the control cell (PBMC) obtained by X^2.

µg/mL	Cell Lines									
	NCIH-292			MCF-7			MOLT-4			PBMC
	DF	PI	*p*	DF	PI	*p*	DF	PI	*p*	DF
Dox 0,04	-	-	-	-	-	-	0,89	1,10	>0,05	0,975
Dox 0,2	-	-	-	0,98	1,02	>0,05	-	-	-	1
Dox 0,5	0,915	1,09	>0,05	-	-	-	-	-	-	1
0,0	0,050	1,98	>0,05	0,8	1,25	>0,05	0,7	1,43	>0,05	1
LA 12,5	0,755	1,26	>0,05	0,955	1,00	>0,05	0,705	1,35	>0,05	0,955
LA 25	0,79	1,26	>0,05	0,955	1,04	>0,05	0,915	1,09	>0,05	0,995
LA 50	0,995	1,01	>0,05	0,965	1,03	>0,05	1	1,01	>0,05	0,99

LA (L-asparaginase); DF (Frequency of genomic damage); PI (Proportionality Index); Accepted level of significance p<0,05.

Through the Micronucleus Test it was observed that at all concentrations there was induction to the formation of micronuclei. The data demonstrate that in the MOLT-4 tumor cell line (Fig. 2) and the PBMC, both the Micronucleated Cells (MNC) and the Micronucleus Frequency (MNF) parameters increased according to the increase of the administered L-asparaginase concentration, noting that the formation of the micronuclei is dependent on the dose that is applied. However, in the NCIH-292 strain the concentration of the highest number of micronucleated cells was 12.5 μg/mL, whereas for the MCF-7 line there was no difference in micronuclei formation when compared to the negative control. It is possible that these cells, when in contact with higher concentrations of L-asparaginase, express membrane receptors that activate efficient defense mechanisms. In relation to the positive control (Doxorubicin), it was observed that L-asparaginase was able to induce greater formation of micronuclei in the NCIH-292 and MOLT-4 lines at concentrations of 12.5 μg/mL and 50 μg/mL, respectively. This fact can also be observed in the PBMC, where the micronuclei induced by the action of the enzyme, were higher than those induced by the substance of the positive control. The exception for this parameter was the MCF-7 line, where L-asparaginase did not induce micronucleus formation higher than Doxorubicin at any of the concentrations. Evidence of micronuclei, such as the number of Micronucleated Cells (MNC) and the Micronucleus Frequencies (MNF), are shown in table 5.

Figure 2: Formation of micronuclei in MOLT-4 tumor cells, when exposed to different concentrations of L-asparaginase in 48 hours. A) negative control; B) concentration of 12.5 μg/mL; C) concentration of 25 μg/mL; D) concentration of 50 μg/mL. The arrows indicate micronuclei formation.



The comparative analysis of the frequency of micronucleated cells in tumor cell lines relative to healthy PBMC cells, considering the different treatments, showed that there were no significant differences (Table 6). This shows that the degree of genotoxicity was similar among the studied lines.

The three methodological parameters used in the study - Damage Index, of Damage Frequency and Micronucleated Cells were also evaluated for the analysis of Damage Proportionality Indexes, which was calculated through the correlation between the negative control and the treatment with different concentrations of L -asparaginase and between the positive control (Doxorubicin) and treatment with different concentrations of L-asparaginase. The highest proportionality indexes were observed for the parameter micronucleated cells, as shown in table 7.

Table 5. Evidence of micronuclei observed in normal and tumor cell lines after exposure for 48 hours at different concentrations of L-asparaginase (12.5, 25 and 50 μg/mL) and Doxorubicin.

μg/mL	Cell Lines							
	NCIH-292		MCF-7		MOLT-4		PBMC	
	MNC	MNF	MNC	MNF	MNC	MNF	MNC	MNF
Dox 0,04	-	-	-	-	22±2,8	0,022	12,5±2,1	0,0125
Dox 0,2	-	-	23±1,4	0,023	-	-	19±5,6	0,019
Dox 0,5	29,5±2,1	0,0295	-	-	-	-	19,5±2,1	0,0195
0,0	3±1,4	0,003	13,5±0,7	0,0135	3,5±35	0,0035	1±1,4	0,001
LA 12,5	45,5±3,5	0,0455	8,5±3,5	0,0085	11±1,4	0,011	14,5±0,7	0,0145
LA 25	30,5±3,3	0,0305	6,5±2,1	0,0065	12,5±6,3	0,0125	19±1,4	0,019
LA 50	38,5±0,7	0,0385	10,5±4,9	0,0105	25,5±3,5	0,0255	24,5±2,1	0,0245

MNC (Micronucleated cells) / MNF (Micronuclei Frequency) / Dox (Doxorubicin) / LA (L-asparaginase)

Table 6. P-values relatives to comparisons of the micronucleated cell frequencies obtained in the different cell lines with the PBMC control obtained by the X^2.

Cell Lines	PBMC				
	Concentrations (μg/mL)				
	0,0	Dox*	12,5	25	50
NCIH-292	*p*>0,05	*p*>0,05	*p*>0,05	*p*>0,05	*p*>0,05
MCF-7	*p*>0,05	*p*>0,05	*p*>0,05	*p*>0,05	*p*>0,05
MOLT-4	*p*>0,05	*p*>0,05	*p*>0,05	*p*>0,05	*p*>0,05

Accepted level of significance p<0,05

*0,5 μg/mL to NICH-292; 0,04 μg/mL to MOLT-4 and 0,2 μg/mL to MCF-7.

Table 7. Proportional indices between the concentration (µg/mL) of L-asparaginase, control and the different treatments, and among the treatments, referring to the methodological parameters in the PBMC line: Damage Index, Damage Frequency and Number of Micronucleated Cells.

Methodological parameters																
		Damage Index					Damage Frequency					Micronucleated Cells				
			PIc	PIt				PIc	PIt				PIc	PIt		
Negative Control	µg/mL	DI	146,1	143,6	186,5	167,6	DF	1	0,96	1	0,99	MNC	1	14,5	19	24,5
	0,0	146,1	-	-	-	-	1	-	-	-	-	1	-	-	-	-
	LA 12,5	143,6	1,02	-	1,30	1,17	0,96	1,05	-	1,04	1,04	14,5	14,5	-	1,31	1,69
	LA 25	186,5	1,28	1,30	-	1,11	1	1,01	1,04	-	1,01	19	19	1,31	-	1,29
	LA 50	167,6	1,15	1,17	1,11	-	0,99	1,01	1,04	1,01	-	24,5	24,5	1,69	1,29	-
Doxorubicin 0,04 µg/mL		DI	150,1	143,6	186,5	167,6	DF	0,98	0,96	1	0,98	MNC	12,5	14,5	19	24,5
	Dox 0,04	150,1	-	-	-	-	0,98	-	-	-	-	12,5	-	-	-	-
	LA 12,5	143,6	1,05	-	1,30	1,17	0,96	1,02	-	1,04	1,03	14,5	1,16	-	1,31	1,69
	LA 25	186,5	1,24	1,30	-	1,11	1	1,02	1,04	-	1,02	19	1,52	1,31	-	1,29
	LA 50	167,6	1,12	1,17	1,11	-	0,99	1,02	1,04	1,01	-	24,5	1,96	1,69	1,29	-
Doxorubicin 0,2 µg/mL		DI	167,8	143,6	186,5	167,6	DF	1	0,96	1	0,99	MNC	19	14,5	19	24,5
	Dox 0,2	167,8	-	-	-	-	1	-	-	-	-	19	-	-	-	-
	LA 12,5	143,6	1,17	-	1,30	1,17	0,96	1,05	-	1,04	1,04	14,5	1,31	-	1,31	1,69
	LA 25	186,5	1,11	1,30	-	1,11	1	1,01	1,04	-	1,01	19	1	1,31	-	1,29
	LA 50	167,6	1,00	1,17	1,11	-	0,99	1,01	1,04	1,01	-	24,5	1,289	1,69	1,29	-
Doxorubicin 0,5µg/mL		DI	181,4	143,6	186,5	167,6	DF	1	0,96	1	0,99	MNC	19,5	14,5	19	24,5
	Dox 0,5	181,4	-	-	-	-	1	-	-	-	-	19,5	-	-	-	-
	LA 12,5	143,6	1,26	-	1,30	1,17	0,96	1,05	-	1,04	1,04	14,5	1,345	-	1,31	1,69
	LA 25	186,5	1,03	1,30	-	1,11	1	1,01	1,04	-	1,01	19	1,026	1,31	-	1,29
	LA 50	167,6	1,08	1,17	1,11	-	0,99	1,01	1,04	1,01	-	24,5	1,256	1,69	1,29	-

PIc (Index of Proportionality in relation to the control); PIt (Proportionality index between treatments); MNC (Number of Micronucleated Cells); Dox (Doxorubicin); LA (L-asparaginase)

Discussion

It has been clarified in the literature that the main mechanism of action of L-asparaginase consists of the hydrolysis of asparagine in aspartic acid and ammonia. The malignant cells of Acute Lymphoblastic Leukemia, by genetic silencing, do not possess L-asparagine-synthetase and need to capture the extracellular L-asparagine that is essential for the synthesis of proteins for their survival. (Narta et al., 2007; Badoei- Dalfard, 2015).

Studies on structure and possible mechanisms of action of L-asparaginases indicate that these enzymes, formed by a tetrameric structure, may have two active interfaces and that each of these active sites be integrated by the catalytic triads I and II (Sanches et al, 2007 Palm et al., 1996). The catalytic triads work simultaneously causing, respectively, hydrolysis of the substrate and release of the product. The mechanism of action, according to Sanches et al. (2007), begins with catalysis, where an acid residue of the triad I activates a base through the extraction of H +. Immediately, as in a cascade, the base activates a nucleophile that covalently binds the substrate to an intermediate acyl-enzyme and releases ammonia in a process called acylation. Concomitantly, another process, deacylation, is initiated where an activated nucleophile of the catalytic triad II activates a water molecule that acts as a second nucleophile and causes the release of the product and free enzyme.

L-asparaginase has been reported as a potent antitumor agent for the treatment of acute lymphoblastic leukemia, and its proven activity and cytotoxicity have been extensively explored (Narta et al., 2007; Mahajan et al, 2014). However, research aimed at clarifying the effects of L-asparaginase at the molecular level is limited and therefore, the safety of administering this substance for a long time cannot be proven. The literature reports that some asparaginases may present toxicity in individuals who use them, causing unwanted reactions caused by side effects. However, the scientific literature is scarce in studies that explain how L-asparaginase exposure causes DNA damage in cancer cells, indicating that it is extremely necessary to conduct a number

of studies to determine the genotoxic mechanism of this drug. Therapeutic exposure to cytostatics constitutes a major exposure factor, so that specifically, many chemical and physical agents used as cytostatics have been shown to induce chromosomal damage both in vitro experiments and in cytogenetic monitoring studies of patients treated with this type of therapy (Sorsa et al., 1985); in addition, many chemotherapeutics influence DNA, and damage to this molecule can produce chromosomal aberrations causing chromosomal instability and mutagenesis (Attia, 2008). Thus, this study using L-asparaginase is one of the pioneers to report the genotoxic analysis of this enzyme and helps to elucidate its possible toxic mechanisms.

The genotoxic potential of several types of compounds can be achieved through in vitro or in vivo experiments in different cell types using various bioassays. The single cell gel electrophoresis assay, known as the alkaline ketone assay, has been widely used to evaluate in vitro genotoxicity of eukaryotic cells, since it is possible to identify the micro-damage, which are primary DNA damage, such as breaks single and / or double chains, alkali-labile lesions and bridges between chains. In this assay, tail length increases with the extent of DNA damage (Tice et al., 2000). To corroborate studies of DNA damage, the formation of micronuclei was also studied. The Micronucleus Test is also used to evaluate genotoxicity in eukaryotic cells, which evaluates genomic macrodynes, such as chromosomal breaks and mitotic dysfunctions (Fenech et al., 1993). The micronuclei and alkaline comet assays have been carried out with great frequency, since these genotoxicity tests present great reliability and sensitivity in the detection of damage to the DNA (Bopp et al., 2008).

In the genotoxic L-asparaginase assay, the Damage Frequency (DF) analysis revealed that this parameter was lower for all tumor lines tested (NCIH-292, MCF-7 and MOLT-4) when compared to healthy PBMC lines. These results indicate that such cells may be developing resistance to genotoxic treatment induced by L-asparaginase. The observation of Damage Indices (DI) reveals that the concentration of 50 μg/mL caused a significant increase of observable damage in the three tumor lines when compared to healthy PBMC cells. The other concentrations did not

present higher damage indexes, except for the MCF-7 lines that showed an effective response at the concentration of 12.5 μg/mL.

The evaluation of macro-damage formation by the expression of micronuclei revealed that only the NCIH-292 line presented higher results than the normal PBMC line in the frequency parameter of micronucleated cells, however, the statistical analyzes prove that none of the lineages, including NCIH -292, did not present significant responses when compared to the PBMC control. These non-significant results from micronucleus analysis may be related to the increase of micronuclei formed by the normal lines when in contact with L-asparaginase. Migliori et al. (1991) Report that the frequency of micronuclei in patients during therapy are higher compared to healthy patients and those who have already completed treatment. The induction of micronuclei also increases according to the prolonged treatment time to which the patients are submitted.

Both micro-damage and macro-damage were found in PBMC cells, when evaluated through genotoxic tests. It was possible to observe a significant result in the damage index at the concentration of 25 μg/mL. The frequency of micronuclei of L-asparaginase may be considered dose-dependent, as the increase in micronuclei frequency increased with the concentration of L-asparagine to which it was subjected.

