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LARISSA CARDOSO CORRÊA DE ARAÚJO VIDERES

Apodanthera congestiflora e *Myracrodruon urundeuva*: Investigação das propriedades biológicas em preparações brutas e produtos isolados

Recife

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como pré-requisito para obtenção do título de Doutor em Ciências Biológicas, Área de concentração – Biotecnologia

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Apodanthera congestiflora E *Myracrodruon urundeuva*: INVESTIGAÇÃO DAS PROPRIEDADES BIOLÓGICAS EM PREPARAÇÕES BRUTAS E PRODUTOS ISOLADOS

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Dedico este trabalho aos meus pais,

pelo amor, dedicação e apoio incondicionais e por sempre terem acreditado em mim.

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RESUMO

Myracrodruon urundeuva (Aroeira do sertão) e *Apodanthera congestiflora* são amplamente utilizadas pela população do nordeste do Brasil. Este trabalho teve como objetivo investigar atividades biológicas de preparações e metabólitos destas plantas. Extratos orgânicos da raiz de *A. congestiflora* foram avaliados quanto à constituição química, atividades antioxidante, citotóxica, hemolítica e antimicrobiana. As frações foram avaliadas quanto à constituição, citotoxicidade e atividade antimicrobiana. A cucurbitacina isolada foi investigada quanto à citotoxicidade. Valores de CI_{50} para células cancerígenas variaram de 3,7 a 15,5 µg/mL (extrato hexânico) e de 18,02 a 32,0 µg/mL (acetato de etila). Na atividade hemolítica valores de CE_{50} variaram de 594,1 a 1.087 µg/mL. As frações exibiram CI_{50} entre 0,54 a 24,35 µg/mL. A cucurbitacina foi citotóxica para células cancerígenas e normais, promovendo em HL-60 apoptose, necrose secundária e despolarização da membrana mitocondrial. A concentração mínima inibitória (CMI) e a concentração mínima microbicida (CMM) dos extratos variaram de 0,23 a 15 mg/mL e 1,87 a 15 mg/mL, respectivamente. Valores de CMI e CMM para as frações variaram de 0,125 a 2,5 mg/mL e de 0,5 a 5 mg/mL, respectivamente. O extrato hidroalcoólico de *A. congestiflora* foi avaliado quanto à constituição, atividades antioxidante, hemolítica, citotóxica, toxicidade aguda, genotoxicidade e mutagenicidade. Identificou-se terpenos, alcalóides e baixa quantidade de compostos fenólicos. O extrato hidroalcoólico apresentou atividade antioxidante (CI_{50} de 81,29 µg/mL- teste do ABTS e de 64,79 µg/mL- teste do DPPH), não apresentou atividade hemolítica e foi potencialmente citotóxico sobre células normais. Na toxicidade, o extrato não promoveu alterações em parâmetros fisiológicos, bioquímicos e hematológicos. Adicionalmente, não foi genotóxico ou mutagênico. Extratos, frações protéicas e lectinas de cerne (MuHL), entrecasca (MuBL) e folha (MuLL) de *M. urundeuva* foram avaliados quanto às atividades citotóxica, hemolítica, genotóxica e mutagênica *in vitro*. Estes extratos e frações inibiram de 2,6 a 55% e de 3,8 a 63,3% a viabilidade de células cancerígenas, respectivamente. Extratos e frações proteicas apresentaram fraca citotoxicidade sobre linfócitos (inibição entre 3,81% e 17,58%). MuBL, MuHL e MuLL reduziram a viabilidade de células cancerígenas variando de 10,51% a 42,36%, 6,35% a 62,56% e 26,8% a 68,2%, respectivamente. MuBL, MuHL e MuLL mostraram CI_{50} sobre linfócitos de 58,41, 43,19 e 62,28 µg/mL, respectivamente, e percentual de hemólise variando de 0 a 11,6. No ensaio cometa a frequência de danos promovida por MuBL, MuHL e MuLL na maior concentração foi 33,5%, 35,67% e 65,2%, respectivamente. O número de micronúcleos na maior concentração foi 7,91, 7,41 e 8,91 para MuBL, MuHL e MuLL, respectivamente. O trabalho revelou que 1) extratos da raiz de *A. congestiflora* são agentes antimicrobianos e extratos, frações orgânicas bem como a cucurbitacina isolada são citotóxicos sobre células cancerígenas; 2) o extrato hidroalcoólico da raiz de *A. congestiflora* é fonte de compostos antioxidantes sem efeitos sistêmicos tóxicos ou dano ao DNA; 3) lectinas de *M. urundeuva* não promovem danos a eritrócitos, são potencialmente citotóxicas a linfócitos humanos, com fraca genotoxicidade e ausência de mutagenicidade. Também foi definido que extratos, frações proteicas e lectinas de *M. urundeuva* apresentaram fraca ou moderada citotoxicidade para células cancerígenas.

Palavras-chave: Apodanthera. Aroeira do sertão. citotoxicidade.

ABSTRACT

Myracrodruon urundeuva (Aroeira do sertão) and *Apodanthera congestiflora* are used by the population of northeastern Brazil. This work aimed to investigate biological activities of preparations and metabolites from these plants. Organic extracts from *A. congestiflora* root were evaluated for chemical composition, antioxidant activity, cytotoxicity, hemolytic and antimicrobial activities. Fractions were evaluated for their constituents, cytoxicity and antimicrobial activity. Cucurbitacin isolated was investigated for cytotoxicity. IC_{50} values on cancer cell lines ranged from 3.7 to 15.5 μg/mL (hexane extract) and from 18.02 to 32.0 μg/mL (ethyl acetate extract). On hemolytic assay, EC_{50} values ranged from 594.1 to 1087 μg/mL. Fractions showed IC_{50} values ranging from 0.54 to 24.35 μg/mL. The cucurbitacin was cytotoxic to cancer and normal cells, promoting on HL-60 apoptosis, secondary necrosis and mitochondrial membrane depolarization. The Minimum inhibitory concentration (MIC) and the Minimum microbicidal concentration (MMC) of the extracts ranged from 0.23 mg/mL to 15 mg/mL and from 1.87 to 15 mg/mL, respectively. MIC and MMC values for fractions ranged from 0.125 to 2.5 mg/mL and from 0.5 to 5 mg/mL, respectively.The hydroalcoholic extract of *A. congestiflora* was evaluated for composition, antioxidant, hemolytic and cytotoxic activities, acute toxicity, genotoxicity and mutagenicity. Were identified terpenes, alkaloids and low amount of phenolic compounds. Hydroalcoholic extract presented antioxidant activity (IC50 of 81.29 μg/mL- ABTS test and 64.79 μg/mL- DPPH test), showed no hemolytic activity and was potentially cytotoxic to normal cells. On acute toxicity, the extract did not promote changes in physiological, biochemical and hematological parameters. Additionally, the hydroalcoholic extract was not genotoxic or mutagenic. Extracts, protein fractions and lectins from heartwood (MuHL), bark (MuBL) and leaf (MuLL) from *M. urundeuva* were evaluated for cytotoxic, hemolytic and *in vitro* genotoxic and mutagenic activities. These extracts and fractions inhibited from 2.6 to 55% and from 3.8 to 63.3% the viability of cancer cells, respectively. Extracts and protein fractions showed weak cytotoxicity on lymphocytes, with inhibition percentage varying between 3.81% and 17.58%. MuBL and MuHL promoted reduction of the viability of the cancer cells ranging from 10.51% to 42.36%, 6.35% to 62.56% and 26.8% to 68.2%, respectively. MuBL, MuHL and MuLL showed IC_{50} lymphocytes of 58.41, 43.19 and 62.28 μg/mL, respectively and percentage of hemolysis ranged from 0 to 11.6. On comet assay, frequency of damage promoted by MuBL, MuHL and MuLL at highest concentration was 33.5%, 35.67% and 65.2%, respectively. The number of micronuclei to the highest concentration tested was 7.91, 7.41 and 8.91 to MuBL, MuHL and MuLL, respectively. This work revealed that 1) *A. congestiflora* extracts are antimicrobial agents and extracts, fractions and the cucurbitacin isolated are cytotoxic against cancer cells; 2) hydroalcoholic extract from *A. congestiflora* is a source of antioxidant compounds without systemic toxic effects or deleterious action on DNA; 3) *M. urundeuva* lectins did not promote damage to erythrocytes, showed to be potentially cytotoxic to human lymphocytes with weak genotoxicity and absence of mutagenicity. Also, was defined that extracts, protein fractions and lectins from *M. urundeuva* presented weak or moderately cytotoxicity on the cancer cell lines tested.

Keywords: Apodanthera. Aroeira do Sertão. cytotoxicity.

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE SIGLAS E ABREVIAÇÕES

CMI	Concentração minima inibitória
CMB	Concentração mínima bactericida
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimetil sulfóxido
DNA	Ácido dexorribonucleico
EROs	Espécies reativas de oxigênio
FPS	Fator de proteção solar
HL-60	Linhagem celular de leucemia promielocítica
HT-29	Linhagem celular de adenocarcinoma de cólon
IC_{50}	Concentração que produz 50 % de inibição no crescimento celular
INCA	Instituto nacional do câncer
IUCN	The World Conservation Union
MCF-7	Linhagem celular de adenocarcinoma de mama humano
MOLT-4	Linhagem celular de leucemia linfoblástica aguda
MTT	brometo de 3-(4,5-dimetiltiazol-2-yl)-2,5-difenil tetrazólio
NAD	Nicotinamida adenina dinucleotídeo
NCI-292	Carcinoma mucoepidermóide de pulmão
OMS	Organização Mundial de Saúde
PBMC	Células Mononucleares de sangue periférico
PBS	Tampão Fosfato Salino pH 7,2
PHA	Fitohemaglutinina
UV	Radiação ultravioleta
UVB	Radiação ultravioleta B

SUMÁRIO

1 INTRODUÇÃO

As plantas são fontes de inúmeras substâncias bioativas, que podem ser empregadas diretamente ou atuar como protótipos para o desenvolvimento de novos produtos, merecendo destaque entre estas substâncias os metabólitos secundários, que possuem inúmeras aplicações, sobretudo para a indústria farmacêutica (TAUR; PATIL, 2011; WANG et al., 2012). Tais substâncias englobam uma ampla gama de moléculas com imensa diversidade estrutural, cuja produção está atrelada a fatores como temperatura, composição atmosférica, radiação ultravioleta (UV), ataque de patógenos, idade do vegetal, água e nutrientes disponíveis (GLOBO-NETO; LOPES, 2007; CHAVES et al., 2013; SCHREINER et al., 2014).

Durante muito tempo os vegetais constituíram a única fonte de substâncias bioativas (KORNBERG, 1997). E apesar do grande número de produtos sintéticos disponíveis atualmente, a natureza permanece como a maior fonte de diversidade estrutural, fornecendo novas substâncias constantemente (CRAGG, GROTHAUS, NEWMAN 2009). De fato, a busca contínua por substâncias bioativas tem impulsionado a realização de pesquisas visando isolar novos produtos ativos no combate às doenças ou com potencial de impedir o desenvolvimento de doenças, associados a baixos danos às estruturas normais.

Dessa forma, em virtude do aumento do número de casos de câncer e dos efeitos deletérios à células normais associados à terapêutica atual, existe uma intensa busca por produtos com atividade anticâncer (TIKOO; SANE; GUPTA, 2010; SENGUPTA et al., 2012; HOLOHAN et al., 2013). Substâncias com atividade antimicrobiana também vêm sendo estudadas, uma vez que a imensa capacidade de adaptação dos microrganismos provoca resistência aos antibióticos (SPELLBERG et al., 2008; GUZMÁN-RODRIGUÉZ et al., 2014; VENUGOPAL; YARLA; UMADEV, 2014). De fato, a descoberta de moléculas bioativas obtidas a partir de plantas agrega valor à biodiversidade.

Neste cenário, merece destaque *Apodanthera congestiflor*a (Cucurbitaceae), conhecida como batata de teiú ou cabeça-de-negro, que é uma planta endêmica do Brasil amplamente distribuída na região nordeste do país (LIMA, 2010). A raiz tuberosa de *A. congestiflora* é utilizada pela população como depurativo do sangue, para tratar manchas de pele e para coceira e no combate a dores de coluna (ROQUE; ROCHA; LOIOLA, 2010; SILVA et al., 2015). A ausência de informações científicas sobre esta

espécie justifica a necessidade de estudos que visem elucidar a composição química e propriedades biológicas de seus tecidos.

Outra espécie encontrada no bioma Caatinga é a *Myracrodruon urundeuva* (Anacardiaceae), conhecida como aroeira do sertão, uma planta endêmica da América do sul, e assim como a *A. congestiflor*a, é amplamente distribuída na região nordeste do país e utilizada pela população para tratar inúmeras desordens, como doenças dermatológicas e ginecológicas e do trato digestório (MATOS, 2002).

O uso de *A. congestiflora* na medicina tradicional justifica a investigação dessa espécie quanto à presença de moléculas com atividades biológicas de interesse para a saúde humana e os possíveis efeitos deletérios associados. Ainda, o uso popular e a presença de proteínas (lectinas) dotadas de ação inseticida e antimicrobiana em *M. urundeuva*, denotam a importância da avaliação da segurança do uso de preparações bioativas em humanos, justificando a investigação do efeito das mesmas em células.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar propriedades biológicas de preparações brutas e produtos isolados de tecidos das espécies *Apodanthera congestiflora* e *Myracrodruon urundeuva.*

2.2 OBJETIVOS ESPECÍFICOS

- Determinar o perfil fitoquímico, atividade antioxidante, citotoxicidade e atividade hemolítica dos extratos em hexano, acetato de etila e metanol da raiz tuberosa de *A. congestiflora*, bem como das frações orgânicas obtidas a partir do extrato mais ativo.

- Avaliar a atividade antimicrobiana dos extratos em hexano, acetato de etila e metanol da raiz tuberosa de *A. congestiflora* sobre bactérias gram-negativas, gram-positivas, ácido-álcool resistente e levedura;

- Analisar a ação antimicrobiana das frações obtidas a partir do extrato mais ativo sobre linhagens de *S. aureus* provenientes de isolados clínicos;

- Investigar o princípio ativo isolado a partir da fração mais ativa da raiz de *A. congestiflora* quanto à citotoxicidade em células cancerígenas e normais, bem como analisar as alterações morfológicas promovidas na linhagem leucêmica HL-60, através de microscopia óptica, e bioquímicas por citometria de fluxo (externalização da fosfatidilserina e potencial de membrana mitocondrial).

- Investigar o extrato hidroalcoólico da raiz tuberosa de *A. congestiflora* quanto à constituição fitoquímica, atividade antioxidante, citotoxicidade *in vitro* sobre células normais, atividade hemolítica e toxicidade aguda, genotoxicidade e mutagenicidade *in vivo*.

- Determinar a toxicidade *in vitro*, atividade hemolítica e atividades genotóxica e mutagênica *in vitro*, dos extratos salinos, frações protéicas e lectinas isoladas do cerne (MuHL), entrecasca (MuBL) e folha (MuLL) de *Myracrodruon urundeuva*.

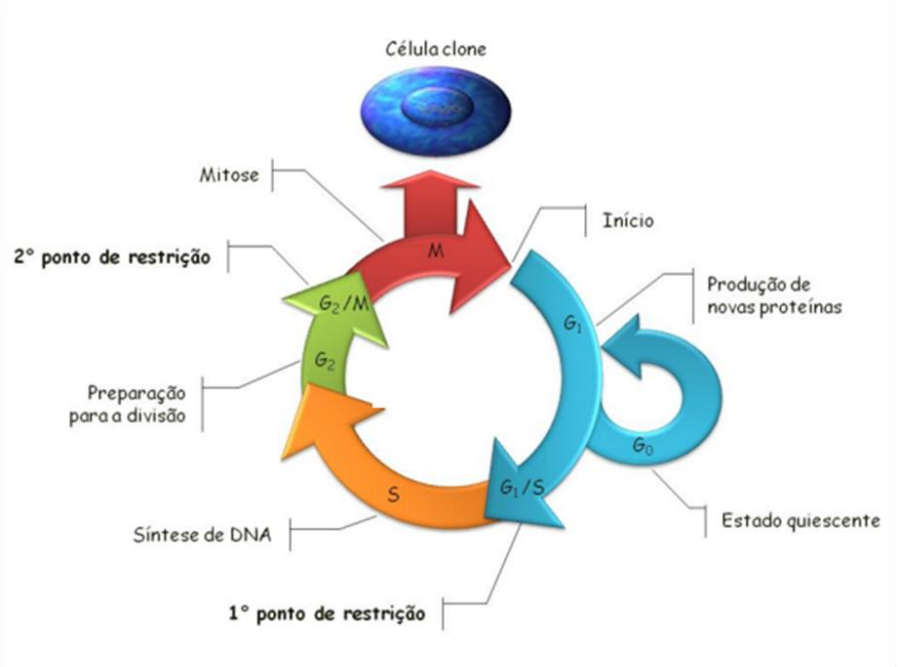
3 REFERENCIAL TEÓRICO

3.1 CICLO CELULAR

Uma nova célula surge a partir da duplicação de uma célula existente, dando origem a duas células-filhas geneticamente idênticas à original, uma vez que neste processo os cromossomos devem ser duplicados, sendo cada cópia enviada para uma célula filha diferente (YANG; HERRUP, 2007).

O ciclo celular (Figura 1) corresponde a todo o processo que envolve um período de crescimento seguido por um período de divisão da célula, podendo, portanto, ser dividido em duas etapas, a intérfase e a mitose (fase M). Na intérfase a célula está com metabolismo elevado, se preparando para a etapa seguinte (a mitose). Esta etapa de intenso metabolismo é dividida em três fases: G1, S e G2. Na fase G1 há síntese de RNA e proteínas, com aumento do citoplasma. Essa fase é a que apresenta maior duração. Ainda em G1, em um ponto de checagem, a célula pode entrar em um estado quiescente (G0) e não prosseguir a divisão, ou continuar e seguir para a fase S. Na fase S ocorre a replicação do DNA e a célula segue para a fase G2, na qual se prepara para a mitose através da síntese de proteínas e de crescimento celular. Posteriormente, na mitose, a cromatina se condensa progressivamente e os cromossomos duplicados são separados, através das fibras do fuso, em pólos opostos em dois conjuntos e são divididos de forma equitativa nas duas células formadas. Em seguida, ocorre a citocinese, que é o processo de divisão do citoplasma da célula original, para formação das duas novas células filhas (ALBERTS, 2010).

Figura 1. Ilustração esquemática do ciclo celular



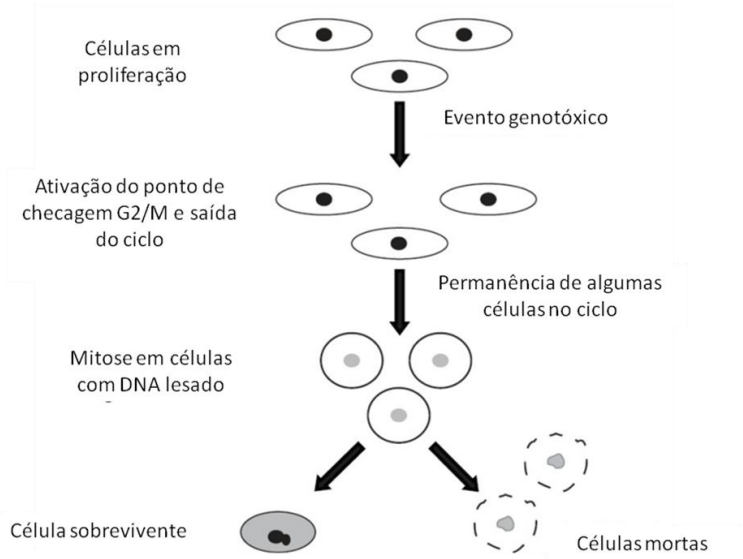
Fonte: ROBINSON; OSORIO (2001).

Ao longo do ciclo há alguns pontos de checagem que controlam a progressão da célula, permitindo ou não o seu avanço no ciclo. Em cada ponto de checagem há a verificação da integridade do DNA, com a ativação da parada do ciclo e acionamento de mecanismos de reparo no caso de identificação de alterações. Estes pontos ocorrem na transição G1/S e G2/M. Ainda, durante a anáfase também há um ponto de checagem, com bloqueio do ciclo caso as cromátides não estejam corretamente montadas no fuso mitótico. Essa checagem acontece normalmente em células normais, mas em células cancerígenas essa capacidade é perdida, fazendo com que a célula prolifere de forma descontrolada (HANAHAN; WEINBERG, 2011, BERTOLI, SKOTHEIM; BRUIN, 2013).

Quando a célula é exposta a um agente genotóxico, que cause, por exemplo, quebras na dupla fita do DNA, antes que a mitose seja iniciada, é essencial que no ponto de checagem G2/M haja desligamento do ciclo celular. Caso contrário, durante a mitose ocorrerão rearranjos cromossômicos errados e separação não equitativa dos cromossomos entre as células filhas. Muitas vezes não há reparo de todas

as quebras, mas a célula continua no ciclo e entra em mitose. Nesse caso, após a divisão celular, a maioria das células morre, mas outras podem sobreviver com alterações em seu genoma, como indica a Figura 2 (SWIFT; GOLSTEYN, 2014).

Figura 2. Proliferação celular após exposição a eventos genotóxicos



Fonte: SWIFT; GOLSTEYN (2014).

Esses pontos de checagem são controlados por proteínas quinase dependentes de ciclina (CDKs) e seus inibidores (inibidores de quinase dependente de ciclina – CDIs). Há um complexo constituído por uma subunidade catalítica (CDK) e uma subunidade reguladora (ciclina), que atua permitindo a progressão do ciclo celular. No entanto, na presença de danos as CDI atuam se ligando a este complexo, inativando as CDKs, de modo a promover a parada no ciclo celular. Há duas famílias de CDIs: a INK4, onde se inserem as proteínas p15, p16, p18 e p19 e a família CIP/KIP, onde estão incluídas as proteínas p21, p27 e p57. O gene 53 também possui papel primordial na regulação do ciclo celular da seguinte maneira: na presença de dano DNA, a proteína codificada por este gene, a p53 atua como fator de transcrição, levando à síntese da proteína p21, que ao se ligar ao complexo CDK2-ciclina, promove a parada do ciclo celular em G1. O gene p53 e outros genes associados à inibição do ciclo celular, quando mutados permitem que células danificadas prossigam no ciclo celular. Em virtude disso, o p53 está mutado na maioria dos tipos de câncer, indicando sua marcante atuação no processo

de proliferação descontrolada das células cancerígenas (PUCCI; KASTEN; GIORDANO, 2000; CHIM et al., 2006).

3.2 CÂNCER

O câncer, segundo a Organização Mundial de Saúde (OMS), é um problema de saúde pública global, associado a elevados índices de mortalidade, morbidade e altos custos econômicos. Além disso, estima-se que a partir de 2020, cerca de 20 milhões de pessoas, a cada ano, serão vítimas desta doença e em 2030 haverá 17 milhões de mortes anuais em sua decorrência.

Dados da OMS indicam que os tipos mais incidentes e responsáveis pelo maior número de mortes são os cânceres de pulmão, mama, cólon, fígado e estômago. O câncer de mama é o mais prevalente entre mulheres, sendo responsável pelo maior número de óbitos por esta doença para o sexo feminino. Enquanto entre os homens, a maior taxa de mortalidade é provocada pelo câncer de pulmão.

No Brasil, espera-se para o biênio 2016-2017 a ocorrência de cerca de 600 mil novos casos da doença, em que os tipos mais incidentes para homens serão os de próstata (28,6% dos casos), pulmão (8,1% dos casos), intestino (5% dos casos), estômago (6,0 % dos casos) e cavidade oral (5,2% dos casos) enquanto para mulheres os tipos mais incidentes serão mama (28,1% dos casos), intestino (8,6% dos casos), colo do útero (7,9% dos casos), pulmão (5,3% dos casos) e estômago (3,7% dos casos) (INCA, 2016). Para o estado de Pernambuco, segundo a estimativa realizada pelo Instituto Nacional do Câncer (INCA) (2016), espera-se mais de 2000 novos casos de câncer de próstata e de câncer de mama feminina, seguidos de 970 novos casos de câncer de colo do útero e 970 novos casos de câncer de traqueia, brônquio e pulmão.

O câncer caracteriza-se como uma doença genética, decorrente do acúmulo de alterações no DNA, como inativação de genes supressores de tumor, ativação de oncogenes, inativação de genes responsáveis pela apoptose (morte celular programada), bem como mutações ocasionadas por agentes químicos, físicos ou biológicos, com a consequente perda de controle da proliferação celular e dos mecanismos de homeostase da célula (DRAKE et al., 2012; LEE et al., 2012).

Entre os agentes físicos capazes de desencadear o processo de carcinogênese está a radiação ultravioleta (UV), cuja ação direta sobre a pele promove a formação de

dímeros entre bases pirimidinas, interações cruzadas entre DNA e proteínas, danos oxidativos a bases e quebras de fita simples (BRASH et al., 1991; GRUIJL; KRANEN; MULLENDERS, 2001). A radiação UVB (280-320 nm) é amplamente responsável pela formação desses dímeros e pela promoção de deleções e aberrações cromossomais (EMRI et al., 2000; HORIGUSHI et al., 2001 RASTOGI et al., 2010; KIM; HE, 2014). Além disso, esta radiação promove a produção de espécies reativas de oxigênio (ROS), que estão associadas ao desenvolvimento de câncer (WARIS; AHSAN, 2006; HOU et al., 2012).

Entre os agentes biológicos associados à carcinogênese, é possível destacar alguns vírus, como o Epstein-Barr, que está associado ao desenvolvimento de linfoma de Burkitt e linfoma de Hodgkin, os vírus de hepatite B e C, associados a carcinoma hepatocelular, o vírus HIV 1, associado a sarcoma de Kaposi, linfoma de Hodgkin e câncer cervical e o papilomavírus humano tipo 16 (HPV-16), associado a carcinoma cervical, de vulva, vagina, pênis, ânus e da cavidade oral (BOUVARD et al., 2009). Bactérias e platelmintos também têm sido descritos como agentes associados a carcinogênese, como por exemplo, a bactéria *Helicobacter pylori,* que está relacionada ao desenvolvimento de câncer de estômago, enquanto os platelmintos *Clonorchis sinensis* e *Schistosoma haematobium* estão associados a carcinomas de vias biliares e câncer de bexiga, respectivamente (WROBLEWSKI; PEEK, JR; WILSON, 2010; ZAGHLOUL, 2012; QUIAN et al., 2012).

Os agentes químicos associados à promoção de alterações no DNA são encontrados em grande número e podem ser de origem sintética ou natural. Entre os de origem natural, destaca-se a aflatoxina, produzida pelos fungos *Aspergillus flavus* e *Aspergillus parasiticus,* que está associada ao desenvolvimento de carcinoma hepatocelular (LIU; WU, 2010). Um dos agentes químicos sintéticos mais comuns e que está relacionado ao desenvolvimento de câncer e pulmão é o benzopireno, um hidrocarboneto aromático policíclico encontrado na fumaça de cigarros (LOEB; HARRIS, 2009).

Para que ocorra o desenvolvimento do câncer é necessário que ocorra uma série de alterações na fisiologia celular, cujos efeitos se somam, resultando na progressão tumoral. Tais alterações proporcionam o surgimento de características como capacidade

replicativa ilimitada, perda de sensibilidade a sinais de inibição do crescimento, evasão da apoptose, capacidade de realizar invasão tecidual, metástase (capacidade de colonizar novas áreas) e angiogênese (formação de novos vasos para levar oxigênio e nutrientes ao tumor). Todas estas alterações ocorrem devido à expressão de inúmeras proteínas como, por exemplo, o fator de crescimento endotelial vascular (VEGF), que consiste no principal indutor da angiogênese (processo essencial para o crescimento do tumor e metástase), survivina e C-MYC (HANAHAN; WEINBERG, 2000).

Às características citadas anteriormente, deve-se adicionar a) reprogramação do metabolismo celular, que permite à célula cancerosa seu desenvolvimento pleno; b) capacidade de fuga ao ataque do sistema imune, proporcionando às células cancerígenas a capacidade de saírem ilesas ao ataque de células de defesa como linfócitos e macrófagos; c) instabilidade genômica que permite que o acúmulo de mutações leve à progressão tumoral e d) resposta inflamatória associada ao tumor, que permite que células do sistema imune produzam moléculas que induzem a imunidade tumoral e o crescimento do tumor. As alterações envolvidas com o processo de progressão tumoral descritas estão apresentadas na Figura 3 (HANAHAN; WEINBERG, 2011).

Como mencionado na seção anterior, uma das proteínas chave para a manutenção adequada do processo de proliferação celular é a proteína p53, que na normalidade, atua como fator de transcrição de genes que impedem a proliferação de células com danos genéticos, como por exemplo, aquele da proteína p21, que promove a saída do ciclo celular na fase G1/S. A relevância da p53 é evidente ao se observar que entre 50 a 55% dos cânceres em humanos ocorre mutação no gene p53 e consequente proliferação das células com dano no material genético (MENDOZA-RODRÍGUEZ; CERBÓN, 2001; ZILFOU; LOWE, 2009). A proteína p53 também está relacionada à apoptose e atua como fator de transcrição do gene pró-apoptótico bax, podendo a sua mutação ser associada à fuga da apoptose (FRIDMAN; LOWE, 2003).

Figura 3. Alterações necessárias para a progressão tumoral.

Fonte: HANAHAN; WEINBERG, 2011.

As alterações observadas na Figura 3 representam possíveis alvos terapêuticos no combate ao câncer. Assim, inúmeras pesquisas visando à obtenção de produtos naturais ou sintéticos com atividade anticâncer têm empregado diferentes abordagens. Em virtude da manutenção da sinalização proliferativa e da evasão da apoptose observadas para as células transformadas, diversos trabalhos tem buscado por produtos que inibam a proliferação celular através da saída da célula cancerígena do ciclo celular, bem como através da ativação de vias pró-apoptóticas (HO et al., 2005; CHOI et al., 2011; RUSSO et al., 2012). Além disso, a investigação da capacidade de inibir a migração das células cancerígenas e de atuar na inibição da angiogênese, tem se mostrado cruciais para a descoberta de produtos com ação anti-metastática (LÓPEZ-MARURE; CONTRERAS; DILLON, 2011; EL-KENAWI; EL-REMESSY, 2013).

A quimioterapia é amplamente empregada na terapia do câncer por ser um tratamento sistêmico. O seu surgimento se deu com a descoberta de que a mostarda nitrogenada, um agente alquilante do DNA, era capaz de destruir as células de um tumor linfoide (CHABNER; ROBERTS, 2005). A partir deste momento, o número de novas drogas quimioterápicas tem crescido constantemente, de modo que os quimioterápicos podem ser agrupados de acordo com seu modo de ação. Os agentes alquilantes, como

ciclofosfamida e cisplatina, inserem um grupamento alquil no lugar antes ocupado por um hidrogênio, se ligando à molécula de DNA de modo a impedir a separação das cadeias. Os agentes antimetabólitos, como o metotrexato e o fluoracil, impedem a formação de componentes do DNA ou RNA. Os antibióticos antitumorais, como doxorrubicina e bleomicina, inibem a síntese do DNA ou de proteínas. Os inibidores da topoisomerase, como o etoposídeo, inibem a ação da enzima topoisomerase, relacionada ao relaxamento da molécula de DNA, que é essencial para a replicação. Ainda, os inibidores mitóticos, como o paclitacel e o docetacel, atuam sobre a proteína tubulina, impedindo que a célula complete a metáfase (BRANDÃO et al., 2010; PALUMBO et al., 2013).

Apesar do elevado número de quimioterápicos disponíveis, é preciso destacar algumas limitações relacionadas a este tipo de tratamento, que está associado à promoção de danos às células normais, com toxicidade aguda, mas reversível (WANG et al., 2004; PALUMBO et al., 2013). Ainda, o desenvolvimento de resistência à quimioterapia por parte das células cancerígenas e a ocorrência de recidivas são fatores limitantes (REBUCCI; MICHIELS, 2013; WANG et al., 2013).

A busca por novas substâncias que promovam danos mínimos às estruturas normais é imprescindível, assim como a realização de investimentos em tratamento e diagnóstico, capacitação de recursos humanos e educação. Entre os estudos realizados, existem aqueles que promovem modificações estruturais nos compostos ativos de origem vegetal para obter derivados mais potentes e com menor toxicidade. Sagar et al. (2014) avaliaram quatro derivados da substância isolada das raízes de uma planta da família Plumbaginaceae conhecida como Plumbagina (5-hidroxi-2-methil-1,4-naftoquinona), e observaram que um dos derivados apresentou menor toxicidade para células normais em comparação com a plumbagina.

Os derivados semi-sintéticos de um produto isolado das raízes de *Podophyllum peltatum* e *Podophyllum emodii*, o etoposídeo e o tenoposídeo, atuam inibindo a ação da enzima topoisomerase e efetuam bloqueio do ciclo celular nas fases S e G2, sendo empregados para linfomas, câncer nos brônquios e testículos (ROWINSKY et al., 1992; HARVEY, 1999; CRAGG; NEWMAN, 2005). A substância 4-hidroxiderricina, isolada das raízes de *Angelica keiskei*, promoveu inibição do crescimento do tumor, bem como

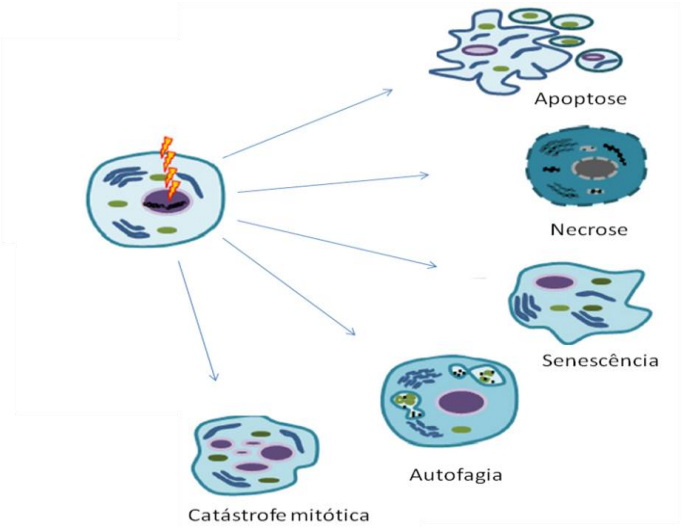
a metástase do carcinoma pulmonar de Lewis em camundongos, atuando como inibidor da angiogênese e modulador do sistema imune (KIMURA, TANIQUCHI, BABA, 2004).

3.2.1 Vias de morte celular no câncer

Um rigoroso sistema de regulação genética permite que as células do corpo continuem se dividindo ou morram, com a finalidade de evitar que células danificadas ou que tenham sofrido mutações se multipliquem, de modo a garantir a homeostasia. Caso a divisão celular seja superior à quantidade de células que morrem, devido a mau funcionamento dos genes envolvidos no controle, instaura-se um processo de proliferação descontrolada (ZIMMERMAN et al., 2013).

Na busca de melhor compreensão acerca das patologias, alguns tipos de morte celular já foram descritos, a saber: apoptose, necrose, autofagia, catástrofe mitótica e senescência (Figura 4), levando em consideração aspectos morfológicos, bioquímicos imunológicos e funcionais (BLANK; SHILOH, 2007). A Tabela 1 mostra as principais alterações morfológicas que ocorrem na membrana, no citoplasma e no núcleo das células em cada tipo de morte descrito.

Figura 4. Tipos de morte celular



Fonte: Adaptado de MINAFRA; BRAVATÁ (2014).

Tabela 1. Características morfológicas de cada via de morte celular.

Alterações	Tipo de morte				
	Apoptose	Autofagia	Necrose	Senescência	Catástrofe mitótica
Membrana	Blebbing, integridade de membrana	Blebbing	Perda de integridade de membrana	Aplanamento; Aumento no tamanho da célula	-
Núcleo	Condensação da cromatina, frgmentção nuclear, fragmentação do DNA	Condensação parcial da cromatina, ausência de fragmentação de DNA	Degradação aleatória do DNA	Acúmulo de focos de heterocromatina	Segregação incorreta durante a citocinese; micronúcleos
Citoplasma	Fragmentos celulares condensados ligados à membrana	Aumento do número de vesículas autofágicas, degradação de Golgi, polirribossomas e RE	Inchaço de organelas celulares	Granularidade	-

Fonte: Ricci; Zong (2006).

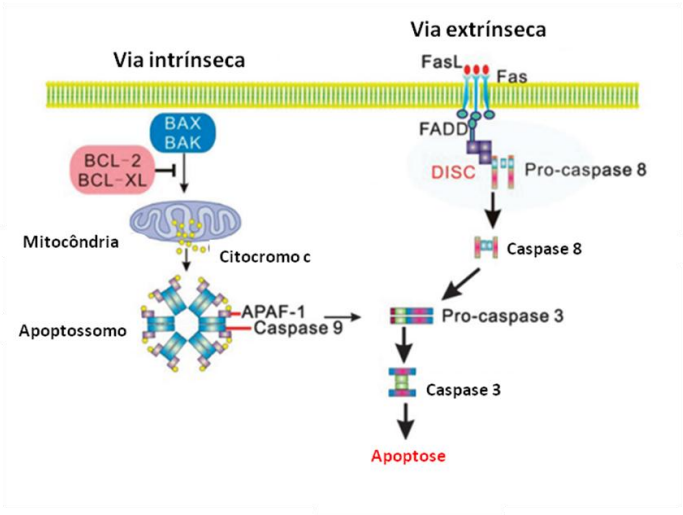
A apoptose é uma morte celular programada, com as seguintes características morfológicas: encolhimento da célula, formação de blebbing de membrana (prolongamentos da membrana plasmática), condensação do núcleo, fragmentação do DNA, fragmentação e destruição celular. Pode ocorrer através de duas vias distintas, dependendo do agente desencadeador deste tipo de morte (GOC et al., 2012). Há a via intrínseca e a via extrínseca (Figura 5). Ambas são finalizadas através da ação de proteases denominadas caspases efetoras. As caspases envolvidas na apoptose são divididas em duas categorias: caspases iniciadoras (2, 8, 9 e 10) e caspases efetoras (3, 6 e 7) (BLANK; SHILOH, 2007).

Na via intrínseca, inicialmente há aumento da permeabilidade da membrana mitocondrial, havendo perda do potencial de membrana e a liberação de proteínas encontradas no espaço intermembranar da mitocôndria, como citocromo c, que se liga a

Apaf-1 (fator de ativação de apoptose 1) no citosol. Este complexo ativa a pró-caspase 9 em caspase 9, ao se ligar a ela e forma o apoptossomo. A caspase 9, por sua vez, cliva as caspases efetoras -3, -6 e -7, que uma vez ativadas, irão atuar na fragmentação do DNA (MINAFRA; BRAVATÁ, 2014).

Na via extínseca, por outro lado, há a ativação de receptores de membrana pertencentes à família de receptores de fatores de necrose tumoral (TNF), também chamados de receptores de morte, entre os quais estão FAS (CD 95) e TRAIL. Estes receptores possuem um domínio extracelular rico em cisteína, no qual seus ligantes se unem, de modo a desencadear a via. Tais receptores apresentam no citoplasma um domínio distinto conhecido como domínio de morte (DD, do inglês *Death Domain*). Após o receptor ser ativado por um ligante, ocorre sua associação com seu DD correspondente, havendo uma mudança conformacional no receptor e o recrutamento de uma proteína adptadora, que se associa ao domínio de morte formando o FADD. O FADD liga-se à pró-caspase 8, transformando-a em caspase 8, que ativa as caspases efetoras -3, -6 e -7 (McILWAIN; BERGER; MAK, 2013).

Figura 5. Representação esquemática da via intrínseca e da via extrínseca da apoptose.



Fonte: TAN et al. (2014).

A catástrofe mitótica ocorre durante a mitose em decorrência de segregação cromossômica anormal ou de dano no DNA, levando à formação de micronúcleos. Este tipo de morte está associado à atuação de agentes genotóxicos. Pode ser definida como

o tipo de morte que ocorre durante a mitose, como resultado da combinação de danos ao material genético e da ausência de verificação adequada nos pontos de controle do ciclo celular (VITALI et al., 2011). As células apresentam-se aumentadas e com micronúcleos ou dois núcleos, além de exibirem durante a mitose defeito no alinhamento dos cromossomos e condensação nuclear incompleta (RICCI; ZONG, 2006).

Alguns autores definem a catástrofe mitótica como um sinal irreversível de morte, e não como uma via de morte. Embora a catástrofe mitótica apresente características presentes na apoptose, como permeabilização da membrana mitocondrial e ativação de caspases, ela pode ser caspase independente (BLANK; SHILOH, 2007).

A senescência foi inicialmente descrita para células normais como um processo de alterações associado ao envelhecimento. As células senescentes mantêm a integridade da membrana celular, mas o seu crescimento para de forma permanente. Elas apresentam-se aumentadas, achatadas e há presença frequente de vacúolos (GOSSELIN et al., 2009). Um dos mecanismos associados à senescência ocorre devido ao encurtamento gradual dos telômeros ao término de cada divisão celular. Quando esse encurtamento chega a um limite mínimo, ocorre uma resposta em virtude ao dano no DNA, promovendo parada no ciclo celular. A enzima telomerase, responsável pela adição de unidades teloméricas no cromossomo, está associada à manutenção da imortalidade das células cancerígenas, permitindo que a célula cancerígena drible a senescência (SHAY; WRIGHT, 2011).

A autofagia está relacionada a estresse metabólico, como privação de nutrientes, bem como desenvolvimento e diferenciação. Ela pode atuar como via de resposta adaptativa, buscando a sobrevivência da célula ou como via de promoção de morte celular independente da ação de caspases. Neste processo, inicialmente porções do citoplasma contendo proteínas ou organelas são englobadas por membranas, dando origem a uma estrutura denominada autofagossomo, que se funde com os lisossomos para a degradação do conteúdo englobado por proteases lisossomais. Na autofagia não é observada fragmentação do DNA e há condensação parcial da cromatina (GLICK; BARTH; MACLEOD, 2010).

Tem sido demonstrada a interação entre vias de controle da autofagia e da apoptose, como indicado por Akar et al. (2008), que demonstraram que o silenciamento da expressão do gene anti-apoptótico bcl-2 induz autofagia através da expressão da proteína autofágica beclina 1.

A necrose ocorre frequentemente como conseqüência de condições patológicas, como inflamação ou infecção. Inicialmente considerado um tipo de morte celular não programada, acidental, tem sido demonstrado que a necrose pode ocorrer de forma programada, regulada geneticamente, sendo neste caso chamada de necroptose (RICCI; ZONG, 2006).

Na necrose as células apresentam tumefação (aumento do volume celular), juntamente com inchaço das organelas, redução do volume nuclear (núcleo picnótico), presença intensa de vacúolos, com a subsequente ruptura da membrana plasmática e extravsamento do conteúdo celular, o que promove danos às células vizinhas e uma reação inflamatória (ORRENIUS; NICOTERA; ZHIVOTOVSKY, 2010). Ao contrário do que pode ser visto na apoptose, na necrose não há condensação da cromatina e fragmentação nuclear. A necrose pode ser ocasionada por desbalanço do fluxo de cálcio, inibidores da produção de energia celular, geração de espécies reativas de oxigênio (EROs) e ativação de proteases não apoptóticas (RICCI; ZONG, 2006).

O início da necroptose se dá através da ativação de receptores comuns a apoptose, como, por exemplo, o receptor do fator de necrose tumoral 1 (TNFR1), cuja interação com seu ligante, o TNF-α, inicia o recrutamento das proteínas de domínio de morte associado à TNFR (TRADD), proteína quinase de interação com receptor-1 (RIP1), proteínas inibidoras de apoptose (IAPs), fator associado a TNFR2 (TRAF-2) e fator associado a TNFR5 (TRAF-5), constituindo o complexo I. Em seguida, RIP1 sofre poliubiquitinação por ação das proteínas IAPs, que ativa o NF-kβ. Após RIP1 sofrer desubiquitinação, o complexo passa por modificação, dando origem ao complexo II ou complexo de sinalização de indução de morte (DISC), que é contituído por RIP1, proteína quinase de interação com o receptor 3 (RIPK3), TRADD, domínio de morte associada ao CD 95 (FADD) e caspase 8. Caso a caspase 8 esteja ativa, RIP1 e RIP3 são desativadas e ocorre a apoptose. Por outro lado, se a caspase 8 não puder ser ativada, há a formação de um complexo denominado necrossoma através da fosforilação de RIP1 e

RIP3. Este complexo estimula a permeabilização da membrana do lisossomo, com a liberação de suas enzimas no citoplasma, alterações metabólicas nas mitocôndrias, promovendo a liberação de EROs, as quais estão associadas a danos ao DNA e outras macromoléculas (GALUZZI et al., 2011; FULDA, 2013).

3.3 ENSAIOS PARA AVALIAÇÃO DE ATIVIDADES BIOLÓGICAS DE COMPOSTOS

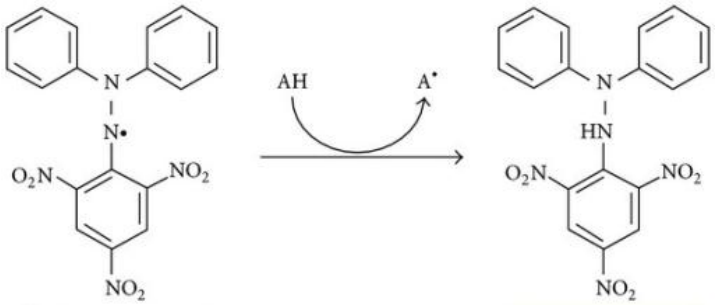
3.3.1 Investigação de atividade antioxidante

Os radicais livres são moléculas que apresentam um elétron não emparelhado e são capazes de receber ou doar um elétron, atuando como oxidantes ou redutores (LOBO et al., 2010). Tais moléculas podem causar danos a células, atacando proteínas, lipídeos e DNA e estão associadas a algumas patologias, como diabetes mellitus, doença de Parkinson e câncer (SARMA et al., 2010; KUMAR et al., 2012; LIU et al., 2013; PETERSEN, 2013).

Diante do envolvimento dos radicais livres com algumas doenças, merecem destaque as substâncias antioxidantes, que se caracterizam pela capacidade de doar elétrons para um radical livre, de modo a impedir os efeitos danosos do mesmo. Ainda, os antioxidantes podem atuar como agentes quelantes de metais envolvidos na formação de radicais livres. Os antioxidantes estão associados à atividade antitumoral, hepatoprotetora, gastroprotetora e antiinflamatória (HSU; FANG; YEN, 2013; ASHRAF et al., 2015; HASSEN; CASABIANCA; HOSNI, 2015).

Entre os métodos empregados para avaliar a atividade antioxidante está o teste do radical 2,2-difenil-1-picril-hidrazila (-DPPH•). Neste ensaio, o -DPPH• é reduzido (Figura 6) na presença de substâncias antioxidantes. O radical, que apresenta coloração violeta, ao ser reduzido passa a apresentar coloração amarela, de modo que a redução da absorbância a 517 nm é proporcional à atividade da amostra testada (ALAM; BRISTI; RAFIQUZZAMAN, 2013). Neste ensaio, é possível calcular a concentração necessária para decrescer a concentração inicial de DPPH em 50% (CE_{50}) para cada amostra (TEOW et al., 2007; MATHANGI; PRABHAKARAN, 2013; XIE; SCHAICH, 2014).

Figura 6. Estrutura do DPPH antes e após reação com substância antioxidante.

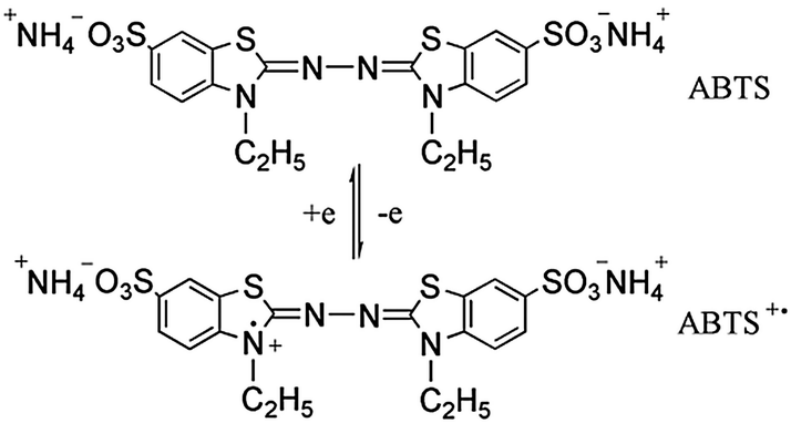


Fonte: TEIXEIRA et al. (2013).

Este método é prático, rápido, sensível e útil para avaliar a atividade antioxidante tanto de substâncias puras, como de extratos, por meio da transferência, principalmente de elétrons, mas também de hidrogênio. Uma das limitações inerentes a este método é a solubilidade do DPPH em solventes alcoólicos (etanol e metanol), o que não permite detectar antioxidantes hidrofílicos (OLIVEIRA, 2015). Outra limitação relacionada ao teste do DPPH é que a atividade antioxidante pode ser subestimada, em virtude da interferência de coloração, em amostras que apresentem compostos coloridos (ARNAO, 2000).