When L-asparaginase catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia, normal tissues tend to compensate and perform L-glutamine conventions as an amine acid donor (He et al., 2014). Some L-asparaginases possess this lateral glutaminase activity and may, even in small amounts, hydrolyse glutamine into L-glutamic acid and ammonia. Both glutamine and asparagine are precursors of purine and pyrimidine biosynthesis for DNA replication. The depletion of these amino acids by the action of L-asparaginase can lead to significant inhibition of protein and DNA synthesis and the cells stop in the G1 phase of the cell cycle, leading to the decline of mitotic cells. In addition, ammonia resulting from the hydrolysis increases cellular

toxicity, DNA fragmentation and induction of micronuclei (Avramis et al, 2014; Mallek et al, 2016).

Depending on the dose of the treatment applied, normal cells may present greater genomic damage when compared to tumor cells. This fact may be related to a process of cellular non-selectivity (Lins et al, 2015). Cell non-selectivity is commonly seen in antitumor genotoxic agents that generally target cells that are in the process of cell division. Thus, genotoxic compounds do not distinguish their action in healthy cells from action in tumor cells, which are constantly in division, and causes genomic damage independent of the cell type. This process, therefore, is associated with an increased risk of drug toxicity (Petkova, 2013).

In leukemic cells, toxic effects are classified as effects related to immunological sensitization to a foreign protein and effects related to the inhibition of protein synthesis (Wang et al, 2003). The literature reports that the molecular basis of cell resistance to L-asparaginase is a problem of great clinical relevance, but remains little understood. Some hypotheses have been proposed in an attempt to explain such mechanisms of resistance to *E. coli* L-asparaginase. The first hypothesis is based on the fact that in some cells of acute lymphoblastic leukemia that showed resistance to the drug there was a significant increase in the levels of asparagine synthetase. Another hypothesis suggests the so-called "silent hypersensitivity", a mechanism that induces a host response leading to the production of anti-asparaginase antibodies. Such antibodies act by neutralizing L-asparaginases enzymes resulting in the inhibition of enzymatic activity (Chakrabarti e Schuster, 1997; Asselin, 1999; Woo et al, 2000).

In breast cancer cells, such as MCF-7, multiple drug resistance (MDR) is a major concern and the primary factor in the failure of chemotherapy (Vtorushin et al, 2014). The lower rates of genomic damage observed through the micronucleus assay suggest that these cells express effective resistance mechanisms in their defense. One of the associated mechanisms is the overexpression of P-glycoprotein (P-gp), which is the most frequent cause of MDR in these cells (Xiang and Gao,

2010). P-gp is a membrane carrier encoded by the MDR1 gene, which acts as efflux pumps of drug of the cell membrane (Jiang et al, 2016). Thus, L-asparaginase that could cause some damage at the cellular level when administered could be ejected out of the cell due to such efflux mechanism.

The low genotoxic activity in breast and lung cancer lines may be related to the fact that some types of tumor cells may develop defense mechanisms against the action of chemotherapeutic agents, which induce the cell to resort to the action of membrane proteins forcing The elimination of the drug out of the cell. The mechanism consists of the performance of a protein called MDR1 (Multi-drug Resistance 1) or P-glycoprotein, which acts as an efflux pump facilitating the elimination of substances from the cell, mainly chemotherapeutic drugs, such as topoisomerase inhibitors and antimetabolites (Bao et al., 2012). The presence of this MDR1 protein can be observed in several healthy human and tumor tissues, including lung and breast. Some studies on these cells are described in the literature and the results indicate that the main physiological function of these molecules in these cell types is to provide cellular protection against the action of toxic substances (Wright et al, 1998; Tao et al, 2013).

The genotoxic effect of L-asparaginase on some cell types may be so high that it is difficult to classify damage levels through the comet or micronucleus assay. This is due to the fact that marked cellular damage leads to cell death induced by apoptosis and decreases cell viability through cytotoxic mechanisms (Mehta et al, 2014). The study by Mehta et al (2014) shows that *E. coli* L-asparaginase II induces cell death through the mitochondrial-dependent apoptotic pathway mediated by the release of cytochrome c. It is estimated that other drugs used in the treatment of leukemias may present different forms of induction of cell death, such as cell death by caspase-mediated apoptosis (Suppipat et al, 2012). The action of tumor suppressor genes, such as regulation by p53, induces apoptosis in the G1 phase of the cell cycle, being observed mainly in cells that respond to signs of death induced by DNA damage or deprivation of several growth factors (Meikrantz and Schlegel , 1995).

L-asparaginase from *Streptomyces ansochromogenes* UFPEDA 3420 through this study demonstrated genotoxicity induced in the tested lines. However, it was also possible to observe that both the different tumoral lines and the healthy lines present mechanisms of resistance to drugs that can effectively act in the decrease of the activity of the L-asparaginase. Thus, it is emphasized the importance of the continuity of the studies, for a specific administration and of greater sensitivity and efficiency of L-asparaginase in the treatment of leukemias and other types of cancers.

Conclusions

L-asparaginase is an enzyme used as the chemotherapeutic agent of choice for the treatment of Acute Lymphoblastic Leukemias. However, in the literature there are no reports of studies evaluating the genotoxic mechanisms of this drug. L-asparaginase produced by *Streptomyces ansochromogenes* UFPEDA 3420 showed genotoxic activity in all the tumor lines tested, which was verified through the comet and micronuclei assay. However, high levels of genomic damage were also observed in healthy cells, mainly in the intermediate concentration compared to the tumor lines, which proves that this enzyme has mechanisms of non-specific target genotoxicity. Evidence for the genotoxic effects observed in this study suggests that new investigations into the genotoxic effects of L-asparaginase on both normal and tumor cells should be undertaken in order to elucidate their real mechanism of action and further suggest future studies to make the action of the L-asparaginase molecule more targeted and studies of the chemical incorporation of this enzyme in order to potentiate its genotoxic action against the tumor cells ensuring their administration in the conventional antineoplastic therapies.

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Artigo 3

6 ARTIGO 3

6.1 L-asparaginase isolated by *Streptomyces ansochromogenes* promotes TH1 profile and activates CD8+ T cells in human PBMC: an in vitro investigation

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L-ASPARAGINASE ISOLATED FROM *Streptomyces ansochromogenes* PROMOTES TH1 PROFILE AND ACTIVATES CD8$^+$ T CELLS IN HUMAN PBMC: AN *IN VITRO* INVESTIGATION

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Abstract

L-asparaginase is an aminohydrolase and is an indispensable component of the chemotherapeutic treatment of Acute Lymphoblast Leukemia (ALL) and Acute Myeloid Leukemia (AML). Currently, L-asparaginase preparations from *Escherichia coli* (Asparaginase®, Kidrolase® and Elspar®) and *Erwinia carotovora* (Erwinase®) are marketed for their action mechanism which hydrolyzes asparagine into aspartic acid and ammonia, leading to nutritional stress in tumor cells. However, the treatment can promote immunosuppression and other side effects. A L-asparaginase produced by *Streptomyces ansochromogenes* UFPEDA 3420 actinobacteria was used in this study against human lymphocyte cultures to evaluate the immunological profile induced by this enzyme. Cultures of PBMC were stimulated for 24 hours with *S. ansochromogenes* L-asparaginase and cytotoxicity, cell viability, cell stimulation and cytokine production were analyzed. This new *S. ansochromogenes* L-asparaginase induced activation and proliferation of the $TCD8^{+}$ lymphocyte subset and produced higher TNF-α, IFN-γ, IL-2 and IL-10 levels in a 24 hour assay. These results show that *S. ansochromogenes* L-asparaginase is a promising molecule to be used in *in vivo* models and to deepen pre-clinical tests.

Keywords: Actinobacteria, L-asparaginase, Lymphocytes, Immunomodulation.

1 Introduction

Actinobacteria are classified as Gram-positive bacteria, have a mycelial lifestyle, and are very important to biotechnology because they produce many natural compounds. Additionally, these bacteria are responsible for producing more than two-thirds of all known secondary metabolites [1,2].

Streptomyces is the most important genus of this group because of its relevant capacity and versatility to produce metabolites such as antibiotics, herbicides, insecticides, antifungals, antitumor or immunosuppressant drugs and anthelmintic agents [3-7]. *Streptomyces ansochromogenes* is a species that can be isolated from the soil environment and is related to producers of antibiotics like Nikkomycin and Tylosin [8-10]. Moreover, bioactive compounds produced by members of the genus *Streptomyces* have been researched and L-asparaginase enzyme production has also been widely explored [11].

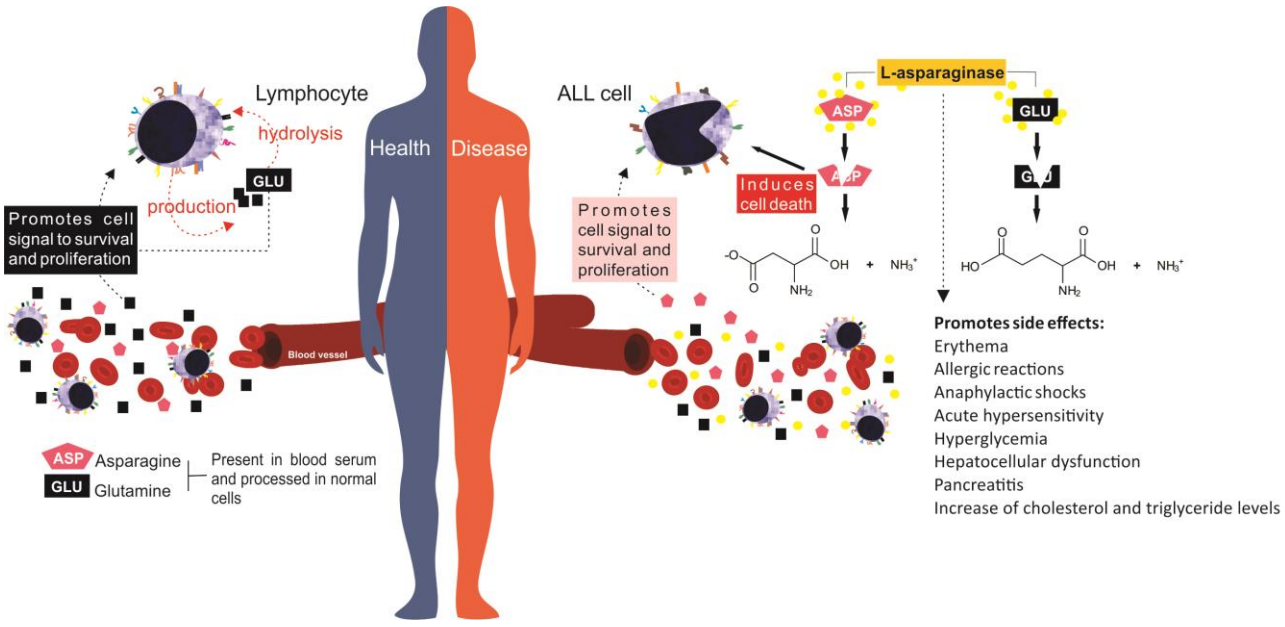
L-asparaginase is an aminohydrolase of great importance used in Acute Lymphoblast Leukemia (ALL) chemotherapy [12] and other T-cell lymphomas, NK-cell tumors and Hodgkin's disease [13]. Currently, L-asparaginase preparations from *Escherichia coli* (Asparaginase®, Kidrolase® and Elspar®) and *Erwinia carotovora* (Erwinase®) are marketed [14].

The action mechanism of the L-asparaginase enzyme involves hydrolyzing asparagine into aspartic acid and ammonia (Figure 1). Cells involved in ALL are dependent on L-asparagine and the absence of this amino acid promotes nutritional stress in these cells [15-16]. Specifically, the absence of L-asparagine affects nucleic acid biosynthesis and protein formation, stopping the cell cycle and cell proliferation [17].

Normal cells have the asparagine synthetase enzyme and can promote the biosynthesis of their own asparagine, but this does not occur in ALL cells [15]. Unfortunately, L-asparagine treatment results in damage due to its toxicity. Studies have shown that this enzyme can promote

hypersensitivity or toxicity reactions due to products resulting from hydrolysis and the inhibition of protein synthesis through the decrease of plasmatic levels of L-asparagine [18-20].

Fig. 1 The action mechanism promoted by L-asparaginase treatment in patients with Acute Lymphoblast Leukemia (ALL) and other T-cell lymphomas. L-asparagine is an amino acid that is used by cells as food for their development. This amino acid is present in blood serum (from diet) and normal cells are capable of synthetizing intracellular amounts of L-asparagine. However, tumor cells are not able to synthetize L-asparagine and require this amino acid from the blood serum for their survival. The L-asparaginase enzyme hydrolyzes asparagine into aspartic acid and ammonia and this process inhibits tumor growth. In addition, L-asparagine also hydrolyzes glutamine into glutamic acid and ammonia. Studies have shown that human and murine treatment using L-asparaginase is promising against Acute Lymphoblast Leukemia but can produce many side effect and compromise the immune system [15,16,18-21]



Immunological studies on L-asparaginase have shown that this enzyme promotes immunosuppressant status *in vivo* in both human and murine models, but these results are

controversial and this status is conditioned by the age of the patients and re-exposure to many doses of L-asparaginase throughout treatment [21,22]. This study aims to evaluate the immunological stimulus, *in vitro*, against human lymphocytes promoted by a new L-asparaginase produced from *Streptomyces ansochromogenes* UFPEDA 3420 actinobacteria.

2 Material and Methods

2.1. L-asparaginase preparation

The L-asparaginase enzyme used for this study was produced by the *Streptomyces ansochromogenes* actinobacteria deposited in the Collection of Microorganisms of the Antibiotics Department with the identification number UFPEDA 3420. The microorganism was reactivated and secondary metabolite production was performed by the fermentative process under pre-established, ideal culture conditions using culture medium M9 ($Na_2HPO_4.2H_2O$, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 0.5 g/L; L-asparagine, 5 g/L; $MgSO_4.7H_2O$, 0.5 g/L; $CaCl_2.2H_2O$, 0.014 g/L; Glucose, 2 g/L; Distilled water, 1 L). Fermentation was carried out for 48 hours at pH 6 and a temperature of 37°C. Determination of enzymatic activity was carried out through the dosage of ammonia formed by Nesslerization [23]. Purification was carried out by means of two chromatographic methods: ion-exchange interaction (DEAE-diethylaminoethyl cellulose chromatography) and gel-filtration chromatography using Sephadex G75 [24]. Molecular weight determination and protein homogeneity were evaluated by SDS-PAGE gel electrophoresis [25]. The data of isolation and characterization of the enzyme L-asparaginase are in the process of publication.