Outra metodologia amplamente empregada para a detecção de atividade antioxidante é o teste do ABTS, que avalia a capacidade de produtos de sequestrar o radical catiônico 2,2'-azinobis-3-etilbenzotiozolino-6-ácido-sulfônico, ABTS•+. Neste ensaio, o ABTS é convertido à forma oxidada através de persulfato de potássio (K_2SO_5), originando o produto ABTS•+, que apresenta coloração azul esverdeada e absorção máxima a 734 nm que, na presença de substâncias antioxidantes, é convertido novamente à sua forma reduzida (incolor), conforme indica a Figura 7 (KONAN; TIEN; MATEESCU, 2016).

Figura 7. Estrutura química das espécies ABTS e ABTS•+ e suas transferências de elétrons.



Fonte: CHEN; YIN (2014).

O ensaio é bastante utilizado devido à solubilidade do radical tanto em meio aquoso, quanto em meio orgânico, sendo capaz de detectar a ação antioxidante de substâncias hidrofílicas e lipofílicas, o que confere uma vantagem em termos operacionais para este método (KUSKOSKI et al., 2005). O teste do ABTS é uma ferramenta útil na detecção de compostos fenólicos e vitamina C, sendo amplamente utilizado como padrão deste ensaio um análogo estutural da vitamina E, o trolox (FERREIRA et al., 2016).

Também bastante utilizado é o ensaio do fosfomolibdênio, que avalia a atividade antioxidante total através da formação do complexo fosfomolibdênio. Este teste baseia-se na redução do molibdênio (Mo) Mo (VI) em Mo (V) pela amostra e formação do complexo fosfato/Mo (V), de coloração verde. Trata-se de um ensaio de baixo custo, rápido e simples. Este método está baseado na transferência de elétrons ou hidrogênio, sendo bastante utilizado para avaliar a atividade antioxidante total (COULIBALY et al., 2014). Este método é útil na detecção de ácido ascórbico, compostos fenólicos, vitamina E e aminas aromáticas (PRIETO PINEDA; AGUILAR, 1999).

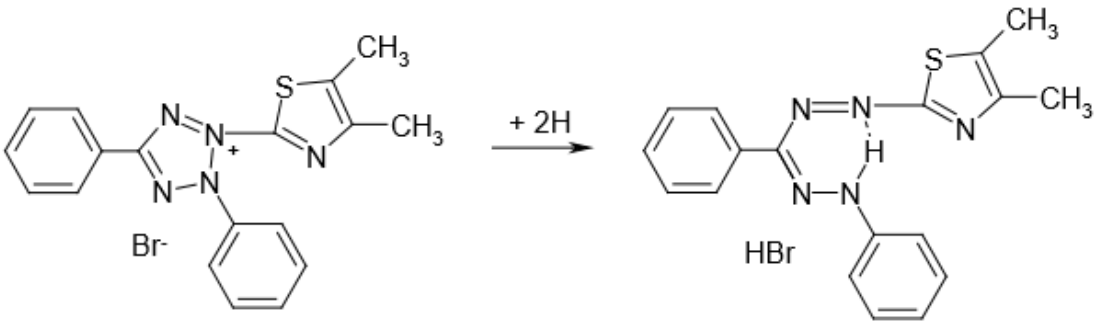
3.3.2 Investigação de toxicidade

Os ensaios de citotoxicidade envolvendo células normais e cancerígenas são amplamente utilizados no processo de triagem de substâncias bioativas. Deste modo, a verificação de danos a células normais causados por potenciais ferramentas farmacológicas são imprescindíveis. O estudo da citotoxicidade de produtos naturais ou sintéticos sobre células cancerígenas proporciona resultados rápidos e confiáveis, além de fornecer subsídios para o prosseguimento dos estudos, evitando o uso desnecessário de modelos animais (VEIGA-JUNIOR; PINTO; MACIEL, 2005; SARZAEEM et al., 2013; PAYDAR et al., 2014).

Embora alguns estudos indiquem o possível uso seguro de produtos naturais que não apresentam efeitos citotóxicos ao material genético de células normais, diversos trabalhos relatam produtos naturais que se apresentam citotóxicos tanto para células cancerígenas como para células normais (BOEIRA et al., 2009; NAKAJIMA et al., 2009; BECHELLI et al., 2011; SERPELONI et al., 2011; CALHELHA et al., 2014).

Entre os testes mais utilizados na avaliação da citotoxicidade, estão o ensaio da timidina tritiada, o teste do MTT e o teste do Alamar blue. O teste do MTT (brometo de {3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio}) consiste em avaliar o efeito de produtos sobre a viabilidade celular (YAN et al., 2009; GRAIDIST; MARTLA; SUKPONDMA, 2015). Neste ensaio, a atividade da enzima succinil-desidrogenase, presente na mitocôndria das células viáveis catalisa a formação de cristais de formazan (Figura 8), um produto de coloração roxa, a partir do MTT (MOSMANN, 1983; ALLEY et al., 1988).

Figura 8. Redução (brometo de {3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazólio}) (MTT) por enzimas mitocondriais a formazan.

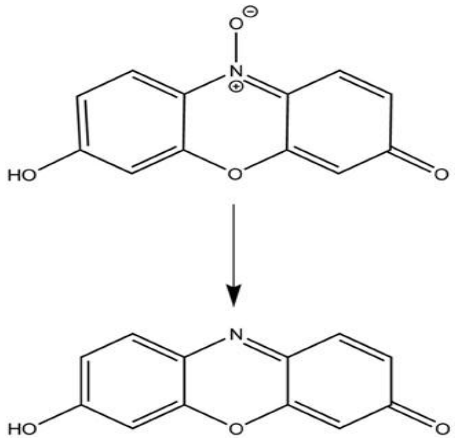


MTT Formazan

Fonte: Aula et al. (2015).

O Alamar blue, ou resazurina-7-hidróxi-3H-fenoxazin-3-ona10-óxido, vem sendo empregado em testes sobre a viabilidade de diversos tipos celulares, como linfócitos, fibroblastos e células cancerígenas, caracteriza-se como um ensaio simples, barato, sensível e com boa reprodutibilidade (O'BRIEN et al., 2000; BORRA et al., 2009; LOOI et al., 2013; FERREIRA et al., 2015). Este ensaio tem como princípio a redução da resazurina, que possui coloração púrpura e apresenta pico de absorção a 600 nm, à resofurina, com coloração rosa e pico de absorção a 570 nm (Figura 9). O mecanismo envolvido no processo de redução a resazurina envolve a atuação do corante como aceptor de elétrons, de modo que é reduzido por NADPH, FADH, FMNH, NADH e citocromos. Ainda, a resazurina pode ser reduzida por outras enzimas mitocondriais e do citoplasma (BORRA et al., 2009; RAMPERSAD, 2012). Vale salientar que este ensaio apresenta boa equivalência com os resultados obtidos no teste do MTT (LOOI et al., 2013).

Figura 9. Estrutura da Resazurina e da Resofurina.



Fonte: Borra et al. (2009).

O ensaio utilizando a timidina tritiada (3HTdR), possibilita a incorporação da timidina radioativa pelas células viáveis, com a subsequente leitura da radioatividade. Apesar de apresentar boa reprodutibilidade, tem como inconveniente a manipulação de material radioativo, bem como a necessidade de destino adequado para este material após o teste (WANG et al., 1996).

Entre os ensaios que investigam a toxicidade de substâncias *in vitro*, a atividade hemolítica é amplamente empregada como teste de triagem, devido à similaridade da membrana dos eritrócitos com outras membranas (ZOHRA; FAWZIA, 2014). Este ensaio avalia a capacidade de um extrato ou substância de promover lise nas hemácias, especificamente pela ação de compostos sobre a membrana celular, levando à formação de poros ou a ruptura total da hemácia (COSTA-LOTUFO et al., 2005; HASSAN et al., 2010; LIU et al., 2012).

A membrana do eritrócito apresenta elevada quantidade de ácidos graxos poli-insaturados e proteínas (WU et al., 2010) e o principal constituinte do eritrócito é a hemoglobina, que, devido à presença do grupo heme, absorve fortemente nos comprimentos de onda de 540-560 nm. No ensaio de atividade hemolítica, as amostras teste são colocadas em contato com os eritrócitos e centrifugadas, de modo a formar um pellet de células. A quantidade de hemoglobina liberada, que permanece no sobrenadante, é mensurada em espectrofotômetro e utilizada como parâmetro para avaliar a hemólise (WU et al., 2010; EVANS et al., 2013).

3.3.3 Investigação de genotoxicidade e mutagenicidade

A molécula de DNA pode sofrer alterações na sua sequência de bases, diferenciando-a da molécula original, proporcionando ou não modificação na funcionalidade da molécula (FLANNICK et al., 2014). A essas alterações dá-se o nome de mutação, que pode ocorrer tanto em células somáticas como em células germinativas, neste último caso, transmitida para os descendentes. As mutações podem estar associadas a adaptação ao ambiente, bem como a desordens como câncer, distúrbios metabólicos e doenças neurológicas (PODURI et al., 2013).

Apesar de a célula possuir um refinado sistema de identificação e correção de erros na molécula de DNA, a exposição contínua a agentes que causam danos à molécula promove elevação da mutagênese em virtude de uma maior probabilidade de ocorrência de erros na replicação de uma fita molde alterada (SOMERS et al., 2002).

Um agente químico, físico ou biológico capaz de danificar o DNA ou alterar a sequência da molécula causando mutação é considerado um agente genotóxico, sendo a expressão dessa mutação prevenida pela célula através de reparo do DNA ou apoptose. Por outro lado, um agente capaz de induzir alterações permanentes e transmissíveis na estrutura do material genético é considerado um agente mutagênico. Com isso, é importante considerar que todos os mutágenos são genotóxicos, mas nem todos os produtos genotóxicos são mutagênicos (NAGARATHNA et al., 2013).

Entre os principais ensaios empregados na avaliação da genotoxicidade e da mutagenicidade, há ensaios realizados com microrganismos, que permitem uma avaliação do dano promovido por um determinado agente ao DNA, ensaios com células humanas em cultura e ensaios empregando animais de laboratório, que apresentam a vantagem de reproduzir tantos as condições de exposição, como a metabolização (KIRKLAND et al., 2011; BALMUS et al., 2015). Merecem destaque o ensaio cometa, amplamente utilizado para avaliação da genotoxicidade e o teste do micronúcleo, empregado no estudo da mutagenicidade de diversos agentes.

O ensaio cometa permite a detecção de lesões genômicas que podem originar mutações, mas que são passíveis de correção, promovidas por diversos produtos, como

poluentes, pesticidas, metais pesados e medicamentos, mesmo com a exposição a baixas concentrações. Há ainda trabalhos que utilizam este ensaio para biomonitoramento de danos em humanos decorrentes de exposição ocupacional (NAGARATHNA et al., 2013).

O teste consiste em um ensaio rápido, simples, bastante sensível para detectar danos em células individualizadas de diversos tipos (JIANG et al., 2008; MURANLI; KANEV; OZDEMIR, 2015; SHAH; LAKKAD; RAO, 2016). Este teste permite detectar quebras de fita simples e de fita dupla, bem como sítios alcali-lábeis (sítios apurínicos/apirimídicos) (ANDERSON; LAUBENTHAL, 2013) e representa um aliado aos testes de citotoxicidade, uma vez que um produto pode promover alterações no DNA, mesmo em concentrações não citotóxicas (MARINS et al., 2012).

No ensaio cometa, as células provenientes do sangue periférico, cultivo celular primário ou permanente ou de órgãos, após serem misturadas a agarose e colocadas sobre uma lâmina coberta com agarose padrão, sofrem lise e o DNA ocupa o espaço da célula no gel de agarose em uma estrutura denominada de nucleoide, formado por alças superenoveladas de DNA, que ficam aderidas à matriz nuclear residual. Ao submeter o nucleoide à eletroforese, ocorre a migração em direção ao polo positivo (ânodo). O padrão apresentado pelo nucleoide após a migração permite identificar a presença de lesões genômicas, uma vez que, se existirem quebras, as alças de DNA sofrem desenovelamento, formando um rastro após a migração. Quando este rastro está presente, o nucleoide assemelha-se a um cometa, com cabeça (região nuclear) e cauda, de modo que quanto maior o dano, maior será a cauda. A migração do DNA é detectada através de agentes intercalantes do DNA, como brometo de etídeo e iodeto de propídeo (COLINS, 2004; SINGH et al, 2010). A análise dos nucleoides pode ser feita de forma visual ou automatizada, havendo boa correlação entre os dados fornecidos pelas duas análises.

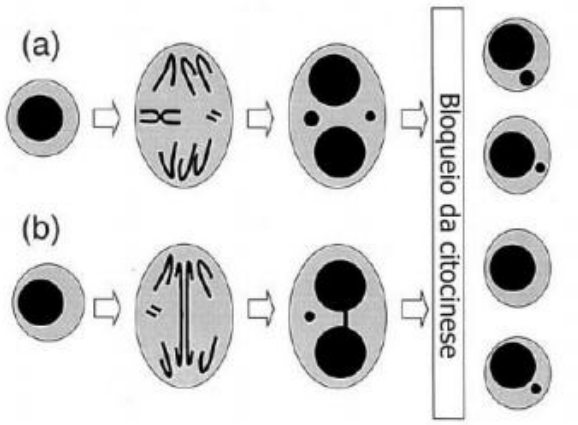
O teste do micronúcleo é um ensaio que avalia o potencial mutagênico de diversos agentes. As versões *in vitro* e *in vivo* são capazes de detectar danos cromossômicos através da presença de estruturas denominadas micronúcleos, que são pequenos corpúsculos nucleares, delimitados por membrana e separados do núcleo. A formação desta estrutura se dá durante a telófase, seja da mitose ou da meiose, no

momento em que ocorre a reconstituição do envelope nuclear em torno dos cromossomos das células-filhas (FENECH et al., 2011; MASER et al., 2015). O teste do micronúcleo fornece evidências do potencial mutagênico de substâncias, assim como oferece suporte à indicação da ausência de mutagenicidade, quando realizado dentro dos critérios adequados, sendo bem aceito por agências internacionais como um método eficiente de estudo de dano ao material genético (OECD, 2007; ARALDI, 2015).

Normalmente, após a replicação do material genético durante a divisão celular, há uma distribuição equitativa desse material para duas células filhas. Quando ocorrem erros através da ação de agentes sobre o DNA, gerando quebras de cromossomos, ou sobre o fuso mitótico, originando perdas de cromossomos inteiros, o material fragmentado não é incorporado a nehuma das células filhas (BRYCE et al., 2014). Os micronúcleos representam os cromossomos inteiros ou fragmentos de cromossomos que não foram incluídos no núcleo ao término da divisão celular. É, portanto, importante, que as células analisadas estejam sofrendo divisão celular e tenham passado por pelo menos um ciclo celular (FENECH, 2007).

Ao realizar o teste *in vitro*, sugere-se o emprego da citocalasina B para promover o bloqueio da citocinese, sem que haja bloqueio da mitose. Portanto, após a adição de citocalasina, as células apresentam-se binucleadas, fornecendo garantia de que as mesmas passaram por um ciclo de divisão (FENECH, 2000). A Figura 10 mostra um micronúcleo proveniente de um cromossomo inteiro e um micronúcleo oriundo de um fragmento de cromossomo acêntrico (a), enquanto em (b) há um micronúcleo derivado de um fragmento de cromossomo e uma ponte citoplasmática em células binucleadas.

Figura 10. Ilustração esquemática da origem de micronúcleos



Fonte: FENECH (2000).

No que diz respeito à avaliação *in vivo*, este teste é comumente realizado em eritrócitos da medula óssea de camundongos e ratos, nos quais a presença de micronúcleos em eritrócitos jovens (eritrócitos policromáticos – PCE) e em eritrócitos maduros (eritrócitos normocromáticos – NCE) é avaliada após exposição a um tratamento. O tempo de vida de um eritrócito policromático é relativamente curto, razão pela qual os micronúcleos presentes nesta célula são provenientes de danos cromossômicos induzidos recentemente (VASQUEZ, 2009). Neste ensaio, poucas horas após as últimas mitoses, os eritrócitos expelem seus núcleos, mas os micronúcleos permanecem no citoplasma. Por um período de 24 horas os eritrócitos jovens (PCEs) possuem coloração diferenciada (basofílicos), em relação às células maduras, uma vez que os seus ribossomos permanecem por, aproximadamente 24 horas após a expulsão do núcleo (SILVA; NEPOMUCENO, 2010).

3.3.4 Investigação de atividade antimicrobiana

Os antibióticos podem ser definidos como produtos de origem natural ou sintética capazes de promover a morte (microbicidas) ou inibir o crescimento (microbiostáticos) de microrganismos (KANG et al., 2011). A habilidade dos antibióticos de inibir o crescimento ou matar microrganismos revolucionou o tratamento

das doenças infecciosas e aumentou a expectativa de vida, permitindo reduzir drasticamente o número de mortes associadas a tais doenças, como ocorreu para a tuberculose e a pneumonia, tratadas com estreptomicina e penicilina, respectivamente (COLE, 2014). Adicionalmente, alguns antibióticos como eritromicinas, tetraciclinas e cloranfenicol apresentam como um efeito colateral a inibição da biogênese mitocondrial e tem sido demonstrado que esta ação pode ser usada na terapêutica do câncer para erradicar células cancerígenas provenientes de diversas linhagens (LAMB et al., 2015).

Mais de 20 classes de antibióticos foram inseridas no mercado entre 1930 e 1962, entre as quais os aminoglicosídeos como a neomicina, as cefalosporinas como a cefalexina, glicopeptídeos como a vancomicina, macrolídeos como a eritromicina, penicilinas como a amoxicilina, quinolonas como a ciprofloxacina, sulfonamidas como o sulfametizol, tetraciclinas e cloranfenicol. Estes antibióticos agem em bactérias gram-negativas, gram positivas ou em ambas atuando através de mecanismos como inibição da síntese da parede celular, inibição da síntese protéica nos ribossomos, inibição da síntese ou dano à membrana plasmática, alterações na síntese dos ácidos nucléicos ou alteração no metabolismo celular (COATES; HALLS; HU, 2011; ADZITEY, 2015).

Após o período de intensa descoberta de agentes antibióticos, observou-se o uso indiscriminado desses agentes pela população, bem como ausência de novas classes de antibióticos no mercado. Esses dois fenômenos contribuíram largamente para o surgimento de microrganismos resistentes, que não são afetados pelo tratamento (COATES; HALLS; HU, 2011). O *Staphylococcus aureus* resistente a meticilina, amplamente conhecido como MRSA (do inglês *Methicillin-resistant S. aureus*), está associado a infecções hospitalares que podem, inclusive, levar à morte (RIBEIRO; PINTO; PEDROSA, 2009; HEMACHANDRAN, 2011). Também tem sido detectado o aumento da taxa de infecções com *Pseudomonas aeruginosa* MDR (do inglês *multidrug-resistant*) e linhagens resistentes de *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* e *Mycobacterium tuberculosis* (HIRSCH; TAM, 2010; TANWAR et al., 2014)

Segundo a Organização Mundial de Saúde (OMS), em 2014 ocorreram cerca de 480.000 novos casos de tuberculose multiresistente. Diante da atual situação, a OMS indica que a resistência microbiana se transformou em um problema de saúde pública, sendo o custo com os cuidados dos pacientes com infecções resistentes superior ao

daqueles com infecções não resistentes. Assim, a dificuldade associada ao desenvolvimento de novos agentes para o tratamento de infecções bacterianas pode estar relacionada a fatores como a existência de mecanismos associados à resistência, como bombas de efluxo e enzimas inativadoras de fármacos, além da promoção de efeitos secundários (SOUSA et al., 2015).

A imensa biodiversidade existente no Brasil pode ser aliada à necessidade da busca por novos agentes antimicrobianos. Neste sentido, muitas espécies vegetais representam fontes de antimicrobianos naturais, sendo muitas vezes empregadas pela população no tratamento de infecções. Adicionalmente, preparações de tecidos vegetais ou produtos isolados de diversas plantas tem demonstrado ação antimicrobiana sobre microrganismos resistentes, como o extrato metanólico de *Psidium guajavaatum* ativo sobre *S. aureus* MDR, uma fração orgânica de *Prosopis juliflora*, ativa sobre Acinetobacter e o flavonoide apigenina, isolado de *Portulaca oleracea* ativo contra *Proteus mirabilis* e *Salmonella typhimurium* (ABDALLAH, 2011; NAYAKA et al., 2014).

A pesquisa de novos agentes antimicrobianos está atrelada a vários testes, entre os quais merecem destaque o método da difusão em ágar e o método da diluição em caldo (macrodiluição e microdiluição) (BALOUIRI; SADIKI; IBNSOUDA, 2016).

O teste de difusão em ágar é um método qualitativo que avalia se há ou não o crescimento do microrganismo quando este é colocado em contato com uma substância teste em meio de cultura sólido, sendo o método padrão pelo NCCLS (National Committe for Clinical Laboratory Standards), de baixo custo e fácil execução e ideal para microrganismos de crescimento rápido (HOLASOVA et al., 2007). A substância é colocada em discos de papel de filtro de pequeno diâmetro, que são posicio superfície do meio de cultura, previamente inoculado com o microrganismo, em placas de petri. Neste ensaio, a partir do disco há uma área em que não há crescimento de microrganismos, chamada de halo de inibição (BALOUIRI; SADIKI; IBNSOUDA, 2016). Assim, o ensaio possibilita relacionar o tamanho do halo de inibição com a eficiência antimicrobiana da substância testada através da medição partindo da circunferência do disco até o ponto em que começa a ocorrer o crescimento microbiano, permitindo a identificação do microrganismo como altamente sensível, moderadamente

sensível ou resistente (MANIKANDAN; AMSATH, 2013). Apesar das vantagens associadas ao método, a principal desvantagem é o risco de erro ao avaliar produtos que não apresentem fácil difusão através do ágar (SOKOVIĆ et al., 2010).

O método da diluição em caldo também é amplamente difundido, sobretudo por permitir uma avaliação quantitativa e não ser influenciado pela velocidade de crescimento do microrganismo. Neste método, o produto teste é adicionado ao meio de cultura líquido com o microrganismo inoculado, permitindo após a incubação, avaliar o crescimento microbiano através de leitura visual direta ou por meio de espectrofotômetro, considerando a turbidez, que aumentará conforme há multiplicação dos microrganismos. No caso da leitura visual, é comum a utilização de resazurina devido a alteração de cor, uma vez que na presença de células microbianas viáveis este indicador de coloração púrpura se oxida e adquire coloração rosa. Assim, este método relaciona a proporção de crescimento do microrganismo com a concentração do produto teste em meio líquido (KANG et al., 2011; BALOUIRI; SADIKI; IBNSOUDA, 2016).

O teste de diluição apresenta dois tipos de metodologias: a macrodiluição e a microdiluição utilizadas para avaliar a ação antimicrobiana de diversos produtos. A primeira é realizada em tubos de ensaio e apresenta como desvantagens a baixa praticidade e a produção de elevada quantidade de resíduos para um número baixo de replicatas. A microdiluição, por sua vez, é realizada em microplacas de 96 poços e apresenta-se como um método de baixo custo e de boa reprodutibilidade. Por isso, tem sido amplamente utilizada para determinação da menor concentração dos produtos testados capaz de inibir a multiplicação de microrganismos (CMI - concentração mínima inibitória), que é utilizada para avaliar a efetividade de um produto. Ainda, a partir dos poços nos quais não há crescimento bacteriano visível, retira-se uma alíquota que é inoculada na superfície do ágar. Nesse caso, é possível determinar a menor concentração capaz de romover a morte do inóculo, que é denominada concentração mínima bactericida (CMB) para bactérias ou concentração mínima fungicida (CMF) para fungos (KLANCNIK et al., 2010; RISTIVOJEVIĆ, et al., 2016).

3.4. METABÓLITOS PRIMÁRIOS E SECUNDÁRIOS DE PLANTAS

3.4.1 Metabólitos primários

3.4.1.1 Lectinas

Lectinas constituem um grupo de proteínas com imensa diversidade estrutural e que se ligam, com elevada especificidade, a carboidratos presentes em glicolipídios, glicoproteínas e polissacarídeos de forma reversível. As forças que estabelecem esta interação são pontes de hidrogênio, interações hidrofóbicas e interações de Van der Walls (GOLDSTEIN; HAYES, 1978; RINI, 1995; ELGAVISH; SHAANAN, 1997). As lectinas podem ser encontradas em animais, plantas, fungos e bactérias (YAGI et al., 2000; SHARON; LIS, 2004; LAKHTIN et al., 2007; VASCONCELOS et al., 2015; XIU et al., 2015).

As lectinas podem ser classificadas em merolectinas, hololectinas, quimerolectinas e superlectinas. As primeiras possuem um único sítio de ligação a carboidratos, sendo, portanto desprovidas da capacidade de aglutinar células. As hololectinas, por sua vez, embora possuam apenas um domínio ligante de carboidrato, apresentam também outros sítios de ligação, o que lhes confere a capacidade de aglutinar células. As quimerolectinas possuem pelo menos um domínio ligante de carboidrato, além do qual há outro sítio que atua de forma independente ao sítio de ligação a carboidratos e que é dotado de atividade biológica. Por fim, as superlectinas apresentam no mínimo dois sítios de ligação a carboidratos, que possuem estrutura distinta e se ligam a carboidratos diferentes. É importante destacar ainda que várias lectinas não têm afinidade or monossacarídeos, sendo mais esecíficas ara oligossacarídeos complexos (PEUMANS; VAN DAMME, 1998; PEUMANS et al., 2001).

A capacidade de se ligar de modo específico a carboidratos também permite agrupar as lectinas levando em consideração o monossacarídeo pelo qual têm afinidade. Portanto, podem ser classificadas em como lectinas ligadoras de glicose/manose, ligadoras de fucose, lectinas ligadoras de galactose/N-acetilgalactosamina, lectinas ligadoras de N-acetilglucosamina e lectinas ligadoras de ácido siálico (PEUMANS; VAN DAMME, 1995).

O grupo mais extensamente estudado para obtenção de lectinas tem sido os vegetais, nos quais é possível obter lectinas de entrecasca, folhas, flores, frutos, raízes e sementes (OLIVEIRA et al., 2011; SOUSA DE ARAÚJO et al., 2012; JIMENEZ et al., 2013; PATIL; DESHPANDE, 2015; ABUDAYEH et al., 2016). A abundância destas macromoléculas nos tecidos vegetais está associada aos mecanismos de defesa das plantas contra insetos, fungos, bactérias e infecção viral. A ativação de genes ligados à expressão destas proteínas pode ser decorrente da ação de hormônios vegetais produzidos contra o ataque de insetos (CHEN et al., 2002; DE VOS et al., 2005).

Costa et al. (2010) demonstraram que a lectina isolada de folhas de *Phthirusa pyrifolia* apresentou atividade antimicrobiana contra as bactérias gram-positivas *Staphylococcus epidermidis*, *Streptococcus faecalis* e *Bacillus subtilis*, bem como para a bactéria gram-negativa *Klebsiella pneumoniae*. Carvalho et al. (2015) identificaram a atividade antibacteriana da lectina de sementes de *Apuleia leiocarpa* contra bactérias gram-positivas (*Bacillus cereus*, *Bacillus subtilis*, *Enterococcus faecalis* e *Micrococcus luteus*) e gram-negativas (*Klebsiella pneumoniae* e *Xanthomonas campestres*).

A atividade inseticida também tem sido relatada para lectinas. Oliveira et al. (2011) demonstraram que a lectina cMoL, isolada das sementes de *Moringa oleifera*, promoveu a morte da traça da farinha (*Anagasta kuehniella*). Paiva et al. (2011) determinaram a atividade termiticida da lectina de cladódios de *Opuntia fícus* contra *Nasutitermes corniger* e Oliveira et al. (2016) revelaram a atividade larvicida da lectina isolada da torta de sementes de *M. oleifera* contra *Aedes aegypti*.

As lectinas são conhecidas também por sua capacidade de interagir diferentemente com células normais e cancerígenas, devido ao padrão de glicosilação exibido na membrana celular. Liu et al. (2009) relataram elevada citotoxicidade da lectina isolada de *Polygonatum cyrtonema* sobre células de melanoma, sem causar a morte de melanócitos normais. Uma vez que células cancerosas apresentam padrão de glicosilação distinto das células normais, as lectinas podem ser acopladas a fármacos, garantindo sua liberação no local alvo (BIES; LEHR; WOODLEY, 2004). A interação de lectinas com as glicoproteínas de uma célula cancerosa é capaz de desencadear a ativação de vias que levam a célula à destruição, seja por apoptose, autofagia ou necrose (CAO et al., 2010; LIU; BIAN; BAO, 2010; ARANDA-SOUZA et al., 2014). Além

disso, tem sido demonstrado que lectinas inibem a migração de células tumorais (OCHOA-ALVAREZ et al., 2012).

A lectina isolada da vagem de *Lotus corniculatus* promoveu apoptose das células cancerosas humanas HOP62 (câncer de pulmão), HCT116 (câncer de cólon) e THP1 (leucemia) e a isolada das folhas de *Morus alba* L promoveu a morte de células de câncer de mama (MCF-7) e de cólon (HCT-15) por apoptose (DEEPA et al., 2012; RAFIQ et al., 2013).

Silva et al. (2014) demonstraram que a lectina isolada de *Canavalia brasiliensis* promoveu a morte por apoptose das células B16F10, além de estimular a produção da citocina IL-12, que possui ação antiangiogênica. Yan et al. (2009) revelaram que a lectina isolada das raízes de *Astragalus mongholicus* promoveu apoptose nas células HeLa (carcinoma cervical humano), com saída do ciclo celular na fase S, bem como na linhagem K562 (leucemia humana). Aranda-Souza et al. (2014) demonstraram que a lectina isolada do veneno de *Bothrops leucurus* promoveu a morte por necrose em células de melanoma murino B16F10.

Diante dos inúmeros relatos acerca da ação anticâncer de lectinas, estudos têm sido desenvolvidos envolvendo a microencapsulação destas moléculas para a aplicação terapêutica. El-Aassar et al. (2014) demonstraram que a lectina de *Pisum sativum* encapsulada promoveu a superexpressão do gene p53 e promoveu morte celular por apoptose. Neste cenário, é e grande interesse a contínua realização de pesquisas que busquem investigar o potencial anticâncer de lectinas, fortalecendo estas moléculas como potenciais ferramentas farmacológicas.

3.4.2 Metabólitos secundários

Os metabólitos secundários são classificados como um conjunto de substâncias com estrutura complexa e baixo peso molecular que não são estritamente essenciais para a sobrevivência da planta. A produção destas substâncias é amplamente influenciada por fatores, tais como temperatura, composição atmosférica, radiação UV, ataque de patógenos, idade do vegetal e disponibilidade de água e nutrientes (PICHERSKY; GANG, 2000; GLOBO-NETO; LOPES, 2007).

Os terpenos ou terpenóides constutuem a maior classe de metabólitos secundários em plantas e até o ano de 2014 mais de 40 mil tipos de terpenos haviam sido descritos (BOUTANAEV et al., 2014). Os terpenos são constituídos por unidades de isopreno, com fórmula geral $(C_5H_8)_n$. De acordo com a quantidade de carbonos presentes na estrutura, os terpenos podem ser classificados em hemiterpenos (com 5 carbonos), monoterpenos (com 10 carbonos), sesquiterpenos (15 carbonos), diterpenos (20 carbonos), triterpenos (30 carbonos) e tetraterpenos (40 carbonos) (DEWICK, 2009).

Os terpenos apresentam atividade antioxidante, leishmanicida, antimicrobiana, anti-inflamatória, anticâncer, antihiperglicêmica, antiviral, antinociceptiva e antidepressiva (ARRUDA et al., 2005; PADUCH et al., 2007; PERAZZO et al., 2007; PERGENTINO et al., 2007; GONZÁLEZ-BURGOS; GÓMES-SERRNILLOS, 2012). A atividade antimicrobiana de terpenos está descrita para os fungos *Aspergilus niger*, *C. albicans* e as bactérias *Staphylococcus aureus*, *Bacillus cereus*, *Eschechia coli*, *Pseudomonas aeruginosa* (SARAC; KALRA, 2011; SARAC et al., 2014). Entre os terpenos já utilizados na terapêutica, a artemisina, um sesquiterpeno isolado de *Artemisia annua,* é amplamente empregado no tratamento da malária (RO et al., 2006).

Os mecanismos envolvidos com a atividade anticâncer dos triterpenos são indução da célula à saída do ciclo celular, indução de apoptose e modulação do sistema imune. É interessante destacar a capacidade que alguns triterpenos possuem de inibir a angiogênese, migração e metástase (LIU, 2005; PADUCH et al., 2007). A relevância destas substâncias como agentes anticâncer fica evidente com o fato de muitas prosseguiram para fase de estudos clínicos.

As substâncias que possuem pelo menos um anel aromático com, no mínimo, um hidrogênio substituído por um grupamento hidroxila são conhecidas como compostos fenólicos e são derivados do ácido chiquímico e do ácido mevalônico. Esta classe engloba substâncias conhecidas como fenóis simples, ácidos fenólicos, lignanas, taninos, cumarinas e flavonoides (SOARES, 2002). Os ácidos fenólicos apresentam atividade antioxidante, pois são capazes de se ligar aos radicais livres (BREWER, 2011).

Os taninos são compostos fenólicos que destacam-se por sua capacidade de precipitar proteínas e alcaloides (HASLAM, 1996). As moléculas pertencentes a esta

classe apresentam estrutura constituída por vários anéis aromáticos (de 5 a 7) e com 12 a 16 grupos fenólicos. Dentro desta classe estão os taninos condensados (proantocianidinas) e os taninos hidrolisáveis. Os taninos condensados (gálicos ou elágcico) são polímeros de flavonoides (2 a 50 unidades) unidos por ligações entre carbonos, enquanto os taninos hidrolisáveis possuem no seu centro uma molécula de carboidrato, cujos grupamentos hidroxila encontram-se ligados a grupamentos fenólicos por ligação éster (ASHOK; UPADHYAYA, 2012). Entre as atividades biológicas descritas para esta classe de taninos estão a atividade antimicrobiana, a atividade antioxidante, atividade antimutagênica, antitumoral, anti-inflamatória, antiúlcera e antidiarréica (SULAIMAN et al., 2011; BRUYNE et al., 1999).

Entre os taninos hidrolisáveis, merece destaque o ácido tânico, que, embora tenha se mostrado citotóxico para células de mama normais, mostrou-se mais efetivo sobre células de câncer de mama, promovendo apoptose nas células transformadas. O potencial deste tanino foi demonstrado por Tikoo; Sane; Gupta (2010) ao evidenciar a redução da cardiotoxicidade promovida pela doxorrubicina, bem como aumento da atividade deste fármaco, quando usada em associação ao ácido tânico.

Reddy et al. (2007) demonstraram a atividade antimicrobiana de taninos contra *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Mycobacterium intracellulare*, bem como contra a cepa de *Staphylococcus aureus* resistente ao antibiótico meticilina. Adicionalmente, Akiyama et al. (2001) sugeriram o uso do ácido tânico como adjuvante no tratamento de infecções causadas por *S. aureus*.

Os flavonoides possuem um esqueleto com 15 átomos de carbono, que consiste em dois anéis aromáticos unidos por um anel heterocíclico oxigenado (HEIM et al., 2002). De acordo com as modificações no esqueleto estrutural, surgem os diferentes tipos de flavonoides, como flavonas, flavononas, flavonóis, antocianidinas, isoflavonas, catequinas, auronas, leucocianidinas e chalconas (HAVSTEEN, 2002). A presença destes compostos está associada à proteção contra radiação ultravioleta e ataque de patógenos nas plantas (HARBORNE; WILLIAMS, 2000).

Os flavonoides são amplamente empregados na nutrição humana como também apresentam diversas atividades biológicas incluindo atividade antitumoral e

antimicrobiana. Matsuo et al. (2005) avaliaram a citotoxicidade de nove flavonoides sobre células endoteliais da veia umbilical humana (HUVEC) e sobre fibroblastos pulmonares de embrião humano (TIG-1), observando diferentes efeitos para estas substâncias pertencentes à mesma classe.

Os mecanismos envolvidos na atividade anticâncer de flavonoides englobam atuação sobre a proteína p53, promoção da saída do ciclo celular, bem como inibição da expressão de proteínas envolvidas em diversas vias de manutenção da sobrevivência da célula cancerosa (KUMAR; PANDEY, 2013). Com relação à atividade antimicrobiana, tem sido demonstrada ação contra bactérias gram-negativas e gram-positivas, como *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* e *Pseudomonas aeruginosa*, (HENDRA et al., 2011). Ainda, alguns flavonoides apresentam atividade contra bactérias resistentes a antibióticos comerciais (MBAVENG et al., 2015). Os flavonóides podem atuar como antioxidantes pois são capazes de sequestrar radicais livres ou quelar metais (BREWER, 2011).

Os compostos fenólicos em geral, apresentam também atividade hemolítica (HASSAN et al., 2010; SCHIAR et al., 2007; CAVALHEIRO et al., 2009). Sugere-se que as saponinas promovam hemólise através da interação com o colesterol presente na membrana do eritrócito, formando um complexo insolúvel com este lipídeo, de modo a permitir a formação de poro na membrana celular (GAUTHIER et al., 2009).

A chalcona 4-hidroxiderricina isolada das raízes de *Angelica keiskei* promoveu inibição do crescimento do tumor, bem como a metástase do carcinoma pulmonar de Lewis em camundongos, atuando como inibidor da angiogênese e modulador do sistema imune (KIMURA; TANIQUCHI; BABA, 2004).

As cumarinas (2H-1-benzopiran-2-ona) são provenientes do ácido chiquímico e podem ser encontradas em diversos tecidos vegetais, mas estão em maior quantidade nos frutos e nas raízes (JAIN; JOSHI, 2012). As moléculas desta classe apresentam atividade antimicrobiana, antiviral, anticâncer, anti-inflamatória, antioxidante, anticonvulsivante e atividade protetora sobre o sistema nervoso central (BORGES et al., 2005; VENUGOPALA; RASHMI; ODHAV, 2012).

Cumarinas apresentam atividade antibacteriana sobre as espécies gram-positivas e gram-negativas *Staphylococcus aureus*, *Salmonella thypii*, *Enterobacter cloacae* e

Enterobacter earogenes (BASILE et al., 2009). Estudos envolvendo derivados sintéticos e naturais de cumarinas demonstraram a atividade anticâncer para linhagens de câncer de pulmão, mama, leucemia, bem como para melanoma, próstata, carcinoma hepatocelular e carcinoma renal. Esta atividade envolve diferentes mecanismos de ação, relacionados com a estrutura destas moléculas, como inibição da enzima telomerase, a regulação da expressão de oncogenes, promoção de apoptose e inibição da proliferação celular por parada no ciclo celular nas fases G0/G1 e G2/M (KLENKAR; MOLNAR, 2015). A substância esculetina (6,7-diidroxicumarina), encontrada em várias espécies como *Artemisia capillaries*, *Citrus limonia* e *Euphorbia lathyris*, é um potente agente antiproliferativo e promotor de apoptose em células de melanoma (JEON et al., 2015).

Os alcalóides vegetais são compostos orgânicos cíclicos derivados dos aminioácidos aromáticos triptofano e tirosina ou de aminoácidos alifáticos como a lisina e a ornitina (LU et al., 2012). Estas substâncias estão associadas a um elevado número de propriedades biológicas incluindo as atividades anti-inflamatória, antiúlcera, anticâncer, antimalárica antiplasmodial, antiviral e antimicrobiana (ISHIDA et al., 2001; BARBOSA-FILHO et al., 2006; FALCÃO et al., 2008; LEVERRIER et al., 2015; SWAIN; SAHU; PADHY, 2015; ZHANG et al., 2015). Já foram reportadas atividade antibacteriana contra *Escherichia coli*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* e *Shigella dysenteriae*, e gram-positivas como *Micrococcus lysodeikticus*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis* e *Staphylococcus aureus* e antifúngica contra *Candida albicans*, *Candida tropicalis* e *Candida glabrata* (DENG et al., 2011).

Inúmeros alcaloides apresentam efeitos tóxicos, como a atropina isolada de *Atropa belladonna*, que promove taquicardia, dilatação das pupilas, redução da motilidade gastrointestinal e diminuição da produção de saliva (BEYER; DRUMMER; MAURER, 2009). A atividade citotóxica de alcaloides contra linhagens cancerígenas está relacionada à ação em diversos alvos, pois podem promover a saída do ciclo celular, autofagia, apoptose e inibição da angiogênese através de ação sobre moléculas chave de diversas vias (LU et al., 2012).

As pesquisas envolvendo a ação anticâncer de produtos de origem vegetal foram responsáveis pela introdução de muitos fármacos no mercado. Alguns merecem

destaque como os alcaloides vimblastina e vincristina, que se ligam a proteínas dos microtúbulos e impedem que a célula finalize a metástase. Ambos foram isolados da espécie *Catharanthus roseus* e são empregados no tratamento do linfoma de Hodgkin, sarcoma de Kaposi, câncer de ovário, de testículos e leucemia linfoblástica aguda infantil. A partir de uma modificação na vimblastina, foi desenvolvida a vinfluina, que também age nos microtúbulos, e apresenta ação para câncer de mama metastático (CAMPONE et al., 2006; BRANDÃO et al., 2010).

O taxol (paclitacel), um éster alcaloide isolado de *Taxus brevifolia* atua no fuso mitótico, impedindo a proliferação celular e é empregado no combate ao câncer de mama, de pulmão e de ovário. Os fármacos topotecano e irinotecano, que são análogos estruturais da camptotecina, um alcaloide isolado de *Camptotheca acuminata*, atuam inibindo a enzima topoisomerase I e são utilizados para o tratamento de câncer de cólon, pulmão e de ovário (OBERLIES; KROLL, 2004).

Entre estes os estudos realizados com metabólitos secundários de origem vegetal, existem aqueles que promovem modificações estruturais nos compostos ativos de origem vegetal para obter derivados mais potentes e com menor toxicidade. Sagar et al. (2014), que avaliaram quatro derivados da substância plumbagina (5-hidroxi-2-methil-1,4-naftoquinona) isolada das raízes de uma planta da família Plumbaginaceae e determinaram que um dos derivados apresentou menor toxicidade para células normais em comparação ao composto isolado das raízes.

3.5 *APODANTHERA CONGESTIFLORA*

Apodanthera congestiflora pertence à família Cucurbitaceae, que apresenta cerca de 118 gêneros, 900 espécies e está amplamente distribuída nas regiões tropicais e subtropicais (SIMPSON, 2010). No Brasil são encontrados cerca de 30 gêneros, com 148 espécies (KLEIN; LIMA, 2011). A família Cucurbitaceae engloba plantas trepadeiras ou ervas prostradas com ou sem gavinhas. As folhas são alternas, simples ou lobadas e as plantas desta família podem ser monóicas, dióicas ou ainda andromonóicas, com flores geralmente pentâmeras (BARROSO, 1978; KLEIN; SANTANA, 2009).

A elevada importância econômica desta família é decorrente do emprego de alguns gêneros na produção de alimentos, como a melancia (gênero *Citrullus*) e a abóbora (gênero *Cucurbita*) (BALDIN et al., 2002; DI STASI et al., 2002). Entre as propriedades biológicas descritas para plantas da família Cucurbitaceae estão as atividades hipoglicemiante e hipotensora, anticâncer, antimicrobiana, anti-inflamatória, hepatoprotetora, analgésica e antioxidante (GONZALEZ; DI STASI, 2002; OJEWOLE; ADEWOLE; OLAYIWOLA, 2006; ESEYIN; SATTAR; RATHORE, 2014; TALUKDAR; HOSSAIN, 2014).

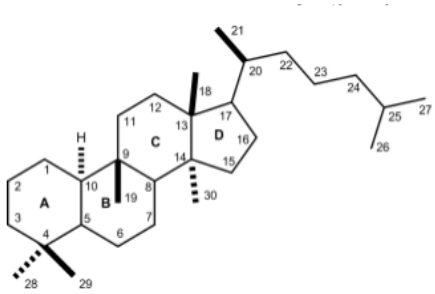
As sementes de espécies do gênero *Curcubita* são ricas em γ-tocoferol, β-sitosterol e os ácidos esteárico, linoleico, oleico e palmítico enquanto foi reportado que o extrato salino das sementes de *Momordica charantia*, *Cucumis sativa*, *Praecitrullus fistulosus*, *Cucurbita pepo* e *Lagenaria siceraria* contem fitosterois, glicosídeos cardíacos, saponinas e terpenos (KIM et al., 2012; SOOD et al., 2012). Foram também identificados ceramidas, esteroides e triterpenos na casca dos frutos de *Luffa operculata*; alcaloides, saponinas, taninos, terpenos e triterpenos nas folhas das cucurbitáceas *Lagenaria vulgaris*, *Luffa acutangula* e *Momordica subangulata* e alcaloides, antraquinonas, taninos, esteroides, flavonoides e saponinas nas partes aéreas de *Telfairia occidentali* (FEITOSA et al., 2011; TUPE et al., 2013; ESEYIN; SATTAR; RATHORE, 2014).

As raízes tuberosas de *Momordica tuberosa* contem esteróis, triterpenos, saponinas e glicosídeos cardíacos, além de carboidratos (KUMAR et al., 2010). Entre os constituintes químicos das raízes de *Momordica dioica* estão alcaloides, esteroides, triterpenos e ácido esteárico. Ainda, as raízes de *Telfairia Occidentali* apresentam taninos, saponinas, triterpenos e esteróis (TALUKDAR; HOSSAIN, 2014).

A família Cucurbitaceae apresenta uma rica constituição química e entre os compostos encontrados, merecem destaque os triterpenos tetracíclicos altamente oxigenados e derivados de um esqueleto cucurbitano, conhecidos como cucurbitacinas (Figura 11). De acordo com as modificações estruturais em torno do esqueleto, é possível identificar mais de 20 tipos de cucurbitacinas distintos (CHEN et al., 2004). Estas moléculas podem ser encontradas em diversas partes da planta, contudo predominam nas raízes e nos frutos (METCALF; METCALF, 1982). Tem sido

reportado que as cucurbitacinas apresentam atividade citotóxica, anti-inflamatória, antitumoral, hepatoprotetora e purgativa (MIRÓ, 1995; HE et al., 2013).

Figura 11. Estrutura geral das cucurbitacinas. Esqueleto cucurbitano: (19-(10→9β)-abeo-10α-lanost-5-ene).



Fonte: Chen et al. (2004).

A atividade anticâncer tem sido amplamente relatada para a cucurbitacina B. Gao et al. (2014) indicaram que esta molécula inibe o crescimento de células de câncer de próstata (PC-3), promovendo apoptose e acúmulo de células na fase G0/G1 do ciclo celular, sem afetar as células epiteliais prostáticas normais. No entanto, outros estudos indicam cucurbitacinas promovendo a saída do ciclo celular na fase G2/M (TANNIN-SPITZ et al., 2007; THOENNISSEN et al., 2009).

A cucurbitcina E também é bastante estudada e trabalhos têm demonstrado a atividade citotóxica seletiva desta molécula, pois inibe o crescimento de células de leucemia promielocítica (HL-60) sem promover a morte de linfócitos (MILITÃO et al., 2012). Adicionalmente, Nakashima et al. (2010) demonstraram atividade antiproliferativa da cucurbitacina E sobre a linhagem U397 (leucemia monocítica humana). Os estudos promissores com esta molécula estimularam a incorporação da cucurbitacina E em lipossomos, passo fundamental para a realização de estudos posteriores visando aumentar a eficácia da ação desta cucurbitacina (HABIB et al., 2013).

Vale destacar ainda o estudo realizado por Sadzuka; Fujiki; Itai (2012), que demonstrou que a associação da cucurbitacina I com o fármaco doxorrubicina

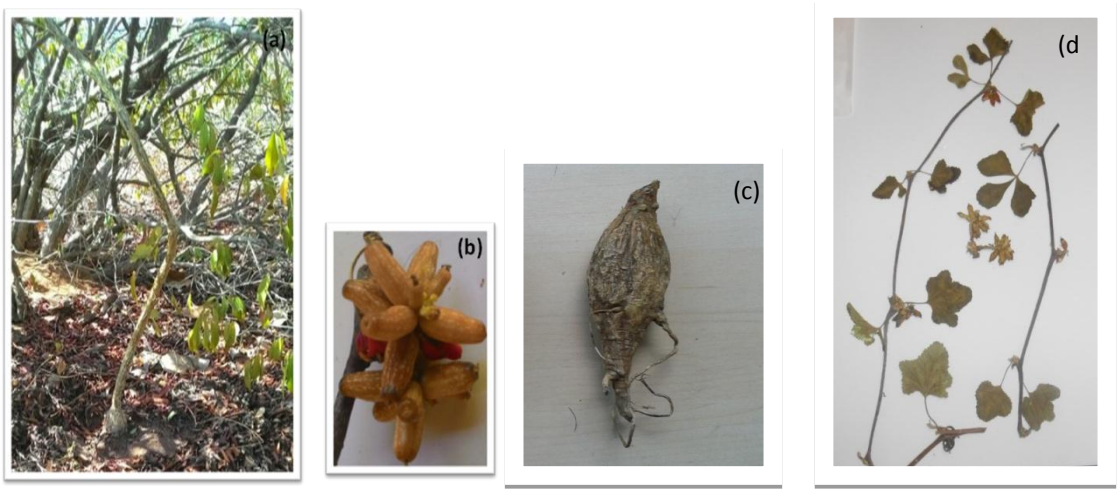
promoveu aumento da citotoxidade sobre células cancerígenas e redução da cardiotoxicidade deste fármaco. Chen et al. (2014) isolaram do rizoma de *Hemsleya amabilis* a cucurbitacina IIa, que demonstrou potente atividade citotóxica sobre células HeLa. A diversidade deste grupo de triterpenos é fator crucial para contínua realização de pesquisas.

O gênero *Apodanthera* possui 40 espécies distribuídas na América tropical e subtropical (POZNER, 1998). O Brasil conta com onze espécies, das quais apenas uma não é endêmica (LIMA, 2010). As espécies deste gênero podem apresentar valor nutricional, como *Apodanthera biflora*, cuja raiz apresenta teores de proteínas, carboidratos, fósforo e cálcio equivalentes ou superiores ao de culturas empregadas na alimentação (CLARK et al., 2012).

Propriedades farmacológicas são também descritas para espécies do gênero *Apodanthera*. Vilar; Carvalho; Furtado (2007) indicaram que o extrato aquoso de *A. villosa* (batata-de-teiu), mas não o de *A. glaziovii* (cabeça-de-negro), foi capaz de retardar a mortalidade de camundongos que receberam veneno de *Bothrops jararaca*. Tal atividade foi atribuída à presença do composto pterocarpano - (-) edunol, um tipo de isoflavonoide. Vale ressaltar ainda o uso para tratamento de câncer, pela população, de *A. smilacifolia*, que é também inclusa na lista da Agência Nacional de Vigilância Sanitária (ANVISA) de fitoterápicos usados em associação no Brasil (CARVALHO et al., 2008; OLIVEIRA; MACHADO; RODRIGUES, 2014).

Apodanthera congestiflora (Figura 12) tem como sinônimo *Melothria congestiflora* e é uma trepadeira endêmica do Brasil, amplamente distribuída na região nordeste, sendo encontrada na Caatinga nos estados da Bahia, Pernambuco, Ceará, Piauí, Rio Grande do Norte, Sergipe, bem como no estado de Minas Gerais (LIMA, 2010). As folhas desta planta podem ser simples ou compostas, trilobadas, com margens sinuosas ou denteadas e faces adaxial e abaxias pubescentes. Há a presença de gavinhas e trata-se de uma espécie dioica. Os frutos (Figura 12 b) apresentam coloração alaranjada a castanha, com estrias brancas longitudinais. As sementes de *A. congestiflora* são obovaladas (MACHADO, 2009; LIMA, 2010).

Figura 12. *Apodanthera congestiflora.* (a) planta inteira, (b) frutos, (c) raiz (d) exsicata.



Fonte: O autor

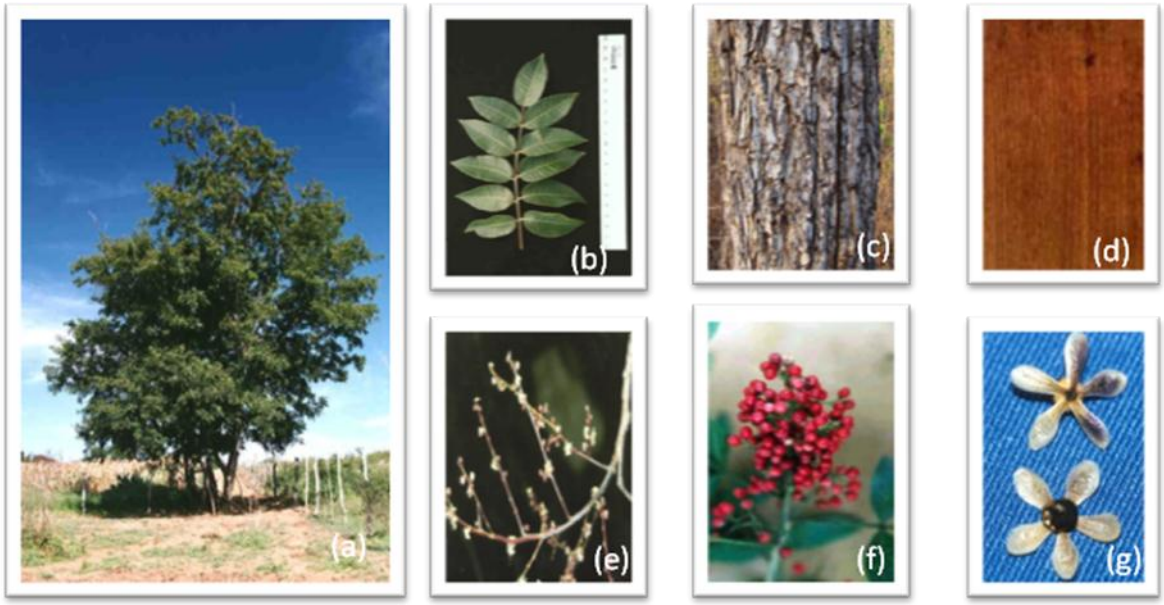
Roque; Rocha; Loiola (2010) demonstraram o uso da raiz de *A. congestiflora* como depurativo do sangue, para tratar manchas de pele e para coceira, enquanto Silva et al. (2015) relataram o uso no combate a dores de coluna, por diferentes populações no nordeste do Brasil.

O imenso potencial da família Cucurbitaceae, bem como a ausência de estudos sobre as propriedades biológicas de *A. congestiflora* evidenciam a necessidade de realização de pesquisas visando à caracterização química da espécie e a descoberta das propriedades biológicas desta planta.

3.6 *MYRACRODRUON URUNDEUVA*

Popularmente conhecida como aroeira do sertão, aroeira preta ou urundeúva, *Myracrodruon urundeuva* (Figura 13), sinônimo *Astronium urundeuva* Fr. All. (QUEIROZ; MORAES; NASCIMENTO, 2002) pertence à família Anacardiaceae e possui ocorrência restrita à América do Sul. No Brasil a espécie pode ser encontrada principalmente na região nordeste, onde é amplamente utilizada devido ao seu valor econômico e medicinal (MONTEIRO et al., 2012; SILVA et al., 2012). *M. urundeuva* caracteriza-se como uma espécie arbórea, podendo alcançar até 30 metros de altura, xerófita seletiva, heliófita, com folhas alternas, compostas e caducifólias, com a queda das folhas ocorrendo entre os meses de julho e setembro. A espécie apresenta frutos do tipo drupa, com uma semente globosa e alada por fruto (LORENZI, 1992; RIZZINI, 1971).

Figura 13. *Myracrodruon urundeuva*. (a) árvore, (b) folhas, (c) casca, (d) madeira, (e) inflorescência, (f) frutos, (g) sementes.



Fonte: Centro Nordestino de Informações sobre Plantas (CNIP); (LORENZI, 2000).

A inclusão desta planta entre as espécies da flora brasileira ameaçadas de extinção na lista elaborada pela IUCN (The World Conservation Union), em decorrência da exploração elevada, evidencia a importância da realização de estudos acerca das propriedades farmacológicas de produtos isolados desta espécie, uma vez que

os metabólitos bioativos estimulam a conservação da planta, além de poderem ser utilizados como protótipos para a síntese de novas substâncias.

Entre os constituintes químicos encontrados em *M. urundeuva*, estão flavonoides, esteroides, alcaloides, xantonas, taninos e saponinas (GOMES et al., 2013; MOTA et al., 2015). Vale salientar que esta planta possui elevado teor de compostos fenólicos, sobretudo de taninos, que estão relacionados à atividade anti-inflamatória da planta (MORAIS et al., 1999, SOUZA et al., 2007). A partir da entrecasca de *M. urundeuva*, foram isoladas chalconas (precursores de flavonoides), sendo estas substâncias, juntamente com os taninos, os constituintes encontrados em maior quantidade no extrato obtido com acetato de etila (VIANA et al., 1997; VIANA; BANDEIRA; MATOS, 2003).

No que se refere aos aspectos toxicológicos e farmacológicos de *M. urundeuva*, o decocto da entrecasca é empregado na medicina popular nordestina para tratar inúmeras desordens, tais como problemas dermatológicos, ginecológicos e distúrbios no sistema digestório, apresentando também atividade cicatrizante e anti-inflamatória (MATOS, 2002). Diante da ampla utilização e propriedades farmacológicas, Resende et al. (2015) investigaram o potencial mutagênico do extrato hidroalcoólico através do teste de Ames, tendo observado a ausência de efeitos mutagênicos, além da ação antimutagênica.

As folhas por sua vez, são utilizadas para tratamento de úlcera (PEREIRA et al., 2014). Ademais, Oliveira et al. (2011) demonstraram que o extrato das folhas possui atividade ovicida contra o nematoide *Haemonchus contortus*, presente no trato gastrointestinal de pequenos ruminantes, atribuindo esta ação aos taninos presentes na planta.

Também foi relatada a atividade anti-inflamatória e analgésica da fração enriquecida de chalconas obtida a partir do extrato em acetato de etila da entrecasca (VIANA; BANDEIRA; MATOS, 2003). Por outro lado, a aplicação tópica do gel contendo o extrato hidroalcoólico (5%) da entrecasca de *M. urundeuva* na gengiva de ratos com doença periodontal induzida promoveu melhora no tecido, além de prevenir o crescimento de microorganismos (BOTELHO et al., 2007).

Estudo realizado por Carlini; Duarte-Almeida; Tabach (2013) demonstrou o potencial tóxico da administração crônica, em ratos, do extrato aquoso obtido por decocção, da entrecasca de *M. urundeuva*. Embora não tenha causado redução no

número de hemácias ou da hemoglobina, o tratamento promoveu redução do hematócrito e redução no peso dos animais, em comparação ao controle. Ainda, os autores observaram aumento do número de malformações ósseas na prole de fêmeas tratadas com o extrato, bem como redução do peso corpóreo de fêmeas grávidas que receberam tratamento com dose elevada do extrato de *M. urundeuva.*

Dentre as atividades biológicas de *M. urundeuva*, tem sido descrita a ação contra células cancerosas. Mahmoud et al. (2011) indicaram que os extratos etanólicos da entrecasca e do cerne, promoveram a morte das linhagens cancerosas HCT-8 (carcinoma humano de cólon), SF-295 (glioblastoma) e MDA-MB-435 (melanoma). Em adição, Jandú et al. (2012) demonstraram que o extrato metanólico da entrecasca não foi citotóxico para a linhagem de células normais VERO (células renais de macaco verde africano).

As lectinas isoladas do cerne (MuHL, 4,4 kDa), das folhas (MuLL, 14,2 kDa) e da entrecasca (MuBL, 14 kDa) de *M. urundeuva* apresentam atividade larvicida contra *Aedes aegypti* (SÁ et al., 2009; NAPOLEÃO et al., 2012). Relatou-se também a atividade termiticida de MuHL, MuLL e MuBL contra *Nasutitermes corniger*, bem como atividade bacteriostática e bactericida contra bactérias encontradas no intestino dessa espécie (SÁ et al., 2008; NAPOLEÃO et al., 2011). As lectinas citadas apresentam características interessantes para o uso biotecnológico das mesmas, como estabilidade a temperaturas elevadas sendo inativadas apenas a 100 °C e atividade em ampla faixa de pH, com MuHL e MuBL sendo inativadas apenas em pH 9 e MuLL em pH 12 (NAPOLEÃO et al., 2011).

4 ARTIGO CIENTÍFICO I

Chemical characterization and biological activities of *Apodanthera congestiflora* roots

A ser submetido no periódico Pharmacological reports

Chemical characterization and biological activities of *Apodanthera congestiflora* roots

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Abstract

Apodanthera congestiflora is an endemic plant from Brazil used by the population as depurative of blood and to treat skin patches, itch and back pain. Due to the absence of studies about the chemical composition and biological activities of this species, this work aimed to evaluate chemical composition, antioxidant activity, cytotoxicity on cancer cells and peripheral blood mononuclear cells (PBMCs), hemolytic activity, as well as the antimicrobial activity of hexane, ethyl acetate and methanol extracts from tuberous roots of *A. congestiflora*. Furthermore, it was evaluated the cytotoxic and antimicrobial activities of organic fractions and the anticancer activity of a cucurbitacin isolated. It was detected on extracts coumarins, alkaloids, terpenes and anthraquinones. *A.congestiflora* extracts showed antioxidant activity, with the best results observed to ethyl acetate on phosphomolybdenum method and for methanol for DPPH assay. The IC_{50} values on cancer cell lines tested ranged from 3.7 to 15.5 μg/mL and from 18.02 to 32.0 μg/mL, for hexane and ethyl acetate, respectively. The methanol extract did not promote reduction in cell viability. The extracts also reduced the viability of PBMCs. On hemolytic assay, the EC_{50} of the extracts ranged from 594.1 to 1.087 μg/mL. The fractions obtained from hexane extract showed IC_{50} values ranging from 0.54 to 24.35 μg/mL. The isolated cucurbitacin from hexane extract was cytotoxic to NCI-H292, HEP-2, HL-60 and K562 (IC_{50} of 1.98, 4.16, 2.95 and 3.22 μg/mL, respectively) and to PBMCs (IC_{50} of 2.31 μg/mL). Effects of the cucurbitacin on morphological aspects of HL-60 showed nuclear fragmentation, as well as pyknosis and membrane disintegration at 8.0 μg/mL. Apoptosis and secondary necrosis were detected, as well as mitochondrial membrane depolarization, suggesting mitochondrial involvement in the apoptosis process. The Minimum inhibitory concentration (MIC) of the extracts ranged from 0.23 mg/mL to 15 mg/mL. The MIC values determined for organic fractions ranged from 0.125 to 2.5 mg/mL.

This study determined the antioxidante, cytotoxic and antimicrobial activities of *A. congestiflora* roots extracts and identified some compounds associated with these effects. The anticancer activity showed by the cucurbitacin isolated suggest involvement of the intrinsic pathway in the apoptosis cell death promoted. However, studies are necessary to verify the deleterious effect of this compound on normal cells.

Keywords: *Apodanthera congesiflora*, cucurbitacin, antimicrobial, apoptosis

Introduction

Plants have important role as source of biologically active substances, which can be directly employed in the treatment of diseases or act as a prototype for the development of drugs [1, 2]. Indeed, several drugs with anticancer activity have been developed from plants, such as vinblastine and vincristine, obtained from *Catharanthus roseus*, vinfluin, developed from a structural modification in vinblastine and paclitacel, isolated from *Taxus brevifolia* [1,3].

Despite the large number of drugs available for cancer treatment, this therapeutics is related with the promotion of damage to normal cells and the development of cancer cells resistance to chemotherapy [4, 5, 6]. Therefore, the search for new active compounds associated to minimal damage on normal structures is essential.

Antibiotics are closely related to cancer treatment and may be used in patients submitted to chemotherapy in order to prevent infections in individuals with low white blood cells [7]. Additionally, antibiotic resistance showed by many pathogenic microorganisms is a global problem and is related to the adaptability of microorganisms [8]. Thus, the high quantity of products with antimicrobial activity obtained from plants indicates the relevance of the studies to find new antibiotics [9].

Cucurbitaceae family has approximately 900 species in 118 genera and is widely distributed in tropical and subtropical regions [10]. Plants belonging to this family have several biological properties, such as hypoglycemic, hypotensive, anticancer, antimicrobial, anti-inflammatory, hepatoprotective, analgesic and antioxidant activities [11, 12, 13, 14]. Among the chemical constituents found in plants of the Cucurbitaceae family are the cucurbitacins, which are tetracyclic triterpenes associated with various biological activities (HE et al., 2013).

Apodanthera congestiflora (*syn. Melothria congestiflora*) is an endemic plant from Brazil, belonging to the Cucurbitaceae family. This plant is widely distributed in the Caatinga biome in states of Bahia, Pernambuco, Ceará, Piauí, Rio Grande do Norte, Sergipe, also being found in the state of Minas Gerais [15]. Despite the use of this plant as depurative of blood and to treat skin patches, itch and back pain by the population, studies involving this species are limited to the field of Ethnobotany [16, 17].

This study aimed to evaluate the chemical composition and investigates the antioxidant, cytotoxic and antimicrobial activities of tuberous roots extracts from *Apodanthera congestiflora*, as well as the anticancer activity of a isolated cucurbitacin.

Material and Methods

Plant material

The tuberous roots from *Apodanthera congestiflora* was collected in Barbalha (Lat.: 7° 16.26' 70.24''; Long.: 39° 15.28' 26.36''), between the months of September and December (2015) at state of Ceará (Brazil) and a voucher specimen was deposited with the number 899985 in the Herbarium *Instituto Agronômico de Pernambu*co (IPA).

Cell lines and cell culture

The cancer cell lines used for the *in vitro* cytotoxicity tests were: NCI-H292 (human pulmonary mucoepidermoid carcinoma), HT-29 (human colon adenocarcinoma), HEp-2 (human larynx epidermoid carcinoma), MCF-7 (human breast adenocarcinoma cell line), (MOLT-4 (human acute lymphoblastic leukemia cell line), K562 (human erythromyeloblastoid leukemia cell line) and HL-60 (promyelocitic leukemia). These cells were grown in DMEM - Dulbecco's Modified Eagle's Medium or RPMI medium, supplemented with 10% FBS, 1% of penicillin–streptomycin solution, 1% of glutamine, at 37 °C with a 5% CO_2 atmosphere. The cells were obtained from the Cell Bank of Rio de Janeiro, Brazil and maintained in the Laboratory of Cell Culture, *Departamento de Antibióticos*, *Universidade Federal de Pernambuco* (UFPE), Brazil. Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood from healthy volunteer donors (18 to 30 years old) (Experiments involving human blood were approved by the Ethics Comitee on Research Involving Human

Beings from Federal University of Pernambuco under the number 60107916.8.0000.5208).

Microorganisms

The strains of bacteria and one yeast used were obtained from the Collection of Microorganisms of the *Departamento de Antibióticos, Universidade Federal de Pernambuco* (UFPEDA), Brazil. For this test were used four gram-positive bacteria: *Staphylococcus aureus* (UFPEDA 01), *Micrococcus luteus* (UFPEDA 100), *Bacillus subtilis* (UFPEDA 86) and *Enterococcus faecalis* (UFPEDA 138); three gram-negative bacteria: *Escherichia coli* (UFPEDA 224), *Serratia marcescens* (UFPEDA 352) and *Pseudomonas aeruginosa* (UFPEDA 39); an acid-fast bacteria: *Mycobacterium smegmatis* (UFPEDA 71) and the yeast *Candida albicans* (UFPEDA 1007). Additionally, clinical isolates from *Staphylococcus aureus* were obtained from urine (UFPEDA 671), purulent exudates (UFPEDA 709), operative wound (oxacillin resistant- ORSA) (UFPEDA 719) and catheter tip (UFPEDA 725).

Tuberous roots extracts

The roots from *A. congestiflora* were dried at room temperature and powdered. The extraction was performed in a soxhlet apparatus using hexane, ethyl acetate and methanol, in this order during six hours for each extract. The obtained extracts were concentrated to near dryness on a rotary evaporator and the yield was calculated from the ratio of the mass of dry crude extract and the initial mass of the plant.

Thin layer chromatography

The obtained extracts were applied in chromatography using silica gel 60 plates (0.20 mm) as fixed phase and pre-selected organic solvents as mobile phase. Then, the plates were irradiated with 365 nm UV light, using specific reagents for each class of compounds. Anisaldehyde-sulfuric acid and ceric sulphate were used to terpenoid compounds and flavonoids. Potassium hydroxide in ethanol was used to coumarins and anthraquinones. The Lieberman-Burchard reagent it was used to detect steroids/triterpenoids. Drangendorff was the reagent used to alkaloids identification and ferric chloride solution was used to tannins [18].

Quantification of total and residual phenols

Quantification of total phenols was carried out according to Amorim et. al. [19] An aliquot (0.2 mL) of the *A. congestiflora* extracts (1 mg/mL) was transferred to a test tube, to which was added 0.5 mL of Folin-Ciocalteu reagent, 1 mL of sodium carbonate and distilled water. After 30 minutes it was read the absorbance at 760 nm. The calibration curve was prepared using tannic acid (0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5 and 10 µg/mL) and total phenols content was expressed as milligrams equivalent of tannic acid per gram of extract (mg TAE/g).

To quantify the residual phenols, the extracts (6 mL, 1 mg/mL) were mixed with casein (1 g) and distilled water (12 mL). After three hours of reaction with stirring, the material was filtered to quantification by the Folin-Ciocalteu method. The tannin content was calculated as the difference between total and residual phenols after filtration.

Flavonoid content

The test was performed according to Peixoto-Sobrinho et al. [20] with modifications. The extracts (0.2 mL; 1 mg/mL) were mixed with glacial acetic acid (120 µL), pyridine (2 mL; 20% v/v in ethanol), aluminum chloride (0.5 mL; 5% p/v in distilled water) and the volume was completed with distilled water until 10 mL. After incubation (30 minutes) in the dark, the absorbance was measured at 420 nm. A standard curve (0.05-2µg/mL) was prepared using rutin. The content of flavonoid in extracts was expressed as milligrams equivalent of rutin per gram of extract (mg RUE/g). The test was performed in triplicate.

Coumarin content

The assay was performed according to Osorio and Martins [21] with modifications. *A. congestiflora* extracts (0.5 mL; 1 mg/mL) were transferred to test tubes and to each tube were added distilled water (2 mL) and lead acetate (500 µL). After stirring and addition of distilled water (7 mL), an aliquot of this solution was added to 8 mL of hydrochloric acid. After 30 minutes, the absorbance of the solution was measured at 320 nm. A standard curve was used with different concentrations of

coumarin (0.4-16 mg/mL) and results were expressed as milligrams equivalent of coumarin per gram of extract (mg CE/g). The tests were performed in triplicate.

Fractionation of hexane extract

Hexane extract (1g), the most active on cancer cells, mixed with Silica Gel 60 (Merck) was placed on the top of a glass column (30 cm x 3.5 cm) using Silica Gel 60 (Merck) as stationary phase. The column was eluted with hexane, hexane-ethyl acetate (8:2), hexane-ethyl acetate (7:3), hexane-ethyl acetate (6:4), hexane-ethyl acetate (5:5) and ethyl acetate, in this order. The fractions obtained were kept in a desiccator until constant weight. Thin layer chromatography was performed for each eluted fraction for banding pattern analysis. Fractions with similar banding profile were pooled, yelding 13 fractions.

Gas chromatography-mass spectrometry (GC-MS)

The fractions (1 mg) were diluted in dichloromethane and analyzed in a gas chromatograph coupled with a mass detector equipped with a capillary column ValcoBond VB-5 (length of 30 m, 0.25 mm internal diameter and 0.25 μm of film thickness). GC-MS operating conditions were as follows: the column temperature was set at 70℃ and held for 40 min, increased to 280℃ at a rate of 4℃/min, and held at this temperature for 15 min. The detector was maintained at 280°C. Split injection was performed with a spilt ratio of 65:1. Helium at 0.8 mL/min was used as carrier gas. In the end of the process, the spectrum of the unknown components of each fraction analyzed was compared with known components stored in the library using NIST mass spectrum database.

Isolation of a cucurbitacin from the roots of *A. congestiflora*

The most active fraction on cancer cells was submitted to a new fractionation to increase purity. The column chromatography was performed in a glass column, using Silica Gel 60 (Merck) as stationary phase. The fraction 13, obtained with ethyl acetate was used for this. The column was eluted with hexane-ethyl acetate (5:5), ethyl acetate and ethyl acetate-methanol (9:1), in this order. The fractions obtained were kept in a desiccator until constant weight. Thin layer chromatography was performed for each

eluted fraction for banding pattern analysis and those with similar banding profile were pooled. Then, majoritarian fraction was recrystallized using ethanol. The crystals were diluted in deuterated chloroform and evaluated for nuclear magnetic resonance spectra of hydrogen (1H NMR) and carbon-13 (13C-NMR), including two-dimensional NMR (HMBC, HSQC and COSY). The hydrogenation pattern of the carbons was determined by using DEPT (Distortionless Enhancement by Polarization Transfer);

Total antioxidant activity by Phosphomolybdenum method

The test is based on reduction of the Mo (VI) to Mo (V) by the extract and formation of a green phosphate/Mo (V) [22]. For this assay, 0.1 mL of *A. congestiflora* extracts (1 mg/mL) or the standard ascorbic acid was mixed with 1 mL of the reagent (0.6 M sulfuric acid, ammonium molybdate and 4 mM sodium phosphate, 28 mM). The tubes were closed and incubated in water bath at 95 °C for 90 minutes, for later reading of the absorbance at 695 nm. The results were expressed as µg of ascorbic acid equivalent (AAE) per mg of extract.

Free radical scavenging activity by DPPH 2,2-Diphenyl-1-picrylhydrazyl assay

The test is based on electron transfer by an antioxidant substance. The DPPH, which presents purple color, is reduced to diphenyl-picryl hydrazine, with yellow coloration. The extracts were tested at concentrations ranging from 62.5 to 1000 µg/mL. and ascorbic acid (0.3, 0.5, 1, 2, 3, 4 and 5 µg/mL) was used as standard and 270 µL of the DPPH solution was added to each sample, using ethanol as white. After 30 minutes, the reduction of DPPH was determined by colorimetric change at 517 nm [23]. The antioxidant concentration required to decrease 50% of the DPPH present (IC_{50}) was determined.

Evaluation of cytotoxicity of extracts and fractions on cancer cell lines

Cell viability was measured by MTT (3-(4, 5-dimethyl-2-thiazole)-2, 5-diphenyl-2H-tetrazolium bromide) reduction assay, which is based on the conversion of the salt MTT to a formazan product by the action of the enzyme succinyl dehydrogenase present in mitochondria of viable cells [24, 25]. The cancer cell lines NCI-H292, HT-29, HEp-2, MCF-7 cells (10^5 cells/mL), MOLT-4 (10^6 cells/mL) in DMEM medium,

K562 and HL-60 ($0.3x10^6$ cells/mL) in RPMI medium were plated in 96-well microplates and incubated during 24 h at 37 °C. After this period, 10 μL of hexane, ethyl acetate or methanol extracts (50 μg/mL) or 10 μL of fractions (25 μg/mL) obtained by column chromatography were added to each well and incubated for 72 h. Next, MTT dye (25 μL; 0.5 mg/mL) was added to the wells and the assay was incubated for more 3 h. After this period, the medium was removed and dimethylsulfoxide (DMSO, 100 μL) was added to the wells for solubilization of the generated formazan salts. The optical density of the wells was measured at 540 nm and compared to that in the control (cells incubated only with medium). Two independent experiments were performed in triplicate. The IC_{50} value was calculated to extracts and fractions with inhibition of cell viability above 70%. To this, the extracts and fractions were tested again in serial concentrations ranging from 0.39 to 50 μg/mL for extracts and 0.195 to 25 μg/mL for fractions. The concentration that reduced cell viability by 50% (IC_{50}) was calculated by nonlinear regression using the software *GraphPad* Prism v. 5.0.

Evaluation of cytotoxicity of *A. congestiflora* extracts on peripheral blood mononuclear cells (PBMCs)

PBMCs were purified from human blood by gradient separation using the solution for cell separation Histopaque-1077 (Sigma–Aldrich). Cells were isolated from a 6 mL blood sample in 5 mL of phosphate buffered saline (PBS) to which was added 3 mL of Histopaque-1077 (Ethics Committee 60107916.8.0000.5208). After centrifugation at 1500 rpm for 30 min, the mononuclear cells (present in the intermediate region between the red cells and serum) were aspirated. The lymphocyte suspension was transferred to another tube to which PBS was added until the volume of 11 mL and centrifuged for 20 min at 1000 rpm. Then, the supernatant was discarded and the lymphocyte pellet ressuspended to a final concentration of 10^6 cells/mL in RPMI 1640 medium supplemented with 20% fetal bovine serum, 1% of a penicillin-streptomycin solution. Lymphocytes proliferation was induced by addition of 3% phytohemagglutinin (PHA), a mitogen which acts fundamentally on T lymphocytes becoming they the predominant cell population in this assay (LIU et al., 2004). The cells in RPMI complete medium (with PHA) were plated in 96-well microplates and incubated during 24 h at 37 °C.

To determine the cytotoxicity on PBMCs, hexane, ethyl acetate or methanol extracts from *A. congestiflora* (100µL; 50 µg/mL) were added to cell and incubated for 72 h at 37 °C with a 5% CO_2 atmosphere. MTT dye (25 µL; 0.5 mg/mL) was added to the wells and the assay was incubated for more 3 h. After this period, the medium was removed and DMSO (100 µL) was added. The optical density of the wells was measured at 540 nm and compared to that in the control.

Hemolytic Assay using human erythrocytes

The hemolytic assay was performed to hexane, ethyl acetate and methanolic extracts and to fractions which were active on cancer cells. The assay was performed in 96-well microplates. Each well received a 0.85% NaCl solution containing 10 mM $CaCl_2$. Samples (100 µL, in DMSO 5%) of the extracts were added to the first well of each column in triplicate, from which 100 µL were transferred to the second well to performed a serial dilution (final concentrations ranging from 15.62 to 2000 µg/mL).. Each well received 100 µL of a 2% (v/v) suspension of human erythrocytes in 0.85% saline containing 10 mM $CaCl_2$ (Ethics Committee 30731814.8.0000.5208). In negative controls, 100 µL of the saline solution plus 50 µL of the saline solution or 50 µL of 5% DMSO were plated. Positive control (to obtain 100% hemolysis) contained 80 µL of saline solution plus 20 µL of 0.1% Triton X-100 in 0.85% saline. After centrifugation during 1 h followed by incubation for 1 h at 27 °C, the supernatant was discarded, and the released hemoglobin was measured by absorbance at 450 nm. Two independent experiments were performed in duplicate.

Cytotoxicty of cucurbitacin isolated on PBMCs and cancer cell lines

The IC_{50} of the cucurbitacin isolated from active fraction on PBMCs and cancer cell lines was determined using the MTT assay, as previously described. Additionally, the cytotoxicity of the compound after 24 and 48 h of treatment was evaluated.

Morphological Analysis on HL-60 cells treated with cucurbitacin

Morphological analysis was performed by Panotic staining after incubation of HL-60 cells ($3x10^5$ cells/mL) with cucurbitacin (2.0, 4.0 and 8.0 µg/mL) during 48 h. Cells were examined for morphological changes using light microscopy (Leica

DM500). To analysis of the morphology, cells were harvested, transferred to cytospin slides and staining with panotic.

Investigation of cell death

Cell death was investigated after treatment of HL-60 cells ($3x10^5$ cells/mL) with cucurbitacin (2.0, 4.0 and 8.0 µg/mL) during 48 h. Quantification of apoptotic or necrotic cells was measured using Guava Nexin reagent, which uses Annexin V to detect the phosphatidylserine on the external membrane of apoptotic cells and as membrane-impermeant dye, 7-amino-actinomycin D (7-AAD). Briefly, cells were centrifugated (5 min, 1500 rpm) and ressuspended with PBS. Next, e25 µl of each sample was suspended in a mixture of 25 µl Annexin V-PE and 7-ADD binding buffer. After incubation at room temperature for 20 min, samples were analyzed by flow cytometry (Amnis Image Stream Mark II, EMD Millipore Corp.).

The population was separated into four groups: viable cells (annexin V-/7AAD), early apoptosis (annexin V+/7AAD-), late apoptosis (annexin V+/7AAD+) and necrotic (annexin V-/7AAD+).

Measurement of mitochondrial membrane potential (ΔΨm) by flow cytometric analysis with JC-1 staining

JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red/orange, which can thus be used as an indicator of mitochondrial potential. In this study, HL-60 ($3×10^5$ cells/mL) was treated with cucurbitacin (2.0, 4.0 and 8.0 µg/mL) during 48 h. The medium was removed, by centrifugation (5 min, 1500 rpm) and cells were incubated with 3 µg/ml of JC-1 in PBS, for 30 min. After incubation, cells were washed two times (5 min, 1500 rpm) with PBS. Both orange and green fluorescence emissions were analyzed by flow cytometry (Amnis Image Stream Mark II, EMD Millipore Corp.).

Antimicrobial activity

Minimum inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

The susceptibility test methodology of the microdilution broth was carried out to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) according to NCCLS (2002; 2003). Hexane, ethyl acetate and methanol extracts of tuberous roots from *A. congestiflora* (90 µL in serial concentrations from 0.02 to 15 mg/mL, diluted in DMSO) or medium Müller Hinton Broth (MHB) or Sabouraud (90 µL) were plated in 96-well plates. Then, suspensions of the microorganisms were prepared at 0.5 McFarland (concentrations of 1.5 x 10^8 CFU/mL to bacteria and 1.5 x 10^5 CFU/mL for yeasts). Thereafter, it was added an aliquot of 10 µL of the microorganism suspension to each well. Plates were incubated during 24 hours (37 ° C) to bacteria and 48 hours (28 ° C) to yeast. Then all wells received 30 µL of Rezasurin for quantitative analysis of microbial growth. To determine the Minimum Bactericidal Concentration (MBC) or Minimal fungicidal Concentration (MFC) 5 µL of the concentrations that were active in the MIC plate were placed into Petri dishes containing Mueller Hinton Agar (AMH) or Sabouraud. These plates were incubated at 37 ° C during 24 hours to bacteria and at 28 °C during 48 hours to yeasts. The MBC or MFC corresponds to the lowest concentration of the extract which did not promote cell growth on the surface of AMH.

Results and Discussion

Chemical composition and antioxidant activity

The elucidation of the chemical composition of *Apodanthera congestiflora* is essential since the literature about this plant discusses only Ethnobotanical aspects [16, 17]. This study evaluated the yield, chemical profile, and biological activities of extracts and organic fractions obtained from the plant, as well as investigated the anticancer activity of a cucurbitacin of the roots from *A. congestiflora*.

The determination of extraction yield for each solvent revealed that the highest yield was detected using methanol (3.83%), followed by ethyl acetate (3.77%) and hexane (0.32%). The extraction method used in this work is inexpensive and largely employed. However, the biggest limitation of extraction by soxhlet is the risk of thermal

decomposition of the compounds, once that this extraction occurs at the boiling point of the solvent [26].

It was observed by thin layer chromatography analysis that all the extracts contain terpenes and did not present tannins and flavonoids. Coumarins and anthraquinones were detected only in the hexane extract and alkaloids in the ethyl acetate extract. The classes of secondary metabolites detected in *A. congestiflora* extracts had already been described for other plants of the family Cucurbitaceae. Shri et al. [27] showed the presence of alkaloids on ethyl acetate extract, obtained by soxhlet, of the roots and rhizomes from *Corallocarpus epigaeus*, but did not find saponins, tannins, phenolic compounds and flavonoids. Another plant belonging to the Cucurbitaceae family, *Momordica dioica*, has triterpenes, alkaloids and steroids in methanolic extract from roots; and alkaloids, saponins, triterpenoids, flavonoids and steroids on extract from fruits [13]. *Momordica tuberosa*, another plant belonging to Cucurbitaceae family, presents triterpenes in petroleum ether, chloroform, and ethanol extracts, sterols in petroleum ether, chloroform, as well as saponins, cardiac glycosides and carbohydrates in ethanol and water extracts of its tubers [28]. Thus, among the secondary metabolites presents on Cucurbitaceae, terpenes and alkaloids are present in several species and were also found in *A. congestiflora* roots. The difference on chemical constitution observed to species of this family probably is due to climatic and geographic factors, as temperature, latitude, longitude and sunshine duration and because they are different species [29].

Table 1 shows the content of total phenols as well as phenolic compounds flavonoids, coumarins and tannins in the *A. congestiflora* extracts.

Table 1. Contents of secondary metabolites in *A. congestiflora* extracts.

Extract	Total phenols	Flavonoids	Coumarins	Tannins
Hexane	20.09 ± 1.19	ND	12.03 ± 0.36	ND
Ethyl acetate	31.46 ± 3.64*	44.06 ± 8.10 *	9.26 ± 0.06	ND
Methanol	19.33 ± 0.91	18 ± 1.39	8.36 ± 0.10	ND

Data presented as tannic acid equivalents per extract gram (mg TAE/g) for total phenols and tannins, equivalent milligrams of rutin per gram of extract (mg RUE/g) and equivalent milligrams of coumarin by extracts of gram (mg CE/g) (mean ± standard deviation). ND- Not detected. *statistically different, $p<0.05$.

Total phenols were found in higher quantities in ethyl acetate extract. Flavonoids were not detected in hexane extract, while the flavonoid concentration in ethyl acetate extracts was higher than in the methanol extract. Coumarins were detected in all extracts and hexane extract was the best solvent to extract this secondary metabolite. Tannins were not detected in the extracts confirming the results from thin layer chromatography assay.

The column chromatography of the hexane extract allowed the obtention of fractions. GC-MS analysis showed the presence of fatty acids in all fractions obtained. Results are showed in Table 2. To fractions from 8 to 13, there was not volatilization of any substance. Therefore, the constituents of these fractions were not detected by GC-MS. Palmitic acid, a saturated fatty acid, was found in several fractions. Similarly, other plants belonging to Cucurbitaceae family contains palmitic acid, as *Ibervillea sonorae*, which possess this molecule in its roots, and *Cucurbita pepo*, which present palmitic acid in its seeds [30, 31, 32].

Table 2. Compounds identified by GC-MS in *A. congestiflora* fractions from hexane extract.

Retention time (min)	Area (%)	Compound
Fraction 1 (hexane-ethyl acetate, 8:2)		
38.70	23.51	Palmitic acid
42.83	6.09	Cis-vaccenic acid
43.43	4.92	Stearic acid
50.99	46.51	Aspidofractinine 3 methanol
51.87	6.5	Behenic acid
68.91	9.0	2-hexyl-1-octanol
70.41	2.36	7,22-Ergostadienone
Fraction 2 (hexane-ethyl acetate, 8:2)		
38.70	15.74	Palmitic acid
68.96	84.26	Heptanoic acid
Fraction 5 (hexane-ethyl acetate, 8:2)		
38.70	86.21	Palmitic acid
69.25	13.79	7,22-Ergostadienol
Fraction 6 (hexane-ethyl acetate, 8:2)		
38.70	95.41	Palmitic acid
69.22	4.59	NI
Fraction 7 (hexane-ethyl acetate, 7:3)		
38.70	100	Palmitic acid

*NI- not identified

Palmitic acid exhibits a wide range of biological properties including antitumor, antinociceptive and antibacterial activities [33, 34, 35]. The majoritarian compound founded in fraction 1 was aspidofractinine-3-methanol or kopsinyl alcohol, an indole alkaloid which is related chemically to kopsiflorine, other indole alkaloid which possess anticancer activity on cells resistant to vincristine [36]. Additionally, heptanoic acid, other saturated fatty acid was the majority constituent of fraction 2 and there is no biological activity reported to this compound [37].

The antioxidant activity of the extracts was tested *in vitro* using the phosphomolybdenum assay (Table 3), which is a widely used test to evaluate the total antioxidant capacity of extracts due to be a method simple, quick, cheap and widely associated with phenolic compounds [38]. Ethyl acetate showed antioxidant activity higher than hexane and methanol extracts ($p<0.05$), probably due to the higher number of total phenolic compounds of this extract in relation to the others. However, it is known that the phosphomolybdenum method presents activity not only to polyphenols but also to others substances, which are not phenolic compounds, such as vitamin E, ascorbic acid and carotenoids [22].

Table 3. Antioxidant activity by the phosphomolybdenum and DPPH methods.

Extract	**Phosphomolybdenum method (mg GAE/g)**	**DPPH (IC_{50} µg/mL)**
Hexane	13.02±0.60	100 ± 2.30
Ethyl acetate	26.4±0.84	28.4 ± 1.08
Methanol	20.41 ±0.05	21.16 ± 0.42

The extracts were also evaluated for antioxidant activity by DPPH assay, which is associated to hydrogen and electron transfer from antioxidant compounds to an organic chemical compound [39]. Methanol extract was more active than the others extracts since showed the lowest IC_{50} value ($p<0.05$). The antioxidant activity detected in the DPPH test is related to the presence of phenolic compounds, due to scavenging of the radical by hydrogen donation [38].

The antioxidant activity of extracts reveals the potential use of *A. congestiflora* tuberous roots as source of compounds for the treatment of various disorders, since substances with antioxidant activity possess the ability to scavenge free radicals such as hydroxyl radicals ($OH^{\bullet}$) or superoxide radical ($O_2^{\bullet-}$). These reactive oxygen species (ROS) are involved in several disorders, such as Alzheimer's disease and cardiovascular diseases [40, 41, 42].

Cytotoxicity of *A. congestiflora* extracts on cancer and normal cell lines

Hexane, ethyl acetate and methanol extracts (50 μg/mL) were evaluated for cytotoxicity on cancer cell lines. Methanol extracts did not reduce the percentage of viable cells over than 70% for any of the tested cell lines. IC_{50} values were then determined for the hexane and ethyl acetate extracts (Table 4). According to the U.S. NCI plant screening program, a crude extract is considered cytotoxic when IC_{50} values are ≤ 30 μg/mL [43]. In this study, hexane extract was considered cytotoxic for all cancer cell lines tested and ethyl acetate extract was not active only for HEP-2.

Table 4. Cytotoxicity of *Apodanthera congestiflora* extracts on cancer and normal cells.

Cell	Treatment		
	Hexane	Ethyl acetate	Doxorrubicin
NCI-H292	3.75 (3.4 – 4.1)	18.02 (16.6 – 19.5)	0.03 (0.01 – 0.05)
HEP-2	4.2 (3.6 – 5.0)	32.0 (27.3 – 37.4)	0.4 (0.30 - 0.50)
HL-60	12.5 (10.5-14.9)	20.32 (18.4-23.3)	0.06 (0.05 – 0.08)
K562	15.53 (13.5-17.9)	20.95 (15.22-28.3)	0.24 (0.19 – 0.29)
MOLT-4	14.08 (11–18.92)	19.56 (17.3-20.7)	0.38 (0.34 – 0.44)
HT29	>50	>50	0.40 (0.30 – 0.50)
PBMC	20.0 (15.0-26.3)	32.1 (20.8-49.6)	0.86 (0.63 – 1.19)

Data showed as IC_{50} and respective confidence interval.

The comparison of IC_{50} values reveals that the hexane extract was the most active extract on cancer cell lines tested with IC_{50} values ranging from 3.75 to 4.2 μg/mL, while the ethyl acetate extract showed IC_{50} values ranging from 18.02 to 32.0 μg/mL. This result may be due to the presence in hexane extract of coumarins and

anthraquinones, which are compounds with anticancer activity and may act inhibiting the proliferation of cancer cells by cell cycle arrest and promoting apoptosis [44].

A. congestiflora extracts contain alkaloids and terpenoids and these compounds can be associated with the cytotoxicity of them on cancer cells. Alkaloids from plants exhibit activity against cancer cells by acting on various routes and may promote cell cycle arrest, autophagy, apoptosis and inhibition of angiogenesis [46]. Yet, plants belonging to Cucurbitaceae family are source of specific triterpenes group, known as cucurbitacins, which exert potent anticancer action on several cancer lines through mechanisms involving cell cycle arrest and apoptosis promotion [47, 48, 49].

The effect of *A. congestiflora* extracts on mononuclear cells from human peripheral blood (PBMCs) was determined since it is important that compounds active on cancer cells promote minimal damage to normal cells. The IC_{50} values of the extracts on PBMCs were 20.0 and 32.1 μg/mL for hexane and ethyl acetate, respectively, being considered potentially cytotoxic for these cells, which are key immune cells for the maintenance of homeostasis, essentials in combating microorganisms that promote infections, such as *Pneumocystis pneumonia* [46, 47, 48].

Hemolytic Assay using human erythrocytes

The potential of the extracts to promote damage to erythrocyte membrane was evaluated using the hemolytic assay, which allows investigate the ability of a product to promote the formation of pores or cause rupture of the membrane of erythrocytes.

CE_{50} was 1087±50.27, 594.1±42.9 e 987.8±68.05 μg/mL for hexane, ethyl acetate and methanol extracts, respectively. To be considered hemolytic, a product must present $CE_{50} \leq 200$ μg/mL [49]. Thus, all the extracts did not cause hemolysis. This assay is essential to contribute to the investigations about the safe use of products. Saponins, tannins, phenols and chalcones are widely associated with the promotion of hemolysis. The roots from *A. congestiflora* presented saponins as indicated by the qualitative test. So, despite having saponins and phenolic compounds, *A. congestiflora* extracts promotes hemolysis only at high concentrations.

Extract fractionation allows greater separation of chemical constituents of plants and is a key step to isolation of the active product. In this sense, the hexane extract was

then fractionated by column chromatography resulting in 13 fractions that showed distinct cytotoxicity on cancer cell lines (Table 6). The fractions from 1 to 6 were eluted with hexane-ethyl acetate (8:2); fractions from 7 to 10 were eluted with hexane-ethyl acetate (7:3); fraction 11 was eluted with hexane-ethyl acetate (6:4); fraction 12 was eluted with hexane-ethyl acetate (5:5); and fraction 13 was eluted with ethyl acetate. Fractions 1 to 6 and 8 did not promote reduction of cell viability above 70 % in at least two cell lines, thus, the IC_{50} was calculated for the fractions 7, 9, 11, 12 and 13 (Table 7), which exhibited a percentage inhibition greater than 70% in at least two cell lines.

Table 6. Percent of growth inhibition on cancer cells of fractions of *Apodanthera congestiflora*.

	Cell lines GI %						
Fraction	**NCI-H292**	**HEP-2**	**HL-60**	**K562**	**MOLT-4**	**HT29**	**MCF-7**
1	30.2±2.0	33.0±1.3	71,6±0.2	64.2±1.7	36.0±0.7	-	30.4±2.6
2	37.0±0.9	28.2±1.8	57+9±0.7	53.9±0.9	26.5±2.0	-	4.53±0.3
3	30.2±0.9	32.6±2.3	61.2±0.2	63.2±1.6	12.9±1.6	-	-
4	51±1.1	26.0±1.3	31.2±1.2	75.4±1.8	20.5±1.25	52.0±5.6	-
5	53.0±2.1	49.7±0.9	67.1±3.0	62.9±2.4	27.1±0.13	20.4±1.0	5.83±0.45
6	50.1±2.4	65.6±1.9	58.8±0.9	54.8±1.2	49.4±1.5	23.3±1.7	19.2±0.43
7	60.4±2,4	74.1±0.9	92.0±1.5	71.3±3.3	50.17±1.8	10.8±0.23	37.6±1.15
8	6.0±0.24	23.3±1.6	33.8±1.4	58.6±1.0	25.6±1.46	-	31.0±1.0
9	76.3±1.74	78.6±2.5	92.3±0.9	53.8±0.6	66.0±2.6	54.5±0.5	40.8±0.33
10	78.8±1.2	66.3±1.9	97.0±0.3	73.8±0.9	41.1±0.64	13.3±0.11	25.5±0.9
11	80.6±1.27	95.7±0.5	93.0±0.1	93.7±0.8	34.36±1.8	70.4±1.2	53.92±2.0
12	88.5±1.65	94.4±0.9	97.2±0.16	65.4±1.3	59.16±0.6	59.4±1.5	54.9±1.1
13	88.5±1.65	96.0±0.8	97.8±0.2	97.7±0.1	48.13±1.9		61.1±1.3

Data showed as menan±standard deviation. (-) Did not reduce cell viability.

Table 7 shows the IC_{50} values of the active fractions. The fractions 11, 12 and 13 were identified as cytotoxic fractions for NCI-H292, HEP-2, K562 and HL-60 cell lines, with IC_{50} after 72 hours of treatment ranging from 0.54 to 3.7 μg/mL. According to National Cancer Institute (NCI) guidelines, pure compounds are considered actives when their IC_{50} is 4 μg/mL or less. The IC_{50} values indicate the fractions 12 and 13 as

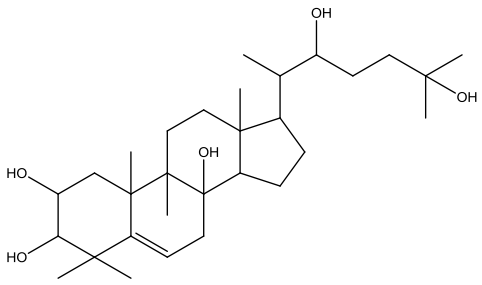
potential sources of molecules for the development of new drugs with anticancer activity.

Table 7. IC_{50} of *Apodanthera congestiflora* fractions on cancer cells.

Cell Line	IC_{50} (µg/mL)					
	7	**9**	**10**	**11**	**12**	**13**
NCI-H292	NT	24.3 21.2-28.0	20.5 19.4-22.3	8.0 6.5-9.5	0.54 0.45-0.64	1.0 0.72-1.33
HEP-2	18.9 17.7-19.2	11.2 9.0-13.8	NT	7.1 5.4-9.3	1.2 0.9-1.5	1.7 1.4-2.0
HT-29	NT	NT	NT	23.5 22.3-26.0	NT	11.3 6.8-18.7
HL-60	10.5 8.7-12.7	8.2 7.3-9.2	8.4 7.1-9.8	8.8 7.4-10.4	1.4 1.1-1.6	2.4 2.0-3.0
K562	22.3 20.4-25.8	NT	21.7 19.4-23.3	8.1 6.4-10.2	NT	3.7 3.3-4.2

Data showed as IC_{50} and respective confidence interval. *NT= not tested.