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

Forty milliliters of blood from 6 voluntary donors were collected in heparin tubes (Vacuette) to obtain PBMCs through a concentration gradient technique (Ficoll-Paque™ Plus, GE Healthcare Life Sciences, Sweden). Cells were washed 2x with sterile 1mL of PBS and after centrifugation (400 *g*, 30 min, 20ºC), a layer of PBMCs was obtained, collected and cells were counted in a Neubauer chamber. Cell viability was evaluated by the trypan blue (Sigma-Aldrich, USA) exclusion method. Cells were only used when viability was >98%. Samples were collected after all volunteers signed a Free and Informed Consent form and experimental protocols were approved by the Federal University of Pernambuco Research Ethics Committee (UFPE, Brazil nº 1.870.360/2016).

2.3. Culture of lymphocytes and *in vitro* stimulus

PBMC were cultured in 1mL of RPMI 1640 medium (Sigma-Aldrich, USA) with 10% (w/v) fetal bovine serum (Sigma-Aldrich, USA) in 24-well plates (TPP Techno Plastic Products, Switzerland) at a density of 10^6 cells/well. PBMC was used to evaluate cell viability and CD4+ and CD8+ proliferation and activation assays. Supernatant of PBMC cultures were used for measure cytokine and chemokines production and nitric oxide (NO) release. All cultures were stimulated at the concentration of 6 µg/mL to 50 µg/mL of L-asparaginase and remained with this stimulus for 24 hours of incubation time. Controls were performed using untreated cells in culture medium.

2.4. Analysis of cell viability

Lymphocytes from PBMC treated with L-asparaginase for 24 hours of incubation as well as untreated cells were centrifuged at 450 *g* and 4°C for 10 min. After discarding the supernatant, 1 mL of PBS 1X was added to the precipitate and after ressuspension, the cells were centrifuged again (450 *g*, 4ºC, 10 min). The pellet was resuspended in 300µL of binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$), transferred to a labelled

cytometer tube, and Annexin V conjugated with Fluorescein Isothiocyanate (FITC) (1:500) and Propidium Iodide (PI, 20 µg⁄mL) were added. Flow cytometry was performed on a FACSCalibur platform (BD Biosciences, San Jose, USA) and the results were analyzed using CellQuest Pro software (BD Biosciences). Annexin-FITC negative/PI positive cells were considered necrotic and Annexin-FITC positive/PI negative cells were considered in the early stage of apoptosis. Double negatives were considered viable cells.

2.5. Measurement of cytokine and chemokine production in PBMC cultures

Supernatants of cultures treated or not with L-asparaginase (12.5 µg/mL) for 24 h were collected for quantification of cytokines using the CBA (Cytometric Bead Array) Human Th1/Th2 Cytokine Kit II (Becton Dickinson Biosciences, USA) for simultaneous detection of interleukins (IL-2, IL-4, IL-6, IL-10, IL-17), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ). The assays were performed according manufacturer's instructions and data were acquired on FACSCalibur platform. Six individual cytokine standard curves (0–5000 pg/mL) were run in each assay. The range of detection was between 3000 pg/mL and 5000 pg/mL. Chemokines assessment was carried out using the CBA Human Chemokine Kit (Becton Dickinson Biosciences, USA) for simultaneous detection of CCL5, CCL2, CXCL8, CXCL9 and CXCL10 in culture supernatants. The cells were analyzed using the FACSCalibur platform according to manufacturer's instructions. Five individual chemokine standard curves (0–5000 pg/mL) were run in each assay and the range of detection was between 1500 pg/mL and 3000 pg/mL.

2.6. *In vitro* nitrite analysis

The supernatants from cell cultures incubated or not incubated with L-asparaginase at 12.5µg/mL for 24 h were used for nitrite analysis by the colorimetric Griess method [26]. NO

concentration was estimated using a standard curve (3.12–100 µmol/mL). The reading was carried out in a microplate spectrophotometer (Thermo Scientific Multiskan FC, Waltham-USA) at 595nm.

2.7. Lymphocyte immunophenotyping assay

Lymphocytes present in PBMC were cultured for 24 h in 24-well plates (TPP Techno Plastic Products, Trasadingen, Switzerland) at a density of 10^6 cells/well, in the presence or absence of L-asparaginase at 12.5 µg/mL and RPMI. After this incubation time, cells were removed from plates using ice-cold 1% PBS-Wash and inserted into 15 mL polypropylene tubes (BD Biosciences) with 6 mL of PBS-Wash for centrifugation (400 x g for 10 min). After discarding the supernatant, cells were washed with 2 mL of PBS-Wash and centrifuged (400 x g for 5 min). Supernatant was discarded and surface monoclonal antibodies were added to tubes and incubated for 30 minutes. After this time, two washing steps were performed with 1 mL of PBS-Wash followed by centrifugation (400 x g for 5 min). Supernatants were discarded and cells were fixed for 15 minutes with 150 mL of Cytofix solution (BD Biosciences). Cells were washed with 2 mL of PBS-Wash followed by centrifugation (400 x g for 5 min). After discarding the supernatant, 300 µL of PBS-Wash and monoclonal antibodies were added to each tube, which were loaded onto the FACSCalibur platform. The monoclonal antibodies used in this assay were anti-CD4-PerCP, anti-CD8-FITC, anti-CD-28-PE and anti-CTLA-4-APC (BD Biosciences).

2.9. Statistical analysis

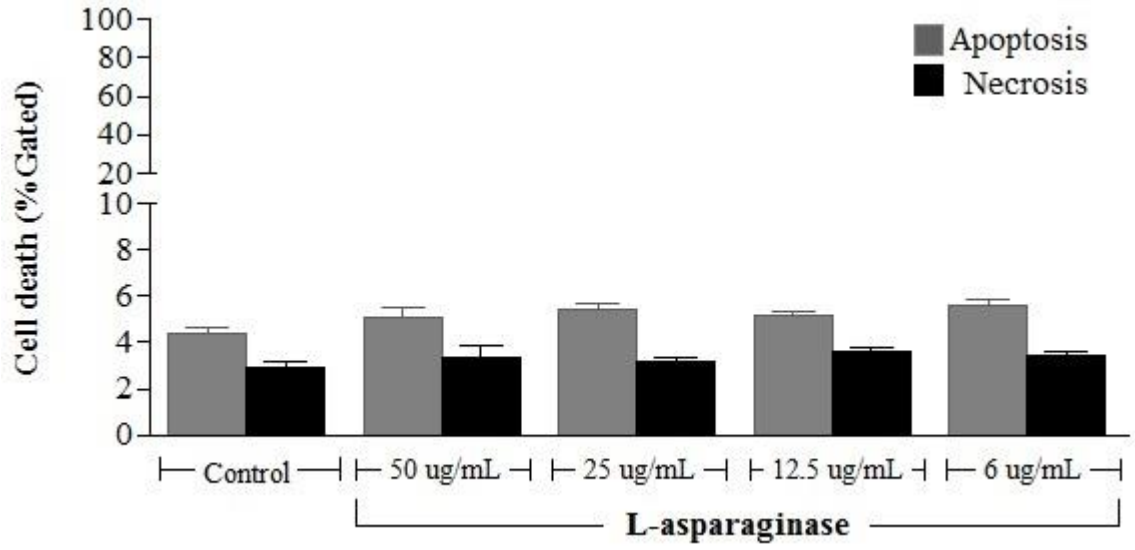
Data were analyzed using non-parametric tests. To detect the differences between groups, the Wilcoxon test was used. The Student's *t*-test was used to analyze the results from the cell viability assay. All results were expressed as mean ± SD and a value of $p < 0.05$ was considered statistically significant.

3 Results

This study evaluated the behavior of a new L-asparaginase isolated from *Streptomyces ansochromogenes* UFPEDA 3420 actinobacteria against human lymphocytes in an *in vitro* assay. Our major aim was to understand the cytotoxicity level promoted by this L-asparaginase and how this enzyme behaves in human lymphocyte cultures during 24 hours of stimulus.

Results showed that L-asparaginase isolated from *S. ansochromogenes* UFPEDA 3420 promoted higher cell viability (95%) in all concentrations tested. Furthermore, L-asparaginase did not induce significant cytotoxicity against human lymphocytes at all concentrations tested in relation to control cells (Figure 2).

Figure 2: Effects of L-asparaginase isolated from *Streptomyces ansochromogenes* UFPEDA 3420 actinobacteria against human lymphocytes. At all concentrations, L-asparaginase did not induce statistical and significant cell death in human lymphocytes. Apoptotic and necrotic values found in L-asparaginase-treated cells were similar to control cells (cells+medium). Horizontal bars represent the average of six independent experiments performed in duplicate.



After the cytotoxicity assay, the L-asparaginase concentration used to stimulate human lymphocytes in 24-hour cultures was 12.5µg/mL. Human lymphocytes were cultivated *in vitro* for 24 hours with 12.5µg/mL of L-asparaginase. Cell activation and proliferation were evaluated. Finally, culture supernatant was collected to investigate cytokine and chemokine production and nitric oxide release promoted by this enzyme.

In the immunological assays production was evaluated for seven cytokines: IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ. CCL5, CCL2, CXCL8, CXCL9 and CXCL10 inflammatory chemokines were also evaluated. Inflammatory chemokines were not produced at higher concentrations by immune cells stimulated with L-asparaginase. However, cytokine production in lymphocytes stimulated by L-asparaginase showed a Th1 profile through higher concentrations of TNF-α, IFN-γ and IL-2. In fact, TNF-α was measured at higher values in relation to the control (Figure 3) and IFN-γ was produced in greater amounts by lymphocytes stimulated by L-asparaginase than in control cells (Figure 4A). In addition, nitric oxide release, stimulated by elevated IFN-γ amounts, was higher in relation to the control in 24 hours of assay (Figure 4B).

Figure 3: TNF-α cytokine production in human lymphocytes stimulated by L-asparaginase in 24-hour cultures using a 12.5µg/mL concentration or only with cells and culture medium (control). Horizontal bars represent the average of six independent experiments performed in duplicate. The average of L-asparaginase was statistically significant in relation to the control with p values equal to 0.01.

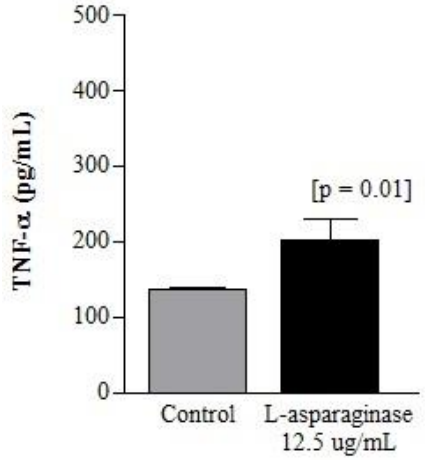
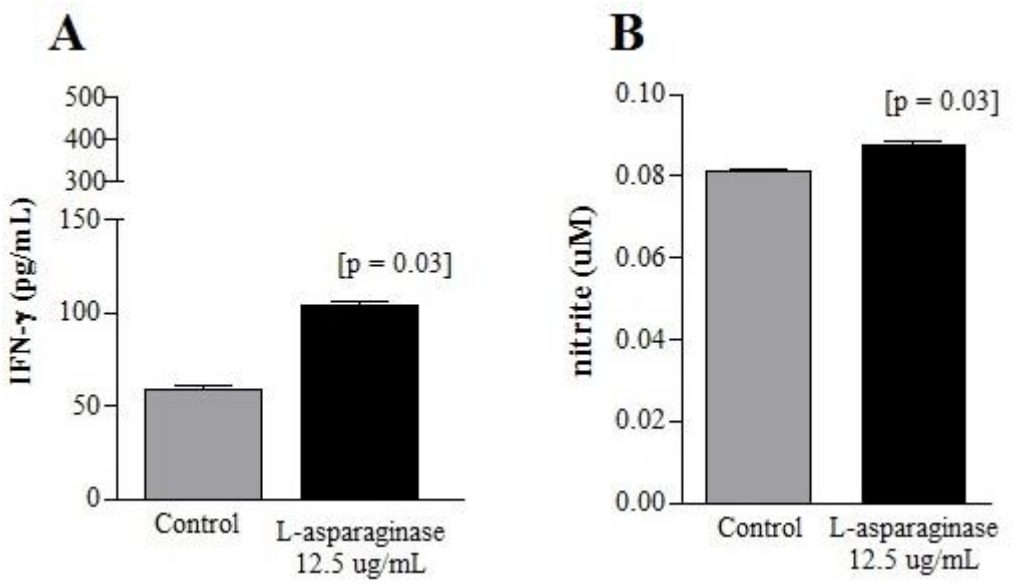


Figure 4: IFN-γ and nitric oxide production of human lymphocytes stimulated by L-asparaginase in 24-hour cultures using a 12.5µg/mL concentration or only with cell and culture medium (control). **A** – IFN-γ was produced at higher concentrations in relation to the control. **B** – Nitric oxide release by human lymphocytes stimulated by L-asparaginase was higher in relation to the control. Horizontal bars represent the average of six independent experiments performed in duplicate. The average of L-asparaginase was statistically significant in relation to the control with a p value equal to 0.03.



IL-2 is a major proliferative cytokine produced by immune cells. The action mechanism of this cytokine is directed at T lymphocytes. IL-2 produced by lymphocytes stimulated by L-asparaginase in this study showed higher values in relation to control cells and this result was corroborated by higher proliferation found in T lymphocytes, specifically $TCD8^{+}$ subsets (Figure 5A and B). IL-10 was the only Th2 cytokine that was produced in higher values by lymphocytes stimulated by L-asparaginase in relation to the control (Figure 6).

Figure 5: IL-2 production promoted by L-asparaginase in stimulated lymphocytes and proliferation of $TCD4^+$ and $TCD8^+$ subsets. **A** – IL-2 was produced in higher values by lymphocytes stimulated with 12.5µg/mL of L-asparaginase in relation to control cells. **B** - $TCD8^+$ subsets showed higher values in lymphocytes stimulated with L-asparaginase than in control cells. Horizontal bars represent the average of six independent experiments performed in duplicate. The average of L-asparaginase was statistically significant in relation to the control for IL-2 production and $TCD8^+$ subset proliferation with a p value equal to 0.02.

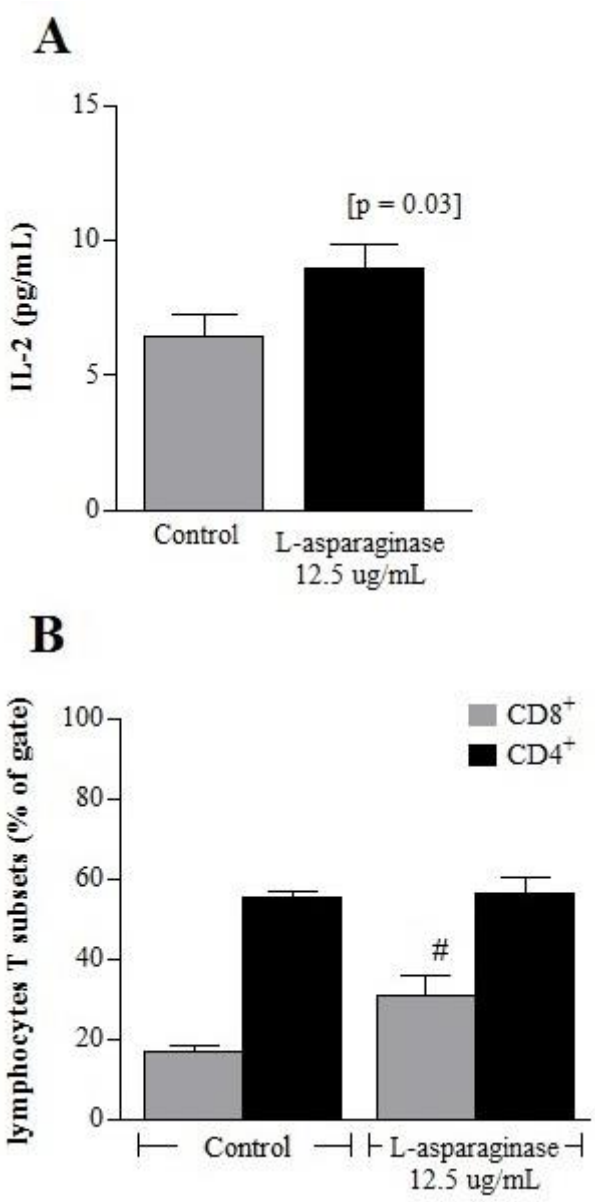
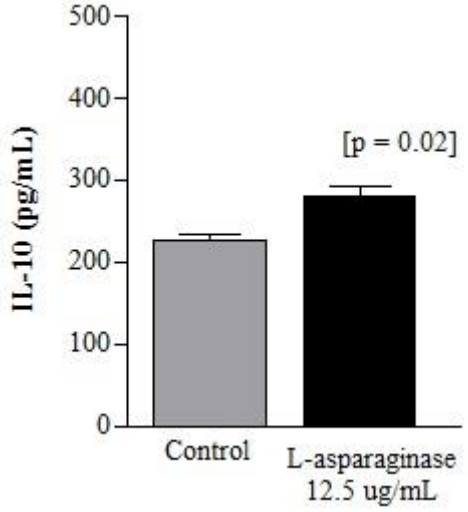
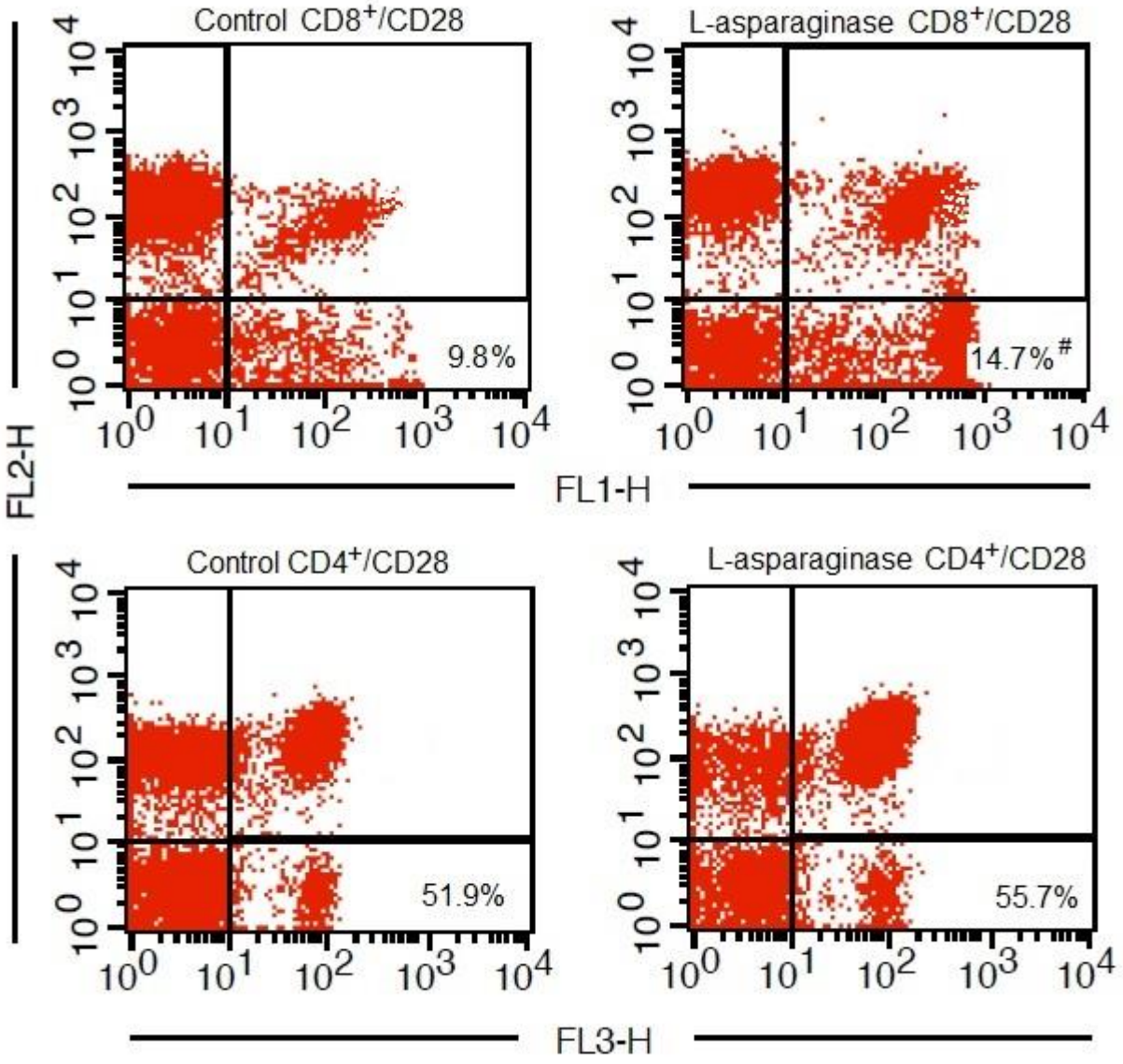


Figure 6: IL-10 cytokine production in human lymphocytes stimulated by L-asparaginase in 24-hour cultures using a 12.5µg/mL concentration or only with cell and culture medium (control). Horizontal bars represent the average of six independent experiments performed in duplicate. The average of L-asparaginase was statistically significant in relation to the control with a p value equal to 0.02.



In order to evaluate if L-asparaginase isolated from *S. ansochromogenes* UFPEDA 3420 is able to activate T lymphocytes, the assay was performed using the costimulatory molecules CD28 and CTLA-4 associated with T lymphocyte CD8 and CD4 subsets. Results showed that the new L-asparaginase was able to promote both $TCD4^+$ and $TCD8^+$ subset activation at higher values in relation to the control. However, only in the $TCD8^+$ subset were these values statistically significant (Figure 7). Results of CTLA-4 did not show higher or significant differences among L-asparaginase-stimulated lymphocytes and control cells. The average of this parameter in CD4/CTLA-4 was 1.2±0.4 x 1.2±0.3 for the control and L-asparaginase-stimulated lymphocytes, respectively, while the average in CD8/CTLA-4 was 3.4±0.4 x 3.2±0.4 for the control and L-asparaginase-stimulated lymphocytes, respectively.

Figure 7: Dot plot of T lymphocyte activation promoted by L-asparaginase isolated from *S. ansochromogenes* UFPEDA 3420 stimulus. L-asparaginase was able to stimulate the TCD8+ subset at higher concentrations in relation to control cells (p value equal to 0.02). Although TCD4+ lymphocytes stimulated with L-asparaginase showed higher values in relation to control cells, these results were not statistically significant. The dot plots represent the average of six independent experiments performed in duplicate.



4 Discussion

Bioactive molecules from many microorganisms, such as the L-asparaginase enzyme, have been isolated and investigated [15,27-28]. L-asparaginase is an enzyme used as a first-line treatment of patients with acute lymphoblastic leukemia (ALL) and pediatric acute myeloid leukemia (AML) [29]. However, it has been demonstrated that L-asparaginase possesses different effects depending on which microorganism it is extracted from [15,30].

Our study evaluated a new L-asparaginase enzyme extracted from *Streptomyces ansochromogenes* UFPEDA 3420 actinobacteria. *Streptomyces* genomes revealed that a large number of antibiotic biosynthetic gene clusters are present and potential biomolecules such as Nikkomycin [8] and Tylosin analogues [9] could be extracted from this microorganism. However, it was not possible to find studies on the immunological action promoted by metabolites of *S. ansochromogenes* and all studies of L-asparaginase action show potential immunosuppression promoted by this enzyme in *in vivo* assays.

It is worth highlighting that our results, performed using an ultrasensitive technique, flow cytometry, showed higher immunological activation promoted by our new L-asparaginase and this stimulation was sufficient to induce a Th1 profile in an *in vitro* microenvironment. The action mechanism promoted by *S. ansochromogenes* L-asparaginase can be explained by its chemical property of hydrolyzing asparagine and glutamine *in vitro*.

Many microbial L-asparaginase enzymes possess both asparaginase and glutaminase activities yet these activities differ among them. In fact, Narta et al. [15] showed that *E. coli* and *Erwinia* L-asparaginase enzymes presented 2% and 10% glutaminase activity. These findings are directly related to studies by Paquement et al. [31] who demonstrated, in lymphoblastic non-Hodgkin lymphoma treatment, that *E. coli* L-asparaginase showed a significantly higher overall survival in comparison to *Erwinia*-treated patients (100 x 74%, for *E. coli* and *Erwinia*, respectively).

Studies related to acute lymphoblastic leukemia (ALL) show that asparagine depletion of the plasma was complete in more than 90% of cases medicated with Asparaginase Medac™, 60% with Crasnitin™ and only 26% with Erwinase™ [32]. However, the main side effects observed in L-asparaginase-treated patients are with *E. coli* L-asparaginase and to ease this problem, polyethylene glycol (PEG)-conjugated enzymes are usually employed in the event of hypersensitivity reactions to the native forms or, alternatively, a mutant wild-type is used [21,33-35].

Compared to *Erwinia* L-asparaginase, *S. ansochromogenes* L-asparaginase showed higher glutaminase activity (20%) and thermal stability (data not shown). Moreover, compared to other L-asparaginase enzymes, *S. ansochromogenes* L-asparaginase promoted higher immunological activation.

L-asparaginase can be administered by the intramuscular or intravenous route. While there is no difference in clinical efficacy between these two routes, they make all the difference in anaphylactic reaction [32]. In fact, the intravenous route can trigger an anaphylactic reaction while the intramuscular route may result in hypersensitivity reactions [36]. These results can be explained by the fact that commercially available asparaginases possess 3–10% intrinsic glutaminase activity [37] and this property may induce several harmful side effects including pancreatitis, hyperglycemia, neurological seizures, as well as various hypersensitivity [38] and immunosuppression reactions [21, 39-40].

Since 1961, therapies using amino acid depletion have been demonstrated [41] and L-asparaginases extracted from both *Escherichia coli* and *Erwinia chrysanthemi*, which also present intrinsic glutaminase activity, have been used for clinical purposes [42,43]. Studies investigating the immunological mechanisms involved in hypersensitivity reactions during L-asparaginase treatment have shown an increase in immunoglobulin classes, especially IgG1, IgG3, IgG4 and IgE antibodies. Furthermore, Fabry et al. [44] suggest that the anaphylaxis mechanism may be complement- rather than IgE-mediated, inducing changes in the components of the complement system in 25% of children having anaphylactic reactions.

Although we have not tested antibody changes in our assays, chemokines analyzed in this study showed that *S. ansochromogenes* L-asparaginase induced neither complement activation nor the initial chemical signal observed in inflammation.

Alterations in the cellular metabolism of amino acids seem to constitute a nearly universal feature in many types of diseases. Therapies that promote amino acid depletion in blood serum are attractive as they leave normal cells unchanged since these cells are less demanding and/or can synthesize these compounds in sufficient amounts for their needs by alternative pathways. As a result, the principal difference between the nutritional needs of tumors and normal tissues creates a metabolic vulnerability of tumors that can be taken advantage of to preclude their survival or proliferation. Exploitation of this vulnerability is the basis of amino acid depletion therapies [30].