Based on these results and considering the amount of fraction available, fraction 13 was subjected to a new fractionation on a silica column. After recrystallization of the fraction obtained in greater quantity, RMN analysis allows to identify a compound with thirty carbons, specifically a triterpene belonging to the group of cucurbitacins, a group that possess a characteristic tetracyclic cucurbitane nucleus skeleton, and are widely described as anticancer agents [54]. This is the first time that is reported the isolation of a cucurbitacin in the roots of *A. congestiflora*. The probable structure of this molecule is showed in Figure 1.



C (6)	CH (7)	CH_2 (9)	CH_3 (8)
141,25	121,38	41,41	28,00
73,11	76,68	34,83	27,25
60,38	75,01	32,27	26,63
48,78	70,283	31,26	25,42
46,55	43,64	30,42	23,87
34,53	42,40	28,87	17,97
	37,82	26,88	15,41
		24,36	12,52
		21,11	

Figure 1. Probable structure of the cucurbitacin from *A. congestiflora* roots.

Therefore, it was evaluated its cytotoxic action of the cucurbitacin obtained on cancer cells after 24, 48 and 72 hours of treatment (Table 8).

Table 8. IC_{50} of the cucurbitacin isolated from *Apodanthera congestiflora* on cancer cells after 24, 48 and 72 hours of treatment.

	IC_{50} (µg/mL)		
Cell	**24 h**	**48 h**	**72 h**
NCI-H292	7.41 ± 0.24	3.50 ± 0.17	1.98 ± 0.15
HEP-2	14.12 ± 1.0	4.85 ± 0,64	4.16 ± 0.32
HL-60	7,60 ± 0.8	4,07 ± 0,08	2.95 ± 0.76
K562	12.73 ± 0.8	4.15 ± 0.34	3.22 ± 0.54

Data showed as mean ± standard deviation.

The value of IC_{50} was lower than 10 µg/mL for all cell lines tested. Thus, the compound it was considered very toxic [47]. After 24 h the lowest IC_{50} values were obtained for NCI-H292 and HL-60 cell lines.

Despite of the promising result for cancer cell lines tested, it was verified that the cucurbitacin isolated from the roots of *A. congestiflora* shows to be very cytotoxic to PBMCs, with IC_{50} of 2.31 ± 0.04 µg/mL after 72h of treatment. This suppressor effect of some cucurbitacins on PBMCs has already been described previously, as observed to cucurbitacin B, which inhibited PBMC activation by phytohemaglutinin (PHA) [55]. Present data indicates the necessity of *in vivo* toxicological investigations in order to evaluate the damage promoted after hepatic metabolization.

Cucurbitacins are widely described for its cytotoxic action on cancer cells. Dantas et al. [56] demonstrated cytotoxic action of three cucurbitacins of the fruits from *Cayaponia racemosa* on cell lines HL-60, MCF-7, CEM, B16, and HCT-8. For cucurbitacin E, it was demonstrated ability to inhibit the proliferation of the cell lines A549, Hep3B, and SW480 [57]. In addition, several studies have been performed to identify the type of death promoted by this group of triterpenes [58].

In this work, investigation of the type of death was performed to HL-60 after 48 h of treatment. For this, the compound it was tested at three concentrations: 2.0, 4.0 and 8.0 µg/mL.

Morphological analysis

Figure 2 shows the effect of the tested compound on morphology of HL-60 cell line after 48 h of treatment. Control cells showed typical morphology of nonadherent cells, such as intact membrane, pleomorphic cells and clear visualization of both plasma and nuclear membranes.

Moreover, it was noticed most of morphological changes associated with apoptosis like nuclear condensation, membrane blebbing and nuclear fragmentation were observed in the treated cells. Analysis of cells of control and those treated with the compound on concentration of 2.0 µg/mL showed several mitotic figures, indicating that the lower concentration did not promote significant changes in cell proliferation. This treatment also promoted nuclear fragmentation and reduction of the cytoplasmic volume, in relation to the control. On the other hand, cells treated with concentration of 4.0 µg/mL showed an accentuated decrease on these mitotic figures, nuclear fragmentation and increase of cell debris. To cells treated with 8.0 µg/mL of the compound, mitotic figures were not observed, but characteristics of apoptosis, such as nuclear fragmentation and necrosis, as pyknosis and plasma membrane disintegration were observed too.

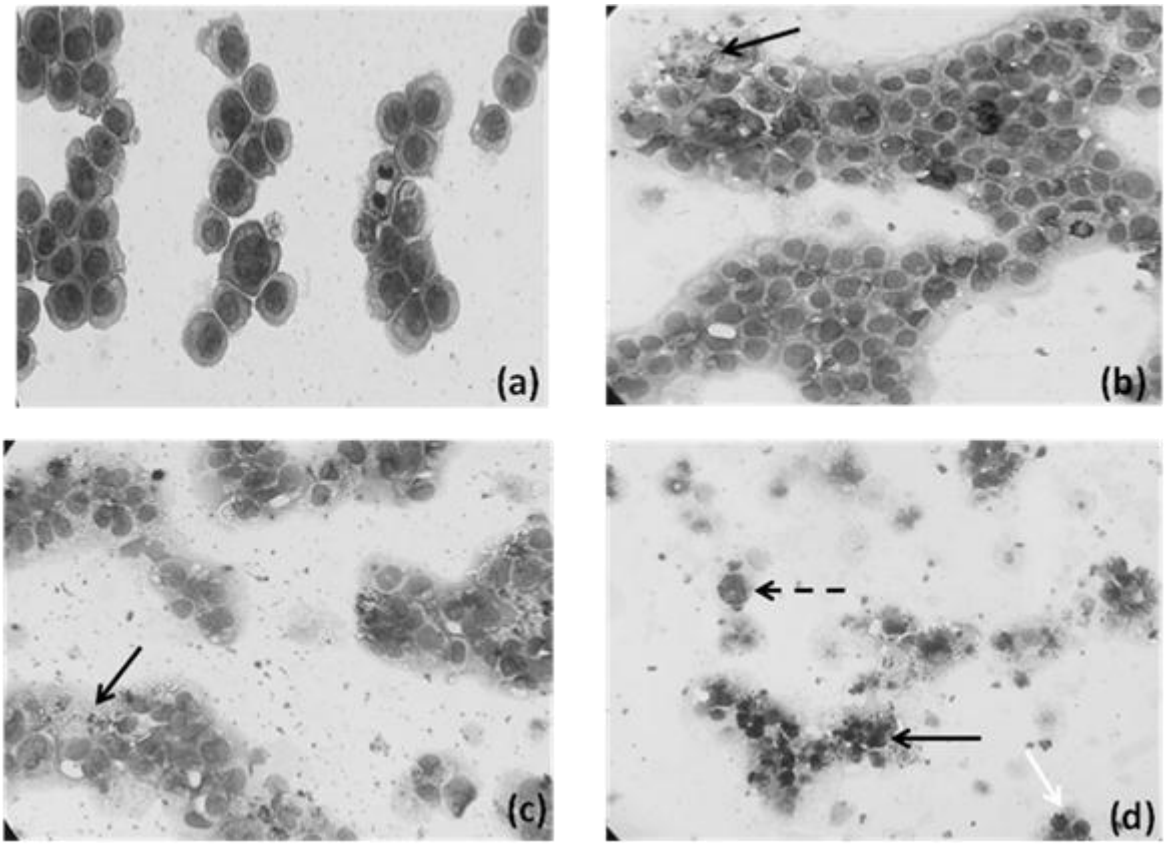
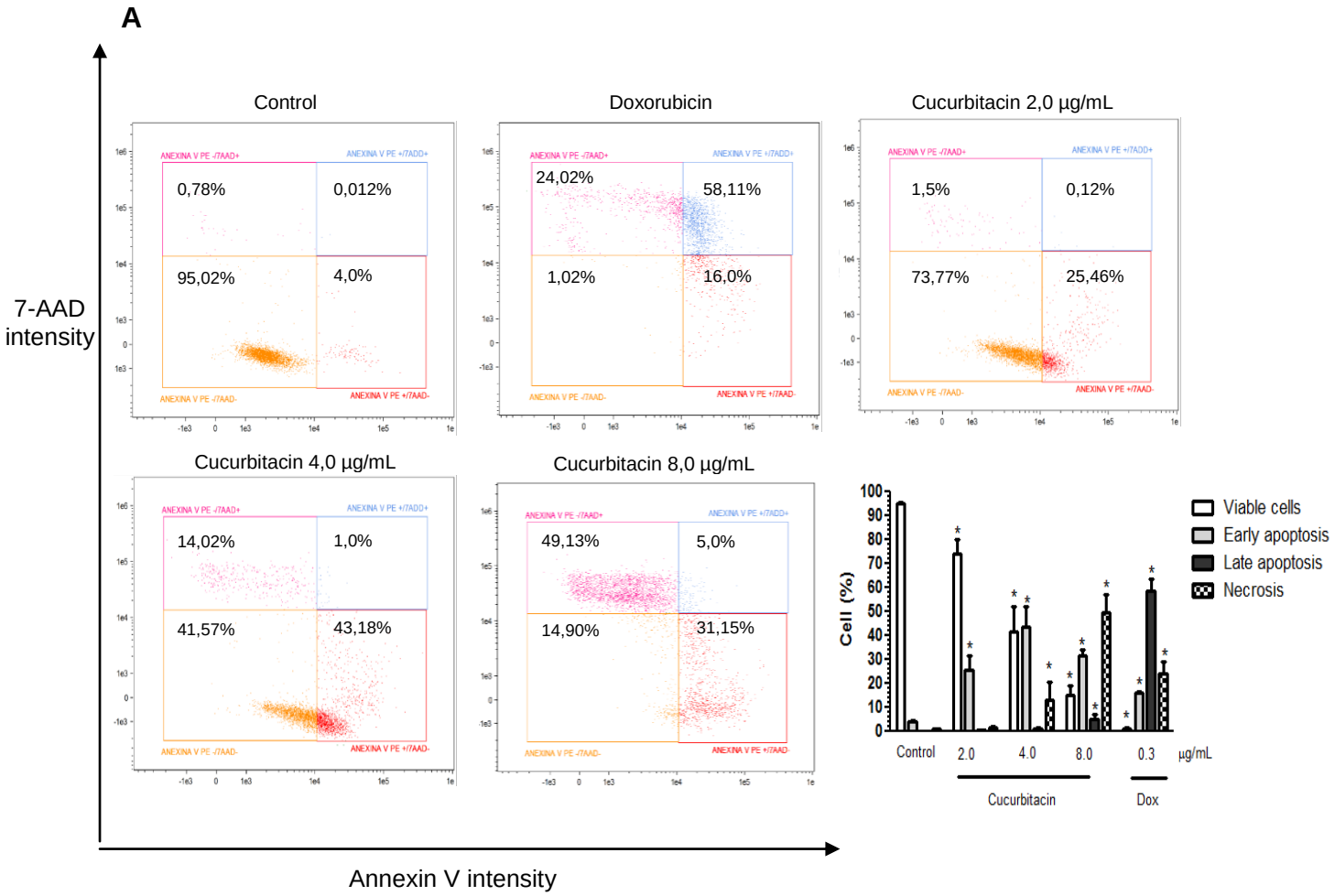
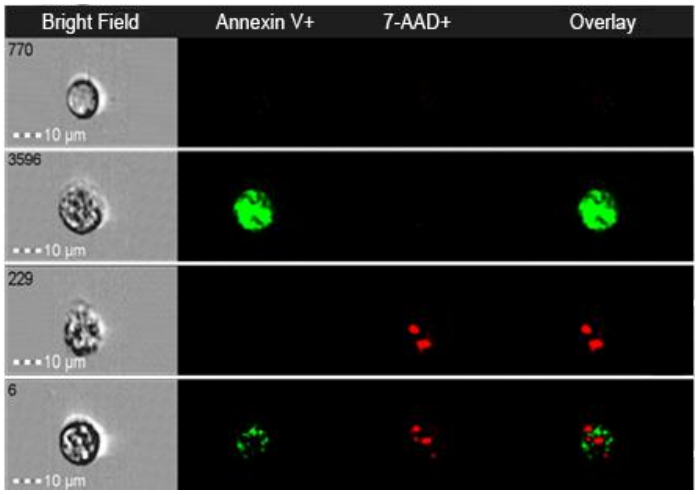


Figure 2. HL-60 cell morphology after treatment with a cucubirtacin from the roots of *A. congestiflora* (a) Control cells, (b) cucurbitacin 2.0 µg/mL, (c) cucurbitacin 4.0 µg/mL, (d) cucurbitacin 8.0 µg/mL. Black arrow - nuclear fragmentation, White Arrow – pyknosis, dashed Arrow - Membrane disintegration.

Investigation of cell death

To validate the results observed on morphological analysis and examine the involvement of apoptosis or necrosis on cell death, HL-60 cells were treated during 48 h and stained with annexin V and 7-AAD. The results of flow cytometry allowed the visualization of four distinct populations: viable cells (annexin V-/7AAD-), early apoptosis (annexin V+/7AAD-), late apoptosis (annexin V+/7AAD+) and necrotic (annexin V-/7AAD+) cells, as showed in Figure 3.





Annexin V- / 7-AAD-

Annexin V+ / 7-AAD-

Annexin V- / 7-AAD+

Annexin V+ / 7-AAD+

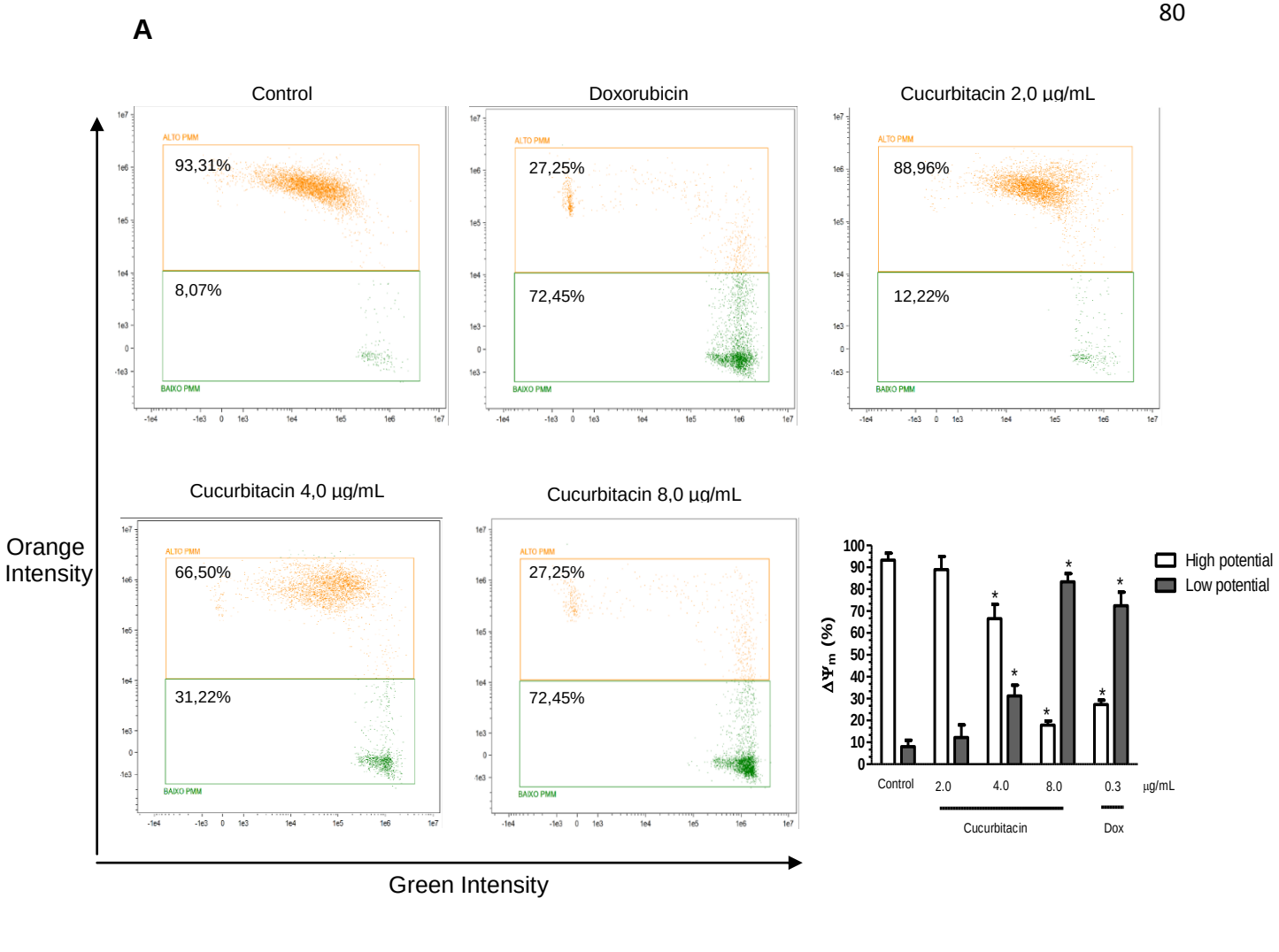
Figure 3 Cell death investigation by Annexin V and 7-AAD. Flow cytometry analysis of HL-60 cells treated with 2.0, 4.0 and 8.0 µg/mL of cucurbitacin. (A) Dot plot and percentage of population of viable, early apoptotic, early apoptotic and necrotic cells after treatment; bar chart showing increased proportion of early apoptotic cells in concentrations of 2.0 and 4.0 and increased on necrotic cells to concentration of 8.0 µg/mL. Data represented as mean ± SD of two independent experiments (*$p<0.05$). (B) Representative figures showing

population of viable (annexin V-/PI-), early apoptotic (annexin V+/PI-), c (annexin V+/PI+) and necrotic (annexin V-/PI+) cells.

After 48 h of treatment with tested compound, a decreased number of viable cells was observed in a dose-dependent manner. Also, it was observed an increased number of apoptotic cells for the treatments with 2.0 and 4.0 µg/mL. Treatment with 8.0 µg/mL showed cells in apoptosis and in necrosis. These results suggest that the compound induces apoptosis followed by secondary necrosis, which is the natural outcome of fully developed apoptosis and it may called "postapoptotic necrosis" [61, 62]. The tested cucurbitacin was able to reduces viability and cell proliferation, through the induction of cell death by apoptosis, followed by necrosis in HL-60.

Membrane mithocondrial despolarization

Mitochondrial membrane permeabilization is widely associated to apoptosis, specifically with intrinsec pathway. In order to confirm the results obtained previously, the effect of the cucurbitacin on mitochondrial membrane potential $\Delta\Psi_m$ was evaluated by JC-1 staining to determine whether mitochondrial dysfunction was involved in the apoptosis. Results are showed in Figure 4.



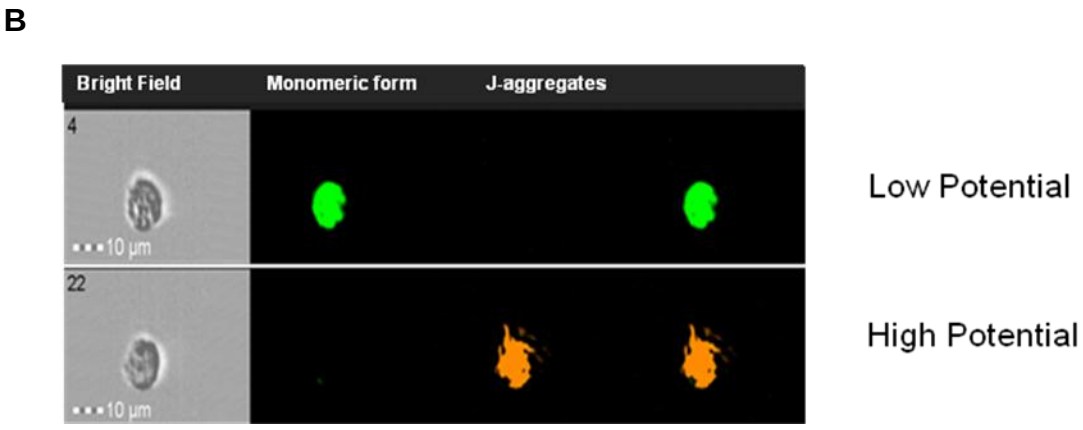


Figure 4 Mitochondrial membrane potential in HL-60 cells treated with isolated cucurbitacin. (A) Flow cytometry fluorescence intensity Dot plot and bar chart of cells stained with JC-1 (B) (C) Representative image to each population. Green cells has ow potential and Orange cells has high potential. Data showed as mean ± SD of two independent experiments. (*p<0.05).

In normal cells, JC-1 concentrates in the mitochondrial matrix and forms J-aggregates, which emit red/orange fluorescence. When the mitochondrial membrane potential is dissipated (depolarized mitochondria), there is no accumulation of JC-1 in

the mitochondria and thus, the dye is dispersed, remaining in the monomeric form, with green fluorescence [63].

The results indicate that there was no significative difference between control and the lower concentration of the tested compound, with the majority of cells presenting high potential. On the other hand, concentrations of 4.0 μg/mL and 8.0 μg/mL promoted depolarization of the mitochondrial membrane (Figure 4).

The increased permeabilization of the mitochondrial membrane promotes a collapse of the mitochondrial membrane potential and consequent release of several apoptogenic proteins from the mitochondrial intermembrane space, as cytochrome c and the apoptosis inducing factor. In the cytoplasm, cytochrome c binds to APAF 1 to promote activation of caspase 9, which will activate effector caspases 3 and 7 to continue the cascade of apoptosis events [64].

Indeed, the ability of modify mitochondrial trans-membrane potential is attributed for the group of cucurbitacins as a significant mechanism with regard to the apoptotic effects of these compounds. This may be observed for example to cucurbitacin E, which induces apoptosis in human oral squamous cell carcinoma cell line (SAS), with significantly reduction in the mitochondrial membrane potential of SAS cells [54, 65].

The anticancer activity it was already described for several compounds which possess cucurbitane skeleton characteristic of cucurbitacins as demonstrated by Sikander et al. [66] detecting that cucurbitacin D induces apoptosis in cervical cancer cells. Similarly, the results obtained indicates an anticancer activity for the cucurbitacin of the roots from *A. congestiflora*.

Most of the cucurbitacins are selective inhibitors of the JAK/STAT pathways. Cucurbitacin B, for example, promotes apoptosis on pancreatic cancer cells by inhibition of JAK2, STAT3, and STAT5 [52].

Signal transducers and activators of transcription (STATs) are a family of seven proteins including STAT 1, 2, 3, 4, 5a, 5b, and 6, which are transcription factors that relay signals generated by cytokine receptor into the nucleus. STATs are present in inactive forms in the cytoplasm and after stimulation by receptor-mediated

growth factor, such as epidermal growth factor (EGF), STAT are activated by association with the cell membrane receptors via phosphorylation at conserved tyrosine 705 residues either directly by receptor tyrosine kinases, or indirectly by non-receptor tyrosine kinases, for example, Janus kinases (JAKs). This phosphorylation promotes STAT dimerization, nuclear translocation, and DNA binding at STAT-specific sequence in target genes, stimulating their transcription [54, 67].

STAT3 regulates the expression of genes involving cell proliferation and in normal physiological conditions, the period of activation of STAT3 is regulated. However, in several types of cancer, as observed in human leukemia HL-60, STAT3 is constitutively activated [68]. Thus the present results allows to suggest that a possible pathway involving in apoptosis promotion in HL-60 cells by the cucurbitacin isolated from *A. congestiflora* roots is through STAT inhibition.

Antimicrobial activity

A. congestiflora extracts were also evaluated for antimicrobial activity (Table 9) against gram-negative bacteria (*Escherichia coli*, *Serratia marcescens* and *Pseudomonas aeruginosa*), gram-positive bacteria (*Staphylococcus aureus*, *Micrococcus luteus*, *Bacillus subtilis* and *Enterococcus faecalis*), acid-fast bacterium (*Mycobacterium smegmatis*) and yeast (*Candida albicans*).

Table 9. Antimicrobial activity of extracts from *A. congestiflora*.

Microorganism	Extract					
	Hexane		Ethyl acetate		Methanol	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC
Staphylococcus aureus	1.56	7.5	1.87	3.75	6.25	15
Micrococcus luteus	3.12	3.75	1.87	1.87	6.25	3.75
Enterococcus faecalis	0.94	7.5	0.94	3.75	1.87	7.5
Bacillus subtilis	0.94	7.5	1.87	7.5	NA	NA
Escherichia coli	3.75	7.5	7.5	7.5	7.5	15
Pseudomonas aeruginosa	0.23	7.5	0.94	3.75	3.75	7.5
Serratia marcescens	1.87	7.5	3.75	15	NA	NA
Candida albicans	0.94	1.87	0.94	3.75	15	7.5
Mycobacterium smegmatis	0.94	1.87	0.94	7.5	3.75	15

Data showed as mg/mL. NA- not active.

According Aligiannis et al. [69] extracts with MIC values up to 500 µg/mL are potent inhibitors, while those with MIC between 600 and 1500 µg/mL are moderate inhibitors and those with MIC above 1600 µg/mL are weak inhibitors. The hexane extract showed the lowest MIC to *Pseudomonas aeruginosa* and *Bacillus subtilis*. The ethyl acetate extract showed the lowest MIC for *Pseudomonas aeruginosa*, *Candida albicans*, *Mycobacterium smegmatis* and *Enterococcus faecalis*. Coumarins, terpenes, quinones, phenolic compounds, alkaloids, flavonoids and tannins from plants exhibit antimicrobial activity [70] and thus, the antimicrobial activity of the extracts may be due to the presence of these comounds in the extracts from *A. congestiflora* tuberous roots.

Pseudomonas aeruginosa is a gram-negative bacterium which is largely associated to nosocomial infections in intensive care units, as well as antibiotic resistance [71]. This bacterium plays roles in a great amount of disorders such as pneumonia, otitis, infections of the urinary tract and meningitis [72, 73]. The ability to resist to antimicrobial therapy demonstrates the relevance of the development of new products able to act against this bacterium. The hexane extract from *A. congestiflora* may be considered a potent inhibitor of the growth of this bacterium and a promising source of molecules with antimicrobial activity. *Mycobacterium smegmatis* is a rare pathogen that is associated with skin infections, bone infections and lung infections in immunocompromised patients [74]. The hexane extract presented as moderate inhibitor of this microorganism. *Enterococcus faecalis* is a gram-positive bacterium that is associated, especially, to infections of the urinary tract, as well as endocarditis, bacteremia, and wound infections. This bacterium is resistant to antibiotics, making difficult to treat disorders caused by it [75]. The yeast *Candida albicans*, in turn, is widely associated with infections in genitourinary tract, causing candidiasis or candiduria [76]. Hexane and ethyl acetate extracts were moderate inhibitors for *Enterococcus faecalis* and *Candida albicans*.

Staphylococcus aureus is founded as commensal, as part of the normal flora of human body, but is also a pathogen to humans, widely associated to bacteremia [77]. According to World Health Organization (WHO), most of *Staphylococcus aureus* infections are caused by resistant strains, thus the treatment with standard antibiotics does not work. Therefore, and due to fact that antibiotics may be used to complement cancer treatment by prevention of infections in patients with low white blood cells due to the use of chemotherapeutic drugs, it was evaluated the antibacterial activity of the fractions obtained from the hexane extract on clinical isolates of *Staphylococcus aureus* (Table 10).

Table 10. Antimicrobial activity of fractions from hexane extract of *A. congestiflora* roots against clinical isolates *Staphylococcus aureus.*

Fraction	Isolates							
	Purulent exudate		Operative wound		Catheter tip		Urine	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	2.5	5.0	2.5	2.5	2.5	5.0	2.5	5.0
2	1.25	1.25	1.25	1.25	1.25	2.5	1.25	2.5
3	1.25	1.25	1.25	1.25	1.25	1.25	1.25	2.5
4	0.312	1.25	1.25	2.5	0.625	0.625	1.25	5.0
5	0.25	0.25	0.25	0.5	0.25	0.5	0.5	0.5
6	0.625	2.5	0.625	1.25	1.25	1.25	1.25	1.25
7	0.5	1.0	0.5	0.5	0.5	0.5	0.5	1.0
8	0.5	1.0	NA		1.0	2.0	NA	
9	1.25	5.0	2.5	2.5	2.5	2.5	2.5	5.0
10	1.25	5.0	2.5	2.5	2.5	5.0	2.5	5.0
11	0.125	0.5	NA		0.5	0.5	NA	
12	0.375	0.75	0.75	0.75	0.375	1.5	0.75	1.5
13	0.625	2.5	2.5	2.5	2.5	5.0	2.5	5.0

Data showed as mg/mL. *NA- not active.

The fraction containing the cucurbitacin (13) showed only moderate activity against *S. aureus* isolated from purulent exudate, exhbiting weak activity for the others clinical isolates.

The fraction 5, which contains palmitic acid and 7,22-ergostadienol was the most active, showing MICs up to 0.5 mg/mL on *S. aureus* from different sites.

Additionally, this fraction showed MIC value of 0.25 mg/mL to a oxacillin resistant *S. aureus* (ORSA) isolated from operative wound. 7,22-ergostadienol is a sterol and the antimicrobial activity has already been described to this group, as observed to 24-propylidene cholest-5-en-3β-ol isolated from *Laurencia papillosa* red seaweed, that showed high antibacterial activity on clinical isolates, including *S. aureus* [78, 79]. Palmitic acid has been reported as antibacterial agent, but no so potent as long-chain unsaturated fatty acids (ZHENG et al., 2005; CHOI et al., 2012). Thus, the antibacterial effect on several clinical isolates may be attributed to the synergistic action of palmitic acid and 7,22-ergostadienol founded in *A. congestiflora* roots.

Conclusions

Extracts from *A. congestiflora* are source of phenolic compounds, including flavonoids and coumarins, with ability to reduce the viability of cancer cells, with more selectivity in relation to normal cells, besides to present antimicrobial activity, being the hexane extract the most promising. The hexane extract fractionation allowed the obtention of fractions containing active antimicrobial compounds. The cucurbitacin isolated from the most cytotoxic fraction promotes HL-60 death by apoptosis. However is necessary caution, due to the cytotoxicity of this compound to PBMCs.

References

[1] Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. Journal of Ethnopharmacology 2005; 100(1-2): 72-79.

[2] Dias DA, Urban S, Roessner U. A Historical Overview of Natural Products in Drug Discovery. Metabolites 2012; 2 (2): 303-336.

[3] Brandão HN, David JP, Couto RD, Nascimento JAP, David JM. Química e farmacologia de quimioterápicos antineoplásicos derivados de plantas. Química Nova 2010; 33(6): 1359-1369.

[4] Palumbo R, Sottotetti F, Riccardi A, Teragni C, Pozzi E, Quaquarini E, Tagliaferri b, Bernardo A. Which patients with metastatic breast cancer benefit from subsequent lines of treatment? An update for clinicians. Therapeutic Advances in Medical Oncology 2013; 5(6): 334-350.

[5] Rebucci M, Michiels C. Molecular aspects of cancer cell resistance to chemotherapy. Biochemical Pharmacology 2013; 85(9): 1219-1226.

[6] Wang B, Zhang S, Yue K, Wang XD. The recurrence and survival of oral squamous cell carcinoma: a report of 275 cases. Chinese Journal of Cancer 2013; 32(11): 614-618.

[7] Gafter-Gvili A, Frase A, Paul M, Vidal L, Lawrie TA, van de Wetering MD, Kremer LCM, Leibovici L. Antibiotic prophylaxis for bacterial infections in afebrile neutropenic patients following chemotherapy. The Cochrane Database of Systematic Reviews, 2012; doi: 10.1002/14651858.

[8] SpellberG B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, Bartlett JG, Edwards JJr. INFECTIOUS DISEASES SOCIETY OF AMERICA. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. Clinical Infectious Diseases 2008; 46(2): 155-164.

[9] Savoia D. Plant-derived antimicrobial compounds: alternatives to antibiotics. Future Microbiology 2012; 7(8): 979-90.

[10] Simpson MG. London, Academic Press: Plant systematics. London, 2010.

[11] Ojewole JA, Adewole SO, Olayiwola P. Hypoglycaemic and hypotensive effects of Momordica charantia Linn (Cucurbitaceae) whole-plant aqueous extract in rats. Cardiovascular Journal of South Africa 2006; 17(5): 227-232..

[12] Gonzalez FG, Di-Stasi LC. Anti-ulcerogenic and analgesic activities of the leaves of Wilbrandia ebracteata in mice. Phytomedicine 2002; 9(2): 125-134.

[13] Talukdar SN, Hossain MN. Phytochemical, Phytotherapeutical and Pharmacological Study of Momordica dioica. Evidence-Based Complementary and Alternative Medicine, 2014. doi:10.1155/2014/806082.

[14] Eseyin OA, Sattar MA, Rathore HA. A Review of the Pharmacological and Biological Activities of the Aerial Parts of *Telfairia occidentalis* Hook.f. (Cucurbitaceae). Tropical Journal of Pharmaceutical Research, 2014; 13(10): 1761-1769.

[15] Lima LFP. Estudos taxonômicos e morfopolínicos em Cucurbitaceae brasileiras. 2010. 232f. Tese (Doutorado em Botânica). Universidade Federal do Rio Grande do Sul, Rio Grande do Sul.

[16] Roque AA, Rocha RM, Loiola LMB. Uso e diversidade de plantas medicinais da Caatinga na comunidade rural de Laginhas, município de Caicó, Rio Grande do Norte (nordeste do Brasil). Revista Brasilera de Plantas Medicinais 2010; 12(1): 31-42.

[17] Silva CG, Marinho MGV, Lucena MFA, Costa JGM. Levantamento etnobotânico de plantas medicinais em área de Caatinga na comunidade do Sítio Nazaré, município de Milagres, Ceará, Brasil. Revista Brasileira de Plantas Medicinais 2015; 17(1): 133-142.

[18] Wagner H, Bladt S, Plant drug analysis - a thin layer chromatography atlas. Berlin, Germany; 1995.

[19] Amorim ELC, Nascimento JE, Monteiro JM, Peixoto-Sobrinho TJS, Araújo TAS, Albuquerque UP. A simple and accurate procedure for the determination of tannin and flavonoid levels and some applications in ethnobotany and ethnopharmacology. Functional Ecosystems and Communities 2008; 2: 88-94.

[20] Peixoto-Sobrinho TJS, Gomes TLB, Cardoso KCM, Albuquerque UP, Amorim ELC. Total flavonoid content in products containing "pata-de-vaca" (Bauhinia L.) sold in pharmacies in Recife/PE. Revista Brasileira de Plantas Medicinais, 2008. http://dx.doi.org/10.1590/S1516-05722012000400003.

[21] Osorio AC, Martins JLS. Determinação de cumarina em extrato fluido e tintura de guaco por espectrofotometria de primeira ordem. Brazilian Journal of Pharmaceutical Sciences 2004; 40(4): 481-486.

[22] Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical Biochemistry 1999; 269(2): 337-341.

[23] Blois MS. Antioxidant determinations by the use of a stable free radical. Nature 1958; 181: 1199–1200.

[24] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 1983; 65(1-2): 55-63.

[25] Alley MC, Scudiere DA, Monks A, Hursey ML, Czerwinski MJ, Fine DL, Abbott BJ, Mayo JG, Shoemaker RH, Boyd MR. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research 1988; 48(3): 589-601.

[26] Wang G, Su P, Zhang F, Hou XY, Yang Y, Gou Z. Comparison of microwave-assisted extraction of aloe-emodin in aloe with Soxhlet extraction and ultrasoundassisted extraction. Science China Chemistry 2011; 54(1): 231–236.

[27] Shri CN, Balaji J, Venkatramanan S, Madhumathi KL. Pharmacognostical and preliminary phytochemical screening of the root and rhizome of Corallocarpus epigaeus. International Journal of Pharmaceutical and Biomedical Research 2010; 1(1): 24-27.

[28] Kumar P, Devala RG, Lakshmayya B, Ramachandra SS. Pharmacognostical studies on tubers of*Momordica tuberosa* Cogn., Cucurbitaceae. Revista Brasileira de Farmacognosia 2010; 20(1): 7-11.

[29] Guo LP, Wang S, Zhang J, Yang G, Zhao MX, WeiFeng MA, Zhang XB, Li X, Han BX, Chen NF, Huang LQ. Effects of ecological factors on secondary metabolites and inorganic elements of Scutellaria baicalensis and analysis of geoherblism. Science China 2013; 56(11): 1047-1056.

[30] Hernández-Galicia E, Calzada F, Roman-Ramos R, Alarcón-Aguilar FJ. Monoglycerides and fatty acids from Ibervillea sonorae root: isolation and hypoglycemic activity. Planta Medica 2007; 73(3): 236-240.

[31] Kim MY, Kim EJ, Kim YN, Choi C, Lee BH. Comparison of the chemical compositions and nutritive values of various pumpkin (Cucurbitaceae) species and parts. Nutricion Research and Practice 2012; 6(1): 21-27.

[32] Freije A, Alkhuzai K, Al-Laith A. Fatty acid composition of three medicinal plants from Bahrain: New potential sources of -linolenic acid and dihomolinolenic. Industrial Crops and Products 2013; 43: 218-224.

[33] Harada H, Yamashita U, Kurihara H, Fukushi E, Kawabata J, Kamei Y. Antitumor activity of palmitic acid found as a selective cytotoxic substance in a marine red alga. Anticancer Research 2002; 22(5): 2587-2590.

[34] Déciga-Campos M, Montiel-Ruiz RM, Navarrete-Vázquez G, López-Muñoz FJ. Palmitic acid analogues exhibiting antinociceptive activity in mice. Proceedings of The Western Pharmacology Society 2007; 50: 75-77.

[35] Zheng CJ, Yoo JS, Lee TG, Cho HY, Kim, YH, Kim WG. Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. Febbs Letters 2005; 579 (23): 5157-5162.

[36] Kam TS, Subramaniam G, Kooi-Mow S, Yoganathan K, Toyoshima TKM, Mun-Chual R, Masahiko H, Kanki K. Reversal of multidrug resistance (MDG) by Aspidofractinin-type indole alkaloids. Bioorganic & Medicinal Chemistry Letters 1998; 8: 2769–2772.

[37] Elezabeth VD, Arumugam S. GC-MS analysis of bioactive constituents of Indigofera suffruticosa leaves. Journal of Chemical and Pharmaceutical Research 2014; 6: 294-300.

[38] Coulibaly AY, Hashim R, Sulaiman SF,Sulaiman O, Ang LZP, Ooi KL. Bioprospecting medicinal plants for antioxidant components. Asian Pacific Journal of Tropical Medicine 2014; 1(1): 553-559.

[39] Muanda F, Koné D, Dicko A, Soulimani R, Younos C. Phytochemical composition and antioxidant capacity of three malian medicinal plant parts. Evidence-based Complementary & Alternative Medicine (eCAM**)** 2011. http://dx.doi.org/10.1093/ecam/nep109

[40] Smith JV, Luo Y. Elevation of oxidative free radicals in Alzheimer's disease models can be attenuated by Ginkgo biloba extract EGb 761. Journal of Alzheimers Disease 2003;5(4): 287-300.

[41] Sugamura K, Keaney JF. Reactive oxygen species in cardiovascular disease. Free Radical Biology and Medicine 2001; 51(5): 978-992.

[42] Gorrini C, Harris IS, Mak TW. Modulation of oxidative stress as an anticancer strategy. Nature Reviews. Drug Discovery 2013; 12(12): 931-947.

[43] Suffness M, Pezzuto JM. Academic Press: Assays related to cancer drug discovery. In Methods in Plant Biochemistry: Assay for Bioactivity. London; 1990.

[44] Klenkar J, Molnar, M. Natural and synthetic coumarins as potential anticancer agents. Journal of Chemical and Pharmaceutical Research 2015; 7(7): 1223-1238.

[45] Grotzke JE, Lewinsohn DM. Role of CD8+ T lymphocytes in control of Mycobacterium tuberculosis infection. Microbes and Infection 2005; 7(4): 776–788.

[46] Lu JJ, Bao JL, Chen XP, Huang M, Wang YT. Alkaloids Isolated from Natural Herbs as the Anticancer Agents. Evidence-Based Complementary and Alternative Medicine, 2012. doi:10.1155/2012/485042, 2012.

[47] Tannin-Spitz S, Grossman S, Dovrat HE, Gottlieb M. Bergman,Growthinhibitory activity of cucurbitacin glucosides isolated from Citrullus colocynthison human breast cancer cells, Biochemistry Pharmacology 2007; 73(1):56–67.

[48] Thoennissen NH, Iwanski GB, Doan NB, Okamoto R, Lin P, Abbassi S, Song JH, Yin D, Toh M, Xie WD, Said JW, Koeffler HP. Cucurbitacin B inducesapoptosis by inhibition of the JAK/STAT pathway and potentiates antiprolifer-ative effects of gemcitabine on pancreatic cancer cells. Cancer Research 2009; 69(14):5876–5884.

[49] Militao G, Dantas IN, Ferreira PM, Alves AP, Chaves DC, Monte FJ, Pessoa C, Moraes MO, Costa-Lotufo LV. In vitro and in vivo anticancerproperties of cucurbitacin isolated from Cayaponia racemosa. Pharmaceutical Biology 2012; 50(12):1479–1487.

[50] Ampasavate C, Okonogi S, Anuchapreeda S. Cytotoxicity of extracts from fruit plants against leukemic cell lines. African Journal of Pharmacy and Pharmacology 2010; 4: 013-021.

[51] Ruan S, Samuelson DR, Assouline B, Morre M, Shellito JE. Treatment with IL-7 restores host defense against*Pneumocystis* in CD4+ T-lymphocyte depleted mice. Infection and Immunity 2015; 89(15): 1-9.

[52] Costa-Lotufo LV, Khan MTH, Wilke AADV, Jimenez PC, Pessoa C, Moraes ME, Moraes MO. Studies of the anticancer potential of plants used in Bangladeshi folk medicine. Journal of Ethnopharmacology 2005; 99(1):21-30.

[53] Alghasham AA. Cucurbitacins – A Promising Target for Cancer Therapy. International Journal of Health Sciences 2013; 7(1):77-89.

[54] U-pratya Y, Jiratchariyakul W, Kummalue T. Anti-proliferative effects of Pouzolzia indica on acute promyelocytic cell lines: NB4 and HT93A. Asian Journal of Traditional Medicines 2008; 3(4):124-133.

[55] Dantas IN, Gadelha GC, Chaves DC, Monte FJ, Pessoa C, de Moraes MO, Costa-Lotufo LV. Studies on the cytotoxicity of cucurbitacins isolated from Cayaponia racemosa (Cucurbitaceae). Zeitschrift fur Naturforschung C 2006; 61(9-10):643-646.

[56] Feng H, Zang L, Zhao ZX, Kan QC. Cucurbitacin-E inhibits multiple cancer cells proliferation through attenuation of Wnt/β-catenin signaling. Cancer Biotherapy and Radiopharmaceuticals 2014; 29(5):210-214.

[57] Chung SO, Kim YJ, Park SU. An updated review of Cucurbitacins and their biological and pharmacological activities. Experimental and Clinical Sciences 2015, doi: 10.17179/excli2015-283.

[58] Chen SP, Yang HL, Lin HY, Chen MC, Wu JL, Hong JR. Enhanced viability of a nervous necrosis virus-infected stable cell line over-expressing a fusion product of the zfBcl-xL and green fluorescent protein genes. Journal of Fish Diseases 2006; 29(6):347-354.

[59] Silva MT. Secondary necrosis: The natural outcome of the complete apoptotic program. FEBS Letters 2010; 584(22):4491-4499.

[60] Gac M, Bigda J, Vahlenkamp TW. Increased mitochondrial superoxide dismutase expression and lowered production of reactive oxygen species during rotavirus infection. Virology 2010; 404(2):293-303.

[61] Minafra L, Bravatà V. Cell and molecular response to IORT treatment. Translational Cancer Research 2014; 13(1):32-47.

[62] Hung CM, Chang CC, Lin CW, Ko SY, Hsu YC. Cucurbitacin E as Inducer of Cell Death and Apoptosis in Human Oral Squamous Cell Carcinoma Cell Line SAS. International Journal of Molecular Sciences 2013; 14(8):17147-17156.

[63] Sikander M, Hafeez BB, Malik S, Alsayari A, Halaweish FT, Yallapu, MM, Chauhan SC, Jaggi M. Cucurbitacin D exhibits potent anti-cancer activity in cervical cancer. Scientific Reports 2016, doi: 10.1038/srep36594.

[64] Chen H, Sun H, You F, Sun W, Zhou X, Chen L, Yang J, Wang Y, Tang H, Guan Y. Activation of STAT6 by STING is critical for antiviral innate immunity. Cell 2011; 147(2):436–446.

[65] Zhao J, Xu Y, Zong Y, Zhang S, Song Y, Yu K, Li Z, Ji Y, Qiu Y, Chen F. Inhibition of Stat3 expression induces apoptosis and suppresses proliferation in human leukemia HL-60 cells. Hematology 2011; 16(4):232-235.

[66] Aligiannis N, Kalpotzakis E, Mitaku S, Chinou IB. Composition and antimicrobial activity of the essential oil of two Origanum species. Journal of Agricultural and Food Chemistry 2001; 49(9):4168-4170.

[67] Cowan MM. Plant products as antimicrobial agents. Clinical Microbiology Reviews 1999; 12(4):564-582.

[68] Peng B, Su YB, Li H, Han Y, Guo C, Tian YM, Peng XX. Exogenous alanine and/or glucose plus kanamycin kills antibiotic-resistant bacteria. Cell Metabolism 2015; 21(2):249, 261.

[69] Mesaros N, Nordmann P, Plésiat P, Roussel-Delvallez M, Van EJ, Glupczynski Y, Van LY, Jacobs F, Lebecque P, Malfroot A, Tulkens PM, Van BF. Clinical Microbiology and Infection 2007; 13(6):560-578.

[70] Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K. Urinary tract infections caused by Pseudomonas aeruginosa: a minireview. Journal of Infection and Public Health 2009; 2(3):101-111.

[71] Fowler J, Mahlen SD. Localized Cutaneous Infections in Immunocompetent Individuals Due to Rapidly Growing Mycobacteria. Archives of Pathology & Laboratory Medicine 2014; 138(8):1106-1109.

5 ARTIGO CIENTÍFICO II

Phytochemical investigation and evaluation of genotoxicity, mutagenicity, cytotoxicity and acute toxicity of a hydroalcoholic extract from *Apodanthera congestiflora* roots

A ser submetido no periódico Phytomedicine

Phytochemical investigation and evaluation of genotoxicity, mutagenicity, cytotoxicity and acute toxicity of a hydroalcoholic extract from *Apodanthera congestiflora* roots

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Abstract

Background: *Apodanthera congestiflora* is a plant endemic from Brazil, founded in the Caatinga biome. The root is used in folk medicine but there are no studies about chemical constituents and biological activities. This work investigated the hydroalcoholic extract from *A. congestiflora* root for secondary metabolites, antioxidant and hemolytic activities, cytoxicity to normal cells and toxicity to Swiss mice, as well as *in vivo* genotoxicity and mutagenicity to mice.

Methods: Phytochemical characterization was performed by thin layer chromatography, quantification of total phenol, flavonoids, tannins and coumarins as well as liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS). The antioxidant activity was evaluated by DPPH and ABTS methods. Cytotoxicity was determinated on human Peripheral Blood Mononuclear Cells (PBMCs), 3T3-L1 and VERO. Hemolytic Assay was performed to verify the ability of the extract to damage cell membrane. Acute toxicity was performed according to OECD and were analyzed hematological, biochemical and histological parameters. Genotoxicity and mutagenicity were evaluated *in vivo* by comet assay and micronucleus test, respectively. 24 hours after treatment, blood was collected from tail and was performed the alkaline comet assay. The same animals were euthanized to collect bone marrow to evaluate presence of micronucleus in polychromatic erythrocytes (PCE) and the ratio between them and normochromatic (NCE) erythrocytes.

Results: Thin layer chromatography showed presence of terpenes and alkaloids. LC–ESI-MS analysis allows to suggest presence of cucurbitacins (triterpenes) in the hydroalcoholic extract. Quantitative analysis revealed that the hydroalcoholic extract contains low amount of total phenols and flavonoids, absence of tannins and coumarins and present antioxidant activity. The hydroalcoholic extract was potentially cytotoxic to 3T3-L1, VERO and PBMCs and was not considered hemolytic. In acute toxicity assay there was no mortality, toxic signs or changes in hematological and biochemical parameters at high dose. The comet assay and micronucleus test showed absence of genotoxicity and mutagenicity. However, there was a reduction in PCE/NCE ratio, indicating cytotoxicity.

Conclusions: Hydroalcoholic extract from *A. congestiflora* roots is a source of antioxidant compounds without systemic toxic effects or deleterious action on DNA. However it is necessary caution in prolonged use due to its cytotoxic effect.

Keywords: *Apodanthera congesiflora*, cytotoxicity, terpene, toxicity

Abreviations

ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

CPA, Cyclophosphamide

DMSO, Dimethyl sulfoxide

EC_{50}, effective concentration that promotes 50% of hemolysis

DPPH, 2,2-diphenyl-1-picrylhydrazyl

EDTA, Ethylenediamine tetraacetic acid

FBS, Fetal Bovine Serum

$H_{max,}$ maximum percentage of hemolysis

$IC_{50,}$ concentration that inhibited 50% of the cell growth compared with the control

NCE, normochromatic erythrocytes

PBMC, peripheral blood mononuclear cell

PCE, polychromatic erythrocytes

UV, ultraviolet

Introduction

Medicinal plants are continuously used by population to treat various disorders and their pharmacological activities are due to chemical constituents present in extracts and other preparations commonly used. The use of herbal medicines is widespread in development countries due to the easy access and the belief on the safety in use due to the natural origin (LEMOS et al., 2011; PINHO et al., 2014).

However, in some cases, this use may be associated with toxicity and risks to human health (DIALLO et al., 2015; ETEBARI et al., 2016). Thus, in addition to the study of the chemical and biological properties of medicinal plants, it is essential to determine the safety or risk of their use.

Apodanthera congestiflora (syn. *Melothria congestiflora*) is an endemic vine of Brazil, belonging to the Cucurbitaceae family and popularly know as "batata de teiú" or "cabeça de negro". This plant is widely distributed in the Brazilian Caatinga in states of Bahia, Pernambuco, Ceará, Piauí, Rio Grande do Norte, Sergipe, also being found in the state of Minas Gerais (LIMA et al., 2010). Root preparations of this plant including hydroalcoholyc extract, infusion, bath, tea, syrup and powder are used as depurative of blood and to treat skin patches, itch and back pain (ROQUE et al., 2010). Despite of this, there is a lack of scientific reports to support the pharmacological potential and the safety of the use of this plant. Studies involving this species have been limited to the field of Ethnobotany (ROQUE et al., 2010; SILVA et al., 2015).

In this context, this study aimed to investigate a hydroalcoholyc extract from the roots of *A. congestiflora* for antioxidant activity, cytotoxic activity on normal cells as well as acute toxicity and *in vivo* genotoxicity and mutagenicity to mice.

Material and Methods

Plant material

Roots from *Apodanthera congestiflora* Cogn. were collected in Barbalha (Lat.: 7° 16.26' 70.24''; Long.: 39° 15.28' 26.36''), at state of Ceará (Brazil) and a voucher specimen was deposited with the number 899985 in the Herbarium of the *Instituto Agronômico de Pernambuco* (IPA), Recife, Brazil.

Cell lines and cell culture

Cell lines used for the *in vitro* cytotoxicity tests, 3T3-L1 (mouse embryonic fibroblast) and VERO (African green monkey kidney cell line), were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 100 µg/mL of penicillin–streptomycin-amphotericin B solution at 37°C with a 5% CO_2 atmosphere. The cells were obtained from the *Banco de Células do Rio de Janeiro* (Rio de Janeiro, Brazil) and maintained in the Laboratory of Cell Culture, *Departamento de Antibióticos, Universidade Federal de Pernambuco* (UFPE). The peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood from healthy volunteer donors (18 to 30 years old) (as approved by the Ethics Comitee on Research Involving Human Beings from UFPE under the number 60107916.8.0000.5208).

Animals

Female Swiss mice (25-30 g) were obtained from the vivarium of the *Departamento de Antibióticos* from UFPE. Before use in the experiments, the mice were housed under standardized conditions (22±2ºC, 12-12 h light/dark photoperiod, and 50–60% humidity) and fed a standard mouse diet with water available *ad libitum*. This study was approved by the Committee for Ethics in Animal Research of the UFPE (process number 23076.043015/2016-50) and the experiments were performed in accordance with the rules of the International Council for Laboratory Animal Science (ICLAS) and the ethical principles of the Brazilian Society of Laboratory Animal Science (SBCAL). The experiments involving animals were performed using methods designed to minimize pain and suffering.

***Apodanthera congestiflora* extract**

The roots of *A. congestiflora* were dried at room temperature and powdered. For preparation of the extract, powdered roots (130 g) were added to ethanol 70% and maintained in a closed recipient protected from light during 3 days. After this period, the extract was filtered and concentrated to near dryness on a rotary evaporator.

Evaluation for saponin

Saponin detection was performed by qualitative test (Kokate, 1999). The powdered root was vigorously agitated for two minutes followed by rest for two hours. It was

considered as a positive result the persistence of foam at the end of the rest period. Also, it was performed the test of capillary flow to compare the flow of the sample with distilled water. In this method, samples with flow greater than that of water are considered postive for presence of saponins.

Thin layer chromatography

Phytochemical composition of the hydroalcoholyc extract was evaluated as decribed by Wagner and Bladt (1995). The extract was applied in chromatographic silica gel 60 plates (0.20 mm) and organic solvents were used as mobile phase. Then, the plates were sprayed using specific reagents for each class of compounds and observed under 365 nm UV light. Anisaldehyde-sulfuric acid and ceric sulphate were used to terpenoid compounds and flavonoids. Potassium hydroxide in ethanol was used to coumarins and anthraquinones. The Lieberman-Burchard reagent was used to detect steroids/triterpenoids. Drangendorff's reagent was used to alkaloids identification. Ferric chloride solution was used to tannins.

Quantification of total phenols and tannins

Quantification of total phenols was carried out according to Amorim et al. (2008). An aliquot (0.2 mL) of the *A. congestiflora* extract (1 mg/mL) was transferred to a test tube, to which was added 0.5 mL of Folin-Ciocalteu reagent, 1 mL of sodium carbonate and distilled water. After 30 min the absorbance at 760 nm was read. A calibration curve was prepared using tannic acid (0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5 and 10 µg/mL) and total phenols content was expressed as milligrams equivalent of tannic acid per gram of extract (mg EAT/g).

To quantify the tannins, the extract (6 mL, 1 mg/mL) was mixed with casein (1 g) and distilled water (12 mL). After 3 h of reaction with stirring (to precipitate tannins with the protein), the material was filtered to quantification of residual phenols by the Folin-Ciocalteu method. The tannin content was calculated as the difference between total and residual phenols.

Flavonoid content

The test was performed according to Peixoto-Sobrinho et al. (2008), with modifications. The extract (0.2 mL; 1 mg/mL) was mixed with glacial acetic acid (120

µL), pyridine (2 mL; 20% v/v in ethanol), and aluminum chloride (0.5 mL; 5% p/v in distilled water) and the volume was completed with distilled water until 10 mL. After incubation (30 min) in the dark, the absorbance was measured at 420 nm. A standard curve was prepared using rutin (0.05–2 µg/mL). The flavonoid content was expressed as milligrams equivalent of rutin per gram of extract (mg RU/g). The test was performed in triplicate.

Coumarin content

The assay was performed according to Osorio and Martin (2004), with modifications. *A. congestiflora* extract (0.5 mL; 1 mg/mL) was transferred to test tubes and distilled water (2 mL) and lead acetate (500 µL) were added to each tube. After stirring and addition of distilled water (7 mL), an aliquot of this solution was added to 8 mL of hydrochloric acid. After 30 minutes, the absorbance of the final solution was measured at 320 nm. A standard curve was used with different concentrations of coumarin (0.4–16 mg/mL) and results were expressed as milligrams equivalent of coumarin per gram of extract (mg CE/g). The tests were performed in triplicate.

Liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS)

The LC-ESI-MS spectra were obtained in positive and negative ion mode using an Esquire 3000 Plus-Bruker Daltonics (Bruker Corp., USA) with the conditions: 4000V capillary, 27 psi nebulizer, 7 l/min dry gas and 320 RC dry temp (FERREIRA et al., 2014).

Antioxidant activity

Free radical scavenging activity by DPPH (2,2-Diphenyl-1-picrylhydrazyl assay)

This test is based on electron transfer by an antioxidant substance. The DPPH, which presents purple color, is reduced to diphenyl-picryl hydrazine, with yellow coloration. The extract was tested at concentrations ranging from 15.62 to 1000 µg/mL. Ascorbic acid (0.3, 0.5, 1, 2, 3, 4 and 5 µg/mL) was used as standard. In each assay, 270 µL of the DPPH solution was added to each sample (in varying volume from stock solution, to obtain the required tested concentrations). After 30 min, the reduction of DPPH was determined by colorimetric change at 517 nm using ethanol as blank

(BLOIS, 1958). The antioxidant concentration required to derease 50% of the DPPH present (IC_{50}) was determined.

ABTS assay

ABTS assay is based on transfer of electrons. In this method, the radical $ABTS^{+}$ is generated by oxidation of ABTS solution (7 mM) with 2.45 mM potassium persulfate solution, originating a blue/green solution. that has maximum absorption at 734 nm. In presence of an antioxidant agent, $ABTS^{+}$ is reduced to ABTS, promoting the loss of the medium staining. The mixture of ABTS solution (7 mM) with 2.45 mM potassium persulfate solution was allowed to react for 12 h in the dark at 25°C before use. For the test, the $ABTS^{+}$ stock solution (1 mL) was diluted in 60 mL of methanol to obtain an absorbance of 0.70 ±0.02 at 734 nm. Next, 2.7 mL of this solution was added to different volumes of stock solution of Trolox (standard) to obtention of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 µg/mL, prepared in methanol or 15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/mL, prepared in methanol. The absorbance was taken 6 min after the adding of the radical at 734 nm.The test was performed in triplicate to determination of the the percentage inhibition of ABTS radical by the extract as well as the concentration required to derease 50% of the $ABTS^{+}$ present (IC_{50}), by linear regression.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were purified from human blood by gradient separation using the solution for cell separation Histopaque-1077 (Sigma–Aldrich, USA). Cells were isolated from a 6-mL blood sample in 5 mL of phosphate buffered saline (PBS) to which was added 3 mL of Histopaque-1077. After centrifugation at 1500 rpm for 30 min, the mononuclear cells (present in the intermediate region between the red cells and serum) were aspirated. The lymphocyte suspension was transferred to another tube to which PBS was added until the volume of 11 mL and centrifuged for 20 min at 1000 rpm. Then, the supernatant was discarded and the lymphocyte pellet ressuspended to a final concentration of 10^{6} cells/mL in 5 mL of RPMI 1640 medium supplemented with 20% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin. Lymphocytes proliferation was induced by addition of 3% phytohemagglutinin, a mitogen which acts fundamentally on T lymphocytes becoming they the predominant cell population in this assay (LIU et al., 2004).

Cytotoxicity on normal cells

Cell viability was measured using a method based on the conversion of 3-(4,5-dimethyl-2-thiazole)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a formazan product by the action of the enzyme succinyl dehydrogenase, which is present in the mitochondria of viable cells (Mosmann 1983, Alley et al., 1988). 3T3-L1 and VERO cells (10^5 cells/mL, in DMEM medium) or lymphocytes (10^6 cells/mL, in RPMI medium) were plated in 96-well microplates and incubated for 24 h at 37°C. After this period, 10 μL of the *A. congestiflora* root extract (0.39–50 μg/mL) was added to each well and incubated for 72 h. Next, the MTT dye (25 μL; 0.5 mg/mL) was added to the wells, and the assay was incubated for another 3 h. After this period, the medium was removed and 100 μL of dimethylsulfoxide (DMSO) was added to the wells to solubilize the formazan salts. The optical density of the wells was measured at 540 nm, and was compared to those in the control wells (cells incubated only with medium). Two independent experiments were performed in duplicate.

Hemolytic assay using human erythrocytes

The *A. congestiflora* extract was evaluated for hemolytic activity by assay performed in 96-well microplates. Each well received a 0.85% NaCl solution containing 10 mM $CaCl_2$. Samples (100 μL, in DMSO 5%) of the extract were added to the first well of each column in triplicate, from which 100 μL were transferred to the second to performed the serial dilution (final concentrations ranging from 15.62 to 2000 μg/mL) Each well received 100 μL of a 2% (v/v) suspension of erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. In negative controls, 100 μL of the saline solution plus 50 μL of the saline solution or 50 μL of 5% DMSO were plated. Positive control (to obtain 100% hemolysis) contained 80 μL of saline solution plus 20 μL of 0.1% Triton X-100 in 0.85% saline. After centrifugation during 1 h followed by incubation for 1 h at 27°C, the supernatant was discarded, and the released hemoglobin was measured by absorbance at 450 nm. Three independent experiments were performed in triplicate.

Evaluation of the acute toxicity of the hydroalcoholic roots extract from *A. congestiflora*

The acute toxicity of the extract was determined according to the guidelines of the Organization for Economic Cooperation and Development (OECD) for the testing of chemicals, nº 423, adopted on December 17, 2001. Female Swiss albino mice (n=3 for each group) received a single dose of the aqueous extract (2000 mg/kg) or saline solution as control. The animals were observed individually during the first hour and at 2, 12 and 24 h after administration to determine the time of death or to investigate possible toxic effects. For the following 14 days the animals were observed once daily. A range of signs such as general activity, irritability, touch response, contortions, tremors, convulsions, tachycardia, piloerection, stereotyped movements, somnolence, defecation, diarrhea and miction were analyzed. The daily consumption of water and food as well as the body weight of the mice during the experiment were also recorded. On the 14th day after administration of the extract, the animals were anesthetized with 0.2 mL/100 g of ketamine and xylazine (2:1), and blood was collected by cardiac puncture and placed in tubes with anticoagulant (EDTA). Hematological index were determined using an automated Horiba ABX Micros 60-Horiba analyzer. The organs (liver, kidney and spleen) were analyzed macroscopically, dried and weighed to calculate the index of organs, which is given by the following formula: *Organ weight (mg)/ Body Weight (g)*. Fragments of the liver, spleen and kidney were collected for histopathological analysis.

Comet assay

The dose used in this test was chosen according the results of the acute toxicity test. Female Swiss albino mice (n=5 for each group) were divided into three groups, which received extract at 2000 mg/kg or saline solution, both by oral route, or cyclophosphamide (CPA) at 25 mg/kg, by intraperitoneal injection. After treatments, 60 µL of total blood was collected from tail by vein puncture and it was performed the alkaline version of comet assay, according Singh et al., (1988), with modifications.

In each comet assay, 20 µL of blood was mixed with 110 µL of 0.5% low melting point agarose at 37°C, placed on fully frosted slides covered with a thin layer of normal-melting point agarose and coated with a coverslip. The slides remained at 4°C for 10 min to allow the agarose to solidify. Then, the slides were immersed in lysis

solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, with adjustment of pH to 10.0 using NaOH) at 4°C for 1 h. After lysis, the slides were placed on a horizontal electrophoresis chamber containing cold electrophoresis alkaline buffer (300 mM NaOH and 1 mM EDTA solution, pH13) during 20 min for DNA denaturation. Electrophoresis was carried out at 40 V and 300 mA for 20 min. The slides were imersed in a neutralization buffer (0.4 M Tris, pH 7.5) for 15 min and fixed with cold absolute ethanol for 5 min. The whole procedure was carried out in dim light to minimize artefactual DNA damage. To stain DNA, propidium iodide was added to each slide (50 µL, 20 µg/mL). Next, the slides were analyzed with a Zeiss fluorescent microscope. Two slides were examined for each animal, analyzing 100 nucleoids for slide, observed at 40× magnification. The score of each treatment was verified by multiplying the number of nucleoids observed in each damage class by the value of the class (0, 1, 2, 3 or 4). Frequency of damage (%) was calculated based on the number of cells with tail in relation to cells with 0 damage to each treatment. Two independent experiments were performed

Micronucleus test

The animals from the same experiments used for comet assay were used in the micronucleus test. After the vein puncture to collect the blood for comet assay, the bone marrow was collected. To this, both femurs of each animal were excised, dissected and cut at the proximal epiphyses. FBS was injected (1 mL/femur) with a syringe (3 mL) and needle (tuberculin type) into the medullary canal of the femur in order to remove the medullary content. The lavage fluid was collected in a test tube containing 2 mL FBS. Cell suspension was homogeneized and centrifuged at 1000 rpm for 5 min. The supernantant was discarded, leaving approximately 1 mL for tube. After resuspension of the pellet, 3 drops were used to make smears on two clean and dried slides by sliding one over the other. After 60 min drying at room temperature, the slides were fixed with methanol during 10 min and stained with Leishman's methylene blue dye (Schmid, 1975). To investigate the genotoxic potential of the extract, 2000 polychromatic erythrocytes (PCE) were analyzed per animal and the frequence of micronucleated erythrocytes PCE (MNPCE) was recorded. The cytotoxic effects were investigated by determining the ratio between PCE and NCE (normochromatic erythrocyte) numbers

after analyzing 200 erythrocytes/animal. All cells were observed and quantified using an optical microscope (10 × 100×) magnification (Schmid, 1975).

Statistical analysis

For evaluation of the cytotoxicity, the IC_{50} was determined by nonlinear regression using GraphPad Prism v. 5.0 software (GraphPad Software, Inc., USA). For acute toxicity the results were compared by T test. For genotoxicity and mutagenicity assays, one-way ANOVA followed by the Newman-Keuls test was used to evaluate the differences among the treatments. P values < 0.05 were considered to be statistically significant.

Results and discussion

Thin layer chromatography and liquid chromatography–electrospray ionisation mass spectrometry (LC–ESI-MS)

Thin layer chromatography analysis of the hydroalcoholic extract showed presence of terpenes and alkaloids and absence of tannins, flavonoids, coumarins and anthraquinones. These secondary metabolites have been reported in root extracts of other plants of the Cucurbitaceae family. Uma and Sekar (2014) found alkaloids, flavonoids and terpenes in roots of *Citrullus colocynthis* and Khatua et al. (2016) detected flavonoids and alkaloids in aqueous extract of the roots from *Trichosanthes dioica*. Also, methanolic extract from *Momordica dioica* roots contains triterpenes, alkaloids and steroids (TALUKDAR and HOSSAIN, 2014).

The triterpenoid substances called cucurbitacins found in Cucurbitaceae plants are divided in twelve categories which present in common the cucurbitane skeleton (DHIMAN et al., 2012). Cucurbitacins are related to several biological properties, including anti-inflammatory, antidiabetic and anti-artherosclerotic activities (SAHRANAVARD et al., 2012; CHEN et al., 2014; KAUSHIK et al., 2015). Cucurbitacins are also associated to cytotoxic activity on cancer and normal cells (DANTAS et al., 2006) and according to KAUSHIK et al. (2015), there are reports of the toxicity of cucurbitacins to humans, related to signs including the gastrointestinal tract and promotion of death.

The LC–ESI-MS analysis of the hydroalcoholic extract from *A. congestiflora* roots revealed three absorption peaks in the corresponding UV spectra analysis with the

majoritary peak showing absorption at 232.53 nm (Figure 1). Since the cucurbitacins usually shows maximum absorption between 228 and 234 nm (KAUSHIK et al., 2015), the compound corresponding to this majoritary peak probably belongs to this group.

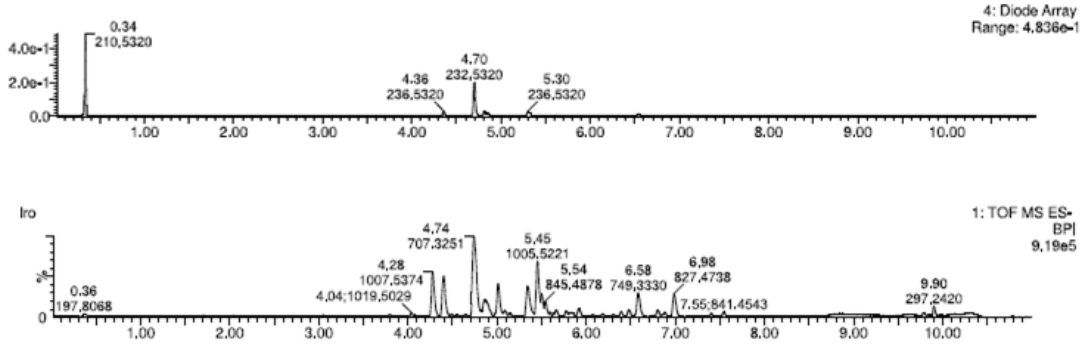

Figure 1. LC-UV-MS spectra of hydroalcoholic extract from *A. congestiflora* roots.

Total phenolic and flavonoid contents and antioxidant activity

The amounts of total phenolic and flavonoids in *A. congestiflora* hydroalcoholic extract were 14.89±1.87 mg ETA/g and 11.83±1.94 mg ER/g, respectively. Tannins and coumarins were again not detected. The amounts of phenolic compounds (6.74 mg GAE/g) and flavonoids (4.42 mg CE/g) present in the hydromethanolic root extract of Cucurbitaceae *Ecballium elaterium* (Abbassi et al., 2014) were lower than that of *A. congestiflora* hydroalcoholic extract. Also the total phenol amount in *Coccinia cardifolia, Ligenaria siceraria, Cucumis sativus, Lufa actangula* and *Momordica charantia*, five edible Cucurbitaceae plants, ranged from 4.54 to 10.13 mg TA/g (Debnath et al., 2015).

Biological properties of plant extracts are related to the chemical constituents of them. The antioxidant activity reported for plants of the cucurbitaceae family has been mainly related to polyphenols and flavonoids, which are powerful antioxidants due to their free radical scavenger ability (Vamanu and Nita, 2013; Yasir et al., 2016). Thus, we evaluated the antioxidant activity of the hydroalcoholic extract from *A. congestiflora* by ABTS and DPPH methods (Table 2). ABTS method allows determining antioxidant activity of both hydrophilic and hydrophobic compounds, whereas DPPH is more appropriate to hydrophobic systems (Palombini et al., 2013; Valantina and Neelamegam, 2015). The results showed that the extract is capable of scavenging these

free radicals, which is probably due to the presence of phenolic compounds and flavonoids.

Table 2. Antioxidant activity of the hydroalcoholyc extract from *A. congestiflora*.

Sample	IC_{50} (µg/mL)	
	ABTS method	**DPPH method**
Hydroalcoholic extract	81.29 ± 4.48	64.79± 4.50
Trolox	2.32 ± 0.01	-
Ascorbic acid	-	0.64 ± 0.05

Data showed as mean ± standard deviation (n=3).

According to Fridianny et al. (2015), a sample with IC_{50}< 50 µg/mL is very strong antioxidant, IC_{50} ranging from 50 to 100 µg/mL is a strong antioxidant, IC_{50} ranging from 101 to 150 µg/mL is a moderate antioxidant and IC_{50}>150 µg/mL is a weak antioxidant. Then, hydroalcoholic extract of *A. congestiflora* roots is considered a strong antioxidant.

Radical scavenging activity is very important due to the deleterious effect of the free radicals in the body, which are associated to several diseases, such as atherosclerosis, diabetes and Alzheimer's disease (Zhao and Zhao, 2013; Singh et al., 2015). Thus, hydroalcoholic extract from *A. congestiflora* may help to reduce the oxidative damage.

Cytotoxicity on normal cells

The IC_{50} value of the extract for 3T3-L1, VERO cells and PBMCs was 40.67±2.34, 39.15±1.58 and 42.62±2.96, respectively. According to Ampasavate et al. (2010), IC_{50} values in MTT assay between 10 and 100 µg/mL are considered indicative of potential cytotoxicity. Thus, hydroalcoholic extract of the roots from *A. congestiflora* was potententially cytotoxic to 3T3-L1 and VERO cells as well as to PBMCs.

In regard to capacity of promoting damage to erythrocytes membrane, the extract showed EC_{50}>2000 µg/mL and presented maximum percentage of hemolysis (H_{max}) of 29.96%. Since to be considered hemolytic a sample must present EC_{50}< 200 µg/mL (Costa-Lotufo et al., 2005), the *A. congestiflora* extract was not considered

hemolytic. The percentage of hemolysis still observed may be due to the presence of the saponins identitified.

Similarly to results obtained in this work, methanolic extract of roots from *Bryonia aspera*, another Cucurbitaceae, was potentially cytotoxic with IC_{50} of 51.05 µg/mL on bovine's kidney epithelial cells (MDBK) (Sahranavard et al., 2012). On the other hand, hydroalcoholic extract of the leaves from *Cucurbita pepo*, widely used in folk medicine, showed IC_{50} of 239.2 and 241.4 µg/mL to Chinese hamster ovarian cells (CHO) and rat fibroblast, respectively (Shokrzadeh et al., 2010), being a weak cytotoxic agent.

Several plants are used by population as medicine and this fact shows the need for studies investigating possible deleterious effects of them. The results from cytotoxicity assays stimulate the evaluation of these effects. Additionally, cytotoxicity on PBMCs allows investigating the effect on human T lymphocytes, since they are the predominant cell population in this assay (Liu et al., 2004).

Acute toxicity

Medicinal plants have been widely used in developing countries to treat diseases (Ngarivhume et al., 2015; Somade et al., 2017). However, there is a lack of scientific studies to many plants regarding their toxicity and adverse effects, which may limit their use. In this work the *A. congestiflora* hydroalcholic extract was evaluated for acute toxicity aiming to determine the safety of use of it.

In the first hours and for the following 14 days after administration of the extract (2000 mg/kg), the mice did not show signs of systemic toxicity and all the animals survived. Figure 2 shows that there were no significant differences ($p<0.05$) in body weight between the control and treated groups. Thus, it is possible to suggest that the extract did not interfere with normal metabolism of the animals. Additionally, there was no change in the consumption of water, but animals which received extract presented an increase of feed intake in comparison with control (Table 3). The organs indices were not different between the groups.

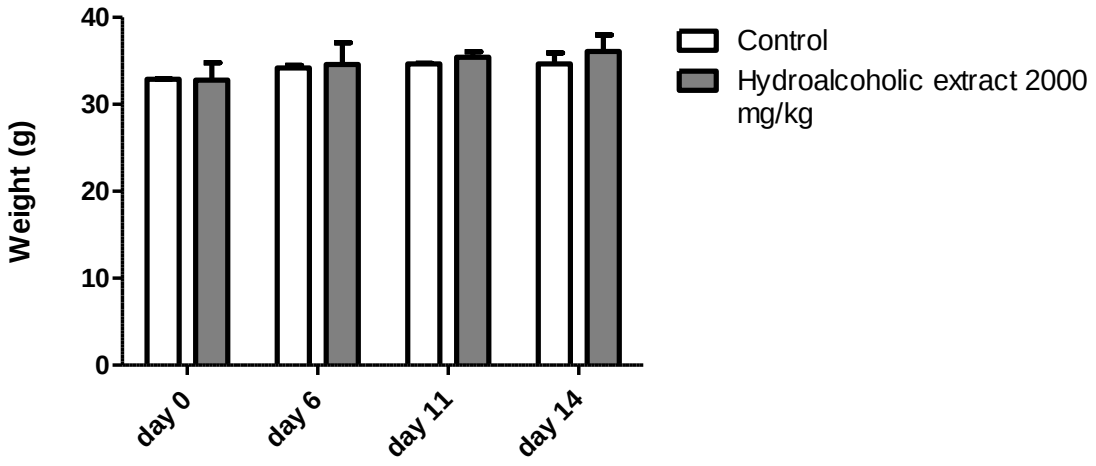

Figure 2. Effect of oral administration of *A. congestiflora* extract (2000 mg/kg) on weight gain by mice in acute toxicity assay.

Evaluation of hematological parameters is essential to identify the toxic effect of a treatment with a potential drug. The hematological parameters of animals from oral acute toxicity assay are shown in Table 4. Animals treated with the extract did not present alterations in red blood cells, leukocytes, platelets, hemoglobin and hemotocrit in comparison to control.

Table 3. Effect of *A. congestiflora* extract (2000 mg/kg) on physiological parameters of mice 14 days after administration.

Group	Feed intake (g)	Water consumption (mL)	Index of organs (mg/g)		
			Liver	Kidney	Spleen
Extract	20.92±1.31*	29.28±1.01	6.26±0.98	0.59±0.05	0.61±0.20
Control	17.31±1.11	27.14±2.52	5.86±0.7	0.62±0.08	0.58±0.08

Data are presented as mean ± standard deviation, with 3 animals per group. *$p<0.05$ when compared to control by t test.

Table 4. Effect of hydroalcoholic extract from *A. congestiflora* (2000 mg/kg) on hematological parameters of mice 14 days after administration.

Parameter	Extract	Control
Red blood cells (10^6/mm^3)	8.63±0.46	8.42±0.58
Leukocytes (10^3/mm^3)	4.8±1.21	3.92±0.63
Platelets (10^3/mm^3)	824.4±115.10	808.66±44.76
Hemoglobin (g/dL)	12.6±0.61	11.98±0.82
Hematocrit (%)	47.05±2.64	44.8±3.10

Data are presented as mean ± standard deviation, with 3 animals per group.

It was also evaluated biochemical parameters, which allow investigating the functionality of the organs, with regard to renal and hepatic functions. The results (Table 5) show that the oral administration of the extract did not cause significant changes in the plasma biomarkers of renal damage. In relation to biomarkers of hepatic damage, there was decrease of AST (aspartate aminotransferase), ALT (alanine aminotransferase), alkaline phosphatase and total protein levels, while no statistical difference was observed for albumin and globulins, in comparison with control.

Hepatic enzymes AST and ALT are usual indicatives of hepatic functionality, whose increase in plasma levels is associated with damage to liver. Despite the difference in relation to control group of AST values, the levels of this hepatic enzyme are within normality in both control and extract treatments, which range from 54 to 298 U/L for mice (Hismiogullari et al., 2011). Similarly, it was observed a slight reduction to ALT levels of the treated animals, however, the result was also within normality values (Ozmen and Yurekli, 1998).

Table 5. Effect of hydroalcoholic extract from *A. congestiflora* (2000 mg/kg) on biochemical parameters of mice 14 days after administration.

Parameter	Extract	Control
Urea (mg/dL)	46.8±5.63	49.83±3.81
Creatinine (mg/dL)	0.31±0.04	0.28±0.03
AST (U/L)	156.0±85.49	215.0±79.51
ALT (U/L)	22.2±2.94*	28.0±3.39
Alkaline phosphatase (U/L)	72.2±8.84*	93.2±9.73
Total protein (g/dL)	3.78±0.33*	4.33±0.28
Albumin (g/dL)	2.55±0.16	2.53±0.10
Globulins (g/dL)	1.2±0.38	1.72±0.14

Data are presented as mean ± standard deviation, with 3 animals per group. *$p<0.05$ when compared to control by t test.

Histological examination showed no detectable alterations on spleen, liver and kidney of the treated animals in relation to control group. Liver of both control and treated groups showed hepatic cells with normal morphology. Similarly, kidney of both groups showed normal glomerulus and tubular structures. These results corroborate the results obtained in the biochemical evaluation and indicate the absence of damages associated to the use of the hydroalcoholic extract of *A. congestiflora* root.

Several Cucurbitacean plants have been used in folk medicine and the roots are much employed. Acute toxicity assay using the ethanolic extract of *Siolmatra brasiliensis* roots revealed that the dose 2000 mg/kg revealed adverse behavioural effects, mortality and morphological organ alterations (Lima et al., 2010). On the other hand, the aqueous extract from the roots of *Dicoma anomala* did not present toxic effects at the dose of 2000 mg/kg for Wistar rats (Balogun and Ashafa, 2016). This study indicated that animals treated with the hydroalcoholic extract (2000 mg/kg) from the roots of *A. congestiflora* did not showed signs of toxicity, mortality or organ alterations.

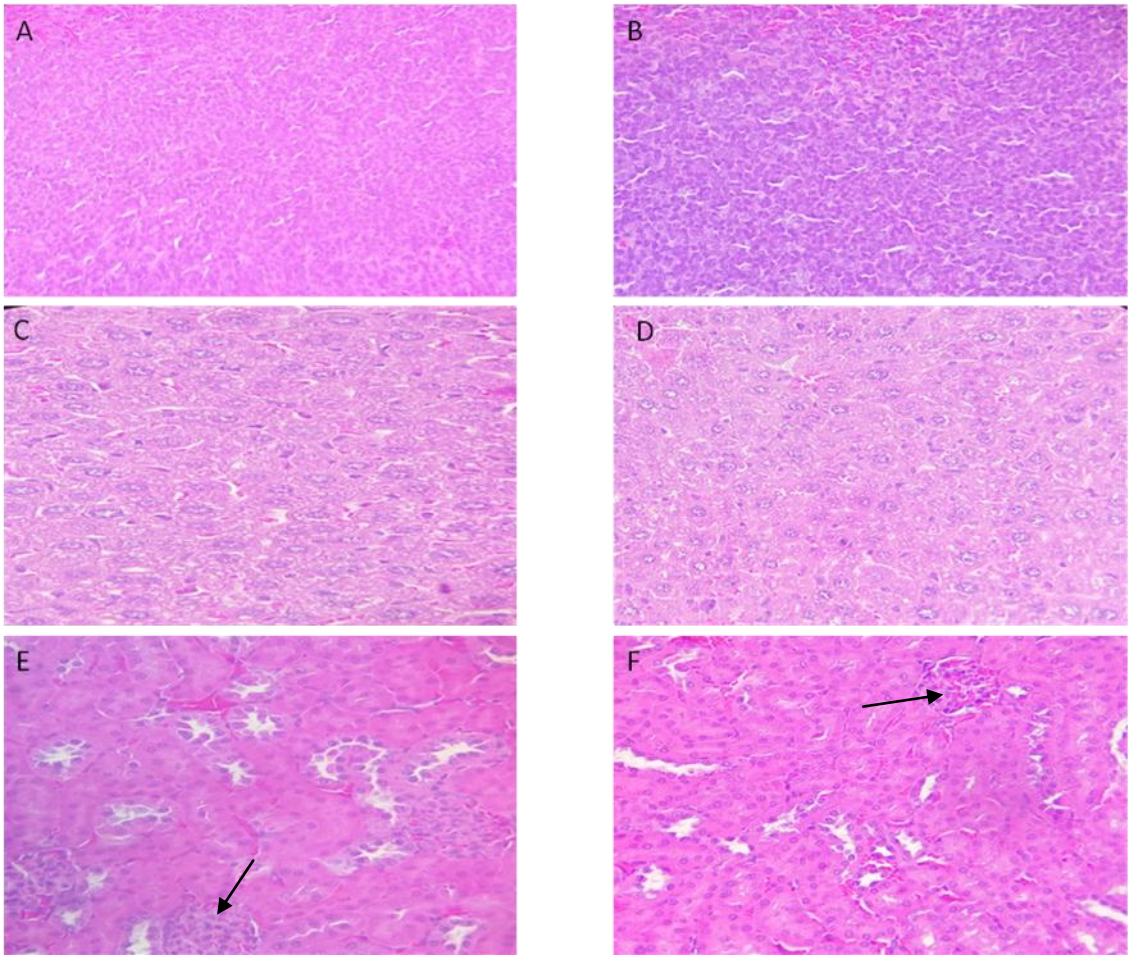

Figure 3. The effects of the hydroalcoholic root extract (2000 mg/kg) on spleen, liver and kidney. The histological sections were stained with hematoxylin-eosin (x 400). (A) Spleen from control group. (B) Spleen from treated group. (C) Liver from control group. (D) Liver from treated group. (E) Kidney from control group and (F) kidney from treated group. Black arrow –glomerulus.

The results obtained on acute toxicity indicates that the chemical constituents present in the extract do not promote acute toxicity when orally administered, but it is necessary caution in use for long periods of time due to the lack of scientifc reports about the continued use of this plant, such as the possibility of chronic toxicity developing with continual use.

Comet assay

Some plants used in tradional medicine may present genotoxic or mutagenic effects (Sousa and Viccini, 2011). Comet assay is a useful tool to investigate the genotoxic potential of a product, for being a quick, cheap and reliable test (Recio et al.,

2010). To evaluate whether the hydroalcoholic extract of *A. congestiflora* contain substances potentialy genotoxic, it was performed the comet assay *in vivo*.

Effect of the extract on DNA of white blood cells is shown in Table 6. Animals treated with extract showed higher number of nucleoids belonging to class 0 (90.5), similarly to control (90.0), whereas those treated with cyclophosphamide (positive control) presented higher number of nucleoids belonging to class 1 (53.83) and class 2 (23.3).

Table 6. Index of damage to each class in comet assay after 24h of treatment with the hydroalcoholic extract of the root from *A. congestifllora.*

	Class					**Score**
Treatament	0	1	2	3	4	
Control	90.0±1.26	8.50±1.37	1.25 ± 0,5	1.33± 0,57	0	12.16±2.71
CPA	19.83±2.13*	53.83±1.72*	23.3±2.4*	2.5±1.04*	0	109±5.86*
Extract	90.5 ±1.04	7.5±0.83	1.33±0.51	1.33±0.57	0	12.16±2.22

Data showed as mean ± standard deviation. * $p < 0.05$ in relation to control.

Tests for detection of genotoxic substances in plant used to traditional folk medicine allow identifying potential risk to human health (Shabbar and Maslat, 2006). Comet assay is a sensitive method which detects a large spectrum of DNA lesions, including double and single strand breaks (Tice et al., 2000). As is possible to observe in Table 7, there was no significant difference between the frequency of damage presented by the animals that received the extract and those which received vehicle. Thus, the results indicate absence of genotoxic activity to *A. congestiflora* extract also suggeting the safe on use of the plant roots.

Table 7. Frequency of damage to white blood cells treated with the hydroalcoholic extract (2000 mg/kg) of the root from *A. congestifllora.*

Treatment	Frequency of damage (%)
Control	10.0 ± 1.26
CPA	80.16 ± 2.13*
Extract	9.5 ± 1.04

Data showed as mean ± standard deviation* p< 0.05 in relation to control.

There is a lack of studies involving the genotoxic investigation of plants belonging to genus *Apodanthera*. However, studies with other Cucurbitaceae plants have evidenced genotoxicity, as demonstrated for fruits of *Ecballium elaterium* (Celik and Aslanturk, 2009) and for a triterpenoid-enriched extract from roots of *Trichosanthes dioica* containing cucurbitacins, which showed antimitotic and genotoxic effects on *Allium cepa* (Bhattacharya and Haldar, 2012).

Micronucleus test

The mutagenic and genotoxic investigation using *in vivo* models are important evaluations since some substances may suffer alterations after metabolization, originating toxic forms. Micronucleus test is well accepted by international agencies as an efficient method of studying damage to genetic material *in vivo* (OECD, 2007). Table 8 shows the frequency of micronucleated polychromatic erythrocytes (PCEs) in 2000 cells and a cytototoxicity parameter evaluation, the PCE/NCE ratio. In relation to induction of micronucleus, treatment with the extract did not show significative differences in comparison with control group. However, there was a decrease in ratio of PCE/NCE, which indicates that the extract promoted cytotoxicity, due to reduction of the production of new erythrocytes.

This result is in agreement with those obtained in comet assay, indicating the absence of damage to genetic material of the hydroalcoholic extract from the roots of *A. congestiflora*. However, there was a reduction in the number of immature erythrocytes (PCEs) in comparison with the number of mature erythrocytes (NCEs). This occurs when the normal proliferation of bone marrow cells is affected by a cytotoxic product (SANTOS et al., 2016). Thus, despite the lack of genotoxicity and acute toxicity, in this

test it was observed a discreet cytotoxicity *in vivo*, suggesting caution in use of *A. congestiflora* preparations.

Table 8. Micronucleated polychromatic erythrocytes (MN PCEs) frequence in 2000 cells and PCE/NCE ratio in bone marrow of mice treated with *A. congestiflora* extract.

Treatment	MN PCEs (mean± standard deviation)	PCE/NCE ratio
Control	4.5 ± 1.0	1.91 ± 0.05
CPA	53.75 ± 4.92*	0.78 ± 0.007*
Extract	4.0 ± 0.81	1.82 ± 0.08*

Data showed as mean ± standard deviation* $p < 0.05$ in relation to control.

Conclusions

In conclusion, this work reveals that hydroalcoholic extract from *A. congestiflora* root containing phenolic compounds, flavonoids and antioxidant activity does not cause damage to genetic material neither alterations in organs as liver, kidney and spleen. However, caution is required in prolonged use of this extract due to a potential cytotoxic effect.

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References

Abbassi, F., Ayari, B., Mhamdi, B., Toumi, L., 2014. Phenolic contents and antimicrobial activity of squirting cucumber (Ecballium elaterium) extracts against food-borne pathogens. Pakistan Journal of Pharmaceutical Sciences, 27, 475-479.

Alley, M.C., Scudiere, D.A., Monks, A., Hursey, M.L., CZERWINSKI, M.J., FINE, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., Boyd, M.R., 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research, 48, 589-601.

Amorim, E.L.C., Nascimento, J.E., Monteiro, J.M., Peixoto-Sobrinho, T.J.S., Araújo, T.A.S., Albuquerque, U.P., 2008. A simple and accurate procedure for the determination of tannin and flavonoid levels and some applications in ethnobotany and ethnopharmacology. Functional Ecosystems and Communities, 2, 88-94.

Ampasavate, C., Okonogi, S., Anuchapreeda, S., 2010. Cytotoxicity of extracts from fruit plants against leukemic cell lines. African Journal of Pharmacy and Pharmacology, 4, 013-021.

Balogun, F.O., Ashafa, A.O.T., 2016. Acute and Subchronic Oral Toxicity Evaluation of Aqueous Root Extract of Dicoma anomala Sond. in Wistar Rats. Evidence-Based Complementary and Alternative Medicine, 2016, http://dx.doi.org/10.1155/2016/3509323.

Bhattacharya, S., Haldar, P.K., 2012. Evaluation of Antimitotic and Genotoxic Effects of the Triterpenoid Enriched Extract from Trichosanthes dioica Root. American-Eurasian Journal of Toxicological Sciences, 4, 20-23.

Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature, 181, 1199–1200.

Celik, T.A., Aslanturk, O.S., 2009. Investigation of cytotoxic and genotoxic effects of Ecballium elaterium juice based on Allium test. Methods and Findings in Experimental and Clinical Pharmacology, 31, 591-596.

Chawech, R., Jarraya, R., Girardi, C., Vansteelandt, M., Marti, G., Nasri, I., Racaud-Sultan, C., Fabre, N., 2015. Cucurbitacins from the Leaves of Citrullus colocynthis (L.) Schrad. 20, doi:10.3390/molecules201018001.

Chekroun, E., Benariba, N., Adida, H., Bechiri, A., Azzi, R., Djaziri, R., 2015. Antioxidant activity and phytochemical screening of two Cucurbitaceae: Citrullus colocynthis fruits and Bryonia dioica roots. Asian Pacific Journal of Tropical Disease, 5, 632-637.

Costa-lotufo, L.V., Khan, M.T.H., Wilke, A.A.D.V., Jimenez, P.C., Pessoa, C., Moraes, M.E., Moraes, M.O., 2005. Studies of the anticancer potential of plants used in Bangladeshi folk medicine. Journal of Ethnopharmacology, 99, 21-30.

Chen, X.B., Chen, G.Y., Liu, J.H., Lei, M., Meng, Y.H., Guo, D.A., Liu, X., 2014. Cytotoxic cucurbitane triterpenoids isolated from the rhizomes of *Hemsleya amabilis.* Fitoterapia, 94, 88-93.

Dantas, I.N., Gadelha, G.C., Chaves, D.C., Monte, F.J., Pessoa, C., de Moraes, M.O., Costa-Lotufo, L.V., 2006. Zeitschrift fur Naturforschung C, 61, 643-646.

Debnath, B., Uddin, Md.J., Patari, P., Das, M., Maiti, D., Manna, K., 2015. estimation of alkaloids and phenolics of five edible cucurbitaceous plants and their antibacterial activity. International Journal of Pharmacy and Pharmaceutical Sciences, 12, 223-227.

Dhiman, K., Gupta, A., Sharma, D.K., Gill, N.S., Goyal. A., 2012. A Review On The Medicinally Important Plants Of The Family Cucubitaceae. Asian Journal Of Clinical Nutrition, 4, 16-26.

Diallo, A., Darre, P., Metowogo, K., Lawson-evi, P., Selva, D., Potchoo, Y., Creppy, E., Ramanathan, M., 2016. Fetal Toxicity and Cytotoxicity of Lannea kerstingii Engl and Krause Stem Bark (Anacardiaceae), 15, 253-258.

Etebari**,** M., Sajjadi, S. E., Jafarian-Dehkordi**,** A., Nazmakanipour S., 2016. Genotoxicity evaluation of hydroalcoholic and aqueous extracts of *Dorema aucheri* by the comet assay. Advanced Biomedical Research, 5, doi: 10.4103/2277-9175.190993.

Ferreira, R.O., Junior, A.R.C., Silva, T.M.G., Castro, R.N., da Silva, T.M.S., de Carvalho, M. G., 2014. Distribution of metabolites in galled and non-galled leaves of *Clusia lanceolata* and its antioxidant activity. Revista Brasileira de Farmacognosia, 24, 617–625.

Fidrianny, I., Fikayuniar, L., Insanu, M., 2015. Antioxidant activities of various seed extracts from four varieties of rambutan (*Nephelium lappaceum*) using 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) assays. Asian Journal of Pharmaceutical and Clinical Research, 5, 215-219.

Hagerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W., Riechel, T.L., 1998. High molecular weight plant polyphenolics (tannins) as biological antioxidants. Journal of Agricultural and Food Chemistry. 46, 1887–1892.

Hismiogullari, A. A., Hismiogullari, S.E., Yavuz, O., Essiz, D., Rahman, K., 2011. Evaluation of biochemical findings in mice exposed to thiamphenicol treatment. African Journal of Pharmacy and Pharmacology, 5, 428-431.

Kabir, M.G., Rahman, M.M., Ahmed, N.U., Fakruddin, M., Islam, S., Mazumdar, R.M., 2014. Antioxidant, antimicrobial, toxicity and analgesic properties of ethanol extract of Solena amplexicaulis root. Biological Research, 47, doi: 10.1186/0717-6287-47-36.

Kaushik, U., Aeri, V., Mir, S.R., 2015. Cucurbitacins – An insight into medicinal leads from nature. Pharmacognosy Reviews, 9, 12-18.

Khatua, S., Pandey, A., Biswas, S. J., 2016. Phytochemical evaluation and antimicrobial properties of Trichosanthes dioica root extract. Journal of Pharmacognosy and Phytochemistry, 5, 410-413.

Kokate A (1999) Phytochemical Methods. Phytotherapy, 78: 126-129.

Lemos, M., Santin, J.R., Júnior, L.C., Niero, R., Andrade, S.F., 2011. Gastroprotective activity of hydroalcoholic extract obtained from the leaves of Brassica oleracea var. acephala DC in different animal models. Journal of Ethnopharmacology, 138, 503-507.

Lima, A.P., Barbosa, C.E.S., Pereira, F.C., Vilanova-Costa, C.A.S.T., Ribeiro, A.S.B.B., Silva, H.D., Azevedo, N.R., Gomes-Klein, V.L., Silveira-Lacerda, E.P., 2010. Siolmatra brasiliensis (Cogn.) Baill., Cucurbitaceae, acute toxicity in mice. Revista Brasileira de Farmacognosia, 20, 917-921.

Lima, Luis Fernando Paiva Estudos taxonômicos e morfopolínicos em Cucurbitaceae brasileiras. 2010. 232f. Tese (Doutorado em Botânica). Universidade Federal do Rio Grande do Sul, Rio Grande do Sul.

Liu, C.P., Tsai, W.J., Lin, Y.L., Liao, J.F., Chen, C.F., Kuo, Y.C., 2004. The extracts from Nelumbo Nucifera suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells. Life Sciences, 75, 699-716.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunology Methods, 65, 55-63.

Ngarivhume, T., Van't, K.C.I., Jong, J.T., Van der Westhuizen, J.H., 2015. Medicinal plants used by traditional healers for the treatment of malaria in the Chipinge district in Zimbabwe. Journal of Ethnopharmacology, 159, 224-237.

OECD - GUIDELINE FOR THE TESTING OF CHEMICALS.,2007. DRAFT PROPOSAL FOR A NEW GUIDELINE 487: In Vitro Mammalian Cell Micronucleus Test (mnvit).

Osório, A. C., Martins, J.L.S., 2004. Determination of coumarin in fluid extract and tinture of "guaco" by first derivative spectrophotometry. Revista Brasileira de Ciências Farmacêuticas, 40, http://dx.doi.org/10.1590/S1516-93322004000400005.

Ozmen, M., Yurekli, M., 1998. Subacute toxicity of uranyl acetate in Swiss-Albino mice. Environmental Toxicology and Pharmacology, 6, 111-115.

Palombini, S.V., Claus, T., Maruyama, S.A., Gohara, A.K., Souza, A.H.P., Souza, N. E., J.V., Visentainer, Gomes, S.T.M., Matsushita, M., 2013. Evaluation of nutritional compounds in new amaranth and quinoa cultivars. Food Science and Technology, 32, 339,344.

Peixoto-Sobrinho, T.J.S., Gomes, T.L.B., Cardoso, K.C.M., Albuquerque, U.P., Amorim, E.L.C, 2008. Total flavonoid content in products containing "pata-de-vaca" (Bauhinia L.) sold in pharmacies in Recife/PE. Revista Brasileira de Plantas Medicinais, 14, http://dx.doi.org/10.1590/S1516-05722012000400003.