Glutamine is the most abundant amino acid in blood plasma and muscular tissue [45]. It is related with many cellular processes, such as cell functioning of lymphocytes, monocytes [46] and granulocytes [47] and cytokine production both in *in vitro* and *in vivo* environments [48]. Moreover, studies with different cell types have shown that an increase of cell proliferation and maintenance of cell integrity can occur when glutamine is present in the culture medium [49].

Avramis and Tiwari [21] related that it was necessary for the enzyme to persist sufficiently long in the circulation of the recipient animal for effective anti-tumor action. Consequently, multiple doses of L-asparaginase are administered in a time- and dose-dependent manner. Side effects are directly correlated to this exposure, as L-asparaginase depletes circulating asparagine, inhibiting tumor growth but also depletes circulating glutamine, promoting down-regulation signal to immunological cells [22].

In fact, based on many studies, the immunosuppressive effects of L-asparaginase are a result of decreased glutamine levels, leading to metabolic stress. As a result, use is made of glutaminase-free asparaginase [50] or increasing the supply of glutamine in the diet of L-asparaginase-treated patients [22], which appear to be promising alternatives. However, as stated earlier, the majority of studies on the immunosuppressive effects of L-asparaginase were performed *in vivo* and report the

percentage of depletion of L-glutamine in blood serum, which depends on the L-asparaginase source, on the size and frequency of treatment doses and on the antigenicity of the enzyme [33,35].

Considering that we performed our assay using lymphocytes in RPMI medium, which contains 300 mg of L-glutamine and 40 mg of L-asparagine in its constitution, and all results showed immunological activation and stimulation, which would be controversial if glutamine medium was depleted significantly, this new *S. ansochromogenes* L-asparaginase appears to be a promising molecule to be used in *in vivo* models and for further pre-clinical tests. Therefore, we conclude that the *S. ansochromogenes* L-asparaginase used in this study was shown to be an immune-stimulating enzyme in an *in vitro* assay and to promote lymphocyte activation, proliferation and cytokine production. Therefore, it can be considered a promising molecule to be used in future *in vivo* models.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflicts of interest.

Ethical approval

All the researches using the patient's material were approved by the Federal University of Pernambuco Research Ethics Committee (UFPE, Brazil nº 1.870.360/2016) and the consent was provided by the participant of the study informed by written.

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Conclusão

7 CONCLUSÃO

Streptomyces ansochromogenes UFPEDA 3420 é uma actinobactéria isolada da rizosfera de *Terminalia fagifolia*, uma planta do Bioma Caatinga, Bioma exclusivamente brasileiro. Os primeiros estudos com este micro-organismo demonstra potencial atividade antifúngica e através desta nova pesquisa pode-se constatar que *Streptomyces ansochromogenes* UFPEDA 3420 apresenta-se mais uma vez como um micro-organismo promissor na produção de metabólitos secundários de importância biológica.

Parâmetros de otimização, como tempo, pH e temperatura foram avaliados para determinação da máxima produção e atividade da enzima. A enzima L-asparaginase foi produzida por *Streptomyces ansochromogenes* UFPEDA 3420, purificada e caracterizada, sendo este o primeiro relato na literatura da produção de L-asparaginase por esta espécie de actinobactéria. A enzima apresenta comportamento Michaeliano, elevada atividade específica e atividade citotóxica frente à linhagem de leucemia.

Essa L-asparaginase de *Streptomyces ansochromogenes* UFPEDA 3420 apresentou atividade genotóxica frente à linhagens de câncer de pulmão, câncer de mama e células de leucemia linfoblática humana, quando expostas a diferentes concentrações da substância por 48 horas. Porém, também houve efeito em células mononucleadas de sangue periférico sadias, sugerindo que a enzima executa mecanismo de genotoxicidade não específico, que ainda devem ser monitorados e estudados para elucidação do real mecanismo de ação.

L-asparaginase de *Streptomyces ansochromogenes* UFPEDA 3420 também apresentou ação imunomodulatória, sendo reportada como enzima estimuladora da produção de citocinas com um perfil de Th1 específica (TNF-α, IFN-γ, IL-2 e IL-10) e, ativadora e indutora da proliferação de linfócitos TCD8+.

Diante de todos estes resultados, pode-se afirmar que L-asparaginase de *Streptomyces ansochromogenes* UFPEDA 3420, é uma molécula promissora por ter apresentado também ação imunomodulatória, sendo necessários estudos pré-clínicos detalhados para que seja possível uma futura aplicação para o uso clínico.

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Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[4] Cancer Research UK, Cancer statistics reports for the UK.
http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).
Reference to a dataset:
[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015.
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Title: L-ASPARAGINASE FROM STREPTOMYCES ANSOCHROMOGENES UFPEDA 3420 PURIFICATION, CHARACTERIZATION AND IN VITRO EVALUATION OF CYTOTOXIC POTENTIAL

Journal: Separation and Purification Technology

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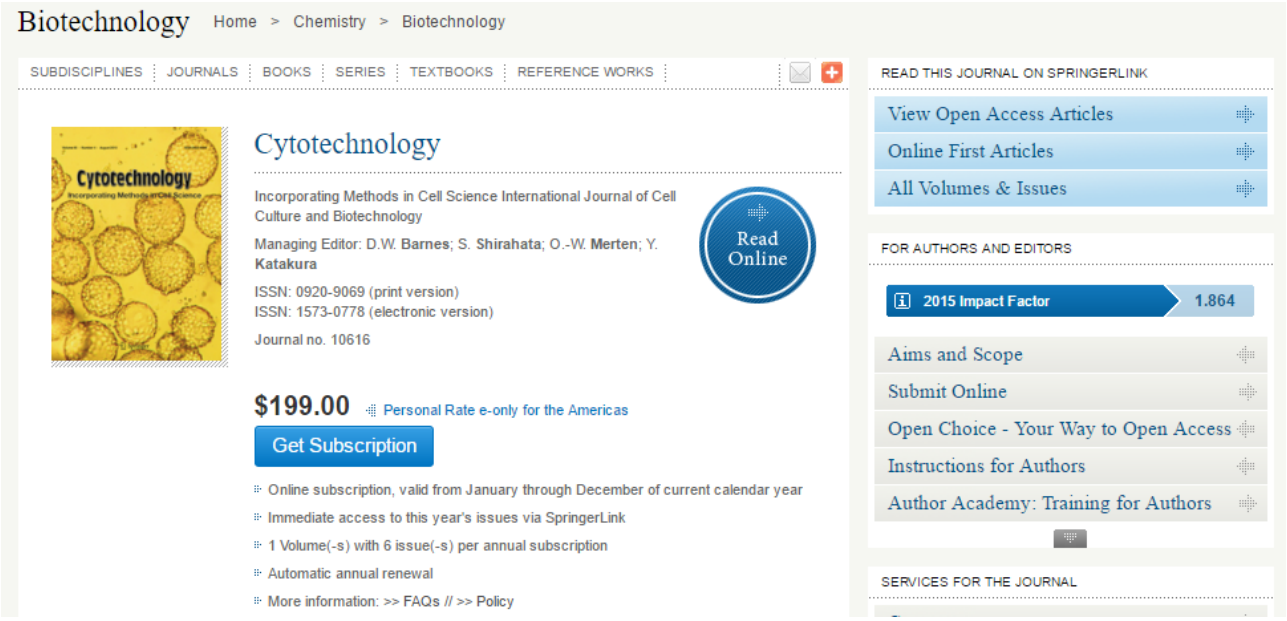
ANEXO 3 - NORMAS DA REVISTA CYTOTECHNOLOGY



Instructions for Authors

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Please provide an abstract of 150 to 250 words. The abstract should not contain any undefined abbreviations or unspecified references.

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- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

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Abbreviations should be defined at first mention and used consistently thereafter.

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Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

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Citation

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- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

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Reference list entries should be alphabetized by the last names of the first author of each work. Order multi-author publications of the same first author alphabetically with respect to second, third, etc. author. Publications of exactly the same author(s) must be ordered chronologically.

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  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329
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  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086
- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257
- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007
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  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

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- ISSN LTWA

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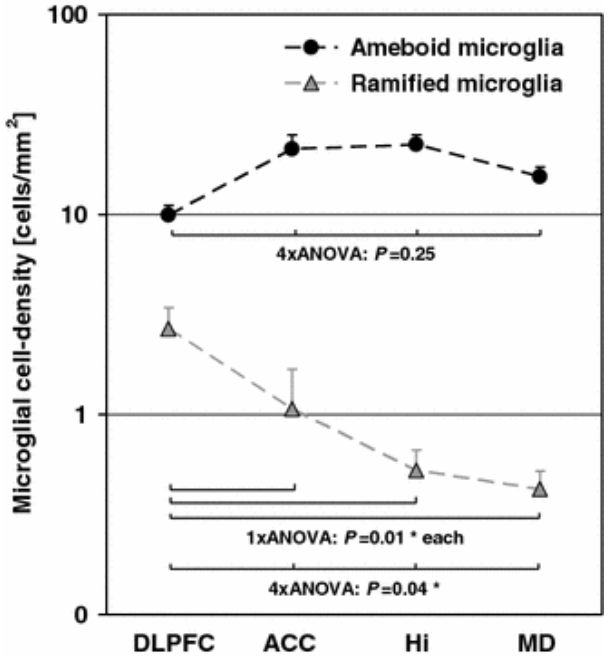

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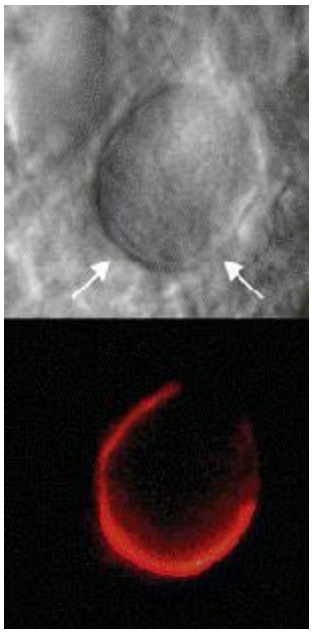

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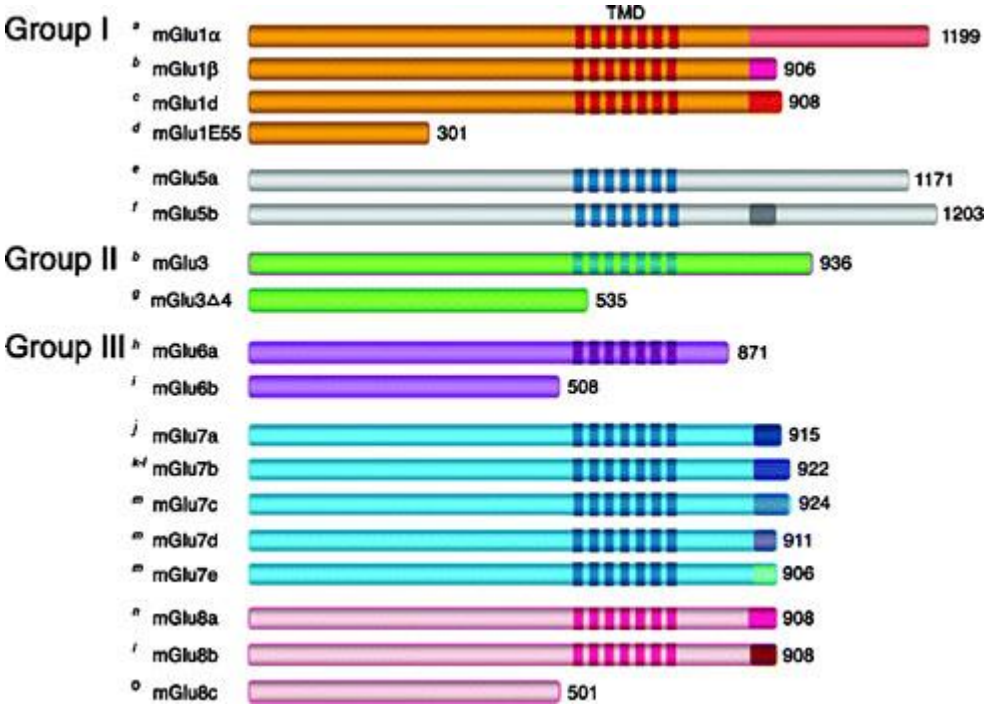

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- Combination artwork should have a minimum resolution of 600 dpi.

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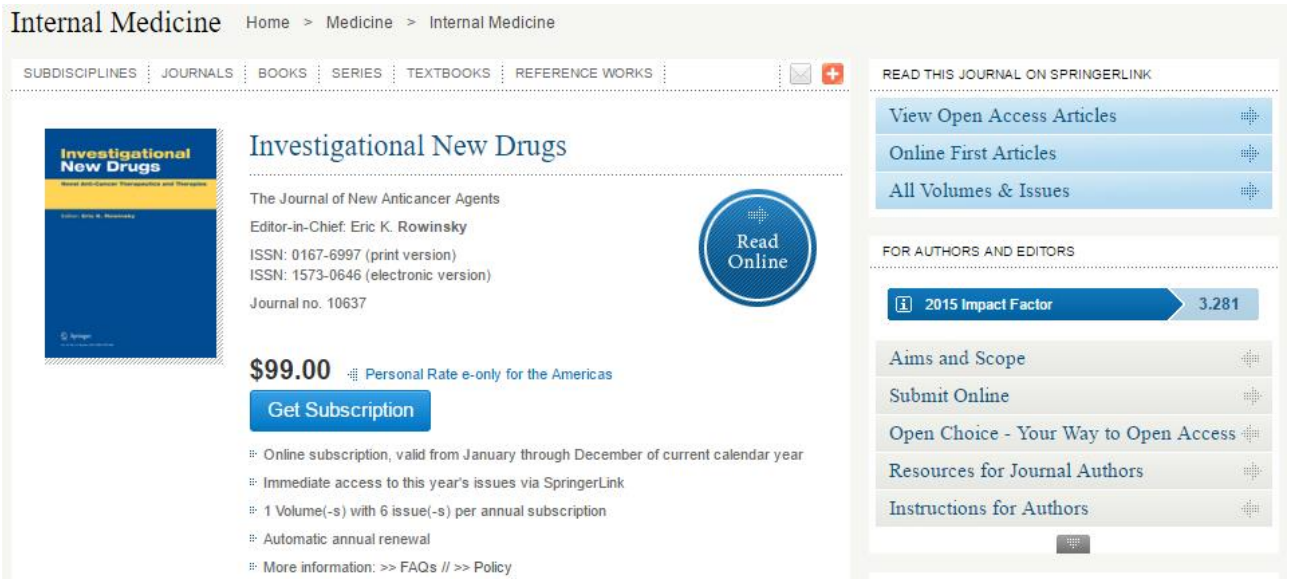

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  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

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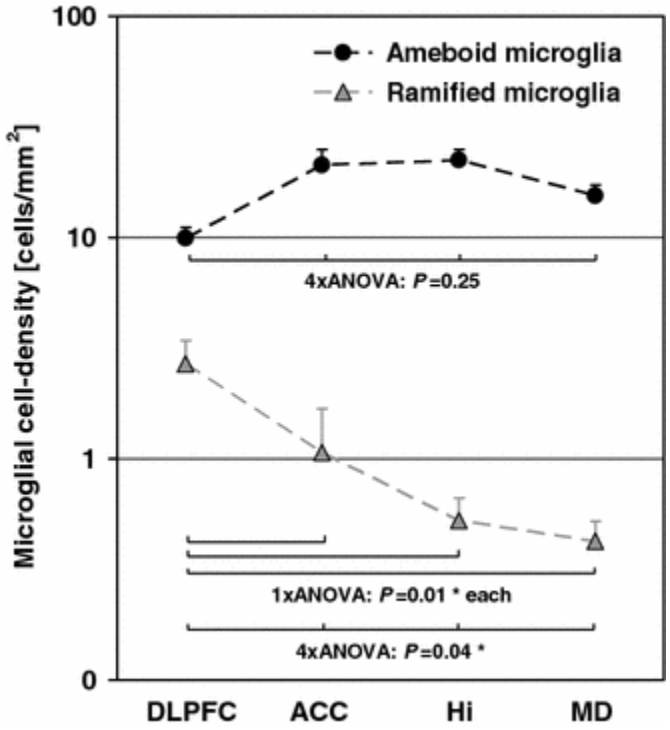

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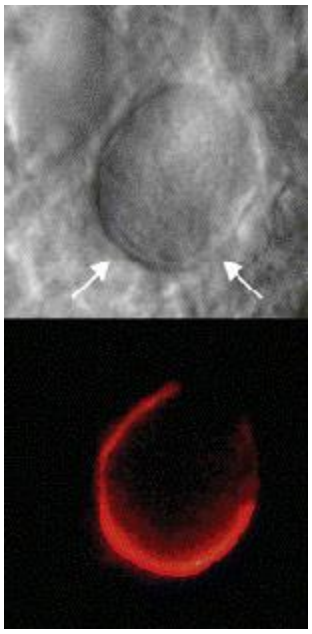

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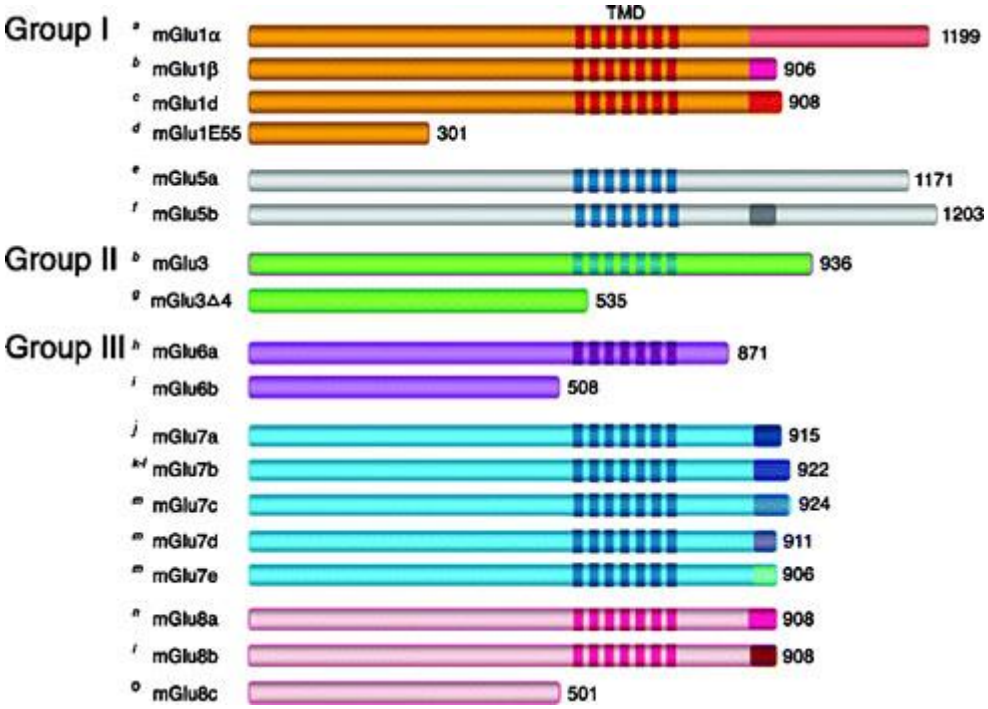

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  - English language tutorial
  - Nature Research Editing Service
  - American Journal Experts

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Authors should include the following statements (if applicable) in a separate section entitled "Compliance with Ethical Standards" when submitting a paper:

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Ethical approval: "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

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"For this type of study formal consent is not required."

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If articles do not contain studies with human participants or animals by any of the authors, please select one of the following statements:

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All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. Hence it is important that all participants gave their informed consent in writing prior to inclusion in the study. Identifying details (names, dates of birth, identity numbers and other information) of the participants that were studied should not be published in written descriptions, photographs, and genetic profiles unless the information is essential for scientific purposes and the participant (or parent or guardian if the participant is incapable) gave written informed consent for publication. Complete anonymity is difficult to achieve in some cases, and informed consent should be obtained if there is any doubt. For example, masking the eye region in photographs of participants is inadequate protection of anonymity. If identifying characteristics are altered to protect anonymity, such as in genetic profiles, authors should provide assurance that alterations do not distort scientific meaning.

The following statement should be included:

Informed consent: "Informed consent was obtained from all individual participants included in the study."

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ANEXO 6 - CARTA DE SUBMISSÃO À REVISTA INVESTIGATIONAL NEW DRUGS

Dear Mrs. Silva Lacerda:

Thank you for submitting your manuscript, "L-asparaginase isolated from Streptomyces ansochromogenes promotes Th1 profile and activates CD8+ T cells in human PBMC: an in vitro investigation", to Investigational New Drugs.

The submission id is: DRUG-D-17-00019

Please refer to this number in any future correspondence.
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The Editorial Office

Investigational New Drugs

ANEXO 7 - PARECER CONSUBSTANCIADO DO CEP - NÚMERO DO PARECER: 1.005.947

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ATIVIDADE CITOTÓXICA, GENOTOXIDADE, EXPRESSÃO DE GENES ENVOLVIDOS NA APOPTOSE E NO CRESCIMENTO CELULAR DE L-ASPARAGINASE PRODUZIDA POR Streptomyces sp.

Pesquisador: Glêzia Renata da Silva

Área Temática:

Versão: 2

CAAE: 41095315.4.0000.5208

Instituição Proponente: CENTRO DE CIÊNCIAS BIOLÓGICIAS

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.005.947

Data da Relatoria: 09/04/2015

Apresentação do Projeto:

Trata-se de um projeto de doutorado apresentado ao programa de Pós-graduação em Ciências Biológicas, pela aluna Glêzia Renata da Silva, sob a orientação da Profª.Drª. Silene Carneiro do Nascimento e co-orientação da Profª Drª Gláucia Manoella de Souza Lima

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar a atividade citotóxica, genotoxidade, expressão de genes envolvidos em processos apoptóticos e de crescimento celular da L-asparaginase produzida por Streptomyces sp. isolado do bioma Caatinga.

Objetivo específicos:

1- Produzir e purificar a enzima L-asparaginase a partir de Streptomyces sp.;

2-Observar o efeito citotóxico em células sadias e tumorais;

3-Analisar o efeito genotóxico provocado nas células sadias e tumorais;

4-Avaliar a expressão de genes apoptóticos e de crescimento celular: p16, TP53, Caspase 8 e 9, bcl-2, bax, bac e birc5 (survivina).

Avaliação dos Riscos e Benefícios:

Riscos: Coleta de sangue- Complicações são raras e geralmente de pequeno porte. Poderá haver alguma dor no momento da coleta e posteriormente levar a pequenos hematomas que desaparecem em poucos dias. No entanto, a coleta será realizada por profissionais capacitados, o que minimiza significativamente a ocorrência de tais riscos.

Benefícios: O presente estudo não possui benefício direto ao paciente. Porém, como benefício indireto, o indivíduo que aceitar participar desta pesquisa estará contribuindo para a detecção de moléculas que possam atuar no tratamento de determinados tipos de cânceres.

Comentários e Considerações sobre a Pesquisa:

A pesquisa é pertinente, poderá trazer grande contribuição nesta área tão importante de combate ao câncer.

A metodologia está clara detalhando todo o experimento e informando também onde e como serão recrutados os 10 indivíduos sadios que participarão da pesquisa.

A pesquisadora também acrescentou a informação que a linhagem de células neoplásicas foi comprada do banco de células do Rio de janeiro.

Considerações sobre os Termos de apresentação obrigatória:

Folha de rosto devidamente assinada e carimbada;

Carta de anuência do Dep de antibióticos devidamente assinada e carimbada;

Lattes dos pesquisadores anexados;

TCLE adequado para a pesquisa;

Projeto adequado em ambos os formatos.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Não há

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

As exigências foram atendidas e o protocolo está APROVADO, sendo liberado para o início da

coleta de dados. Informamos que a APROVAÇÃO DEFINITIVA do projeto só será dada após o envio do Relatório Final da pesquisa. O pesquisador deverá fazer o download do modelo de Relatório Final para enviá-lo via “Notificação”, pela Plataforma Brasil. Siga as instruções do link “Para enviar Relatório Final”, disponível no site do CEP/CCS/UFPE. Após apreciação desse relatório, o CEP emitirá novo Parecer Consubstanciado definitivo pelo sistema Plataforma Brasil.

Informamos, ainda, que o (a) pesquisador (a) deve desenvolver a pesquisa conforme delineada neste protocolo aprovado, exceto quando perceber risco ou dano não previsto ao voluntário participante (item V.3., da Resolução CNS/MS Nº 466/12).

Eventuais modificações nesta pesquisa devem ser solicitadas através de EMENDA ao projeto, identificando a parte do protocolo a ser modificada e suas justificativas.

Para projetos com mais de um ano de execução, é obrigatório que o pesquisador responsável pelo Protocolo de Pesquisa apresente a este Comitê de Ética relatórios parciais das atividades desenvolvidas no período de 12 meses a contar da data de sua aprovação (item X.1.3.b., da Resolução CNS/MS Nº 466/12).

O CEP/CCS/UFPE deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (item V.5., da Resolução CNS/MS Nº 466/12). É papel do/a pesquisador/a assegurar todas as medidas imediatas e adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e ainda, enviar notificação à ANVISA – Agência Nacional de Vigilância Sanitária, junto com seu posicionamento.

RECIFE, 31 de Março de 2015

Assinado por:
Gisele Cristina Sena da Silva Pinho
(Coordenador)

ANEXO 8 - PARECER CONSUBSTANCIADO DO CEP - NÚMERO DO PARECER: 1.870.360

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - CEP - CCS - UFPE

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Investigação imunológica e antitumoral de compostos naturais extraídos de plantas

Pesquisador: Cristiane Moutinho Lagos de Melo
Área Temática:
Versão: 2
CAAE: 62119716.5.0000.5208
Instituição Proponente: CENTRO DE CIÊNCIAS BIOLÓGICIAS
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.870.360

Apresentação do Projeto:
Trata-se de um projeto a ser desenvolvido no departamento de antibióticos desta universidade a ser conduzido pela pesquisadora Cristiane Moutinho Lagos de Melo e oito colaboradores com desenvolvimento das atividades no Laboratório de Imunoparasitologia - LIMP, Departamento de Imunologia do Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz - Pernambuco (CPqAM/FIOCRUZ - PE). No presente projeto, compostos naturais como lectinas, inibidores de proteases e frações isoladas de extratos de plantas serão investigados in vitro quanto à sua ação imunológica e antitumoral frente a linhagens decélulas normais e cancerígenas humanas.

Objetivo da Pesquisa:
Investigar in vitro mecanismos celulares e moleculares de novos compostos naturais candidatos a fármacos imunomoduladores e antitumorais.

Avaliar em cultura de células mononucleares do sangue periférico (PBMC) humano a atividade citotóxica de compostos naturais;

Determinar in vitro a atividade antitumoral das substâncias avaliadas sobre as linhagens de células cancerígenas JURKAT (câncer de próstata) e MDA MB231 (câncer de mama);

Endereço: Av. da Engenharia s/nº - 1º andar, sala 4, Prédio do CCS
Bairro: Cidade Universitária **CEP:** 50.740-600
UF: PE **Município:** RECIFE
Telefone: (81)2126-8588 **E-mail:** cepccs@ufpe.br

Investigar in vitro a liberação de cálcio citosólico e mitocondrial promovida pelos compostos;

Investigar in vitro a liberação de Espécies Reativas de Oxigênio promovida pelos compostos;

Determinar in vitro a liberação de Citocromo c e ativação das Caspases 8, 9 e 3 promovidas pelos compostos;

Quantificar a produção das citocinas IL-1, IL-6, IL-4, IL-12, TNF-e TGF- nos sobrenadantes de cultura de PBMC humano e de linhagens celulares tumorais após estimulação in vitro com os compostos;

Investigar a viabilidade celular das células tumorais tratadas in vitro com oscompostos avaliados.