Pinho, E., Ferreira, I.C.F.R., Barros, L., Carvalho, A. M., Soares, G., Henriques, M., 2014. Antibacterial Potential of Northeastern Portugal Wild Plant Extracts and

Respective Phenolic Compounds. BioMed Research International, v. 2014, http://dx.doi.org/10.1155/2014/814590.

Rakotoarivelo, N.H.,Rakotoarivony, F., Ramarosandratana, A.V., Jeannoda, V.H., Kuhlman, A.R., Randrianasolo, A., Bussmann, R.W., 2015. Medicinal plants used to treat the most frequent diseases encountered in Ambalabe rural community, Eastern Madagascar. Journal of Ethnobiology and Ethnomedicine, 11, DOI 10.1186/s13002-015-0050-2.

Recio, L., Hobbs, C., Caspary, W., Witt, K.L., 2010. Dose-Response Assessment of Four Genotoxic Chemicals in a Combined Mouse and Rat Micronucleus and Comet Assay Protocol. The Journal of Toxicological Sciences, 35, 149-162.

Roque, A., A., Rocha, R.M., Loiola, L.M.B., 2010. Uso e diversidade de plantas medicinais da Caatinga na comunidade rural de Laginhas, município de Caicó, Rio Grande do Norte (nordeste do Brasil). Revista Brasilera de Plantas Medicinais, 12, 31-42,

Santos, N.C., Ramos, M.E., Ramos, A.F., Cerqueira, A.B., Cerqueira, E.M., 2016. Evaluation of the genotoxicity and cytotoxicity of filling pastes used for pulp therapy on deciduous teeth using the micronucleus test on bone marrow from mice (Mus musculus). Mutagenesis, 31, 589-595.

Schmid, W,. The micronucleus test. Mutation Research, 31, 9-15.

Sahranavard, S., Naghibi, F., Ghaffari, S., 2012. Cytotoxic activity of extracts and pure compounds of *Bryonia aspera*. International Journal of Pharmacy and Pharmaceutical Sciences, 4, 541-543.

Shabbar, I.M.S., Masla, A., 2006. Genotoxicity of Ecballium elaterium (L) A Rich Cucurbitaceae Fruit Juice Using Micronucleus Assay and DNA Single Strand Break Techniques. The Internet Journal of Health, 6, 4-15.

Shokrzadeh, M., Azadbakht, M., Ahangar, N., Hashemi, A., Sarav, S.S.S., 2010. Cytotoxicity of hydro-alcoholic extracts of *Cucurbita pepo* and *Solanum nigrum* on HepG2 and CT26 cancer cell lines. Pharmacognosy Magazine, 6, 176-179.

Silva, C.G., Marinho, M.G.V., Lucena, M.F.A., Costa, J.G.M., 2015. Ethnobotanical survey of medicinal plants in the Caatinga area in the community of Sitio Nazaré, Milagres, Ceará, Brazil. Revista Brasileira de Plantas Medicinais, 17, http://dx.doi.org/10.1590/1983-084X/12_055.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research, 175, 184-191.

Singh, R., Devi, S., Gollen, R., 2015. Role of free radical in atherosclerosis, diabetes and dyslipidaemia: larger-than-life. Diabetes/metabolism research and reviews, 31, 113-126.

Somade, O.T., Adeniji, K.D., Adesina, A.-R.A., Olurinde, O.J., 2017 Oral acute toxicity study as well as tissues oxidative stress and histopathological disorders in edible camphor administered rats. Experimental and Toxicologic Pathology, 69, 99-108.

Sousa, S.M., Viccini, L.F., 2011. Cytotoxic and genotoxic activity of Achillea millefolium aqueous extracts. Revista Brasileira de Farmacognosia, 21, 98-104.

Talukdar, S.N., Hossain, M.N., 2014. Phytochemical, Phytotherapeutical and Pharmacological Study of Momordica dioica, 2014, http://dx.doi.org/10.1155/2014/806082.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis, 35, 206-221.

Uma, C., Sekar, K.G., 2014. Phytochemical analysis of a folklore medicinal plant *Citrullus colocynthis* L. (bitter apple). Journal of Pharmacognosy and Photochemistry, 2, 195-202.

Valantina, R.S., Neelamegam, P., 2015. Selective ABTS and DPPH- radical scavenging activity of peroxide from vegetable oils. International Food Research Journal, 22, 289-294.

Vamanu, E., Nita, S., 2013. Antioxidant capacity and the correlation with major phenolic compounds, anthocyanin, and tocopherol content in various extracts from the wild edible Boletus edulis mushroom. Biomed Research International, 2013, http://dx.doi.org/10.1155/2013/313905.

Wagner, H., Bladt, S., 1995. Plant drug analysis - a thin layer chromatography atlas. Berlin, Germany.

Yasir, M., Sultana, B., Nigam, P.S., 2016. Owusu-Apenten, R., Antioxidant and genoprotective activity of selected *cucurbitaceae* seed extracts and LC–ESIMS/MS identification of phenolic components. Food Chemistry, 199, 307-313.

Zhao, Y., Zhao, B., Oxidative stress and the pathogenesis of Alzheimer's disease. 2013. Oxidative Medicine and Cellular Longevity, 2013, http://dx.doi.org/10.1155/2013/316523.

6 ARTIGO CIENTÍFICO III

Evaluation of the cytotoxicity, genotoxicity and mutagenicity of extracts, fractions and lectins from *Myracrodruon urundeuva* tissues

A ser submetido no periódico Phytomedicine

Evaluation of the cytotoxicity, genotoxicity and mutagenicity of extracts, fractions and lectins from *Myracrodruon urundeuva* tissues

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Abstract

Background: *Myracrodruon urundeuva* is an endemic plant of South America with extensive use by the northeastern Brazil population and the lectins MuBL, MuHL and MuLL were isolated from its bark, heartwood and leaf, respectively.

Methods: This study evaluated the extracts, protein fractions and lectins from *M. urundeuva* bark, heartwood and leaf for cytotoxicity on cancer and normal cells. The lectins were also investigated for hemolytic activity, genotoxicity using comet assay, and mutagenicity by micronucleus test.

Results: Bark, heartwood, and leaf extracts inhibited the viability of cancer cells ranging from 2.6 to 55%, whereas the values determined for the protein fractions ranged from 3.8 to 63.3%. The bark extract and the leaf protein fraction were moderately active on cell line HT-29, with inhibition percentage of 55.0 ± 0.49 and 58.6 ± 3.4 respectively, while the bark protein fraction was moderately active on NCI-H292 and HL-60 cancer cell lines (inhibition percentage of 62.94 ± 2.68 and 63.37 ± 2.33 respectively). The extracts and protein fractions showed weak cytotoxicity on lymphocytes, with inhibition percentage varying between 3.81% and 17.58 %. The lectins MuBL and MuHL showed inhibition percentages for cancer cells ranging from 10.51 % to 42.36 % and 6.35 to % 62.56 %, respectively. Only MuHL was moderately active on the HL-60 cell line. MuBL, MuHL and MuLL showed IC_{50} on mononuclear peripheral blood cells of 58.41, 43.19 and 62.28 μg/mL, respectively. The percentage of hemolysis promoted by lectins ranged from 0 to 11.6. Data from comet assay revealed that MuBL, MuHL and MuLL at highest concentration showed frequency of damage of 33.5 %, 35.67 % and 65.2 %, respectively, whereas control showed 16.17 %. On micronucleus test, the number of micronuclei to the highest concentrations tested was 7.91, 7.41 and 8.91 to MuBL, MuHL and MuLL, respectively, whereas to control was 6.91.

Conclusions: Extracts, protein fractions and lectins from *M. urundeuva* presented weak to moderate cytotoxicity on the cancer cell lines tested, weak to potentially cytotoxicity on lymphocytes and did not promote damage to erythrocytes. Lectins from *M. urundeuva* showed to be potentially genotoxic, on the other hand did not showed mutagenic activity.

Keywords: *Myracrodruon urundeuva*, cytotoxicity, genotoxicity, mutagenicity.

Abreviations

EC_{50}, effective concentration that promotes 50% of hemolysis

FBS, Fetal Bovine Serum

$IC_{50,}$ concentration that inhibited 50% of the cell growth compared with the control

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PBS, phosphate buffered saline

PHA, phytohemagglutinin

Introduction

Myracrodruon urundeuva is widely used in Brazil to treat several health problems, such as dermatological and gynecological diseases, disorders in the digestive system, and inflammations (Matos, 2002; Pereira et al., 2014). Among the bioactive compounds identified in *M. urundeuva* tissues are the lectins isolated from bark (MuBL), heartwood (MuHL) and leaf (MuLL) (Sá et al., 2009; Napoleão et al., 2011).

Lectins are proteins that bind specifically and reversibly to carbohydrates, without promoting alterations in the glycosidic bonds and covalent structure (Cao et al., 2010; Lavanya et al., 2014). MuBL, MuHL and MuLL showed larvicidal activity against *Aedes aegypti* and termiticide activity on *Nasutitermes corniger* (Sá et al., 2008, 2009a; Napoleão et. al., 2011, 2012). In addition, MuHL showed antibacterial activity against human pathogenic species and antifungal property against phytopathogenic species of *Fusarium* (Sá et al., 2009b). Until now there are no reports regarding the effects of these lectins on human cells.

In addition to the biological activities described for the lectins from *M. urundeuva*, a diversity of other biological effects have been described for plant lectins, such as anti-inflammatory, antinociceptive and anticancer (Yau et al., 2015; Campos et al., 2016). Lectins have received attention on the search for new anticancer agents due to the fact that malignant transformation is associated with altered cell glycosylation. This alteration allows lectins acting on these cells without promote damage to cells with normal glycosylation pattern. Thus, these proteins represent promising tools in the search for molecules with specificity against cancer cells (Yau et al., 2015).

In this context, the present study evaluated extracts, protein fractions and lectins from *M. urundeuva* bark, heartwood and leaf for effect on the viability of human normal and cancer cells. Before the evaluation of cytotoxic effects, MuBL, MuHL and MuLL were also investigated for their hemolytic, cytotoxicity and genotoxicity to normal cells, in order to evaluate their safety degree.

Material and Methods

Plant material

Bark, heartwood and leaves from *Myracrodruon urundeuva* Allemão were collected in Caxias, Maranhão, northeastern Brazil, with authorization (number 38690) of the *Instituto Chico Mendes de Conservação da Biodiversidade* (ICMBio) from Brazilian Ministry of Environment. A voucher specimen is archived under number 054 at the herbarium Aluisio Bittencourt, *Centro de Estudos Superiores de Caxias*, *Universidade Estadual do Maranhão* (Caxias, Brazil).

Cell lines and cell culture

The lymphocytes were obtained from peripheral blood collected from healthy volunteer donors that signed an informed consent term, with approval (60107916.8.0000.5208) of the Ethics Committee of the *Universidade Federal de Pernambuco* (UFPE), Recife, Brazil.

The human cancer cell lines used for the *in vitro* cytotoxicity assays were NCI-H292 (lung mucoepidermoid carcinoma), HT-29 (colon adenocarcinoma), HEp-2 (larynx epidermoid carcinoma), MCF-7 (breast adenocarcinoma), MOLT-4 (acute lymphoblastic leukemia), K562 (erythroleukemia), and HL-60 (promyelocytic leukemia). The cells were cultivated in DMEM (Dulbecco's Modified Eagle's Medium) or RPMI-1640 (supplemented with 10% fetal bovine serum, FBS, and 100 μg/mL of penicillin–streptomycin-amphotericin B solution) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The cells were obtained from the *Banco de Células do Rio de Janeiro* (Rio de Janeiro, Brazil) and maintained in the Laboratory of Cell Culture, *Departamento de Antibióticos* of UFPE.

Extracts, protein fractions and lectins

The *M. urundeuva* were tissues were dried at room temperature and powdered. The powdered bark, heartwood or leaves (10 g) were separately suspended in 0.15 M NaCl (100 mL). After homogenization in a magnetic stirrer (16 h at 4°C), followed by

filtration through gauze and centrifugation (3000 *g*, 15 min), the clear supernatants obtained corresponded to the crude extracts.

Soluble proteins in the extracts were fractioned with ammonium sulphate to obtain the following protein fractions enriched in lectin: 40% supernatant from bark extract, 40-60% precipitate from heartwood extract, and 60-80% precipitate from leaf extract (Sá et al., 2009; Napoleão et al., 2011). After the treatment with the salt, the fractions were solubilized in 0.15 M NaCl and submitted to dialysis (3,500 Da cut-off membrane, 4°C) against distilled water (4 h) followed by 0.15 M NaCl (4 h).

MuBL and MuHL were isolated according to Sá et al. (2009a) and MuLL was isolated according to Napoleão et al. (2011) starting from the protein fractions described above. The fraction was loaded onto a chitin (Sigma-Aldrich, USA) column (7.5 × 1.5 cm) equilibrated at 20 mL/h flow rate with 0.15 M NaCl (100 mL). MuBL, MuHL and MuLL were eluted with 1.0 M acetic acid and dialyzed against 0.15 M NaCl for eluent elimination.

The extracts, protein fractions and isolated lectins were evaluated for hemagglutinating activity (Paiva and Coelho, 1992) and protein concentration was estimated according to Lowry et al. (1951) using bovine serum albumin (31.25–500 µg/mL) as standard.

Evaluation of cytotoxicity of *M. urundeuva* extracts, protein fractions and lectins on lymphocytes

Lymphocytes were purified from human blood by gradient separation using Histopaque-1077 (Sigma-Aldrich, USA). Cells were isolated from 6-mL blood sample in 5 mL of phosphate buffered saline (PBS) to which was added 3 mL of Histopaque-1077. After centrifugation at 1500 rpm for 30 min the mononuclear cells (present in the intermediate region between the red cells and serum) were aspirated. The lymphocyte suspension was transferred to another tube to which PBS was added until the volume of 11 mL and centrifuged for 20 min at 1000 rpm. Then, the supernatant was discarded and the lymphocyte pellet ressuspended to a final concentration of 10^6 cells/mL in 5 mL of RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin. Lymphocytes proliferation was induced by addition of 3% phytohemagglutinin (PHA), a mitogen which acts fundamentally on T

lymphocytes becoming them the predominant cell population in the assay (Liu et al., 2004). The cells in RPMI complete medium were plated in 96-well microplates and incubated during 24 h at 37°C.

To determine the cytotoxicity on lymphocytes, extracts (50 µg/mL), protein fractions (50 µg/mL), and lectins (6.25–100 µg/mL) were added to cell cultures and incubated for 72 h. To select the concentrations of the lectins used in the genotoxicity and mutagenicity tests, the lymphocytes were treated by 24 h. Incubation occurred at 37°C with a 5% CO_2 atmosphere. Next, 25 µL of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/mL) were added to the wells and the assay was incubated for more 3 h. After this period, the medium was removed and dimethylsulfoxide (DMSO, 100 µL) was added to the wells for solubilization of the generated formazan salts. The optical density of the wells was measured at 540 nm and compared to that in the control (cells incubated only with medium). Three independent experiments were performed in triplicate.

Hemolytic assay

The lectins were also evaluated for hemolytic activity by assay performed in 96-well microplates. Each well received 100 µL of a 0.85% NaCl solution containing 10 mM $CaCl_2$. Samples (100 µL) of MuBL, MuHL and MuLL were added to the first well of the respective treatment, from which 100 µL were transferred to the second to performed the serial dilution (final concentrations ranging from 0.78 to 100 µg/mL in saline solution). Next, it was added 100 µL of a 2% (v/v) suspension of human erythrocytes in saline solution containing 10 mM $CaCl_2$. In negative controls, 100 µL of the saline solution plus 50 µL of the saline solution or 50 µL of 5% DMSO were plated. Positive control (to obtain 100% hemolysis) contained 80 µL of saline solution plus 20 µL of 0.1% Triton X-100 in saline. After centrifugation during 1 h followed by incubation for 1 h at 27°C, the supernatant was discarded, and the released hemoglobin was measured by absorbance at 450 nm. Three independent experiments were performed in triplicate.

Evaluation of cytotoxicity of *M. urundeuva* extracts, protein fractions and lectins on cancer cell lines

Cancer cell lines NCI-H292, HT-29, HEp-2, MCF-7 cells (10^5 cells/mL), MOLT-4 (10^6 cells/mL) in DMEM medium, and K562 and HL-60 (0.3×10^6 cells/mL) in RPMI medium were plated in 96-well microplates and incubated during 24 h at 37°C. After this period, extracts (50 μg/mL), protein fractions (50 μg/mL) or lectins (25 μg/mL) were added to each well and incubated for 72 h. Next, the MTT assay was performed as described in the previous section. Three independent experiments were performed in triplicate. The samples were classified according to the inhibition percent of cell viability in the following categories: inactive (1-20%), weakly active (20–50%), moderately active (50–70%) or highly active (70–100%) (Fouche et al., 2008). The concentration that inhibited 50% of the cell growth (IC_{50}) was determined only for products classified as highly active.

Comet assay

To perform comet assay, it was selected lectin concentrations that did not inhibit the viability of lymphocytes in more than 30% after 24 h of treatment. Lymphocytes were cultured as described in previous section. Cells were plated ($3x10^6$ cells/mL, in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 μg/mL streptomycin and 3% PHA) in 12-well plates and incubated during 24 h at 37 °C. After this incubation, the cells were incubated with MuHL (25, 50 and 100 μg/mL), MuBL or MuLL (12.5, 25 and 50 μg/mL) during 24 h or with positive control methyl methanesulfonate (MMS) 4×10^{-2} M for 4 h. After treatment, it was performed the alkaline version of comet assay (Singh et al., 1988) with modifications. Control and treated cells were harvested and, after centrifugation, the pellet obtained was suspended in 0.5% low-melting point agarose at 37°C, placed on fully frosted slides covered with a thin layer of normal-melting point agarose and coated with a coverslip. The slides remained at 4°C for 10 min to allow the agarose to solidify. Then, the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, with adjustment of pH to 10.0 using NaOH) at 4°C for 1 h. After lysis, the slides were placed on a horizontal electrophoresis chamber containing cold electrophoresis alkaline buffer

(300 mM NaOH and 1 mM EDTA solution, pH 13.0) during 20 min for DNA denaturation. Electrophoresis was carried out at 40 V and 300 mA for 20 min in darkness to avoid DNA damages without relation with the treatments. The slides were immersed in a neutralization buffer (0.4 M Tris, pH 7.5) for 15 min and fixed with cold absolute ethanol for 5 min. To stain DNA, it was added propidium iodide to each slide (20 µg/mL, 50 µL). Next, the slides were analyzed with a Zeiss fluorescent microscope. Two slides were examined for each plate well (six slides for treatment), analyzing 100 nucleoids per slide, observed at 40× magnification. Comets were classified in the following classes: absence of tail or without damage (class 0), tail smaller than the diameter of the head (class 1), tail with up to twice the diameter of the head (class 2), tail larger than twice the diameter of the head (class 3), and comet without head (class 4). The score of each treatment was verified by multiplying the number of nucleoids observed in each class by the value correspondent to each class (0, 1, 2, 3 or 4). Frequence of damage (%) was calculated based on the number of cells with tail in relation to cells with 0 damage to each treatment. Two independent experiments were performed in triplicate.

Cytokinesis block micronucleus assay

This assay was performed as described by Fenech (2007) and it was also selected lectin concentrations that reduced lymphocytes viability in no more than 30%. Lymphocytes were plated ($3x10^6$ cells/mL, $3x10^6$ cells/mL, in RPMI 1640 medium supplemented with 20% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin and 3% PHA) in 6-well plates and incubated during 24 h at 37°C. To evaluate the mutagenicity, the cultured cells were incubated with MuHL (25, 50 and 100 µg/mL), MuBL and MuLL (12.5, 25 and 50 µg/mL) for 24 h or with MMS for 4 h. Cytokinesis was blocked with cytochalasin B exactly 44 h after PHA stimulation. Twenty-eight hours after cytochalasin addition, cells were harvested by centrifugation (1500 rpm, 5 min) and submitted to hypotonic treatment with 0.075 M KCl. Next, cells were fixed by three successive washes (1500 rpm, 5 min) with 5 mL of fixative solution (3:1 methanol:acetic acid) and placed on the slide. Later, slides were staining with 10% Giemsa during 10 min. Five hundred binucleated cells were analysed for each slide,

totalizing 3,000 for each treatment by experiment. Two independent experiments were performed in triplicate.

Statistical analysis

The IC_{50} was calculated by nonlinear regression using GraphPad Prism v. 5.0 software (GraphPad Software, Inc., USA). For the other tests, one-way ANOVA followed by the Newman-Keuls test was used to evaluate the differences among the treatments. P values < 0.05 were considered to be statistically significant.

Results and discussion

Investigations about cytotoxicity, genotoxicity and mutagenicity of natural compounds on normal cells are of extreme importance, because they provide informations on the safety of bioactive products (Calderón-Segura et al., 2012; Pavão et al., 2016).

Table 1 shows the effect of extracts and protein fractions on viability human lymphocytes and cancer cell lines. The extracts and fractions showed inhibition percentage varying between 3.81% and 17.58%, being considered as not toxic to lymphocytes (Fouche et al., 2008). All the extracts inhibited the viability of cancer cells in 2.6% to 55%, whereas the values determined for the fractions ranged from 3.8% to 63.3%. Bark extract and leaf fraction were moderately active against HT-29 while bark fraction was moderately active to NCI-H292 and HL-60. The heartwood and leaf extracts were inactive or weakly active to all cells.

Table 1. Cytotoxicity of *M. urundeuva* extracts and protein fractions on human lymphocytes and cancer cell lines

Cell line	Inhibition of viability (%)					
	Extracts			Protein fractions		
	Leaf	Bark	Heartwood	Leaf	Bark	Heartwood
Lymphocytes	17.58 ± 0.95	5.61 ± 0.23	0.0	3.81±0.64	8.79±0.27	0.0
NCI-H292	24.03 ± 1.8	23.76 ± 1.37	0.0	39.61 ± 1.51	62.94 ± 2.68	0.0
HEP-2	12.38 ± 0.08	9.02 ± 1.36	0.0	29.95 ± 0.66	0.0	11.97 ± 0.66
MCF-7	16.9 ± 0.3	29.08± 2.8	15.19±0.9	35.09 ± 5.05	14.60 ± 0.4	0.0
HL-60	2.63 ± 0.55	23.34 ± 0.6	0.0	15.15 ± 0.38	63.37 ± 2.33	10.23 ± 0.32
HT-29	3.3 ± 0.9	55.0 ± 0.49	0.0	58.6 ± 3.4	42.7 ± 3.7	0.0

Data showed as mean ± standard deviation.

Cytotoxic activity on cancer cells, such as HCT-8 (human colon carcinoma), SF-295 (glioblastoma) and MDA-MB-435 (melanoma), has been described for bark and heartwood extracts from *M. urundeuva* obtained with the organic solvents ethanol and methanol (Mahmoud et al., 2011; Jandú et al., 2012). Such solvents can extract a greater diversity of bioactive compounds than that present in the saline extract, which may explain the results obtained in the present study. On the other hand, the results also show that proteins present in the *M. urundeuva* extracts are cytotoxic agents since this activity was revealed or increased after the treatment with ammonium sulphate, in comparison with the extracts. For this reason, we evaluated whether the *M. urundeuva* lectins would be the active principles involved in cytotoxic property of the fractions.

Before the evaluation of cytotoxicity of *M. urundeuva* lectins to cancer cells, the lectins were evaluated for hemolytic activity and safety to human lymphocytes by different assays. Determination of damage promoted by bioactive compounds from plants to normal structures is essential for the evaluation of chemotherapeutic potential and for definition of potential formulations for humans.

The results of hemolytic assay and the IC_{50} values of MuBL, MuHL and MuLL on lymphocytes are shown in Table 2. The percentages of hemolysis caused by MuBL, MuHL and MuLL indicate low hemolytic activity (Le et al., 2015). According to Ampasavate et al. (2010), lectins with an IC_{50} < 10 μg/mL are considered to be very toxic, those with an IC_{50} between 10 and 100 μg/mL are considered to be potentially toxic, and those with an IC_{50}>100 μg/mL are considered to be non-cytotoxic. In this sense, the *M. urundeuva* were classified as potentially toxic.

Table 2. Cytotoxicity on human peripheral blood mononuclear cells and hemolytic activity of *M. urundeuva* lectins.

Lectin	**Cytotoxicity**	**Hemolytic activity**	
	IC_{50} (μg/mL)	**EC_{50} (μg/mL)**	**H_{MAX} (%)**
MuBL	58.41±1.68	>100	11.6±0.82
MuHL	43.19±2.51	>100	1.8±0.29
MuLL	62.28±1.74	>100	4.73±0.37

Data showed as mean ± standard deviation. IC_{50}: concentration that inhibited 50% of the cell growth in comparison with control. EC_{50}: effective concentration that promotes 50% of hemolysis. H_{MAX}: maximum percentage of hemolysis.

Since MuBL, MuHL and MuLL showed to be potentially cytotoxic to lymphocytes, we investigated if these lectins would be able to cause damages on genetic material of lymphocytes. For this, it was used a genotoxicity test (comet assay) and a mutagenic test (micronucleus test). Comet assay is a very useful tool to investigate DNA damage in individual cells caused by many products, including drugs, pesticides and metals (Jiang et al., 2008; Muranli and Kanev; Ozdemir, 2015; Shah et al., 2016).

Concentrations employed in comet and micronucleus assays were chosen according to the results of cytotoxicity of MuBL, MuHL and MuLL on lymphocytes after 24 h of treatment (Table 3). It was chosen concentrations at which cell viability was at least 70%.

Table 3. Percentage of viabillity of human lymphocytes after 24 h of exposure to *M. urundeuva* lectins.

Concentration (µg/mL)	**Viability (%)**		
	MuHL	**MuLL**	**MuBL**
6.25	87.56±0.8	92.46±1.5	91.22 ±0.4
12.5	82.38±2.4	81.97±2.3	84.58±1.6
25	77.44±2.1	81.46±0.2	81.17±3.5
50	77.77±1.9	75.61±2.8	74.61±6.3
100	38.22±0.7	54.4±2.8	71.38±1.6

Data showed as mean±standard deviation.

In comet assay, nucleoids present an increase in migration of DNA fragments as greater is the damage promoted. Nucleoids with intact DNA do not exhibit fragments. Table 4 summarizes the results from comet assay. Cells treated with all concentrations of MuBL and MuHL present mostly nucleoids belonging to class 0 (absence of damage), followed by class 1. Cells treated with MuLL at concentrations of 12.5 and 25 µg/mL also presented mostly nucleoids belonging to class 0, followed by class 1. The cells treated with MuLL at 50 µg/mL showed mostly nucleoids belonging to class 0 and class 1, without statistical difference between these classes, and also a higher number of nucleoids of class 3 than the treatments with the other concentrations and the other lectins. Additionally, it was determined the score of damage to each treatment (Table 4). MuLL at 50 µg/mL showed the highest score.

Table 4. Indexes of damage to each class in comet assay after 24 h of treatment of human lymphocytes with MuBL, MuHL and MuLL.

Treatment	Class					Score
	0	1	2	3	4	
Control	83.83±3.54	12.8±3.03	2.6±0.54	1.25±0.5	0	18.83±3.76
MuHL						
12.5 µg/mL	84.33±0.81	11.0± 1.29	2.8±0.5	1.6±0.5	1.0±0*	21.66±3.61
25 µg/mL	74.5±1.04*	19.5±1.37*	4.0±0.7*	1.2±0.44	1.25±0.5*	32.5 ± 5.54*
50 µg/mL	64.33±1.75*	26.0±2.0*	4.33±0.51*	3.4±0.54*	1.8±0.44*	50.0 ± 8.87*
MuLL						
12.5 µg/mL	73.83±2.13*	22.0±1.89*	2.0±0.7	1.2±0.44	0.75±0.5	30.33±3.88*
25 µg/mL	64.33±3.01*	26.66±2.16*	6.6±1.14*	2.25±0.5	0	42.16±8.32*
50 µg/mL	34.8±4.02*	39.2±5.54*	11.0±3.57*	20.0±5.77*	2.75 ± 0.5*	116.0±16.76*
MuBL						
25 µg/mL	83.33±1.21	12.5±1.04	2.5±0.54	1.33±0.51	0	22.83 ± 2.48
50 µg/mL	75.33±1.63*	19.16±2.04*	2.4±0.54	2.2±0.44	0.8±0.4	33.5 ± 3.39*
100 µg/mL	66.5±2.25*	20.3±2.87*	7.8±1.09*	3.66±0.81*	2.0±0.7*	52.33 ± 5.6*
MMS	2.50±0.57*	16.41±1.56*	28.5±1.73*	44.08±1.67*	7.66±0.86*	239.0±5.15*

Data showed as mean ± standard deviation. * $p < 0.05$ in relation to control.

Table 5 shows that MuLL promoted a higher frequency of damage (65.2) in comparison to MuBL (33.5) and MuHL (35.67). The positive control MMS promoted the highest frequency of damage due to its action on DNA as alkylating agent, which change guanine and adenine to 7-methylguanine and 3-methlyladenine, respectively, causing erroneous base pairing and replication blocks (Lundin et al., 2005).

Table 5. Frequency of damage caused by MuHL, MuLL and MuBL to lymphocytes in comet assay.

Treatment	Frequency of damage
Control	16.17 ± 3.54
MMS	97.5 ± 0.57*
MuHL	
12.5 µg/mL	15.67 ± 0.81
25 µg/mL	25.5 ± 1.04*
50 µg/mL	35.67 ± 1.75*
MuLL	
12.5 µg/mL	26.17 ± 2.13*
25 µg/mL	35.67 ± 3.01*
50 µg/mL	65.2 ± 4.02*
MuBL	
25 µg/mL	16.67 ± 1.21
50 µg/mL	24.67 ± 1.63*
100 µg/mL	33.5 ± 2.25*

Data showed as mean ± standard deviation. * $p < 0.05$ in relation to control.

Table 6 shows the effect of the lectins from *M. urundeuva* on micronucleus formation in human lymphocytes. Cells treated with MuBL, MuHL and MuLL did not present micronucleus formation significantly higher than in negative control. The numbers of micronuclei in highest concentrations to each lectin were 7.41, 8.91 and 7.91, to MuHL, MuLL and MuBL, respectively. Figure 2 shows a binucleated lymphocyte without micronucleus from control and a binucleated lymphocyte with micronucleus from MMS treatment.

Micronuclei are corpuscles formed when loss of a chromosome fragment or an entire chromosome occurs during cell division. Thus, micronuclei are genetic material which was lost by principal nucleus in consequence of damage to DNA (Fenech et al., 2011) and this assay allows to evaluate the presence of irreversible damages to DNA and to detect clastogenic and aneugenic agents (Bryce et al., 2014). These results show that, although MuLL was potentially genotoxic in comet assay, the damage promoted to this lectin on human lymphocytes may be repaired without resulting in permanent lesions.

Table 6. Micronucleated human lymphocytes after treatment with MuBL, MuHL and MuLL

Treatment	Number of micronucleated cells
Control	6.91 ± 1.72
MMS	40.25 ± 4.30*
MuHL	
12.5 µg/mL	6.5± 1.31
25 µg/mL	7.41 ± 0.90
50 µg/mL	7.41± 1.78
MuLL	
12.5 µg/mL	6.33 ± 1.87
25 µg/mL	7.0 ± 1.27
50 µg/mL	8.91 ± 1.37
MuBL	
25 µg/mL	6.58 ± 1.31
50 µg/mL	7.08 ± 1.67
100 µg/mL	7.91 ± 0.99

Data showed as mean ± standard deviation. * $p < 0.05$ in relation to control.

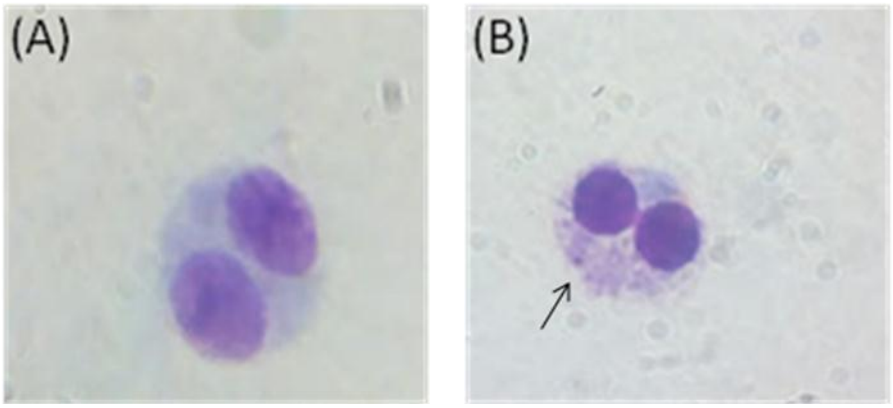

Figure 2. Binucleated lymphocytes (A) without micronucleus and (B) with micronucleus (x 1000). Black arrow indicates micronucleus.

It has been demonstrated that MuHL displayed antibacterial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Streptococcus faecalis* at concentrations of 0.58, 4.68, 1.17 and 2.34 µg/mL, respectively (values of minimal inhibitory concentration) (Sá et al., 2009b). According to the results of this work, the concentrations in which this lectin exhibits antimicrobial activity does not

promote damage on normal human cells, reinforcing the biotechnological potential of this molecule.

The concentration of 25 µg/mL was used to evaluate cytotoxic effects on cancer cell lines since the lectins do not promote hemolysis as well as they were not toxic to lymphocytes or genotoxic to lymphocytes at this dose.

Table 7 shows the effects of the *M. urundeuva* lectins on viability of cancer cells. MuBL inhibited cancer cells growth in 10.51% to 42.36%, MuHL showed inhibition percentages ranging from 6.35% to 62.56%, while MuLL promoted inhibition from 31.0% to 68.2%. MuHL was moderately active on the HL-60 line and MuLL was moderately active on the NCI-H292 and MCF-7 cells. The results showed that MuBL, MuHL and MuLL showed higher cytotoxicity than protein fractions and this fact can be due to concentration of them in the protein fractions or elimination during the chromatography of compounds that are able to reduce the lectin toxicity.

Table 7. Cytotoxicity on human cancer cells of *M. urundeuva* lectins.

Cell line	**Inhibition of viability (%)**		
	MuBL	**MuHL**	**MuLL**
NCI-H292	10.51±1.23	25.10±0.80	63.5±0.4
HEP-2	42.36±3.09	16.59±1.38	49.3±0.2
MCF-7	40.74±0.16	6.35±0.9	68.2±2.8
HL-60	12.42±1.06	62.56±5.8	31.0±0.7
HT-29	22.20 ± 0.29	8.61 ± 0.33	26.8±1.4

Data showed as mean ± standard deviation.

Plant lectins have been widely described as substances with anticancer activity, which is related to promotion of cell death or inhibition of the migration of these cells. Rafiq et al. (2013) reported that the lectin isolated from *Lotus corniculatus* can to promote apoptosis of the human cancer cells HOP62 (lung cancer), HCT116 (colon cancer) and THP1 (leukemia). Other examples are the lectin isolated from the tubers of *Dioscorea opposita*, which inhibits the growth of MCF-7 and nasopharyngeal carcinoma CNE2 cells (Chan and Ng, 2013) and the lectin from *Polygonatum odoratum*, which induces apoptosis and autophagy on lung cancer A549 cells (LI et al.,

2014). Anticancer activity was also described for other *N*-acetylglucosamine binding lectins in addition to MuBL, MuHL and MuLL. For example, the lectin AAL-2 isolated from the fungus *Agrocybe aegerita* induced apoptosis of hepatoma cells and the lectin WGA from *Triticum vulgaris* promoted apoptosis in human pancreatic carcinoma (Jiang et al., 2012; Giacometti, 2015).

Conclusions

MuBL, MuHL and MuLL did not promote damage to erythrocytes, showed to be potentially cytotoxic to human lymphocytes with weak genotoxicity and absence of mutagenicity. These results suggest the need of care in the use of these lectins. Extracts, protein fractions and the lectins presented weak or moderately cytotoxicity on the cancer cell lines tested.

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References

ALLEY, M.C., SCUDIERE, D.A., MONKS, A., HURSEY, M.L., CZERWINSKI, M.J., FINE, D.L., ABBOTT, B.J., MAYO, J.G., SHOEMAKER, R.H., BOYD, M.R. 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Research, 48, 589-601.

AMPASAVATE, C., OKONOGI, S., ANUCHAPREEDA, S., 2010. Cytotoxicity of extracts from fruit plants against leukemic cell lines. African Journal of Pharmacy and Pharmacology, 4, 013-021.

ANDERSON, D., LAUBENTHAL, J., 2013. Analysis of DNA damage via single-cell electrophoresis. Methods in Molecular Biology, 1054, 209-218.

ARAÚJO, S.S., FERNANDES, T.C.C., CARDONA, Y.T., ALMEIDA, P.M., MARIN-MORALES, M.A., SANTOS, A.V., RANDAU, A.P.; BENKO-ISEPPON, A.M., BRASILEIRO-VIDAL, A.C., 2015. Cytotoxic and genotoxic effects of ethanolic

extract of *Euphorbia hyssopifolia* L. on HepG2 cells. Journal of Ethnoparmacology, 170, 16-19.

BIES, C., LEHR, C.M., WOODLEY, J.F., 2004. Lectin-mediated drug targeting: history and applications. Advanced Drug Delivery Reviews, 56, 425-435.

BRYCE, S.M., BEMIS, J.C., MERENESS, J.A., SPELLMAN, R.A., MOSS, J., DICKINSON, D., SCHULER, M.J., DERTINGER, S.D., 2014. Interpreting in vitro micronucleus positive results: Simple Biomarker matrix discriminates clastogens, aneugens, and misleading positive agents. Environmental and molecular mutagenesis, 55, 542-555.

CALDERÓN-SEGURA, M.E., GÓMEZ-ARROYO, S., VILLALOBOS-PIETRINE, R., MARTÍNEZ-VALENZUELA, C., CARBAJAL-LÓPEZ, Y., CALDERÓN-EZQUERRO, M.C., CORTÉS-ESLAVA, J., GARCÍA-MARTÍNEZ, R., FLORES-RAMÍREZ, D., RODRÍGUEZ-ROMERO, M. I., MÉNDEZ-PÉREZ, P., BAÑUELOS-RUÍZ, E., 2012. Evaluation of Genotoxic and Cytotoxic Effects in Human Peripheral Blood Lymphocytes Exposed In Vitro to Neonicotinoid Insecticides News. Journal of Toxicology, 27, 612-647.

CAMPOS, J.K.L., ARAUJO, C.S.F., ARAÚJO, T.F.S., SANTOS, A.F.S., TEIXEIRA, J.A., LIMA, V.L.M., COELHO, L.C.B.B., 2016. Anti-inflammatory and antinociceptive activities of *Bauhinia monandra* leaf lectin. Biochimie Open, 2, 62-68.

CAO, X., HUO, Z., LU, M., MAO, D., ZHAO, Q., XU, C., WANG, C., ZENG, B., 2010. Purification of lectin from larvae of the fly, Musca domestica, and in vitro anti-tumor activity in MCF-7 cells. Journal of Insect Science, 10, 164-169.

CHAN, Y.S., NG, T.B., 2013. A Lectin with Highly Potent Inhibitory Activity toward Breast Cancer Cells from Edible Tubers of Dioscorea opposita cv. Nagaimo. Plos one, 8, e54212.

COSTA-LOTUFO, L.V., KHAN, M.T.H., WILKE, A.A.D.V., JIMENEZ, P.C., PESSOA, C., MORAES, M.E., MORAES, M.O., 2005. Studies of the anticancer potential of plants used in Bangladeshi folk medicine. Journal of Ethnopharmacology, 99, 21-30.

FENECH, M., 2007. Cytokinesis-block micronucleus cytome assay. Nature Protocols, 2, 1084-1104.

FENECH, M., KIRSCH-VOLDERS, M., NATARAJAN, A.T., SURRALES, J., CROTT, J.W., PARRY, J., NORPPA, H., EASTMOND, D.A., TUCKER, J.D., THOMAS, P., 2011. Mutagenesis, 26, 125-132.

FOUCHE, G., CRAGG, G.M., PILLAY, P., KOLESNIKOVA, N., MAHARAJ, V.J, et al., 2008. In vitro anticancer screening of South African plants. Journal of Ethnopharmacology, 119, 455 461.

GIACOMETTI, J., 2015. Plant lectins in cancer prevention and treatment, Medicina fluminensis, 51, 211-229.

HOFFMAN, W., LAKKIS, F.G., CHALASANI, G.B., 2016. Cells, Antibodies, and More. Clinical Journal of the American Society of Nephrology, 11, 137-154.

JANDÚ, J.J.B., DA SILVA, L.C.N., PEREIRA, A.P.C., SOUZA, R.M., JÚNIOR, C.A. S., FIGUEIREDO, R.C.B.Q., ARAÚJO, J.M., CORREIA, M.T.S., SILVA, M.V., 2012. Myracrodruon urundeuva bark: an antimicrobial, antioxidant and non-cytotoxic agent. Journal of Medicinal Plants Research, 7, 413-418.

JIANG, S., CHEN, Y., WANG, M., YIN, Y., PAN, Y., G.U,B., YU, G., LI, Y., 2012. A novel lectin from *Agrocybe aegerita* shows high binding selectivity for terminal N-acetylglucosamine. Biochemical Journal, 15, 369-378.

JIANG, W., LU, Y., CHEN, Z., CHEN, S., ZHANG, M., JIN, L., LOU, J., HE, J., 2008. Studying the genotoxicity of vincristine on human lymphocytes using comet assay, micronucleus assay and TCR gene mutation test in vitro. Toxicology, 252, 113-117.

KOPJAR, N., GARAJ-VRHOVAC, V, 2001. Application of the alkaline comet assay in human biomonitoring for genotoxicity: a study on croatian medical personnel handling antineoplastic drugs. Mutagenesis, 16, 71-78.

LAVANYA, V., ADIL, A.A.M., AHMED, N., JAMAL, S., 2014. Lectins-the promising cancer therapeutics. Oncobiology Targets, 1, 12-15.

LE, C.-F., YUSOF, M.Y.M., HASSAN, H., SEKARAN, S.D., 2015. In vitro properties of designed antimicrobial peptides that exhibit potent antipneumococcal activity and produces synergism in combination with penicillin. Scientific reports, 5, doi: 10.1038/srep09761.

LI, C., CHEN, J., LU, B., SHI, Z., WANG, H., ZHANG, B., ZHAO, K., QI, W., BAO, J., WANG, Y., 2014. Molecular Switch Role of Akt in *Polygonatum odoratum* Lectin-Induced Apoptosis and Autophagy in Human Non-Small Cell Lung Cancer A549 Cells. Plos One, 9, e101526.

LIU, C.P., TSAI, W.J., LIN, Y.L., LIAO, J.F., CHEN, C.F.; KUO, Y.C., 2004. The extracts from *Nelumbo nucifera* suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells. Life Sciences, 75, 699-716.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L., RANDALL, R.J., 1951. Protein measurement with the folin phenol reagent. The Journal of Biological Chemistry, 193, 265-295.

LUNDIN, C., NORTH, M., ERIXON, K., WALTERS, K., JENSSEN, D., GOLDMAN, A.S.H., HELLEDAY, T., 2005. Methyl methanesulfonate (MMS) produces heat-labile DNA damage but no detectable *in vivo* DNA Double-strand breaks. Nucleic Acids Research, 33, 3799-3811.

MAHMOUD, T.S., MARQUES, M.R., PESSOA, C.Ó, LOTUFO, L.V.C., MAGALHÃES, H.I.F., MORAES, M.O., LIMA, D.P., TININIS, A.G., OLIVEIRA, J. E., 2011. *In vitro* cytotoxic activity of Brazilian Middle West plant extracts. Revista Brasileira de Farmacognosia**,** 21, 456-464.

MATOS, F.J.A., 2002. Farmácias vivas. Fortaleza: Editora UFC, 267.

MOSMANN, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays**.** Journal of Immunological Methods, 65, 55-63.

MURANLI, F.D., KANEV, M., OZDEMIR, K., 2015. Genotoxic effects of diazinon on human peripheral blood lymphocytes. Arhive za Higijenu Rada i Toksikologiju, 66, 153-158.

NAPOLEÃO, T.H., GOMES, F.S., LIMA, T.A.; SANTOS, N.D.L., SÁ, R.A., ALBUQUERQUE, A.C., COELHO, L.C.B.B.; PAIVA, P.M.G., 2011. Termiticidal activity of lectins from Myracrodruon urundeuva against Nasutitermes corniger and its mechanisms. International Biodeterioration & Biodegradation, 65, 52-59.

NAPOLEÃO, T.H., PONTUAL, E.V., LIMA, T.A., SANTOS, N.D.L., SÁ, R.A., COELHO, L.C.B.B., NAVARRO, D.M.A.F., PAIVA, P.M.G., 2012. Effect of Myracrodruon urundeuva leaf lectin on survival and digestive enzymes of Aedes aegypti larvae. Parasitology Research, 110, 609–616.

PAIVA, P.M.G., COELHO, L.C.B.B., 1992. Purification and partial characterization of two lectin isoforms from *Cratylia mollis* Mart (camaratu bean). Applied Biochemistry and Biotechnology, 36, 113–118.

PAVÃO, G.B., VENÂNCIO, V.P., OLIVEIRA, A.L.L., HERNANDES, L.C., ALMEIDA, M.R., ANTUNES, L.M.G., DEBONSI, H.M., 2016. Differential genotoxicity and cytotoxicity of phomoxanthone A isolated from the fungus Phomopsis longicolla in HL60 cells and peripheral blood lymphocytes. Toxicology in vitro, 37, 211-217.

PEREIRA, P.S., BARROS, L.M., BRITO, A.M., DUARTE, A.E., MAIA, A.J., 2014. Uso da *Myracroduon urundeuva* Allemão (aroeira do sertão) pelos agricultores no tratamento de doenças. Revista Cubana de Plantas Medicinales, 19, 1.

RAFIQ, S., MAJEED, R., QAZIB, A.K., GANAIA, B.A., WANIA, I., RAKHSHANDAA, S., QURISHIB, Y., SHARMAB, P.R., HAMID, A., MASOODA, A., HAMID, R., 2013. Isolation and antiproliferative activity of Lotus corniculatus lectin towards human tumour cell lines. Phytomedicine, 21, 30-38.

SÁ, R.A., NAPOLEÃO, T.H., SANTOS, N.D.L., GOMES, F.S., ALBUQUERQUE, A.C., XAVIER, H.S., COELHO, L.C.B.B., BIEBER, L.W., PAIVA, P.M.G., 2008. Induction of mortality on Nasutitermes corniger (Isoptera, Termitidae) by Myracrodruon urundeuva heartwood lectin. International Biodeterioration & Biodegradation, 62, 460-464.

SÁ, R.A., SANTOS, N.D.L., SILVA, C.S.B., NAPOLEÃO, T.H., GOMES, F.S., CAVADA, B.S., COELHO, L.C.B.B., NAVARRO, D.M.A.F., BIEBER, L.W., PAIVA, P.M.G., 2009a. Larvicidal activity of lectins from Myracrodruon urundeuva on Aedes aegypti, Comparative Biochemistry and Physiology, 149, 300-306.

SÁ, R.A., GOMES, F.S., NAPOLEÃO, T.H., SANTOS, N.D.L., MELO, C.M.L., GUSMÃO, N.H., COELHO, L.C.B.B., PAIVA, P.M.G., BIEBER, L.W., 2009b. Antibacterial and antifungal activities of Myracrodruon. Wood Science and Technology, 43, 85-95.

SHAH, A.J., LAKKAD, B.C., RAO, M.V., 2016. Genotoxicity in lead treated human lymphocytes evaluated by micronucleus and comet assays. Indian Journal of Experimental Biology, 54, 502-508.

SINGH, R.K., MISHRA, S.K., KUMAR, N., SINGH, A.K., 2010. Assessment of DNA Damage by Comet Assay in Lymphocytes of Workers Occupationally Exposed to Petroleum fumes. International Journal of Genetics, 2, 18-22.

WOOLDRIDGE, L., EKERUCHE-MAKINE, J., VAN DEN BERG, H.A., SKOWERA, A., MILES, J.J., TAN, M.P., DOLTON, G., CLEMENT, M., LLEWELLYN-LACEY, S., PRICE, D. A., PEAKMAN, M., SEWELL, A.K., 2012.
A single autoimmune T cell receptor recognizes more than a million different peptides. The Journal of Biological Chemistry, 287, 1168-1177.

YAU, T., DAN, X., NG, C. C., NG, T.B., 2015. Lectins with potential for anti-cancer therapy. Molecules, 20, 3791-810.

7 CONCLUSÕES

Os extratos hexano, acetato de etila e metanólico da raiz de *A. congestiflora* são fontes de compostos fenólicos e possuem atividades anticâncer e antimicrobiana. A cucurbitacina isolada foi identificada como composto com atividade anticâncer e citotóxico para PBMCs.

Extrato hidroalcoólico da raiz de *A. congestiflora* contendo terpenos e alcalóides foi citotóxico, mas não promoveu lise de eritrócitos bem como toxicidade aguda em camundongos. Células provenientes dos animais tratados não apresentaram dano genético.