Avaliação dos Riscos e Beneficios:

No caso deste projeto de pesquisa os riscos devem ser considerados mínimos, uma vez que para os experimentos de imunomodulaçãodesse projetos estamos requerendo apenas alíquotas sanguíneas de doadores voluntários sadios, para que possamos isolar os linfócitos e testar os compostos isolados in vitro. Neste procedimento acreditamos ser possível haver apenas um desconforto, especialmente psicológico, com a retirada de sangue do voluntário, assim como acontece em um hemograma comum prescrito pro um médico.

Comentários e Considerações sobre a Pesquisa:

Os compostos serão isolados poralguns grupos de pesquisa no estado de Pernambuco, provenientes do Departamento de Bioquímica da Universidade Federal de Pernambuco e Departamento de Biofísica da Universidade Federal Rural de Pernambuco. Além disso, outras atividades biológicas desses compostos já vêm sendo investigadas no Laboratório de Imunoparasitologia do Centro de Pesquisas Aggeu Magalhães/FIOCRUZPE. A pretensão desse estudo é investigar novosfármacos naturais potencialmente imunomoduladores e antitumorais, elucidando parte de seu mecanismo de ação de indução de morte celular. Os resultados deste projeto, se bem sucedido, nos permitirá chegar a uma adequada eleição de protótipos a fármacos antitumorais e imunomoduladores, bem como contribuir para o entendimento do mecanismo de ação destas classes de moléculas,aspectos que são cruciais para a inovação terapêutica. A pretensão inicial é a busca de autênticos compostos naturais candidatos a novos fármacos antitumorais, planejados para atuarem de maneira alvo-específica. Isso significa que os resultados

obtidos poderão permitir novos avanços nas estratégias imunoquimioterápicas e profiláticas (tanto a nível regional como mundial).

Considerações sobre os Termos de apresentação obrigatória:

O projeto tem um orçamento de R$ 15.750,00 e todos os itens econtram-se já adquiridos e recebidos pelos pesquisadores. O cronograma tem previsão para a coleta das amostras em dezembro de 2016 e janeiro de 2017, O projeto apresenta viabilidade para a realização com a estrutura dos laboratórios citados. Os compostos serão isolados poralguns grupos de pesquisa no estado de Pernambuco, provenientes do Departamento de Bioquímica da Universidade Federal de Pernambuco e Departamento de Biofísica da Universidade Federal Rural de Pernambuco. Apresentou carta de anuência do Laboratório de Imunoparasitologia do Centro de Pesquisas Aggeu Magalhães/FIOCRUZ-PE e do Departamento de Bioquímica da Universidade Federal de Pernambuco.

Recomendações:

Sem recomendações

Conclusões ou Pendências e Lista de Inadequações:

Sem pendências

Considerações Finais a critério do CEP:

As exigências foram atendidas e o protocolo está APROVADO, sendo liberado para o início da coleta de dados. Informamos que a APROVAÇÃO DEFINITIVA do projeto só será dada após o envio do Relatório Final da pesquisa. O pesquisador deverá fazer o download do modelo de Relatório Final para enviá-lo via "Notificação", pela Plataforma Brasil. Siga as instruções do link "Para enviar Relatório Final", disponível no site do CEP/CCS/UFPE. Após apreciação desse relatório, o CEP emitirá novo Parecer Consubstanciado definitivo pelo sistema Plataforma Brasil.

Informamos, ainda, que o (a) pesquisador (a) deve desenvolver a pesquisa conforme delineada neste protocolo aprovado, exceto quando perceber risco ou dano não previsto ao voluntário participante (item V.3., da Resolução CNS/MS Nº 466/12).

Eventuais modificações nesta pesquisa devem ser solicitadas através de EMENDA ao projeto, identificando a parte do protocolo a ser modificada e suas justificativas.

Para projetos com mais de um ano de execução, é obrigatório que o pesquisador responsável pelo Protocolo de Pesquisa apresente a este Comitê de Ética relatórios parciais das atividades desenvolvidas no período de 12 meses a contar da data de sua aprovação (item X.1.3.b., da Resolução CNS/MS Nº 466/12).

O CEP/CCS/UFPE deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (item V.5., da Resolução CNS/MS Nº 466/12). É papel do/a pesquisador/a assegurar todas as medidas imediatas e adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e ainda, enviar notificação à ANVISA – Agência Nacional de Vigilância Sanitária, junto com seu posicionamento.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_778348.pdf	10/12/2016 00:41:11		Aceito
Declaração de Pesquisadores	Carta_de_anuencia_Emmanuel.jpeg	10/12/2016 00:40:47	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Instituição e Infraestrutura	carta_de_anuencia_projeto_humanos.pdf	10/12/2016 00:40:32	Cristiane Moutinho Lagos de Melo	Aceito
Outros	resposta_ao_parecerista.docx	10/12/2016 00:40:05	Cristiane Moutinho Lagos de Melo	Aceito
Projeto Detalhado / Brochura Investigador	projeto_final.docx	10/12/2016 00:39:13	Cristiane Moutinho Lagos de Melo	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_final.doc	10/12/2016 00:38:58	Cristiane Moutinho Lagos de Melo	Aceito
Outros	termo_confidencialidadecristiane.docx	18/11/2016 12:14:31	Cristiane Moutinho Lagos de Melo	Aceito
Folha de Rosto	FRCRISTINEMOUTINHO.doc	18/11/2016 12:04:02	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	Curriculo_Vanessa.pdf	17/11/2016 23:22:29	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	Curriculo_Leydianne.pdf	17/11/2016 23:22:17	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	Curriculo_Jessica.pdf	17/11/2016 23:22:04	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	Curriculo_Glezia.pdf	17/11/2016 23:21:54	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	Curriculo_Dayane.pdf	17/11/2016 23:21:40	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	Curriculo_Camila.pdf	17/11/2016 23:21:30	Cristiane Moutinho Lagos de Melo	Aceito

Outros	Curriculo_Bruno.pdf	17/11/2016 23:20:20	Cristiane Moutinho Lagos de Melo	Aceito
Outros	Curriculo_Barbara.pdf	17/11/2016 23:19:58	Cristiane Moutinho Lagos de Melo	Aceito
Outros	lattes_cristiane.pdf	16/11/2016 02:07:15	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	carta_de_anuencia_virginia.jpg	16/11/2016 02:05:46	Cristiane Moutinho Lagos de Melo	Aceito
Declaração de Pesquisadores	carta_de_anuencia_thiago.jpg	16/11/2016 02:05:33	Cristiane Moutinho Lagos de Melo	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

RECIFE, 16 de Dezembro de 2016

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador)

28° Congresso Brasileiro de Microbiologia

De 18 a 22 de Outubro de 2015 | Centro Sul - Centro de Convenções de Florianópolis | Florianópolis - Santa Catarina - Brasil

Certificado

Certificamos que o trabalho intitulado OPTIMIZATION OF L-ASPARAGINASE PRODUCTION OF STREPTOMYCES ANSOCHROMOGENES TUR 10 com a autoria de: SILVA-LACERDA, G.R., ALBUQUERQUE, L.E.F., SILVA, I.D.G, SILVA, W.J.L., SANTANA, R.C.F., SILVA, L.A.O, NASCIMENTO, S.C., LIMA, G.M.S. foi apresentado na forma de pôster durante o 28º Congresso Brasileiro de Microbiologia realizado no Centro de Convenções de Florianópolis, na cidade de Florianópolis, SC, no período de 18 a 22 de outubro de 2015.

Profa. Dra. Marina Baquerizo
Presidente da SBM

Prof. Dr. Gustavo Henrique Goldmann
1º Secretário

Apoio: CAPES, FAPERJ, FAPESP, CNPq

Organização: SBM Sociedade Brasileira de Microbiologia

Eventos simultâneos: III Simpósio de Fermentação Alcoólica - III Simpósio de Microrganismos Fotossintetizantes - IV Simpósio de Escherichia coli Luiz Rachid Trabulsiv

Title: OPTIMIZATION OF L-ASPARAGINASE PRODUCTION OF *STREPTOMYCES ANSOCHROMOGENES* TUR 10

Authors: SILVA-LACERDA, G.R.[1]; ALBUQUERQUE, L.E.F. 1; SILVA, I.D.G. [1]; SILVA, W.J.L. [1]; SANTANA, R.C.F.[1]; SILVA, L.A.O. [1]NASCIMENTO, S.C. [1]; LIMA, G.M.S. [1];

Institution: [1]Universidade Federal de Pernambuco (UFPE), Departamento de Antibióticos (CCB/UFPE), Av. Prof. Moraes Rego - Cidade Universitária, Recife/PE - CEP: 50670-901, Brasil.

Abstract: Streptomyces, one actinobacteria gender, have been studied more each day for being characterized as a major producer of a variety of secondary metabolites, including enzymes such as L-asparaginase. This enzyme hydrolyzes asparagine and releasing ammonia yielding aspartyl enzyme. This intermediate reacts with water to form aspartic acid. Due to this action, L-asparaginase is an important chemotherapeutic agent in the treatment of acute lymphoblastic leukemia (ALL), Hodgkin's disease, tumors NK, T cell lymphomas and some subtypes of myeloid leukemias. The objective of this study was to evaluate the production of L-asparaginase by *Streptomyces ansochromogenes* TUR 10 and optimize the production conditions of this enzyme. The qualitative assay was performed using the standardized inoculum of actinobacteria in media CZ and M9 with the addition of phenol red as an indicator and L-asparagine as substrates. Quantification of L-asparaginase enzyme was determined by analysis of parameters such as: carbon sources (M9 media and TGY), fermentation time (24 to 120 hours), pH (5 to 9) and temperature (25 °C to 50 °C). The enzyme activity peak of each individual standards allow optimization of enzyme production conditions. The enzyme activity was determined by measuring the amount of ammonia formed by nesslerization, where 1 U of L-asparaginase was equal to the amount of enzyme which released 1 μM of ammonia per minute at 37 °C. The qualitative assay showed the production of the enzyme through a pink halo around the colony. Through the quantitative assay was determined that the M9 medium was best suited for production of the enzyme, with 133.9 U/mL, 3 times greater value than in the TGY medium. The kinetics showed a peak enzyme production in 48 hour period with values reached 197.27 U/mL. The pH of 6 was considered optimal for the production of L-asparaginase as compared to other pH, having an amount of 743.29 U/mL. The temperature 35 °C corresponding to more favorable condition for enzyme production among all analyzed temperatures, reaching 382.2 U/mL. The optimized conditions allow this way, the large-scale production to be successful, with levels of L-asparaginase quite high. This study demonstrates the potential of actinomycetes, especially the genus Streptomyces, such as large producers of L-asparaginase enzyme. It is essential to use different sources producing this enzyme, once L-asparaginase is a chemotherapeutic potential used for the treatment of leukemias.

Keywords: Asparaginase; Actinobacteria; Enzyme activity.

Development agency: CAPES

28° Congresso Brasileiro de Microbiologia

De 18 a 22 de Outubro de 2015 | Centro Sul - Centro de Convenções de Florianópolis | Florianópolis - Santa Catarina - Brasil

Certificado

Certificamos que o trabalho intitulado STREPTOMYCES GOUGEROTII C1.129 PRODUCER OF L-ASPARAGINASE com a autoria de: SILVA-LACERDA, G.R., SILVA, I.D.G, ALBUQUERQUE, L.E.F., SILVA, W.J.L., SANTANA, R.C.F., SILVA, L.A.O, NASCIMENTO, S.C., LIMA, G.M.S. foi apresentado na forma de pôster durante o 28º Congresso Brasileiro de Microbiologia realizado no Centro de Convenções de Florianópolis, na cidade de Florianópolis, SC, no período de 18 a 22 de outubro de 2015.

Profa. Dra. Marina Baquerizo
Presidente da SBM

Prof. Dr. Gustavo Henrique Goldmann
1º Secretário

Apoio: CAPES, FAPERJ, FAPESP, CNPq

Organização: SBM Sociedade Brasileira de Microbiologia

Eventos simultâneos: III Simpósio de Fermentação Alcoólica - III Simpósio de Microrganismos Fotossintetizantes - IV Simpósio de Escherichia coli Luiz Rachid Trabulsiv

Title: *STREPTOMYCES GOUGEROTII* C1.129 PRODUCER OF L-ASPARAGINASE

Authors: SILVA-LACERDA, G.R.[1]; SILVA, I.D.G. [1]; ALBUQUERQUE, L.E.F. [1]; SILVA, W.J.L. [1]; SANTANA, R.C.F.1; SILVA, L.A.O. [1]; NASCIMENTO, S.C. [1], LIMA, G.M.S. [1]

Institution: [1]Universidade Federal de Pernambuco (UFPE), Departamento de Antibióticos (CCB/UFPE), Av. Prof. Moraes Rego - Cidade Universitária, Recife - PE - CEP: 50670-901, Recife-PE, Brasil.