Extrato, frações proteicas e lectinas de entrecasca, cerne e folha de *M. urundeuva* foram citotóxicas para células cancerígenas e linfócitos, mas não foram hemolíticas para eritrócitos humanos. As lectinas foram potencialmente genotóxicas, mas não mutagênicas.

REFERÊNCIAS

ABDALLAH, E. M. Plants: An alternative source for antimicrobials. **Journal of Applied Pharmaceutical Science**, v. 1, v. 6, p. 16-20, 2011.

ABUDAYEH, Z. H. M.; AZZAM, K. M. A.; KARPIUK, U.V.; HASSOUNEH, L. K.; ABUIRMEILEH, A. Isolation, identification, and quantification of lectin protein contents in *Chamerion angustifolium* L. Dried raw material and the study of its activity using ratuserytroagglutination. **International Journal of Pharmacy and Pharmaceutical Sciences**, v. 8, n. 2, p. 150-153, 2016.

ADZITEY, F. Antibiotic Classes and Antibiotic Susceptibility of Bacterial Isolates from Selected Poultry; A Mini Review. **World's Veterinary Journal**, v. 5, n. 3, p. 36-41, 2015.

AKAR, U.; CHAVES-REYEZ, A.; BARRIA, M.; TARI, A.; SANGUINO, A.; KONDO, Y.; KONDO, S.; ARUN, B.; LOPEZ-BERESTEIN, G.; OZPOLAT, B. Silencing of Bcl-2 expression by small interfering RNA induces autophagic cell death in MCF-7 breast cancer cells. **Autophagy**, v. 4, n. 5, p. 669-679, 2008.

ALBERTS, B.; JOHNSON, A.; LEWIS, J.; RAFF, M.; ROBERTS, K.; WALTER, P. **Biologia Molecular da célula**. 5ª Ed. Porto Alegre: Artmed, 2010. 1396 p.

AKIYAMA, H.; FUJII, K.; YAMASAKI, O.; OONO, T.; IWATSUKI, K. Antibacterial action of several tannins against Staphylococcus aureus. **The Journal of Antimicrobial Chemoterapy**, v. 48, n. 4, p.487-491, 2001.

ALAM, M. N.; BRISTI, N. J.; RAFIQUZZAMAN, M. Review on in vivo and in vitro methods evaluation of antioxidant activity. **Saudi Pharmaceutical Journal**, v. 21, n. 2, p. 143-152, 2013.

ALLEY, M. C.; SCUDIERE, D. A.; MONKS, A.; HURSEY, M. L.; CZERWINSKI, M. J.; FINE, D. L.; ABBOTT, B. J.; MAYO, J. G.; SHOEMAKER, R. H.; BOYD, M. R. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. **Cancer Research**, n.48, p.589-601, 1988.

ANDERSON, D.; LAUBENTHAL, J. Analysis of DNA damage via single-cell electrophoresis. **Methods in Molecular Biology**, v. 1054, p. 209-218, 2013.

ARALDI, R. P.; MELO, T. C.; MENDES, T. B.; JÚNIOR, P. L. S.; NOZIMA, B. H. N.; ITO, E. T.; CARVALHO, R. F.; SOUZA, E. B.; STOCCO, R. C. Using the comet and micronucleus assays for genotoxicity studies: A review. **Biomedicine & Pharmacotherapy**, v. 72, p. 74-82, 2015.

ARANDA-SOUZA, M. A.; ROSSATO, F. A.; COSTA, R. A. P. ; FIGUEIRA, T. R.; CASTILHO, R. F.; GUARNIERE, M. C.; NUNES, E. S. ; COELHO, L. C. B. B.; CORREIA, M. T. S. ; VERCESI, A. E. A lectin from Bothrops leucurus snake venom raises cytosolic calcium levels and promotes B16-F10 melanoma necrotic cell death via mitochondrial permeability transition. **Toxicon**, v. 82, p. 97-103, 2014.

ARNAO, M. B. Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. **Trends in Food Science & Technology**, v. 11, n. 1, p. 419-421, 2000.

ARRUDA, D. C.; D'ALEXANDRI, F. L.; KATZIN, A. M.; ULIANA. S. R. Antileishmanial activity of the terpene nerolidol. **Antimicrob Agents Chemother**, v. 49, n. 5, p. 1679-1687, 2005.

ASHOK, P. K.; UPADHYAYA, K. Tannins are Astringent. **Journal of Pharmacognosy and Phytochemistry**, v. 1, n. 3, p. 45-50, 2012.

ASHRAF, A.; SARFRAZ, R. A.; Mahmoodc, A.; Dina M. U. Chemical composition and in vitro antioxidant and antitumor activities of Eucalyptus camaldulensis Dehn. Leaves. **Industrial Crops and Products**, v. 74, n. 15, p. 241-248, 2015.

BALDIN, E. L. L.; CAETANO, A. C.; LARA, F. M. Atração e desenvolvimento de Leptoglossus gonagra (Fabr.) (Hemiptera: Coreidae) em cultivares de abóbora e moranga, **Scientia Agricola**, v. 59, n. 1, p. 191-195, 2002.

BALMUS, G.; KARP, N. A.; NG, B. L.; JACKSON, S. P.; ADAMS, D. J.; EMCINTYRE, R. A high-throughput *in vivo* micronucleus assay for genome instability screening in mice. **Nature Protocols,** v. 10, p. 205-215, 2015.

BALOUIRI, M.; SADIKI, M.; IBNSOUDA, S. K. Methods for *in vitro* evaluating antimicrobial activity: A review. **Journal of Pharmaceutical Analysis**, v. 6, n. 2, p. 71-79, 2016

BARBOSA-FILHO, J. M.; MEDEIROS, K. C. P.; DINIZ, M. F. F. M.; BATISTA, L. M.; ATHAYDE-FILHO, P. F.; SILVA, M. S.; CUNHA, E. V. L.; Almeida, J. R. G. S.; Quintans-Júnior, L. J. Natural products inhibitors of the enzyme acetylcholinesterase. **Revista Brasileira de Farmacognosia**, v. 16, n. 2, p. 258-285. 2006.

BARROSO, G.M. **Sistemática de Angiospermas do Brasil**. Livro Técnico e Científico. EDUSP, São Paulo, 255pp, 1978.

BASILE, A.; SORBO, S.; SPADARO, V.; MAURIZIO B.; MAGGIO, A.; FARAONE, N.;ROSSELLI, S. Antimicrobial and Antioxidant Activities of Coumarins from the Roots of Ferulago campestris (Apiaceae). **Molecules**, v. 14, n.3, p. 939-952, 2009.

BECHELLI, J.; COPPAGE, M.; ROSELL, K.; LIESVELD, J. Cytotoxicity of algae extracts on normal and malignant cells. **Leukemia Research and Treatment**, v. 2011, n. 19, p. 37-35, 2011.

BERTOLI, C.; SKOTHEIM, J. M.; DE BRUIN, R. A. M. Control of cell cycle transcription during G1 and S phases. **Nature Reviews Molecular Cell Biology**, v. 14, n. 8 p. 518-528, 2013.

BEYER, J.; DRUMMER, O. H.; MAURER, H. H. Analysis of toxic alkaloids in body samples. **Forensic Science International**, v. 185, n. 1-3, p. 1-9, 2009.

BIES, C.; LEHR, C. M.; WOODLEY, J. F. Lectin-mediated drug targeting: history and applications. **Advanced Drug Delivery Reviews**, v. 56, n. 4, p. 425-435, 2004.

BLANK, M.; SHILOH, Y. Programs for cell death: apoptosis is only one way to go. **Cell Cycle**, v. 6, n. 6, p. 686-695, 2007.

BOEIRA, J. M.; FENNER, R.; BETTI, A. H.; PROVENSI, G.; LACERDA, L. A.; BARBOSA, P. R.; GONZALEZ, F.H.D.; CORREA, A. M. R.; DRIEMEIER, D.; DALL'ALBA, M. P.; PEDROSO, A. P.; GOSMANN, G.; SILVA, J.; RATES, S. M. K. Toxicity and genotoxicity evaluation of Passiflora alata Curtis (Passifloraceae). **Journal of Ethnopharmacology**, v. 128, n. 2, p. 526-532, 2009.

BORGES, F.; ROLEIRA, F.; MILHAZES, N.; SANTANA, L.; URIARTE, E. Simple coumarins and analogues in medicinal chemistry: occurrence, synthesis and biological activity. **Current Medicinal Chemistry**, v. 12, n. 8, p.887-916, 2005.

BOTELHO, M. A.; RAO, V. S.; CARVALHO, C. B.; BEZERRA-FILHO, J. G.; FONSECA, S. G.; VALE, M. L.; MONTENEGRO, D.; CUNHA, F.; RIBEIRO, R. A.; BRITO, G. A. Lippia sidoides and Myracrodruon urundeuva gel prevents alveolar bone resorption in experimental periodontitis in rats. **Journal of Ethnopharmacology**, v. 113, n. 3, p. 471-478, 2007.

BREWER, M.S. Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. **Comprehensive Reviews in Food Science and Food Safety**, v. 10, n. 4, p. 221-247, 2011.

BRUYNE, T.; PIETERS, L.; DEELSTRA, H.; VLIETINCK, A. Condensed vegetable tannins: biodiversity in structure and biological activities. **Biochemical Systematics and Ecology**, v. 27, n. 4, p. 445-459, 1999.

BRYCE, S. M.; BEMIS, J. C.; MERENESS, J. A.; SPELLMAN, R. A.; MOSS, J.; DICKINSON, D.; SCHULER, M. J.; DERTINGER, S. D. Interpreting in vitro micronucleus positive results: Simple Biomarker matrix discriminates clastogens, aneugens, and misleading positive agents. **Environmental and molecular mutagenesis**, v. 55, p. 542-555, 2014.

BORRA, R. C.; LOTUFO, M. A.; GAGIOTI, S. M.; BARROS, F. M.; ANDRADE, P. M. A simple method to measure cell viability in proliferation and cytotoxicity assays. **Brazilian Oral Research**, v. 23, n. 3, p. 255-262, 2009.

BOUTANAEV, A. M.; MOSES, T.; ZI, J.; NELSON, D. R.; MUGFORD, S. T.; PETERS, R. J.; OSBOURN, A. nvestigation of terpene diversification across multiple sequenced plant genomes. **Proceedings of the National Academy of Sciences of the United States of America**, v. 112, n. 1, p. E81-E88, 2014.

BOUVARD, V.; BAAN, R.; STRAIF, K.; GROSSE, Y.; SECRETAN, B.; EL GHISSASSI, F.; BENBRAHIM-TALLAA, L.; GUHA, N.; FREEMAN, C.; GALICHET, L.; COGLIANO, V. A review of human carcinogens – part B: biological agents. **Lancet Oncology,** v. 10, n.4, p. 321–322, 2009.

BRASH, D. E.; RUDOLPH, J. A.; SIMON, J. A.; LIN, A.; MCKENNA, G. J.; BADEN, H. P.; HALPERIN, A. J.; PONTÉN, J. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. **Proceedings of the National Academy of Sciences of the United States of America**, v. 88, n. 22, p. 10124-10128, 1991.

CALHELHA, R. C.; FALCÃO, S.; QUEIROZ, M. J. R. P.; VILAS-BOAS, M.; FERREIRA, I. C. F. R. Cytotoxicity of Portuguese Propolis: The Proximity of the In Vitro Doses for Tumor and Normal Cell Lines. **BioMed Research International**, v. 2014, Artigo ID 897361, doi:10.1155/2014/897361, 2014.

CAMPONE, M.; CORTES-FUNES, H.; VOROBIOF, D.; MARTIN, M.; SLABBER, C. F.; CIRUELOS, E.; BOURBOULOUX, E.; MENDIOLA, C.; DELGADO, F. M.; COLIN, C.; ASLANIS, V.; FUMOLEAU, P. **British Journal of Cancer**, v. 95, n. 9, p. 1161-1166, 2006.

CAO, X.; Huo, Z.; Lu, M.; Mao, D.; Zhao, Q.; Xu, C.; Wang, C.; Zeng, B. Purification of lectin from larvae of the fly, Musca domestica, and in vitro anti-tumor activity in MCF-7 cells. **Journal of Insect Science**, v. 10, n. 164, disponível em: insectscience.org/10.164, 2010.

CARLINI, E. A.; DUARTE-ALMEIDA, J. M.; TABACH R. Assessment of the toxicity of the Brazilian pepper trees Schinus terebinthifolius Raddi (Aroeira-da-praia) and Myracrodruon urundeuva Allemão (Aroeira-do-sertão). **Phytotheraopy Research**, v. 27, n. 5, p. 692-698, 2013.

CARVALHO, A. C. B.; BALBINO, E. E.; MACIELA, A.; PERFEITO, J. P. S. Situação do registro de medicamentos fitoterápicos no Brasil. **Revista Brasileira de Farmacognosia**, v. 18, p. 314-319, 2008.

CARVALHO, A. S.; DA SILVA, M. V.; GOMES, F. S.; PAIVA, P. M.; MALAFAIA, C. B.; DA SILVA, T. D.; VAZ, A. F.; DA SILVA, A. G.; ARRUDA, I. R.; NAPOLEÃO, T. H.; CARNEIRO-DA-CUNHA, M.; CORREIA, M. T. Purification, characterization and antibacterial potential of a lectin isolated from Apuleia leiocarpa seeds. **International Journal of Biological Macromolecules**, v. 75, p. 402-408, 2015.

CAVALHEIRO, M. G.; FARIAS, D. F.; FERNANDES, G. S.; NUNES, E. P.; CAVALCANTI, F. S.; VASCONCELOS, I. M.; MELO, V. M. M.; CARVALHO A. F. U. Biological and enzymatic activities of aqueous extract of seeds from *Caesalpinia ferrea* Mart., Leguminosae. **Revista Brasileira de Farmacognosia**, v. 19, n.2b, p. 586-591, 2009.

CHABNER, B. A.; ROBERTS JR., T. G. Timeline: Chemotherapy and the war on cancer. **Nature Reviews. Cancer**, v. 5, n. 1, p. 65-72, 2005.

CHAVES, T. P.; SANTANA, C. P.; VÉRAS, G.; BRANDÃO, D. O.; FELISMINO, D. C.; MEDEIROS, A. C. D.; TROVÃO, D. M. DE B. M. Seasonal variation in the

production of secondary metabolites and antimicrobial activity of two plant species used in Brazilian traditional medicine. **African Journal of Biotechnology,** v. 12, n. 8, p. 847-853, 2013.

CHEN, J. C.; CHIU, M. H.; NIE, R. L.; CORDEL, G. A.; QIU, S. X. Cucurbitacins and cucurbitane glycosides: Structures and biological activities. **Natural Product Reports**. v. 22, n. 3, p. 386-399, 2005.

CHEN, Y.; PEUMANS, W. J.; HAUSE, B.; BRAS, J.; KUMAR, M.; PROOST, P.; BARRE, A.; ROUGÉ, P.; VAN DAMME, E. J. Jasmonic acid methyl ester induces the synthesis of a cytoplasmic/nuclear chito-oligosaccharide binding lectin in tobacco leaves. **The FASEB Journal**, v. 16, n. 8, p. 905-907, 2002.

CHEN, X.-B.; CHEN, G.-Y.; LIU, J.-H.; LEI, M.; MENG, Y.-H.; GUO, D.-A.; LIU, X. Cytotoxic cucurbitane triterpenoids isolated from the rhizomes of *Hemsleya amabilis*. **Fitoterapia**, v. 94, p. 88-93, 2014.

CHEN, Z.; YIN, G. The reactivity of the active metal oxo and hydroxo intermediates and their implications in oxidations. **Chemical Society Reviews**, DOI: 10.1039/c4cs00244j, 2014.

CHIM, C S; FUNG, T K.; WONG, K F.; LAU, J S.; LAW, M.; LIANG, R. Methylation of INK4 and CIP/KIP families of cyclin- dependent kinase inhibitor in chronic lymphocytic leukaemia in Chinese patients. **Journal of Clinical Pathology,** v. 59, n. 9, p. 921-926, 2006.

CHOI, S.; LIM, M. H.; KIM, K. M.; JEON, B. H.; SONG, W. O.; KIM, T. W. Cordycepin-induced apoptosis and autophagy in breast cancer cells are independent of the estrogen receptor. **Toxicology and Applied Pharmacology**, v. 257, n. 2, p. 165-173, 2011.

CLARK, D.; TUPA, M.; BAZÁN, A.; CHANG, L.; GONZÁLES, W. L. Chemical composition of Apodanthera biflora, a Cucurbit of the dry forest in northwestern Peru. **Revista Peruana de Biologia**, v. 19, n. 2, p. 199-203, 2012.

COATES, A. R. M.; HALLS, G.; HU, Y. Novel classes of antibiotics or more of the same? **Briths Journal of Pharmacology**, v. 163, n. 1, p. 184-194, 2011.

COLE, S. T. Who will develop new antibacterial agents? **Philosofical**

transactions of the Royal society B, v. 369, n. 1645, 2014.

COLINS, A. R. The comet assay for DNA damage and repair: principles, applications, and limitations**. Molecular Biotechnology**, v. 26, n. 3, p. 249-261, 2004.

COSTA, R. M. P. B.; VAZ, A. F. M.; OLIVA, M. L. V.; COELHO, L. C. B. B.; CORREIA, M. T. S.; CARNEIRO-DA-CUNHA, M. G. A new mistletoe *Phthirusa pyrifolia* leaf lectin with antimicrobial properties. **Process Biochemistry**, v. 45, n. 4, p. 526-533, 2010.

COSTA-LOTUFO, L. V.; KHAN, M. T. H.; WILKE, A. A. D. V.; JIMENEZ, P. C.; PESSOA, C.; MORAES, M. E.; MORAES, M. O. Studies of the anticancer potential of plants used in Bangladeshi folk medicine. **Journal of Ethnopharmacology**, v. 99, n. 1, p. 21-30, 2005.

COULIBALY, A. Y.; HASHIM, R.; SULAIMAN, S. F.; SULAIMAN, O.; ANG, L. Z. P.; Ooi, K. L. Bioprospecting medicinal plants for antioxidant components. **Asian Pacific Journal of Tropical Medicine**, v. 1, n. 1, p. S553-559, 2014.

CRAGG, G., M., GROTHAUS, P. G.; NEWMAN, D. J. Impact of Natural Products on Developing New Anti-Cancer Agents. **Chemical Reviews**, v. 109, n.7, p. 3012-3043, 2009.

CRAGG, GM; NEWMAN, DJ Plants as a source of anti-cancer agents. Journal of Ethnopharmacolog, v. 100, n. 1-2, p. 72-79, 2005.

DEEPA, M.; SURESHKUMAR, T.; SATHEESHKUMAR, P. K.; PRIYA, S. Purified mulberry leaf lectin (MLL) induces apoptosis and cell cycle arrest in human breast cancer and colon cancer cells. **Chemico-Biological Interactions**, v. 200, n. 1, p. 38-44, 2012.

DENG, Y.; YU, Y.; LUO, H.; ZHANG, M.; QIN, X.; LI, L. Antimicrobial activity of extract and two alkaloids from traditional Chinese medicinal plant *Stephania dielsiana.* **Food Chemistry**, v. 124, n. 4, p. 1556-1560, 2011.

DE VOS, M.; VAN OOSTEN, V. R.; VAN POECKE, R. M. P.; VAN PELT, J. A.; POZO, M. J.; MUELLER, M. J.; BUCHALA, A. J.; MÉTRAUX, J. P.; VAN LOON, L. C.; DICKE, M.; PIETERSE, C. M. Signal signature and transcriptome changes of

Arabidopsis during pathogen and insect attack. **Molecular Plant-Microbe Interactions**, v. 18, n. 9, p. 923–937, 2005

DEWICK, P. M. **Alkaloids, in Medicinal Natural Products: A Biosynthetic Approach**, 3rd Edição, John Wiley & Sons, Ltd, Chichester, UK. doi: 10.1002/9780470742761. Capítulo 6, 2009.

DI STASI, L. C.; GUIMARÃES, E. M.; SANTOS, C. M.; HIRUMA-LIMA, C. A.; SOUZA BRITO, A. R. M. Fabales medicinais. In: DI STASI, L. C.; HIRUMA-LIMA, C. A. Plantas medicinais na Amazônia e na Mata Atlântica. **São Paulo: Fundação Editora Unesp, São Paulo**, 2002, cap. 18, p. 276-320.

DRAKE, J. M.; GRAHAM, N. A.; STOYANOVA, T.; SEDGHI, A.; GOLDSTEIN, A. S.; CAI, H.; SMITH, D. A.; ZHANG, H.; KOMISOPOULOU, E.; HUANG, J.; GRAEBER, T. G.; WITTE, O. N. Oncogene-specific activation of tyrosine kinase networks during prostate cancer progression. **Proceedings of the National Academy of Sciences of the United States of America.** v. 109, n. 5, p. 1643-1648, 2012.

ELGAVISH S, SHAANAN B. Lectin-carbohydrate interactions: different folds, common recognition principles. **Trends in Biochemical Sciences**, v. 22, p. 462-467, 1997.

EL-AASSAR, M. R.; HAFEZ, E. E.; EL-DEEB, N. M.; FOUDA, M. M. Microencapsulation of lectin anti-cancer agent and controlled release by alginate beads, biosafety approach. **International Journal of Biological Macromolecules**, v. 69, p. 88-94, 2014.

EL-KENAWI, A. E.; EL-REMESSY, A. B. Angiogenesis inhibitors in cancer therapy: mechanistic perspective on classification and treatment rationales. **British Journal of Pharmacology**, v. 170, n. 4, p. 712-729, 2013.

EMRI, G.; WENCZL, E.; VAN E. P.; JANS, J.; ROZA, L.; HORKAY, I.; SCHOTHORST, A. A. Low doses of UVB or UVA induce chromosomal aberrations in cultured human skin cells. **The Journal of Investigative Dermathology**, v. 115, n. 3, p. 435-440, 2000.

ESEYIN, O. A.; SATTAR, M. A.; RATHORE, H. A. A Review of the Pharmacological and Biological Activities of the Aerial Parts of *Telfairia occidentalis* Hook.f.

(Cucurbitaceae). **Tropical Journal of Pharmaceutical Research**, v. 13, n. 10, p. 1761-1769, 2014.

EVANS, B. C.; NELSON, C. E.; YU, S. S.; BEAVERS, K. R.; KIM, A. J.; LI, H.; NELSON, H. M.; GIORGIO, T. D.; DUVALL, C. L. *Ex Vivo* Red Blood Cell Hemolysis Assay for the Evaluation of pH-responsive Endosomolytic Agents for Cytosolic Delivery of Biomacromolecular Drugs. **Journal of Visualized Experiments**, n. 73, e50166, doi:10.3791/50166, 2013.

FALCÃO, H. S.; LEITE, J. A.; BARBOSA-FILHO, J. M.; DE ATHAYDE-FILHO, P. F.; CHAVES, M. C. O.; MOURA, M. D.; FERREIRA, A. L.; DE ALMEIDA SOUZA-BRITO, A. R. M.; DINIZ, A. F. F. M.; BATISTA, L. M. Gastric and duodenal antiulcer activity of alkaloids: a review. **Molecules**, v. 13, n. 12, p. 3198–3223, 2008.

FEITOSA, C. R. S.; SILVA, R. C.; BRAZ-FILHO, R.; MENEZES, J. E. S. A.; SIQUEIRA, S. M. C.; MONTE, F. J. Q. Characterization of Chemical Constituents of Luffa operculata (Cucurbitaceae). **American Journal of Analytical Chemistry**, v. 2011, n. 2, p. 989-995, 2011.

FENECH, M. The in vitro micronucleus technique, Mutation Research, v. 455, n. 1-2, p. 81-95, 2000.

FENECH, M. Cytokinesis-block micronucleus cytome assay. **Nature Protocols**, v. 2, n. 5, p. 1084-1104, 2007.

FENECH, M.; KIRSCH-VOLDERS, M.; NATARAJAN, A.T.; SURRALES, J.; CROTT, J.W.; PARRY, J.; NORPPA, H.; EASTMOND, D.A.; TUCKER, J. D.; THOMAS, P. **Mutagenesis**, v. 26, n. 1, p. 125-132, 2011.

FERREIRA, P. M. P.; DA COSTA, P. M.; COSTA, A. M.; LIMA, D. J. B.; DRUMOND, R. R.; SILVA, J. N.; MOREIRA, D. R. M.; PESSOA, C. Cytotoxic and toxicological effects of phthalimide derivatives on tumor and normal murine cells. **Anais da Academia Brasileira de Ciências,** v. 87, n. 1, p. 313-330, 2015.

FERREIRA, E. A.; SIQUEIRA, H. E.; BOAS,E. V. V.; HERMES,V. S.; RIOS, A. O. Bioactive Compounds And Antioxidant Activity Of Pineapple Fruit Of Different Cultivars. **Revista Brasileira de Fruticultura**, v. 38, n. 3, p. e-146, 2016.

FLANNICK, J.; THORLEIFSSON, G.; BEER, N. L.; JACOBS, S. B. R.; GRARUP, N.; BURTT, N. P.; MAHAJAN, A.; FUCHSBERGER, C.; ATZMON, G.; BENEDIKTSSON, R.; BLANGERO, J.; BOWDEN, D. W.; BRANDSLUND, I.; BROSNAN, J.; BURSLEM, F.; CHAMBERS, J.; CHO, Y. S.; CHRISTENSEN, C.; D. A.; DUGGIRALA, R.; DYMEK, Z.; FARJOUN, Y.; FENNELL, T. D.; Fontanillas**,** P. Loss-of-function mutations in *SLC30A8* protect against type 2 diabetes. **Nature Genetics**, v. 46, p. 357-363, 2014.

FULDA, S. The mechanism of necroptosis in normal and cancer cells. **Cancer Biology and Therapy**, v. 14, n. 11, p. 999-1004, 2013.

FRIDMAN, J. S.; LOWE, S. W. Control of apoptosis by p53. **Oncogene**, v. 22, n. 56, p. 9030-9040, 2003.

GALLUZZI, L.; VANDEN, B. T.; VANLANGENAKKER, N.; BUETTNER, S.; EISENBERG, T.; VANDENABEELE, P.; MADEO, F.; KROEMER, G. Programmed necrosis from molecules to health and disease. **International Review of Cell and Molecular Biology**, v. 289, p. 1-35, 2011.

GAO, Y.; ISLAM, M. S.; TIAN, J.; LUI, V. W.; XIAO, D. Inactivation of ATP citrate lyase by Cucurbitacin B: A bioactive compound from cucumber, inhibits prostate cancer growth. **Cancer Letters**, v. 349, n. 1, p. 15-25.

GAUTHIER, C.; LEGAULT, J.; GIRARD-LALANCETTE, K.; MSHVILDADZE, V.; PICHETTE, A. Haemolytic activity, cytotoxicity and membrane cell permeabilization of semisynthetic and natural lupane- and oleanane-type saponins. **Bioorganic & Medicinal Chemistry**, v. 17, n. 5, p. 2002-2008, 2009.

GLICK, D.; BARTH, S.; MACLEOD, K. F. Autophagy: cellular and molecular mechanisms. **The Journal of pathology,** v. 221, n. 1, p. 3-12, 2010.

GLOBO-NETO, L.; LOPES, N.P. Plantas Medicinais: Fatores De Influência No Conteúdo De Metabólitos Secundários. **Química Nova**, v.30, n. 2, p. 374-381, 2007.

GOC, A.; KOCHUPARAMBIL, S. T.; AL-HUSEIN, B.; AL-AZAYZIH, A.; MOHAMMAD, S.; SOMANATH, P. R. Simultaneous modulation of the intrinsic and extrinsic pathways by simvastatin in mediating prostate cancer cell apoptosis. **BMC Cancer**, v. 12, n. 409, **DOI:** 10.1186/1471-2407-12-409, 2012.

GOLDSTEIN, I. J.; HAYES, C. E. The lectins: carbohydrate-binding proteins of plants and animals. **Advances in carbohydrate chemistry and biochemistry,** v. 25, p. 137-340, 1978.

GOMES, L.; VANGERLAN, T.; CHAVES, T. P.; ALENCAR, L. C. B.; DANTAS, I. C.; MEDEIROS, A. C. D.; FELISMINO, D. C. Antimicrobial activity of natural products from Myracrodruon urundeuva Allemão (Aroeira-do-sertão) / Actividad antimicrobiana de productos naturales de Myracrodruon urundeuva Allemão (Aroeira-do-sertão). **Revista Cubana de Plantas Medicinales**, v. 18, n. 4, p. 529-533, 2013.

GONZÁLEZ-BURGOS, E.; GÓMEZ-SERRANILLOS, M. P. Terpene compounds in nature: a review of their potential antioxidant activity. **Curr Med Chem**, v. 19, n. 31, p. 5319-5341, 2012.

GONZALEZ, F. G.; DI-STASI, L.C. Anti-ulcerogenic and analgesic activities of the leaves of Wilbrandia ebracteata in mice. **Phytomedicine**, v. 9, p. 125-134, 2002.

GOSSELIN, K.; DERUY, E.; MARTIEN, S.; VERCAMER, C.; BOUALI, F.; DUJARDIN, T.; SLOMIANNY, C.; HOUEL-RENAULT, L.; CHELLI, F.; DE LAUNOIT, Y.; ABBADIE, C. Senescent Keratinocytes Die by Autophagic Programmed Cell Death. **The American Journal of Pathology**, v. 174, n. 2, p. 423-435, 2009.

GRAIDIST, P.; MARTLA, M.; SUKPONDMA, Y. Cytotoxic activity of Piper cubeba extract in breast cancer cell lines. **Nutrients**, v. 7, n. 4, p. 2707-2718, 2015.

GRUIJL, F. R.; KRANEN, H. J.; MULLENDERS, L. H. UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer. **Journal of Photochemistry and Photobiology B: Biology**, v. 63, n.1-3, p. 19-27, 2001.

GUZMÁN-RODRÍGUEZ, J. J.; OCHOA-ZARZOSA, A.; LÓPEZ-GÓMEZ, R.; LÓPEZ-MEZA, J. E. Plant Antimicrobial Peptides as Potential Anticancer Agents. **BioMed Research International**, v. 2015, article ID 735087, doi:10.1155/2015/735087, 2015.

HABIB L, KHREICH N, JRAIJ A, ABBAS S, MAGDALOU J, CHARCOSSET C.; GREIGE-GERGES, H. Preparation and characterization of liposomes incorporating cucurbitacin E, a natural cytotoxic triterpene. **International Journal of Pharmaceutics**, v. 448, n. 1, p. 313-319, 2013.

HANAHAN, D., WEINGERG, R.A. The hallmarks of cancer. **Cell**, v.100, n. 1 p.57 – 70, 2000.

HANAHAN, D., WEINGERG, R.A. The hallmarks of cancer: The next generation. **Cell**, v.144, n. 5 p.646 – 674, 2011.

HARBORNE, J. B.; WILLIAMS, C. A. Advances in flavonoid research since. **Phytochemistry**, v. 55, n. 2000, p. 481–504, 1992.
HARVEY, A. L. Medicines from nature: are natural products still relevant to drug discovery? Trends in Pharmacological Sciences, v. 20, n. 5, p. 196-198, 1999.

HASLAM, E. Natural polyphenols (vegetable tannins) as drug: possible modes of action. The Journal of Natural Products, v. 59, p. 205-215, 1996.

HASSAN, S. M.; HAQ, A. U.; BYRD, J. A.; BERHOW, M. A.; CARTWRIGHT, A. L.; BAILEY, C. A. Haemolytic and antimicrobial activities of saponin-rich extracts from guar meal. **Food Chemistry**, v. 119, n. 2, p. 600–605, 2010.

HASSEN, I.; CASABIANCA, H.; HOSNI, K. Biological activities of the natural antioxidant oleuropein: Exceeding the expectation – A mini-review. **Journal of functional foods**, v. 18, parte B, p. 926–940, 2015.

HAVSTEEN, B. H. The biochemistry and medical significance of the flavonoids. **Pharmacology & Therapeutics**, v. 96, n. 2-3, p. 67-202, 2002.

HE, J.; WANG, Y.; XU, L. H.; QIAO, J.; OUYANG, D. Y.; HE, X. H. Cucurbitacin IIa induces caspase-3-dependent apoptosis and enhances autophagy in lipopolysaccharide-stimulated RAW 264.7 macrophages. **International Immunopharmacology**, v. 16, n. 1, p. 27–34, 2013.

HEIM, K. E.; TAGLIAFERRO, A. R.; BOBILYA, D. J. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. **Journal of Nutritional Biochemistry**, v. 13, n. 10, p. 572-584, 2002.

HEMACHANDRAN, K.; BHARATHI, S.; RADHAKRISHNAN, M.; BALAGURUNATHAN, R. Studies on extended beta lactamase producing, biofilm forming clinical bacterial pathogens and its invitro inhibition by actinobacterial extracts. **Journal of Applied Pharmaceutical Sciences**, v.1, n.8, p. 210-213, 2011.

HENDRA, R.; AHMAD, S.; SUKARI, A.; SHUKOR, M. Y.; OSKOUEIAN, E. Flavonoid Analyses and Antimicrobial Activity of Various Parts of Phaleria macrocarpa (Scheff.) Boerl Fruit. **International Journal of Molecular Sciences**, v. 16, n. 6, p. 3422-3431, 2011.

HIRSCH, E. B.; TAM, V. H. Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. **Expert Review of Pharmacoeconomics & Outcomes Research,** v. 10, n. 4, p. 441-451, 2010.

HO, Y. S.; WU, C. H.; CHOU, H. M.; WANG, Y. J.; TSENG, H.; CHEN, C. H.; CHEN, L. C.; LEE, C. H.; LIN, S. Y. Molecular mechanisms of econazole-induced toxicity on human colon cancer cells: G0/G1 cell cycle arrest and caspase 8-independent apoptotic signaling pathways. **Food and Chemical Toxicology**, v. 43, n. 10, p. 1483-1495, 2005.

HOLASOVAL, M.; KARPISKOVA, R.; KARPISKOVA, S.; BABAK, V.; SCHLEGELOVA, J. Comparison of methods for the determination of antimicrobial resistance in Campylobacter spp. human and the food chain isolates. **Veterinarni Medicina**, v. 52, n. 4, p. 169-174, 2007.

HOLOHAN, C.; SCHAEYBROECK, S. V.; LONGLEY, D. B.; JOHNSTON, P. G. Cancer drug resistance: an evolving paradigm. **Nature Reviews Cancer**, v. 13, n. 10, p.714–726, 2013.

HORIGUCHI M, MASUMURA KI, IKEHATA H, ONO T, KANKE Y, NOHMI T. Molecular nature of ultraviolet B light-induced deletions in the murine epidermis. **Cancer Research**, v. 61, n. 10, p. 3913-3918, 2001.

HOU, Y.; OUYANG, X.; WAN, R.; CHENG, H.; MATTSON, M. P.; CHENG, A. Mitochondrial superoxide production negatively regulates neural progenitor

proliferation and cerebral cortical development. **Stem Cells,** v.30, n. 11, p. 2535–2547, 2012.

HSU, C.-L.; FANG, S.-C.; YEN, G.-C. Anti-inflammatory effects of phenolic compounds isolated from the flowers of *Nymphaea mexicana* Zucc. **Food & Function**, v. 4, n. 8, p. 1216-1222, 2013.

HUANG, D.; OU, B.; PRIOR, R. L. The Chemistry behind antioxidant capacity assays. **Journal of Agricultural and Food Chemistry**, v. 53, p. 1841-1856, 2005.

INCA. 2014. **Estimativa 2014: Incidência de Câncer no Brasil** / Instituto Nacional de Câncer – Rio de Janeiro. Disponível em: www.inca.gov.br Acessado em: 09 de outubro de 2015.

ISHIDA, J.; WANG, H. K.; MASAYOSHI, O.; COSENTINO, C. L.; HU, C.Q.; LEE, K. H. Anti-aids agents 46. Anti-HIV activity of harman, an anti-HIV principle from symplocos setchuensis and its derivatives. **Journal of Natural Products,** v. 64, p. 958–960, 2001.

JAIN, P. K.; JOSHI, H. Coumarin: Chemical and Pharmacological Profile. **Journal of Applied Pharmaceutical Science**, v. 2, n. 6, p. 236-240.

JANDÚ, J. J. B.; DA SILVA, L. C. N.; PEREIRA, A. P. C.; SOUZA, R. M.; JÚNIOR, C. A. S.; FIGUEIREDO, R. C. B. Q.; ARAÚJO, J. M.; CORREIA, M.T. S.; SILVA, M. V. Myracrodruon urundeuva bark: an antimicrobial, antioxidant and non-cytotoxic agent. **Journal of Medicinal Plants Research**, v. 7, n. 8, p. 413-418, 2012.

JEON, Y.J.; MIDDLETON, J.; KIM, T.; LAGANA, A.; PIOVAN, C.; SECCHIERO, P.; NUOVO, G. J.; CUI, R.; JOSHI, P.; ROMANO, G.; DI LEVA, G.; LEE, B.K.; SUN, H.L.; KIM, Y.; FADDA, P.; ALDER, H.; GAROFALO, M.; CROCE, C. M. Set of NF-kappaB-regulated microRNAs induces acquired TRAIL resistance in lung cancer. **Proceedings of the National Academy of Sciences of the United States of America**, v. 112, n. 26, p. E3355–E3364. 2015.

JIANG, W.; LU, Y.; CHEN, Z.; CHEN, S.; ZHANG, M.; JIN, L.; LOU, J.; HE, J. Studying the genotoxicity of vincristine on human lymphocytes using comet assay,

micronucleus assay and TCR gene mutation test in vitro. **Toxicology**, v. 252, n. 1-3, p. 113-117, 2008.

JIMENEZ,P.; CABRERO, P.;BASTERRECHEA, J.E.;TEJERO, J.; CORDOBA-DIAZ, D.; GIRBES, T. Isolation and Molecular Characterization of Two Lectins from Dwarf Elder (*Sambucus ebulus* L.) Blossoms Related to the *Sam* n1 Allergen. **Toxins (Basel)**, v. 5, n. 10, p. 1767-1779, 2013.

KANG, C.-G.; HAH, D.-S.; KIM, C.-H.; KIM, Y.-H.; KIM, E.; KIM, J.-S. Evaluation of Antimicrobial Activity of the Methanol Extracts from 8 Traditional Medicinal Plants. **Toxicology Research**, v. 27, n. 1, p. 31-36, 2011.

KIM, I. Y.; HE, Y.-Y. Ultraviolet radiation-induced non-melanoma skin cancer : Regulation of DNA damage repair and inflammation. **Genes & Diseases**, v. 1, n. 2, p. 188-198, 2014.

KIM, M.Y.; KIM, E. J.; KIM, Y.-N.; CHOI, C.; LEE, B.-H. Comparison of the chemical compositions and nutritive values of various pumpkin (Cucurbitaceae) species and parts. **Nutricion Research and Practice**, v. 6, n. 1, p.21-27, 2012.

KIMURA, Y.; TANIGUCHI, M.; BABA K. Antitumor and antimetastatic activities of 4-hydroxyderricin isolated from Angelica keiskei roots. **Planta Medica** v. 70, n.3, p. 211-219, 2004.

KIRKLAND, D.; REEVE, L.; GATEHOUSE, D.; VANPARYS, P.; A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. **Mutation Research**, v. 721, n. 1, p. 27-73, 2011.

KLANCNIK, A.; PISKERNIK, S.; JERSEK, B.; MOZINA, S. S. Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. **Journal of Microbiological Methods,** v. 81, n. 12, p. 121-126, 2010.

KLEIN, V. L. G.; LIMA, L. F. P. Cucurbitaceae. *In*: **Lista de espécies da flora do Brasil**. Jardim Botânico do Rio de Janeiro, 2011. Disponível em: <http://floradobrasil.jbrj.gov.br/2010/FB017036>.

KLEIN, V.L.G. & SANTANA, J.R.O. 2009. Cucurbitaceae. In: Giulietti, A.M.; Rapini, A.; Andrade, M.J.G.; Queiroz, L.P. & Silva, J.M.C.D. **Plantas Raras do Brasil**. Belo Horizonte: Conservação Internacional; Universidade Estadual de Feira de Santana, 496p.

KLENKAR, J.; MOLNAR, M. Natural and synthetic coumarins as potential anticancer agents. **Journal of Chemical and Pharmaceutical Research**, v. 7, n. 7, p. 1223-1238, 2015.

KONAN, K. V.; TIEN, C. L.; MATEESCU, M. A. Electrolysis-induced fast activation of the ABTS reagent for an antioxidant capacity assay. Analytical Methods, v. 28, n. 8, p. 5638-5644, 2016.

KORNBERG, A. Science and medicine at the Millennium. *Brazilian Journal of* **Medical and Biological Research**, v. 30, n.12, p. 1379-1386, 1997.

KUMAR, H.; LIM, H.-W.; MORE, S. V.; KIM, B.-W.; KOPPULA, S.; KIM, I. S.; CHOI, D.-K. The Role of Free Radicals in the Aging Brain and Parkinson's Disease: Convergence and Parallelism. **International Journal of Molecular Sciences's,** v. 18, n. 3, p. 10478-10504, 2012.

KUMAR, P.; DEVALA, R. G.; LAKSHMAYYA, B.; RAMACHANDRA, S. S Pharmacognostical studies on tubers of *Momordica tuberosa* Cogn., Cucurbitaceae. **Revista Brasileira de Farmacognosia**, v. 20, n. 1, p. 7-11, 2010.

KUMAR, S.; PANDEY, A. K. Chemistry and Biological Activities of Flavonoids: An Overview. **The Scientific World Journal**, v. 2013, Artigo ID 162750, doi:10.1155/2013/162750, 2013. **Brazilian Journal of Pharmacognosy**, v. 10, n.1, p. 7-11, 2013.

KUSKOSKI, E.M.; ASUERO, A.G.; TRONCOSO, A.M.; MANCINI-FILHO, J.; FETT, R. Aplicatíon de diversos métodos químicos para determinar actividad antioxidanteen pulpa de frutos. **Ciência e Tecnologia de Alimentos**, v.25, n.4, p.726-732, 2005.

LAKHTIN, V. M.; LAKHTIN, M. V.; POSPELOVA, V. V.; SHENDEROV, B. A. Lectins of lactobacilli and bifidobacteria. II. Probiotic lectins of lactobacilli and bifidobacteria as possible signal molecules regulating inter- and intrapopulation

relationships between bacteria and between bacteria and the host. **Microbial Ecology in Health and Disease**, v. 19, n. 3, p. 153-157, 2007.

LEE, C.; RAFFAGHELLO, L.; BRANDHORST, S.; SAFDIE, F. M.; BIANCHI, G.; MARTIN-MONTALVO, A.; PISTOIA, V.; WEI, M.; HWANG. S.; MERLINO, A.; EMIONITE, L.; DE CABO, R.; LONGO, V. D. Fasting cycles retard growth of tumors and sensitize a range of cancer cell types to chemotherapy. **Science Translational Medicine**, v. 7, n.4, p. 124-127, 2012.

LEVERRIER, A.; BERO, J.; CABRERA, J.; FRÉDÉRICH, M.; QUETIN-LECLERCQ, J.; PALERMO, J. A. Structure-activity relationship of hybrids of Cinchona alkaloids and bile acids with in vitro antiplasmodial and antitrypanosomal activities. **European Journal of Medicinal Chemistry**, v. 100, p. 10-17, 2015.

LAMB, R.; OZSVARI, B.; LISANTI, C. L.; TANOWITZ, H. B.; HOWELL, A.; MARTINEZ-OUTSCHOORN, U. E.; SOTGIA, F.; LISANTI, M. P. Antibiotics that target mitochondria effectively eradicate cancer stem cells, across multiple tumor types: Treating cancer like an infectious disease. **Oncotarget**, v. 6, n. 7, p. 4569-4584, 2015.

LIMA, LUIS FERNANDO PAIVA **Estudos taxonômicos e morfopolínicos em Cucurbitaceae brasileiras**. 2010. 232f. Tese (Doutorado em Botânica). Universidade Federal do Rio Grande do Sul, Rio Grande do Sul.

LIU, B.; CHENG, Y.; ZHANG, B.; BIAN, H.; BAO, J. Polygonatum cyrtonema lectin induces apoptosis and autophagy in human melanoma A375 cells through a mitochondria-mediated ROS–p38–p53 pathway. **Cancer Letters,** v. 275, p. 54-60, 2009.

LIU, B.; BIAN, H. J.; BAO, J. K. Plant lectins: potential antineoplastic drugs from bench to clinic. **Cancer Letters**, v. 287, n. 1, p. 1-12, 2010.

LIU, J. F.; LIU, Y. H.; CHEN, C. M.; CHANG, W. H.; CHEN, C. Y. O. The effect of almonds on inflammation and oxidative stress in Chinese patients with type 2 diabetes mellitus: A randomized crossover controlled feeding trial. **European Journal of Nutrition**, v. 52, n.3, p. 927–935, 2013.

LIU, J. Oleanolic acid and ursolic acid: research perspectives. **Journal of Ethnopharmacology**, v. 100, p. 92–94, 2005.

LIU, L. Z.; LI, N.; GAO, W.; MAN, S.; YIN, S.; LIU, C. Comparative study on hemostatic, cytotoxic and hemolytic activities of different species of Paris. **Journal of Ethnopharmacology** v. 142, n. 3, p. 789–794, 2012.

LIU, Y.; WU, F. Global Burden of Aflatoxin-Induced Hepatocellular Carcinoma: A Risk Assessment. **Environmental Health Perspectives**, v. 118, n. 6, p. 818-824, 2010.

LOBO, V.; PATIL, A.; PHATAK, A.; Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. **Pharmacognosy Reviews**, v. 4, n. 8, 118-126, 2010.

LOEB, L. A.; HARRIS, C. C. Advances in Chemical Carcinogenesis: A Historical Review and Prospective, Cancer Research, v. 68, n. 17, p. 6863-6872, 2009.

LOOI, C. Y.; ARYA, A.; CHEAH, F. K.; MUHARRAM, B.; LEONG, K. H.; MOHAMAD, K.; WONG, W. F.; RAI, N.; MUSTAFA, M. R. Induction of Apoptosis in Human Breast Cancer Cells via Caspase Pathway by Vernodalin Isolated from *Centratherum anthelminticum* (L.) Seeds. **Plos One**, v. 8, n. 2, e56643., 2013.

LÓPEZ-MARURE, R.; CONTRERAS, P. G.; DILLON, J. S. Effects of dehydroepiandrosterone on proliferation, migration, and death of breast cancer cells. **European Journal of Pharmacology**, v. 660, n. 2-3, p. 268-274, 2011.

LORENZI, H. **Árvores brasileiras:** manual de identificação e cultivo de plantas arbóreas nativas do Brasil. São Paulo: Plantarum, 1992. v.1. 368p.

LU, J.-J.; BAO, J.-L.; CHEN, X.-P.; HUANG, M.; WANG, Y.-T. AlkaloidsIsolatedfromNaturalHerbsastheAnticancerAgents. **Evidence-Based Complementary and Alternative Medicine**, v. 2012, Artigo ID 485042, doi:10.1155/2012/485042, 2012.

MACHADO, M. C. Apodanthera- Caduciform Cucumbers of Bahia, Brazil. **Cactus and Succulent Journal**, v. 81, n. 3, p. 147-149, 2009.

MAHMOUD, T. S.; MARQUES, M. R.; PESSOA, C. Ó; LOTUFO, L. V. C.; MAGALHÃES, H. I. F.; MORAES, M. O.; LIMA, D. P.; TININIS, A. G.; OLIVEIRA,

J. E. *In vitro* cytotoxic activity of Brazilian Middle West plant extracts. **Revista Brasileira de Farmacognosia,** v. 21, n. 3, p. 456-464, 2011.

MANIKANDAN, C.; AMSATH, A.; Antibiotic susceptibility pattern of Klebsiella pneumoniae isolated from urine samples. **International ournal of current microbiology and applied sciences**, v. 2, n. 8, p. 330-337, 2013.

MARINS, J. S. R.; SASSONE, L. M.; FIDEL, S. R.; RIBEIRO, D. A. *In vitro* genotoxicity and cytotoxicity in murine fibroblasts exposed to EDTA, NaOCl, MTAD and citric acid. **Brazilian Dental Journal**, v. 23, n. 5, p. 527-533, 2012.

MASER, E.; SCHULZ, M.; SAUER, U. G.; WIEMANN, M.; MA-HOCK, L.; WOHLLEBEN, W.; HARTWIG, A.; LANDSIEDEL, R. *In vitro* and *in vivo* genotoxicity investigations of differently sized amorphous SiO_2 nanomaterials. **Mutation Research/Genetic Toxicology and Environmental Mutagenesis**, v.794, p. 57-74, 2015.

MATHANGI, T.; PRABHAKARAN, P. DPPH Free Radical Scavenging Activity of the Extracts of the Aquatic Fern Marsilea quadrifolia Linn. **International Journal of Current Microbiology and Applied Sciences**, v. 2, n. 10, p. 534-536, 2013.

MATOS, F. J. A. Farmácias vivas. Fortaleza: Editora UFC, 2002, p 267.

MATSUO, M.; SASAKI, N.; SAGA, K.; KANEKO, T. Cytotoxicity of flavonoids toward cultured normal human cells. **Biological & Pharmaceutical Bulletin**, v. 28, n. 2, p. 253-259, 2005.

MBAVENG, A. T.; SANDJO, L. P.; TANKEO, S. B.; NDIFOR, A. R.; PANTALEON, A.; NAGDJUI, B.T.; KUETECORRESPONDING, V. Antibacterial activity of nineteen selected natural products against multi-drug resistant Gram-negative phenotypes. **Springer Plus**, v. 4, n; 823, p. 2-9, 2015.

MCILWAIN, D. R.; BERGER, T.; MAK, T. W. Caspase functions in cell death and disease**. Cold Spring Harbor Perspectives in Biology**, v. 5, n. 4**,** doi: 10.1101/cshperspect.a008656., 2013.

MENDOZA-RODRÍGUEZ, C. A., CERBÓN, M. A. Tumor suppressor gene p53: mechanisms of action in cell proliferation and death. **Revista de Investigacíon Clinica**, v. 53, n. 3, p. 266-273, 2001.

MILITAO, G.; DANTAS, I. N.; FERREIRA, P.M.; ALVES, A.P.; CHAVES, D.C.;

MONTE, F.J.; PESSOA, C.; MORAES, M.O.; COSTA-LOTUFO, L.V.; In vitro and in vivo anticancerproperties of cucurbitacin isolated from Cayaponia racemosa. **Pharmaceutical Biology,** v. 50, p. 1479–1487, 2012.

MINAFRA, L.; BRAVATÀ, V. Cell and molecular response to IORT treatment. **Translational Cancer Research,** v. 13, n. 1, p. 32-47, 2014.

MIRO, M. Cucurbitacins and their pharmacological effects. **Phytotherapy Research**, v. 9, p. 159–68, 1995.

MONTEIRO, J. M.; ARAÚJO, E. L.; AMORIM, E. L. C.; ALBUQUERQUE, U. P. Valuation of the Aroeira (*Myracrodruon urundeuva* Allemão): perspectives on conservation. **Acta Botanica Brasilica**, v.26, n.1, p. 125-132, 2012.

MORAIS, S. A. L.; NASCIMENTO, E. A.; QUEIROZ, C. R. A. A. Studies on polyphenolsand lignin of *Astronium urundeuva* wood. **Journal of Brazilian Chemical Society**, v. 10, n. 6, p. 447 - 452, 1999.

MOSMANN, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays**. Journal of Immunological Methods**, v. 65, p. 55-63, 1983.

MOTA, B. C. F.; ROYO, V. A.; FONSECA, J. M. S. DOS SANTOS, A. C.; JUNIOR, A. F. M.; MENEZES, E. V.; ESPERANDIM, V. R.; LAURENTIZ, R. S. Comprative Studies between the chemical constituents and properties of the extracts from the leaves and barks of Myracrodruon urundeuva Fr. All. **Journal of Medicinal Plants Research**, v. 9, n. 6, p. 159-168, 2015.

MURANLI, F. D.; KANEV, M.; OZDEMIR, K. Genotoxic effects of diazinon on human peripheral blood lymphocytes. **Arhive za Higijenu Rada i Toksikologiju**, v. 66, n. 2, p. 153-158, 2015.

NAGARATHNA, P. K. M.; WESLEY, M. J.; REDDY, P. S.; REENA, K. Review on Genotoxicity, its Molecular Mechanisms and Prevention. **International Journal of Pharmaceutical Sciences Review and Research**, v. 22, n. 1, p. 236-243, 2013.

NAKAJIMA, Y.; NISHIDA, H.; MATSUGO, S.; KONISHI, T. Cancer cell cytotoxicity of extracts and small phenolic compounds from Chaga [Inonotus obliquus (persoon) Pilat. **Journal of Medicinal Food**, v. 12, n. 3, p. 501-507, 2009.

NAKASHIMA, S.; MATSUDA, H.; KURUME, A.; ODA, Y.; NAKAMURA, S.; YAMASHITA, M.; YOSHIKAWA, M. Cucurbitacin E as a new inhibitor of cofilin phosphoryla-tion in human leukemia U937 cells. **Bioorganic & Medicinal Chemistry Letters**, v. 20, p. 2994–2997, 2010.

NAPOLEÃO, T. H.; GOMES, F. S.; LIMA, T. A.; SANTOS,N. D.L.; SÁ, R. A.; ALBUQUERQUE, A. C.; COELHO, L.C.B.B.; PAIVA,P. M.G. Termiticidal activity of lectins from Myracrodruon urundeuva against Nasutitermes corniger and its mechanisms. **International Biodeterioration & Biodegradation**, v. 65, n. 1 p. 52-59, 2011.

NAPOLEÃO, T. H.; PONTUAL, E. V.; LIMA, T. A.; SANTOS, N. D. L.; SÁ, R. A.; COELHO, L. C. B. B.; NAVARRO, D. M. A. F.; PAIVA, P. M. G. Effect of Myracrodruon urundeuva leaf lectin on survival and digestive enzymes of Aedes aegypti larvae. **Parasitology Research**, v. 110, n. 2, p. 609–616, 2012.

NAYAKA, H. B.; LONDONKAR, R. L.; UMESH, M. K.; TUKAPPA, A. Antibacterial Attributes of Apigenin, Isolated from *Portulaca oleracea* L. **International Journal of Bacteriology**. v. 2014, doi:10.1155/2014/175851, 2014.

OCHOA-ALVAREZ, J. A.; KRISHNAN, H.; SHEN, Y.; ACHARYA, N. K.; HAN, M.; MCNULTY, D. E.; HASEGAWA, H.; HYODO, T.; SENGA, T.; GENG, J. G.; KOSCIUK, M.; SHIN, S. S.; GOYDOS, J. S.; TEMIAKOV, D.; NAGELE, R. G.; GOLDBERG, G. S. Plant lectin can target receptors containing sialic acid, exemplified by podoplanin, to inhibit transformed cell growth and migration. **Plos One**, v. 7, n. 7, e41845, 2012.

O'BRIEN, J.; WILSON, I.; ORTON, T.; POGNAN, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. **European Journal of Biochemistry**, v. 267, n. 17, p. 5421-5426, 2000.

OECD - GUIDELINE FOR THE TESTING OF CHEMICALS. DRAFT PROPOSAL FOR A NEW GUIDELINE 487: **In Vitro Mammalian Cell Micronucleus Test (mnvit)**, 2007. Disponível em: < http://www.oecd.org/dataoecd/38/58/39780112.doc>.

OLIVEIRA, L. M.; BEVILAQUA, C. M.; MACEDO, I. T.; MORAIS, S. M.; MACHADO, L. K.; CAMPELLO, C. C.; AQUINO, M. M. Effects of Myracrodruon urundeuva extracts on egg hatching and larval exsheathment of Haemonchus contortus. **Parasitology Research**, v. 109, n.3, p. 893-898, 2011.

OLIVEIRA, C. F. R.; LUZ, L. A.; PAIVA, P. M. G.; COELHO, L. C. B. B.; MARANGONI, S.; MACEDO, M. L. R. Evaluation of seed coagulant Moringa oleifera lectin (cMoL) as a bioinsecticidal tool with potential for the control of insects. **Process Biochemistry**, v. 46, n. 2, p. 498-504, 2011.

OLIVEIRA, G. L. S. Determination in vitro of the antioxidant capacity of natural products by the DPPH•method: review study. **Revista brasileira de plantas medicinais,** v, 17, n. 1, p. 36-44, 2015.

OLIVEIRA, L. A. R.; MACHADO, R. D.; RODRIGUES, J. R. Levantamento sobre o uso de plantas medicinais com a terapêutica anticâncer por pacientes da Unidade Oncológica de Anápolis. **Revista Brasileira de Plantas Medicinais**, v.16, n.1, p.32-40, 2014.

OJEWOLE, J. A.; ADEWOLE, S. O.; OLAYIWOLA, P. Hypoglycaemic and hypotensive effects of Momordica charantia Linn (Cucurbitaceae) whole-plant aqueous extract in rats. **Cardiovascular Journal of South Africa**, v. 17, n. 5, p. 227-232, 2006.

ORRENIUS, S.; NICOTERA, P.; ZHIVOTOVSKY, B. Cell Death Mechanisms and Their Implications in Toxicology. **Toxicological Sciences**, v. 119, n. 1, p. 3-19, 2010.

PADUCH, R.; KANDEFER-SZERSZEŃ, M.; TRYTEK, M.; FIEDUREK, J. Terpenes: substances useful in human healthcare. **Archivum Immunologiae et Therapiae Experimentalis (Warsz)**, v. 55, n. 5, p. 315-327, 2007.

PAIVA, P. M. G.; SANTANA, G. M. S.; SOUZA, I. F. A. C.; ALBUQUERQUE, L. P.; AGRANETO, A. C.; ALBUQUERQUE, A. C.; LUZ, L. A.; NAPOLEÃO, T. H.; COELHO, L. C. B. B. Effect of lectins from Opuntia ficus indica cladodes and Moringa oleifera seeds on survival of Nasutitermes corniger. **International Biodeterioration & Biodegradation**, v. 65, n. 7, p. 982-989, 2011.

PALUMBO, R.; SOTTOTETTI, F.; RICCARDI, A.; TERAGNI, C.; POZZI, E.; QUAQUARINI, E.; TAGLIAFERRI, B.; BERNARDO, A. Which patients with metastatic breast cancer benefit from subsequent lines of treatment? An update for clinicians. **Therapeutic Advances in Medical Oncology**, v. 5, n. 6, p. 334-350, 2013.

PATIL, M. B; DESHPANDE, K. V. Isolation and characterization of lectin from leaves of *Dregea volubilis*. **Journal of Global Biosciences**, v. 4, n. 6, p. 2496-2503, 2015.

PAYDAR, M.; KAMALIDEHGHAN, B.; WONG, Y. L.; WONG, W. F.; LOOI, C. Y.; MUSTAFA, M. R. Evaluation of cytotoxic and chemotherapeutic properties of boldine in breast cancer using in vitro and in vivo models. **Drug Design, Development and Therapy**, v. 8, p. 719-733, 2014.

PERAZZO, F. F.; CARVALHO, J. C.T.; RODRIGUES, M,; MORAIS, E. K. L; MACIEL, M. A. M. Comparative anti-infammatory and antinociceptive effects of terpenoids and an aqueous extract obtained from Croton cajucara Benth. **Revista Brasileira de Farmacognosia,** v.17, p. 521-528, 2007.

PEREIRA, P. S.; BARROS, L. M.; BRITO, A. M.; DUARTE, A. E.; MAIA, A. J. Use of *Myracrodruon urundeuva* Allemão (pepper tree) by farmers to treat diseases. **Revista Cubana de Plantas Medicinales**, v. 19, n. 1, p. 51-60, 2014.

PERGENTINO, S. D.; FARIAS, N. F. F.; ALMEIDA, R. N. Influence of the chirality of (R)-(-)- and (S)-(+)-carvone in the central nervous system: a comparative tudy. **Chirality**, v. 19, p. 264-268, 2007.

PETERSEN, R. C. Free-Radical Polymer Science Structural Cancer Model: A Review. **Scientifica**, v. 2013, artigo ID 143589, 2013.

PEUMANS, W. J.; VAN DAMME, E. J. M. Lectins as PIant Defense Proteins. **Plant Physiology**, v. 109, n. 2, p. 347-352, 1995.

PEUMANS, W. J.; VAN DAMME, E. J. Plant lectins: specific tools for the identification, isolation, and characterization of O-linked glycans. **Critical Reviews in Biochemistry and Molecular Biology**, v. 33, n. 3, p. 209-258, 1998.

PEUMANS, W. J.; VAN DAMME, E. J.; BARRE, A.; ROUGE, P. Classification of plant lectins in families of structurally and evolutionary related proteins. **Advances in Experimental Medicine and Biology**, v. 491, p. 27- 54, 2001.

PICHERSKY, E.; GANG, D. R. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. **Trends in Plants Science**, v. 5, n. 10, p. 1360-1385, 2000.

PODURI, A.; GILAD, D.; EVRONY, X. C.; CHRISTOPHER, A. W. Somatic Mutation, Genomic Variation, and Neurological Disease. **Science**, v. 341, p. 43-51, 2013.

POZNER, R. Revision del Genero Cucurbitella (Cucurbitaceae). **Annals of the Missouri Botanical Garden**, v. 85, n. 3, p. 425-439, 1998.

PRIETO, P.; PINEDA, M.; AGUILAR, M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. **Analytical Biochemistry**, v. 269, n. 2, p. 337-341, 1999.

PUCCI, B.; KASTEN, M.; GIORDANO, A. Cell Cycle and Apoptosis. **Neoplasia,** v. 2, n. 4, p. 291-299, 2000.

QUEIROZ, C. R. A. A.; MORAIS, S. A. L.; NASCIMENTO, E. A. CARACTERIZAÇAO DOS TANINOS DA AROEIRA-PRETA (*Myracrodruon urundeuva*). **Revista Árvore**, v. 26, n. 4, p. 496-497, 2002.

QIAN, M.-B.; CHEN, Y.-D.; LIANG, S.; YANG, G.-J.; ZHOU, X.-N.; The global epidemiology of clonorchiasis and its relation with cholangiocarcinoma. **Infectious Diseases of Poverty**, v. 1, n. 4, p. 1-11, 2012.

RAFIQ, S.; MAJEED, R.; QAZIB, A. K.; GANAIA, B. A.; WANIA, I.; RAKHSHANDAA, S.; QURISHIB, Y.; SHARMAB, P. R.; HAMID, A.; MASOODA, A.; HAMID, R. Isolation and antiproliferative activity of Lotus corniculatus lectin towards human tumour cell lines. **Phytomedicine**, v. 21, n. 1, p. 30-38, 2013.

RAMPERSAD, S. N. Multiple Applications of Alamar Blue as an Indicator of Metabolic Function and Cellular Health in Cell Viability Bioassays. **Molecules**, v. 12, n. 9, p. 12347-12360, 2012.

RASTOGI, R. P.; RICHA; KUMAR, A.; TYAGI, M. B.; SINHA, R. P. Molecular Mechanisms of Ultraviolet Radiation-Induced DNA Damage and Repair. **Journal of Nucleic Acids**, v. 2010, artigo ID: 592980, 2010.

REBUCCI, M.; MICHIELS, C. Molecular aspects of cancer cell resistance to chemotherapy. **Biochemical Pharmacology**, v. 85, n. 9, p. 1219-1226, 2013.

REDDY, M. K.; GUPTA, S. K.; JACOB, M. R.; KHAN, S. I.; FERREIRA, D. Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from Punica granatum L. **Planta Med**, v. 73, n. 5, p. 461-467, 2007.

RESENDE, F. A.; CAMPOS, D. L.; SILVA, V. C.; GRANDIS, R. A.; SOUZA, L. P.; LEONARDO, J. C. S.; ROCHA, C. Q.; SANTOS, L. C.; VILEGAS, W.; VARANDA, E. A. Mutagenicity and chemopreventive activities of Astronium species assessed by Ames test. **Regulatory Toxicology and Pharmacology**, v. 72, n. 3, p. 506-513, 2015.

RIBEIRO, M.; PINTO, I.; PEDROSA, C. Comportamento da população do concelho de Vizela no consumo de antibióticos. **Revista Portuguesa de Saúde Pública**, v.27, n. 2, p. 57-70, 2009.

RICCI, M. S.; WEI-XING, Z. Chemotherapeutic Approaches for Targeting Cell Death Pathways. **Oncologist**, v. 11, n. 4, p. 342-357, 2006.

RINI, J. M. Lectin structure. **Annual Review of Biophysics and Biomolecular Structure**, v. 24, p. 551-577, 1995.

RISTIVOJEVIĆ, P.; DIMKIĆ, I.; TRIFKOVIĆ, J.; BERIĆ, T.; VOVK, I.; MILOJKOVIĆ-OPSENICA, D.; STANKOVIĆ, S. Antimicrobial Activity of Serbian Propolis Evaluated by Means of MIC, HPTLC, Bioautography and Chemometrics, **Plos One**, v. 11, n. 6, e0157097. doi:10.1371/journal.pone.0157097, 2016.

RIZZINI, C.T. Aspectos ecológicos da regeneração em algumas plantas do cerrado. *In* **Anais do III Simpósio sobre cerrado** (M.G. FERRI, coord.). Editora Edgard Blücher, São Paulo, p.61-64, 1971.

RO, D.-K.; PARADISE, E. M.; OUELLET, M.; FISHER, K. J.; NEWMAN, K. L.; NDUNGU, J. M.; HO, K. A.; EACHUS, R. A.; HAM, T. S.; KIRBY, J.; CHANG, M. C. Y.; WITHERS, S. T.; SHIBA, Y.; SARPONG, R.; KEASLING, J. D. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. **Nature**, v. 440, p. 940–943, 2006.

ROBINSON, W. M.; M. R. B. OSÓRIO. **Genética humana**. 2ª ed. RS: Artmed Editora, 2001.

ROQUE, A. A.; ROCHA, R. M.; LOIOLA, L. M. B. Uso e diversidade de plantas medicinais da Caatinga na comunidade rural de Laginhas, município de Caicó, Rio Grande do Norte (nordeste do Brasil). **Revista Brasilera de Plantas Medicinais**, v.12, n.1, p.31-42, 2010.

RUSSO, A.; ESPINOZA, C. L.; CAGGIA, S.; GARBARINO, J. A.; PEÑA-CORTÉS, H.; CARVAJAL, T. M.; CARDILE, V. A new jasmonic acid stereoisomeric derivative induces apoptosis via reactive oxygen species in human prostate cancer cells. **Cancer Letters**, v. 326, n. 2, p. 199-205, 2012.

SÁ, R.A.; NAPOLEÃO, T.H.; SANTOS, N. D.L.; GOMES, F.S.; ALBUQUERQUE, A.C.; XAVIER, H.S.; COELHO, L.C.B.B.; BIEBER, L.W.; PAIVA, P.M.G. Induction of mortality on Nasutitermes corniger (Isoptera, Termitidae) by Myracrodruon urundeuva heartwood lectin. **International Biodeterioration & Biodegradation**, v. 62, p. 460-464, 2008.

SÁ, R. A.; SANTOS, N. D. L.; SILVA,C. S.B.; NAPOLEÃO, T. H.; GOMES, F. S.; CAVADA, B. S.; COELHO, L. C. B. B.; NAVARRO,D. M. A. F.; BIEBER, L. W.; PAIVA, P. M. G. Larvicidal activity of lectins from Myracrodruon urundeuva on Aedes aegypti, **Comparative Biochemistry and Physiology**, Part C, v. 149, p. 300-306, 2009.

SADZUKA, Y.; FUJIKI, S.; ITAI, S. Enhancement of doxorubicin-induced antitumor activity and reduction of adverse reactions by cucurbitacin I. **Food Research International**, v. 47, n. 1, p. 67-69, 2012.

SAGAR, S.; ESAU, L.; MOOSA, B.; KHASHAB, N. M.; BAJIC, V. B.; KAUR, M. Cytotoxicity and Apoptosis Induced by a Plumbagin Derivative in Estrogen Positive MCF-7 Breast Cancer Cells. Anti-Cancer Agents in Medicinal Chemistry, v. 14, n. 1, p. 170-180, 2014.

ŠARAC, Z.; MATEJIĆ, J. S.; STOJANOVIĆ-RADIĆ, Z. Z.; VESELINOVIĆ, J. B.; DŽAMIĆ, A. M.; BOJOVIĆ, S.; MARIN, P. D. Biological activity of Pinus nigra terpenes—Evaluation of FtsZ inhibition by selected compounds as contribution to their antimicrobial activity. **Computers in Biology and Medicine**, v. 54, p. 72-78, 2014.

SARMA, A. D.; MALLICK A. R.; GHOSH, A. K. Free Radicals and Their Role in Different Clinical Conditions: An Overview. **International Journal of Pharma Sciences and Research**, v.1, n. 3, p. 185-192, 2010.

SARZAEEM, A.; MIRAKABADI, A. Z.; MORADHASELI, S.; SAYAD, A. Comparative study for toxic Effects of Camptothecin in Cancer and Normal Cells. **Journal of Biology and today's world**, v. 2, n. 4, p. 188-201, 2013.

SCHIAR, V. P.; DOS SANTOS, D. B.; LÜDTKE, D. S.; VARGAS, F.; PAIXÃO, M. W.; NOGUEIRA, C. W.; ZENI, G.; ROCHA, J. B. Screening of potentially toxic chalcogens in erythrocytes. **Toxicology In Vitro**, v. 21, n. 1, p. 139-145, 2007.

SENGUPTA, S.; MARRINAN, J.; FRISHMAN, C.; SAMPATH, P. Impact of Temozolomide on Immune Response during Malignant Glioma Chemotherapy. **Clinical and Developmental Immunology**, v. 2012, Artigo: ID 831090, doi:10.1155/2012/831090, 2012.

SCHREINER, M.; MARTÍNEZ-ABAIGAR, J.; GLAAB, J.; JANSEN, M. UV-B Induced Secondary Plant Metabolites Potential benefits for plant and human health. **Optic & Photonic**, v. 9, n. 2, p. 34-37, 2014.

SHAH, A. J.; LAKKAD, B. C.; RAO, M. V. Genotoxicity in lead treated human lymphocytes evaluated by micronucleus and comet assays. **Indian Journal of Experimental Biology**, v. 54, p. 502-508, 2016.

SHARON, N.; LIS, H. History of lectins: from hemagglutinins to biological recognition molecules. **Glycobiology**. p. 53–62, 2004.

SHAY, J. W.; WOODRING, E. Wright Role of telomeres and telomerase in cancer. **Seminars in Cancer Biology**, v. 21, n. 6, p. 349-353, 2011.

SINGH, R. K.; MISHRA, S. K.; KUMAR, N.; SINGH, A. K. Assessment of DNA Damage by Comet Assay in Lymphocytes of Workers Occupationally Exposed to **Petroleum fumes. International Journal of Genetics**, v. 2, n. 1, p. 18-22, 2010.

SILVA, C. G.; MARINHO, M. G. V.; LUCENA, M. F. A.; COSTA, J. G. M. Levantamento etnobotânico de plantas medicinais em área de Caatinga na comunidade

do Sítio Nazaré, município de Milagres, Ceará, Brasil. **Revista Brasileira de Plantas Medicinais**, v.17, n.1, p.133-142, 2015.

SILVA, F. DE O.; SANTOS, P. D.; FIGUEIRÔA, E. DE O.; DE MELO, C. M.; DE ANDRADE, L.; NEVES, J. K.; ARRUDA, F. V.; CAJAZEIRAS, J. B.; DO NASCIMENTO, K. S.; TEIXEIRA, E. H.; CAVADA, B. S.; PORTO, A. L.; PEREIRA, V. R. Antiproliferative effect of Canavalia brasiliensis lectin on B16F10 cells. **Research in Veterinary Science**, v. 96, n. 2, p. 276-282, 2014.

SILVA, M. I. G.; MELO, C. T. V.; VASCONCELOS, L. F.; CARVALHO, A. M. R.; SOUSA, F. C. F. Bioactivity and potential therapeutic benefi ts of some medicinal plants from the Caatinga (semi-arid) vegetation of Northeast Brazil: a review of the literature. **Revista Brasileira de Farmacognosia**, v. 22, n.1, p. 193-207, 2012.

SILVA, A. C.; NEPOMUCENO, J. C. Avaliação da frequência de micronúcleos em eritrócitos periféricos de mandi-amarelo (Pimelodus maculatus) do rio Paranaíba. **Revista do Núcleo Interdisciplinar de Pesquisa e Extensão do UNIPAM**, v. 1, n. 7, p. 167-179, 2010.

SIMPSON, M. G. Plant systematics, 2nd. edn. **London, Academic Press**, 2010.

SOARES, S. E. Ácidos fenólicos como antioxidantes *Phenolic acids as antioxidants*, Revista de Nutrição, v. 15, n.2, p. 71-81, 2002.

SOKOVIĆ, M.; GLAMOČLIJA, J.; MARIN, P. D.; BRKIĆ, D.; VAN GRIENSVEN, L. J. L. D. Antibacterial Effects of the Essential Oils of Commonly Consumed Medicinal Herbs Using an In Vitro Model. **Molecules**, v. 15, p. 7532-7546, 2010.

SOMERS, C. M.; YAUK, C. L.; WHITE, P. A.; PARFETT, C. L. J.; JAMES, S. Quinn Air pollution induces heritable DNA mutations. **Proceedings of the National Academy of Sciences of the United States of America**, v.99, n. 25, p. 15904-15907, 2002.

SOOD, A.; KAUR, P.; GUPTA, R. Phytochemical Screening and Antimicrobial Assay of Various Seeds Extract of Cucurbitaceae Family. **International Journal of Applied Biology and Pharmaceutical Technology**, v. 3, n. 3, p. 401-409, 2012.

SOUSA, T.; BRANCO, R.; PIEDADE, A. P.; MORAIS, P. V. Hyper Accumulation of Arsenic in Mutants of *Ochrobactrum tritici* Silenced for Arsenite Efflux Pumps. **Plos One**, v. 10, n. 7, e0131317., 2015.

SOUZA, S. M.; AQUINO, L. C.; MILACH, A. C. Jr.; BANDEIRA, M. A.; NOBRE, M. E.; VIANA, G. S. Antiinflammatory and antiulcer properties of tannins from Myracrodruon urundeuva Allemão (Anacardiaceae) in rodents. **Phytother Res.**, v. 21, n. 3, 220-225, 2007.

SOUZA, DE ARAÚJO, R. M.; DA SILVA, F. R.; NAPOLEÃO, T. H.; CARNEIRO-DA-CUNHA, M. G.; COELHO, L. C. B. B.; CORREIA, M. T. S.; OLIVA, M. L. V.; PAIVA, P. M. G. *Crataeva tapia* bark lectin is an affinity adsorbent and insecticidal agent. **Plant Science**. v. 183, p. 20–26, 2012.

SPELLBERG, B.; GUIDOS, R.; GILBERT, D.; BRADLEY, J.; BOUCHER, H. W.; SCHELD, W. M.; BARTLETT, J. G.; EDWARDS, J. Jr.; INFECTIOUS DISEASES SOCIETY OF AMERICA. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. **Clinical Infectious Diseases**, v. 46, n. 2, p. 155-164, 2008.

SULAIMAN, S.; IBRAHIM, D.; KASSIM, J.; SHEH-HONG, L. Antimicrobial and antioxidant activities of condensed tannin from Rhizophora apiculata barks. **Journal of Chemical and Pharmaceutical Research**, v. 3, n. 4, p. 436-444, 2011.

SWAIN, S. S.; SAHU, M. C.; PADHY, R. N. In silico attempt for adduct agent(s) against malaria: combination of chloroquine with alkaloids of Adhatoda vasica. **Computer Methods and Programs in Biomedicine**, v. 122, n. 1, p. 16-25, 2015.

SWIFT, L. H.; GOLSTEYN, R. M. Genotoxic Anti-Cancer Agents and Their Relationship to DNA Damage, Mitosis, and Checkpoint Adaptation in Proliferating Cancer Cells. **International Journal of Molecular Sciences.** v. 15, n.3, p. 3403-3431, 2014.

TALUKDAR, S. N.; HOSSAIN, M. N. Phytochemical, Phytotherapeutical and Pharmacological Study of Momordica dioica. **Evidence-Based Complementary and Alternative Medicine**, v. 2014, Artigo ID 806082, doi:10.1155/2014/806082, 2014.

TAN, C.-P.; LU, Y-Y.; JI, L-N.; MAO, Z.-W. Metallomics insights into the programmed cell death induced by metal-based anticancer compounds. **Metallomics**, v. 6, n. 5, p. 978-995, 2014.

TANNIN-SPITZ, S.; GROSSMAN, S.; DOVRAT, H. E.; GOTTLIEB, M. Bergman,Growthinhibitory activity of cucurbitacin glucosides isolated from Citrullus colocynthison human breast cancer cells, **Biochemical Pharmacology**, v. 73, p. 56–67, 2007.

TANWAR, J.; DAS, S.; FATIMA, Z.; HAMEED, S. Multidrug Resistance: An Emerging Crisis. **Interdisciplinary Perspectives on Infectious Diseases**, v. 2014, Article ID 541340, 7 pages. doi:10.1155/2014/541340, 2014.

TAUR, D. J.; PATIL, R. Y. Some medicinal plants with antiasthmatic potential: a current status. **Asian Pacific Journal of Tropical Biomedicine**, v. 1, n. 5, p. 413-418, 2011.

TEIXEIRA, J.; GASPAR, A. GARRIDO, E. M.; GARRIDO, J. BORGES, F. hydroxycinnamic acid antioxidants: an electrochemical overview. **Biomed Research International**, v. 2013, artigo ID 251754, doi:10.1155/2013/251754, 2013.

TEOW, C. C.; TRUONG, V.-D.; MCFEETERS, R. F.; THOMPSON, R. L.; PECOTA, K. V.; G. YENCHO, C. Antioxidant activities, phenolic and b-carotene contents of sweet potato genotypes with varying flesh colours. **Food Chemistry**, v. 103, p. 829–838, 2007.

THOENNISSEN, N.H.; IWANSKI, G.B.; DOAN, N.B.; OKAMOTO, R.; LIN, P.; ABBASSI, S.; SONG, J. H.; YIN, D.; TOH, M.; XIE, W. D.; Said, J.W.; KOEFflER, H.P. Cucurbitacin B inducesapoptosis by inhibition of the JAK/STAT pathway and potentiates antiprolifer-ative effects of gemcitabine on pancreatic cancer cells. **Cancer Research**, v. 69, p. 5876–5884, 2009.

TIKOO, K.; SANE, M. S.; GUPTA, C. Tannic acid ameliorates doxorubicin-induced cardiotoxicity and potentiates its anti-cancer activity: potential role of tannins in cancer chemotherapy. **Toxicology and applied Pharmacology**, v. 251, n.3, p. 191-200, 2010.

TUPE, S.B.; PATIL, P. D.; THOKE, R. B.; APARADH, V. T. Phytochemical screening

in some cucurbitaceae members. **International Research Journal of Pharmaceutical and Applied Sciences**, v. 3, n. 1, p. 49-51, 2013.

VASCONCELOS, M. A.; ALVES, A. C.; CARNEIRO, R. F.; DIAS, A. H.; MARTINS, F.W.; CAJAZEIRAS, J. B.; NAGANO, C. S.; TEIXEIRA, E. H.; NASCIMENTO, K. S.; CAVADA, B. S. Purification and primary structure of a novel mannose-specific lectin from Centrolobium microchaete Mart seeds. **International Journal of Biologic Macromolecules**, v. 81, p. 600-607, 2015.

VASQUEZ, M. Z., Combining the in vivo comet and micronucleus assays: a practical approach to genotoxicity testing and data interpretation. **Mutagenesis**, v. 25, n. 2, p. 187-199, 2009.

VEIGA-JUNIOR, V. F.; PINTO, A. C.; MACIEL, M. A. M. Medicinal plants: safe cure? **Química Nova**, v. 28, n. 3, p. 519-528, 2005.

VENUGOPAL, D. V. R.; YARLA, N. S.; UMADEVI, P. Synthesis, of Novel Piperine Analogs of Dipeptidyl Boronic Acid as Antimicrobial and Anticancer Agents, **Medicinal Chemistry**, n. 4, p. 606-610, 2014.

VENUGOPALA, K.N.; RASHMI, V.; ODHAV B. Review on Natural Coumarin Lead Compounds for Their Pharmacological Activity. **BioMed Research International**, v. 2013, Artigo ID 963248, doi:10.1155/2013/963248, 2013.VIANA, G. S.; BANDEIRA, M. A., MATOS, F. J. Analgesic and antiinflammatory effects of chalcones isolated from Myracrodruon urundeuva allemão. **Phytomedicine**, v. 10, n. 2-3, p. 189-195, 2003.

VIANA, G.S.B., BANDEIRA, M.A.M., MOURA, L.C., SOUZA-FILHO, M.V.P., MATOS, F.J.A., RIBEIRO, R.A**.** Analgesic and anti-inflammatory effects of the tannin fraction from *Myracrodruon urundeuva* Fr. All. **Phytotherapy Research**,v. 11, n. 2, p. 118-122, 1997.

VILAR, J. C.; CARVALHO, C. M.; FURTADO, M. F. D. Effects Of The Aqueous Extracts Of Plants Of The Genera Apodanthera (Cucurbitaceae) And Jatropha (Euphorbiaceae) On The Lethality Of The Venom Of Bothrops jararaca (Serpentes, Viperidae). **Biologia Geral e Experimental**, v. 7, n. 2, p. 32-39, 2007.

VITALE, I.; GALLUZZI, L.; CASTEDO, MARIA.; KROEMER, G. Mitotic catastrophe: a mechanism for avoiding genomic instability. **Nature Reviews Molecular Cell Biology**, v. 12, p. 385-392, 2011.

WANG, L.; SUN, J.; HORVAT, M.; KOUTALISTRAS, N.; JOHNSTON, B.; SHEIL, A. G. R. Evaluation of MTS, XTT, MTT and ^{3}HTdR incorporation for assessing hepatocyte density, viability and proliferation. **Methods in Cell Science**, v. 18, n. 3, p. 249-255, 1996.

WANG, H.-M.; LIAO, C.-T.; CHANG, T.-C. J.; CHEN, J.-S.; LIAW, C.-C.; CHEN, I.-H.; TSANG, N.-M. Biweekly paclitaxel, cisplatin, tegafur, and leucovorin as neoadjuvant chemotherapy for unresectable squamous cell carcinoma of the head and neck. **Cancer**, v. 101, n. 8, p. 1818–1823, 2004.

WANG, Q.; KUANG, H.; SU, Y.; SUN, Y.; FENG, J.; GUO, R.; CHAN, K. Naturally derived anti-inflammatory compounds from Chinese medicinal plants. **Journal of Ethnopharmacology**, v. 146, n. 1, p. 9-39, 2013.

WANG, B.; ZHANG, S.; YUE, K.; WANG, X.-D.. The recurrence and survival of oral squamous cell carcinoma: a report of 275 cases. **Chinese Journal of Cancer**, v. 32, n. 11, p. 614-618, 2013.

WARIS, G.; AHSAN, H. Reactive oxygen species: role in the development of cancer and various chronic conditions. **Journal of Carcinogenesis**, v. 5, n. 14, p. 1-8, 2006.

WROBLEWSKI, L.E.; PEEK JR, R.M.; WILSON, K. T. Helicobacter pylori and gastric cancer: factors that modulate disease risk. **Clinical Microbiology Reviews**, v. 23, n. 4, p. 713-139, 2010.

WU, Y.; HUANG, Y. X.; KANG, L. L.; WU, Z. J.; LUO, M. Effect of pH on molecular constitution and distribution of hemoglobin in living erythrocyte. **Biopolymers**, v. 93, n. 4, p. 348-354, 2010.

XIE, J.; SCHAICH, K. M. Re-evaluation of the 2,2-diphenyl-1-radical (DPPH) assay for antioxidant activity. Journal of Agricultural and Food Chemistry, v. 62, n. 19, p. 4251-4260, 2014.

XIU, Y.; HOU, L.; LIU, X.; WANG, Y.; GU, W.; MENG, Q.; WANG, W. Isolation and characterization of two novel C-type lectins from the oriental river prawn, Macrobrachium nipponense. **Fish Shellfish Immunology**, v. 46, n. 2, p. 603-611, 2015
YAGI, F.; SAKAI, T.; SHIRAISHI, N.; YOTSUMOTO, M.; MUKOYOSHI, R. Hemagglutinins (lectins) in fruit bodies of Japanese higher fungi. **Mycoscience**, v. 41, n. 4, p. 323-330, 2000.

YAN, Q.; LI, Y.; JIANG, Z.; SUN, Y.; ZHU, L.; DING, Z. Antiproliferation and apoptosis of human tumor cell lines by a lectin (AMML) of Astragalus mongholicus. **Phytomedicine**, v. 16, n. 6-7, p. 586-593, 2009.

YANG, Y; HERRUP, K; Cell division in the CNS: protective response or lethal event in post- mitotic neurons? **Biochimica at biophysica acta**, v. 1772, n. 4, p. 457-466, 2007.

ZAGHLOUL, M. S. Bladder cancer and schistosomiasis. **Journal of the Egyptian National Cancer Institute**, v. 24, n. 4, p. 151-159, 2012.

ZHANG, P.; SHAO, L.; SHI, Z.; ZHANG, Y.; DU, J.; CHENG, K.; YU, P. Pregnane alkaloids from *Sarcococca ruscifolia* and their cytotoxic activity. **Phytochemistry Letters**, v. 14, p. 31-34, 2015.

ZILFOU, J. T.; LOWE, S. W. Tumor suppressive functions of p53. **Cold Spring Harbor perspectives in biology**, v. 1, n. 5, a001883, 2009.

ZIMMERMAN, M. A.; HUANG, Q.; LI, F.; LIU, X.; LI, C.-Y. Cell death-stimulated cell proliferation: A tissue regeneration mechanism usurped by tumors during radiotherapy. **Seminars in Radiation Oncology,** v. 23, n.4, p. 288-295, 2013.

ZOHRA, M.; FAWZIA, A. Hemolytic activity of different herbal extracts used in Algeria. **International Journal of Pharma Sciences and Research**, v. 5, n. 8, p. 495-500, 2014.

Anexo A

NORMAS DO PERIÓDICO PHYTOMEDICINE

PHYTOMEDICINE
International Journal of Phytotherapy and Phytopharmacology

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

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DESCRIPTION

Phytomedicine is primarily a therapy-oriented Journal. *Phytomedicine* publishes innovative studies on efficacy, safety, quality and mechanisms of action of specified plant extracts, phytopharmaceuticals and their isolated constituents. This includes *clinical, pharmacological, pharmacokinetic, and toxicological studies* of specified herbal medicinal products, herbal preparations and purified compounds which have a defined and consistent quality assuring reproducible pharmacological activity.

Phytomedicine was founded in 1994 to focus and stimulate research in this particular field and to set internationally accepted scientific standards for pharmacological studies, proof of clinical efficacy and safety of phytomedicines.

The main aims of *Phytomedicine* are associated with the integration of phytopreparations into conventional/official medicine.

The journal covers the following sections:

Clinical pharmacology and toxicology (randomized, placebo controlled, double blind, and observational open label studies)Behavioural, mental, affective, and stress-associated disordersAge-associated disordersNeuropharmacology Endocrine pharmacology Metabolic syndrome and obesityCancerImmunopharmacology, inflammationInfectious diseasesPulmonary, gastrointestinal, cardiovascular and urogenital diseasesSystems biologySafety assessment, pre-clinical toxicology, interaction with drugs and adverse events of herbal preparations Pharmacokinetic of natural compoundsStandardization of herbal preparationsLegislation of botanicalsInvited reviews

The directions of *Phytomedicine* are known to provide profound scientific background in Herbal Medicinal Products, their reproducible Quality and evidence based therapeutic efficacy. Since then quality criteria and standardization methods were defined and the European Medical Agency has elaborated numerous guidelines for the conduction of clinical studies and preparation of Herbal Medicinal Products. In total 107 ESCOP monographs have been produced and submitted to EMA. Many new analytical methods and instruments were implemented both for analysis and standardization of herbal Substances, herbal preparations and their bioassays and tremendous work has been carried out to remain aligned with these intentions during the last 18 years.

Nowadays important topics remain to be approached, such as harmonization of the regulatory frameworks in Europe, America, Asia and Australia or the legislation of various "botanicals", where strict differentiation of requirements for health claims of herbal medicinal product, dietary supplements and nutraceuticals are required.

AUDIENCE

Pharmacologists, toxicologists, pharmacists, pharmacognosists, phytotherapists (clinicians), biochemists, botanists, general practitioners

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INTRODUCTION

PHYTOMEDICINE

International Journal of Phytotherapy and Phytopharmacology

Scope

Phytomedicine is primarily a therapy-oriented Journal, which publishes innovative studies on efficacy, safety, quality and mechanisms of action of specified plant extracts, phytopharmaceuticals and their purified constituents. This includes clinical and preclinical studies of properly standardized herbal medicinal products, herbal preparations and isolated compounds, which have reproducible pharmacological activity.

The journal covers the following sections: Trends in Phytopharmacology: innovative technologies and emerging concepts - Reviews Clinical pharmacology and toxicology Pre-clinical pharmacology and toxicology Mechanisms of action of herbal medicines and their active constituents Neuropharmacology Endocrine pharmacology Cancer Inflammation Infectious diseases Cardiovascular diseases Ageing associated disorders Quality of Herbal preparations/botanicals: adulteration, standardization, analysis Legislation of Herbal preparations/botanicals Current issues in Phytomedicine research (various topics which are not covered in all other volumes).

BEFORE YOU BEGIN

Article requirements

Please note the following requirements for consideration of an article, upon submitting your manuscript:

1. Is your article within the scope of Phytomedicine?
Your article must meet the scope of Phytomedicine (please see above). **Articles that are not in the scope, will be rejected immediately!** Articles on the isolation and structure elucidation of novel bioactive compounds or the development of new analytical methods do not fall into the scope of Phytomedicine. However, pharmacological and clinical studies of novel natural products, where new compounds or methods of analysis of active of pharmaceutical ingredients in herbal preparations and biological fluids and tissues are reported (e.g. in pharmacokinetic studies), are welcome. Dietary Supplements, "Botanicals" or "Functional Food" are not within the scope of Phytomedicine unless they are specified/standardized and pharmacologically investigated analogues to herbal drugs and if the evidence presented is comparable to therapeutic outcomes with a positive control. Studies on pure compounds are not accepted if their origin is not clearly related to the plant kingdom. Pharmacological studies of isolated compounds in various forms (salts, ethers, etc.), which do not exist in nature are out of scope of Phytomedicine. Screening results of a large number of plant extracts or plant constituents for pharmacological activities will not be considered unless they are focused on those plants or constituents which show superior activities in comparison with generally accepted positive (reference) compounds.

2. Does your article comply with the standard requirements of Phytomedicine?
Your article must meet the criteria assuring reproducible quality and efficacy of herbal preparations.
Plant name and herbal substance
Latin binomial name and the author, local name and English name and plant part(s) used must by specified for all plants used in the study. It should be stated that the plant name has been checked with http://www.theplantlist.org. The authentication of fresh plants or dried herbal drugs, including those of formulas, must be carried out by means of macroscopic and/or microscopic, molecular biological, chemical, chromatographic and/or other suitable pharmacognostic methods. Voucher specimens of plant materials used for all studies must be deposited and identified with a voucher number, the date and location of collection. The plant material may derive from natural origin, from cultivated plants, or from an herbal drug market. In case of commercially procured material the source, batch number, and quality control data should be specified. All scientific names of the plants must be written in italics through the whole manuscript! Herbal medicinal products and herbal extracts
Herbal medicinal products or herbal preparations must be declared in accordance to EMA guidelines. In particular, herbal extracts must be clearly and comprehensively described with respect to the plant part used, the drug extract ratio, type and concentration of extraction solvent, extraction

conditions etc. They must be sufficiently characterized (e.g. by HPLC fingerprints) and specified for the content of marker compounds to ensure a consistent quality and reproducible pharmacological activity. The choice of marker must be justified. The analytical methods have to be validated for selectivity, accuracy and precision and briefly described, providing the most important information necessary to obtain reproducible results. Traditional and commercial names of herbal preparations should be mentioned in the Introduction of the manuscript, but not in the title. Phytomedicine accepts only international standard terminology – binomial Latin names of the plants and their combinations.
Herbal combinations
Studies with herbal drug combinations (e.g. 2-5 plants) will be accepted only if each herbal drug undergo the same authentication and standardization process as described above, each single herbal extracts is HPLC fingerprinted and relevant marker constituents are quantified before and after the extracts are mixed. A 3-D-HPLC-profile of the multiherbal drug combination must be provided. Authors must clearly demonstrate which analytical marker specifically indicates on the presence each of herbal ingredients in the combination.Additionally, we encourage the use other relevant and validated physiological, biological, or biochemical methods, which ensure reproducible pharmacological activity of multi-herbal drug combinations.
Chemicals, phytochemicals and other purified compounds For purified compounds, please provide chemical names using relevant information from the NCBI PubChem which can be found on the website http://www.ncbi.nlm.nih.gov/pccompound. In studies with purified compounds the evidences of their purity (13C NMR or HPLC peak purity test) are required.

Gene nomenclature Authors should use approved nomenclature for gene symbols. Please consult the appropriate nomenclature data bases for correct gene names and symbols. "Entrez Gene" is a useful resource. Approved human gene symbols are provided by HUGO Gene Nomenclature committee (HGNC): http://www.gene.ucl.ac.uk/nomenclature Approved Mouse symbols are provided by The Jackson Laboratory: http://www.informatics.jaxorg/mgihome/nomen
Approved C. elegans symbols are provided by Caenorhabditis Genetics Center: http://www.cbs.unmn.edu/CGC/Nomenclature/no menguid.htm For approved S. cerevisiae and S. pombe symbols see http://yeastgenome.org/help/yeastGeneNomenclature.shtml and http://www.sanger.ac.uk/Projects/S_pombe/SP_Name_FAQ.shtml, respectively Statistical analysis
Statistical hypothesis and methods should be described in detail. Actual P values should be used unless less than 0.001. Reporting of 95% confidence intervals is encouraged. The choice of appropriate parametric or nonparametric tools has to be justified. Refer to B.S. Evererett. Statistical Methods for Medica Investigations, Oxford University Press, New York, 1989.

3. Is your article approaching new findings?
Scientific novelty of your study must be clearly demonstrated. The articles limited with a repetition of well-known data or identification of only well-known ubiquitous compounds with little or no relation to activity are not acceptable.

4. Is your article relevant to clinical medicine?
Your article must be based on a thorough study, using proper controls and convincing evidences of therapeutic significance and observations.

Not acceptable are: In vitro studies with concentrations of active compounds, which could not be implemented in-vivo and that are not appropriate for further pharmaceutical development. In vitro studies without results on organs, tissues, fluids or cells. In vitro studies without positive control. In vivo single dose studies or studies with one set of experiments and few animals. Studies on antimicrobial activity with only single dose, very high concentration, measuring only inhibition zones without MIC values, without information on type of activity (or growth inhibition) or microorganisms investigation. Pharmacological studies of pure compounds, which are not supported by evidences on pharmacological activity of plant extract where it was identified.

5. Does your article meet the requirements to clinical and pharmacological studies?
Your article must comply with the basic criteria for conducting and reporting clinical and pharmacological studies.

Requirements for clinical studies: Clinical studies must meet the current standards for clinical trials (GCP = Good Clinical Practice), which are equivalent to those required for synthetic drugs. http://www.fda.gov/downloads/Drugs/Guidances/ucm073122.pdf, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002832.pdf
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of Reporting Trials Statement for Herbal Medicinal Interventions (CONSORT), particularly when it comes to description of study medication, which is a strict requirement of acceptance for Phytomedicine. For guidelines and necessary information, please use the following internet addresses: http://www.consort-statement.org, http://www.consort-statement.org/extensions?ContentWidgetId=557, http://www.consort-statement.org/Media/Default/Downloads/Extensions/CONSORTHerbalInterventions.pdf, http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003370.pdf. Use of a CONSORT checklist and flow diagram is recommended for illustration of grouping and flow of patients in all clinical studies, randomized clinical trials as well as other trials. Clinical studies must be approved by an Institutional Ethics Committee or its equivalent. The Methods section must state that the study followed the guidelines of the Declaration of Helsinki and Tokyo for humans!

Requirements for pharmacological studies (in vitro, ex vivo or in vivo): Investigations with animals must state in the Methods section that the research was conducted in accordance with internationally accepted principles for laboratory animal use and care (e.g. European community guidelines/ EEC Directive of 1986 or the US guidelines/ NIH publication). The route of drug administration, different of oral, must be justified. Appropriate and justified statistical methods must be used.Positive controls (reference standards must be included in study design). Many natural compounds are known for their polyvalent (pleiotropic) activities and are only of interest if one or two pharmacological activities are dominant and somehow superior in comparison with generally accepted reference standards/compounds. Their potential therapeutic application must be justified for specified indication. Antimicrobial evaluation of plants are of scientific value only if these plant extracts show superior biological activities in comparison with a synthetic or natural antimicrobial agent standard. It is preferred that in vitro activity (MIC) of an extract in not higher than 100 ig/ ml. For the correct determination of MIC values, standardized methodologies such as those of CLSI or EUCAST are preferred. All articles that are reporting gene expression profiling data (microarray experiments) should comply with the Minimum Information about Microarray Experiments (MIAME, http://www.mged.org/Workgroups/MIAME/miame.html). At least two microarrays should be provided for each experimental condition. Results of selected genes should be validated by a second method (e.g. RT-PCR) or protein data should be provided. In addition functional test (animal experiments/ clinical data) undertaken simultaneously are desirable to allow an appraisement of the biological/ clinical relevance of the data. Alternatively, results of in vivo experiments with comparable dosages can be discussed. The presentation of a sole data collection is not acceptable. Biologically relevant information should be presented. We recommend do not overuse specific names, notions and terms from various theories of traditional medical systems (e.g. TCM, Ayurveda, etc.). That makes articles difficult for perceptions and understanding. The essence of these theories should be translated into internationally accepted scientific theories, while traditional names and terms should be converted to English. Final interpretation of the results of the study must adhere to conventional scientific theories.

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