Abstract: L-Asparaginase is an enzyme of great medical importance, due of its chemotherapeutic action by extensive capacity to hydrolyze the amino acid L-asparagine in aspartic acid and ammonia. A wide variety of micro-organisms such as yeast filamentous fungi, bacteria and actinomycetes are capable of producing L-asparaginase. This enzyme has been widely used against several types of cancers, the main choice for the treatment of acute lymphoblastic leukemia. The objective of this study was to evaluate the production of L-asparaginase by *Streptomyces gougerotii* C1.129 isolated from Caatinga and optimize the conditions of production of this enzyme. Initially a qualitative assay was performed by adding 15 μL of the standardized inoculum of actinobacteria in CZ and M9 media with addition of phenol red as an indicator and L-asparagine as substrates. For the quantitative determination of L-asparaginase, the micro-organism was fermented and some parameters such as carbon sources (M9 media and TGY), fermentation time (24 to 120 hours), pH (5 to 9) and temperature (25 °C to 50 °C) were evaluated for optimization of enzyme production conditions. The quantitative assay parameters were determined by measuring the amount of ammonia formed by nesslerization, where 1 U of L-asparaginase was equal to the amount of enzyme that releases 1 μM of ammonia per minute at 37 °C. The qualitative analysis showed the production of the enzyme through a pink halo around the colony. Through the quantitative assay was determined that the M9 medium was best suited for production of the enzyme, with 101.9 U/ml, a value 3.75 times greater than in the TGY medium. The kinetics showed a peak enzyme production in a 96 hour period with values that reached 189.9 U/ml. The pH of 6 was considered optimal for the production of L-asparaginase as compared to other pH, having an amount of 937.18 U/ml. The 35 °C temperature corresponded to more favorable condition for enzyme production, reaching 273.83 U/ml among the analyzed temperatures. The optimized conditions allow this way, the large-scale production to be successful, with levels of L-asparaginase quite high. The isolated from actinomycetes of Caatinga have shown promise for production of various enzymes including L-asparaginase. The study of bioprospecting of these micro-organisms originating from Brazilian biomes, for their great biotechnological potential and possible use of the product by the pharmaceutical industry is essential.

Keywords: Optimization; Asparaginase; Acute Lymphoblastic Leukemia.

Development agency: CAPES

Antimicrobial potential of actinobacteria isolated from the rhizosphere of the Caatinga biome plant *Caesalpinia pyramidalis* Tul.

G.R. Silva-Lacerda, R.C.F. Santana, M.C.V. Vicalvi-Costa, E.G. Solidônio, K.X.F.R. Sena, G.M.S. Lima and J.M. Araújo

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ABSTRACT. Actinobacteria are known to produce various secondary metabolites having antibiotic effects. This study assessed the antimicrobial potential of actinobacteria isolated from the rhizosphere of *Caesalpinia pyramidalis* Tul. from the Caatinga biome. Sixty-eight actinobacteria isolates were evaluated for antimicrobial activity against different microorganisms by disk diffusion and submerged fermentation, using different culture media, followed by determination of minimum inhibitory concentration (MIC) and chemical prospecting of the crude extract. Of the isolates studied, 52.9% of those isolated at 37°C and 47.05% of those isolated at 45°C had activity against *Bacillus subtilis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Fusarium moniliforme*, and *Candida albicans*. When compared with others actinobacteria, the isolate C1.129 stood out with better activity and was identified by 16S rDNA gene analysis as *Streptomyces parvulus*. The crude ethanol extract showed an MIC of 0.97 µg/mL for MRSA and *B. subtilis*, while the ethyl acetate extract showed MIC of 3.9 µg/mL for *S. aureus* and MRSA, showing the greatest potential among the metabolites produced. Chemical prospecting revealed

CERTIFICADO

Certificamos que o trabalho científico abaixo indicado foi apresentado no **Congresso Nordestino de Biólogos - Congrebio 2016**, promovido pela Rede Brasileira de Informações Biológicas, realizado no período de 19 e 20 de maio de 2016.

ET-09-001 - PARÂMETROS IDEAIS PARA PRODUÇÃO DE L-ASPARAGINASE A PARTIR DE *Streptomyces parvulus* C1.129 - Isllan D'Eric Gonçalves da Silva, Luiz Eduardo Felix de Albuquerque, Hanna Katarina Lopes Ferreira, Wanda Juliana Lopes e Silva, Suellen Emilliany Feitosa Machado, Glêzia Renata da Silva Lacerda, Silene Carneiro do Nascimento, Gláucia Manoella de Souza Lima

João Pessoa, 19 de maio de 2016.

Maria Deise das Dores Costa Duarte
Comissão Organizadora
Congrebio 2016

Pedro Jusselino Filho
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Congrebio 2016

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Congrebio 2016

Verificação de autenticidade: http://congresso.rebibio.net/congrebio2016/trabalhos.html#ET-09-001

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ET-09-001

PARÂMETROS IDEAIS PARA PRODUÇÃO DE L-ASPARAGINASE A PARTIR DE *Streptomyces parvulus* C1.129

Isllan D'Eric Gonçalves da Silva[1], Luiz Eduardo Felix de Albuquerque[1], Hanna Katarina Lopes Ferreira, Wanda Juliana Lopes e Silva[1], Suellen Emilliany Feitosa Machado, Glêzia Renata da Silva Lacerda[1,2], Silene Carneiro do Nascimento[1], Gláucia Manoella de Souza Lima[1]

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RESUMO

A L-Asparaginase é considerada uma enzima importante para tratamento da leucemia linfoblástica aguda. Esta enzima é capaz de lisar a L-Asparagina em ácido aspártico e amônia. Alguns microrganismos são capazes de sintetizar essa substância, como fungos filamentosos, leveduras, bactérias e actinobactérias. O presente estudo refere-se à avaliação das condições ideais para a produção de L-Asparaginase a partir do microrganismo *Streptomyces parvulus* C1.129, que é uma actinobactéria pertencente à Coleção de Cultura de Microrganismos da Universidade Federal de Pernambuco (UFPEDA - 3408). Inicialmente foi realizado um ensaio qualitativo onde foi adicionado o inóculo padronizado em meio ágar M9 e CZ adicionando vermelho de fenol como um indicador e L-asparagina como substrato. O microrganismo foi submetido a fermentações, a fim de quantificar a L-Asparaginase, onde foram avaliados vários parâmetros, tais como fontes de carbono (caldo M9 e TGY), tempo de fermentação (24 a 120 horas), o pH (5-9) e temperatura (25 °C a 50 °C). Os parâmetros do ensaio quantitativo foi avaliado medindo a quantidade de amônia formada pela nesslerização, em que 1 U de L-Asparaginase foi igual à quantidade de enzima que liberou 1 µM de amônia por minuto a 37 °C. A análise qualitativa mostrou a produção da enzima por meio de um halo cor de rosa em torno das colônias. O ensaio quantitativo evidenciou que o meio M9 foi o mais adequado para a produção da L-asparaginase, com 101,9 U/mL de enzima, um valor de 3,75 vezes maior comparado ao meio TGY. As cinéticas de produção de enzima mostrou um pico num período de 96 horas, com quantidade de enzima que atingiu 189,9 U/mL. A temperatura de 35 °C foi considerada ideal para a produção da enzima, chegando a 273,83 U/mL entre as temperaturas testadas. O pH 6 foi considerado o melhor para a produção de L-asparaginase, em comparação com os outros valores de pH, tendo uma quantidade de 937,18 U/mL. A definição das condições ideais de produção da enzima são fatores de grande importância para a produção de L-Asparaginase em larga escala. As actinobactérias isoladas de solo e a raízes das plantas da Caatinga, tem demonstrado uma grande capacidade de produzir diversas enzimas. Dessa forma, o estudo destes micro-organismos oriundos do bioma Caatinga é importante devido ao seu alto potencial biotecnológico e possível aplicação dos seus compostos na indústria farmacêutica.

Palavras-chave: Actinobacteria; L-Asparaginase; Produção.

INTRODUÇÃO

As actinobactérias pertencem a um grupo de bactérias Gram-positivas que está amplamente distribuído na natureza e representam um elemento de grande importância para a

CERTIFICADO

Certificamos que o trabalho científico abaixo indicado foi apresentado no **Congresso Nordestino de Biólogos - Congrebio 2016**, promovido pela Rede Brasileira de Informações Biológicas, realizado no período de 19 e 20 de maio de 2016.

ET-09-002 - AVALIAÇÃO *IN VITRO* DO POTENCIAL ANTIMICROBIANO DE *Streptomyces* sp. G-27 CONTRA MICRORGANISMOS DE INTERESSE CLÍNICO - Hanna Katarina Lopes Ferreira, Suellen Emilliany Feitosa Machado, Raphael Carlos Ferrer de Santana, Luiz Eduardo Felix de Albuquerque, Isllan D'Eric Gonçalves da Silva, Glêzia Renata da Silva-Lacerda, Janete Magali de Araújo, Gláucia Manoella de Souza Lima

João Pessoa, 19 de maio de 2016.

Maria Deise das Dores Costa Duarte
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Congrebio 2016

Pedro Jusselino Filho
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Congrebio 2016

Ronilson José da Paz
Comissão Organizadora
Congrebio 2016

Verificação de autenticidade: http://congresso.rebibio.net/congrebio2016/trabalhos.html#ET-09-002

Título: Avaliação e otimização das condições de produção de L-Asparaginase por *Kribella swartbergensis* G8 (UFPEDA 3417)

Autores: Natália Coimbra da Silva Queiroz[1], Jéssica Martins Fontes[1], Glêzia Renata da Silva Lacerda[1], Janete Magali de Araújo[1], Gláucia Manoella de Souza Lima[1]

Instituição: [1]Universidade Federal de Pernambuco – Recife – PE.

Resumo: O filo das Actinobactérias possui grande importância biotecnológica por produzir diferentes metabólitos secundários, tais como antibióticos, e enzimas. A enzima L-asparaginase é um desses metabólitos, que se destaca por sua atividade antitumoral. O objetivo deste trabalho foi avaliar a produção da L-asparaginase por *Kribella swartbergensis* G8 (UFPEDA 3417) e otimizar as condições de produção desta enzima. O ensaio qualitativo foi feito por meio do inóculo da actinobactéria em placas contendo os meios M9 e CZ acrescidos de vermelho de fenol como indicador e a L-asparagina como substrato. A análise quantitativa da enzima foi feita submetendo o micro-organismo a diferentes parâmetros, como fontes de carbono (M9 e CZ), tempo de crescimento (24 a 120 horas), níveis de pH (5,0 a 9,0) e temperatura (25 a 50ºC). A atividade da enzima foi medida através da quantidade de amônia formada na reação de Nesslerização, na qual 1 U de L-asparaginase é igual a quantidade de enzima que libera 1µM de amônia por minuto a 37ºC. No ensaio qualitativo, foi observada a presença de uma zona rosa ao redor da colônia, indicando a produção de L-asparaginase. A partir do ensaio quantitativo, foi possível observar que o meio M9, após 120 horas de inóculo, foi o mais indicado para a produção da enzima, apresentando 3675 UI/mL. Para o tempo de crescimento, observou-se melhor produção enzimática em 72 horas, com 5722,22 UI/mL. Ao medir os níveis de pH, o micro-organismo desenvolveu-se melhor no pH 8,0, com atividade enzimática de 4875 UI/mL. A temperatura mais favorável foi a de 25ºC, chegando a uma produção enzimática de 1533,95 UI/mL. Com base nesses resultados, é possível afirmar que essa linhagem produz elevados níveis elevados de L-asparaginase, sendo este o primeiro relato de atividade enzimática por *Kribella swartbergensis*.

Palavras-chave: Asparaginase; Actinobactéria.

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Avaliação *in vitro* do potencial antimicrobiano de *Streptomyces* sp G-27 contra microrganismos de interesse clínico

Hanna Katarina Lopes Ferreira*, Suellen Emilliany Feitosa Machado, Raphael Carlos Ferrer de Santana, Luiz Eduardo Felix de Albuquerque, Isllan D'Eric Gonçalves da Silva, Glêzia Renata da Silva-Lacerda, Janete Magali de Araújo, Gláucia Manoella de Souza Lima

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Resumo. Actinobactérias ou actinomicetos são bactérias filamentosas gram-positivas, normalmente isoladas do solo e que constituem um dos maiores filos bacterianos. Possuem grande potencial biotecnológico, pois são reconhecidamente produtoras de enzimas, pigmentos, substâncias com ações antibióticas, antitumoral, anti-helmíntica e antifúngica, entre outros. Apesar da resistência aos antimicrobianos ser considerada um fenômeno natural de adaptação dos microrganismos às drogas, o surgimento de cepas resistentes conduz à ineficácia da terapia medicamentosa. A resistência antimicrobiana é encarada como desafio e, nesse contexto, a exploração dos produtos naturais apresenta-se como alternativa para a descoberta de novos fármacos antimicrobianos e, consequentemente, para o combate a esse tipo de resistência. Assim, este trabalho teve como objetivo investigar o potencial antimicrobiano da cepa *Streptomyces* sp G-27 frente a microrganismos de interesse clínico. O microrganismo foi cultivado em ágar ISP-2, a 37 °C, durante 120 h. Os testes de atividade antimicrobiana foram realizados em bloco de gelose, medindo 8 x 8 mm de diâmetro, frente à bactérias gram-positivas, gram-negativas e levedura. Para estes microrganismos, foram preparadas suspensões com densidade de 0,5 da Escala de McFarland, que foram semeadas em placas contendo Ágar Mueller Hinton para bactérias e Ágar Sabouraud para a levedura. Os blocos de gelose foram colocados sobre as placas inoculadas, as quais foram incubadas a 37 °C por 24 h para bactérias e a 30 °C por 48 h para a levedura. O ensaio foi realizado em triplicata. Após o período de cultivo, o diâmetro dos halos foi medido e os resultados foram obtidos pela média aritmética das triplicatas. *Streptomyces* sp G-27 apresentou atividade contra *Staphylococcus aureus*, *Enterococcus faecium*, *Escherichia coli*, *Klebisiella pneumoniae* e *Candida albicans*. Observou-se que a actinobactéria testada possui atividade antimicrobiana contra cinco dos seis microrganismos teste utilizados, revelando perspectivas sobre o potencial biotecnológico de tal microrganismo, que foi isolado da Caatinga, uma região de microbiota pouco explorada.

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