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JEAN CARLOS VENCIONECK DUTRA

CARACTERIZAÇÃO FISIOLÓGICA, FITOQUÍMICA E DE ATIVIDADES BIOLÓGICAS DE PLANTAS MEDICINAIS COM POTENCIAL PARA PRODUÇÃO DE FITOTERÁPICOS

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Centro de Ciências Humanas e Naturais da Universidade Federal do Espírito Santo como parte dos requisitos exigidos para a obtenção do título de Doutor em Biologia Vegetal.

Área de concentração: Fisiologia Vegetal.

Orientador(a): Prof.ª. Dr.ª Maria do Carmo Pimentel Batitucci

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CARACTERIZAÇÃO FISIOLÓGICA, FITOQUÍMICA E DE ATIVIDADES BIOLÓGICAS DE PLANTAS MEDICINAIS COM POTENCIAL ECONÔMICO PARA PRODUÇÃO DE FITOTERÁPICOS

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Aprovada em 25 de fevereiro de 2019.

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RESUMO

Plantas da Família Cactaceae e Lamiaceae são usadas pela população como alimento e na medicina tradicional para tratar ou prevenir doenças. O objetivo deste trabalho foi avaliar as atividades antioxidantes, citotóxicas, anti-citotóxicas, antiproliferativas e a composição química de extratos vegetais de *Cereus jamacaru* (Cactaceae) e *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Ocimum selloi*, *Plectranthus amboinicus, Plecthanthus amboinicus* "variegata" e *Plectranthus ornatus* (Lamiaceae), relacionado os resultados obtidos aos aspectos fenológicos ou variações entre espécies. O fruto e cladódios de *Cereus jamacaru* (Cactaceae) foram coletados em estádio vegetativo e frutificação e a parte aérea total de *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Ocimum selloi*, *Plectranthus amboinicus, Plecthanthus amboinicus* "variegata" e *Plectranthus ornatus* (Lamiaceae) foram coletados para produção de extratos hidroalcoólicos. Os extratos foram avaliados por ensaios de fitoquímica preliminar; o teor total de flavonoides e ácido rosmarínico foram determinados por técnicas espectrofotométricas e HPLC, respetivamente; a atividade antioxidante foi avaliada pelos ensaios DPPH, ABTS, atividade quelante do íon Fe^{2+} e pelo sistema β-caroteno/ácido linoleico; citotoxicidade e anti-citotoxicidade contra cisplatina em linfócitos humanos e efeito antiproliferativo em células de sarcoma 180 *in vitro* pelo ensaio MTT; e atividade antitumoral e avaliação da mutagenicidade *in vivo*. Os cladódios de *C. jamacaru* foram capazes de inibir danos citotóxicos induzidos pela cisplatina, o fruto e cladódios foram eficazes na promoção de atividade anticâncer *in vitro* e *in vivo*, induzindo significante redução tumoral em roedores sarcoma-induzidos. A investigação da variegação foliar em *Plecthanthus amboinicus* demonstrou que a variegação, além de alterações morfológicas, leva a alterações na composição química e atividades biológicas exibidas por esta planta, conduzindo ao aumento do teor total de flavonoides e ácido rosmarínico, como também do efeito anticâncer *in vitro* de *Plectranthus*

amboinicus “variegata”. A comparação entre as demais plantas da Família Lamiaceae estudadas sugere que a biossíntese de ácido rosmarínico ocorra em detrimento da síntese de flavonoides, como também sugere que *Melissa officinalis* e *Mentha piperita*, espécies vegetais com maior teor de ácido rosmarínico neste estudo, sejam mais promissoras como antioxidantes naturais usados para prevenção de danos citotóxicos e como agentes anticâncer. Esses resultados reforçam o uso dessas plantas tanto como alimento quanto na medicina tradicional e fornecem informações relevantes para o desenvolvimento de novos fármacos.

Palavras-chave: Cactaceae • Lamiaceae • anti-citoxicidade • sarcoma 180 anticâncer • ensaio MTT.

ABSTRACT

Plants of the Cactaceae and Lamiaceae Family are used by the population as food and in traditional medicine to treat or prevent diseases. The objective of this work was to evaluate the chemical composition, antioxidant, cytotoxic, anti-cytotoxic and antiproliferative activities of plant extracts of one plant of the Cactaceae Family and seven plants of the Lamiaceae Family, relating the results obtained to the phenological aspects or variations between species. The fruit and cladodes of *Cereus jamacaru* (Cactaceae) were collected at vegetative and fruiting stage and the total aerial part of *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Ocimum selloi*, *Plecthanthus amboinicus*, *Plectranthus amboinicus* "variegata" and *Plectranthus ornatus* (Lamiaceae) were collected for the production of hydroalcoholic extracts. Extracts were evaluated by preliminary phytochemical tests; the total content of flavonoids and rosmarinic acid were determined by spectrophotometric and HPLC techniques, respectively; the antioxidant activity was evaluated by the DPPH, ABTS, Fe^{2+} ion chelation activity and the β-carotene/linoleic acid system; cytotoxicity and anti-cytotoxicity against cisplatin in human lymphocytes and antiproliferative effects on sarcoma cells 180 *in vitro* by the MTT assay; and antitumor activity and evaluation of mutagenicity *in vivo*. The fruit and cladodes of *C. jamacaru* were effective in promoting anticancer activity *in vitro,* and cladodes were able to inhibit cytotoxic damage induced by cisplatin *in vitro* and induced significant tumor reduction in sarcoma-induced rodents *in vivo*. Investigation on leaf variegation in *Plecthanthus amboinicus* showed that the variegation, in addition to morphological changes, leads to changes in the chemical composition and biological activity exhibited by this plant, leading to an increase in total flavonoid and rosmarinic acid content, as well as increased *in vitro* anticancer effects of *Plectranthus amboinicus* "variegata". The comparison between the other plants of the Lamiaceae Family studied suggests that rosmarinic acid biosynthesis

occurs in detriment of flavonoid synthesis, and also suggests that *Melissa officinalis* and *Mentha piperita*, plant species with higher rosmarinic acid contente in this study, are more promising as natural antioxidants used to prevent cytotoxic damage and as anticancer agents. These results reinforce the use of these plants as much as food as in traditional medicine and provide information relevant to the development of new drugs.

Keywords: Cactaceae • Lamiaceae • anti-cytotoxicity • sarcoma 180 anticancer • MTT assay.

LISTA DE FIGURAS

LISTA DE TABELAS

SUMÁRIO

INTRODUÇÃO GERAL

A utilização de plantas para tratar doenças é uma das práticas mais antigas realizadas pelo homem e, frente à relevância das plantas medicinais para promoção de saúde, inúmeros estudos vêm sendo conduzidos com o objetivo de investigar suas atividades biológicas, eficácia e aplicações terapêuticas.

Os ativos vegetais, substâncias responsáveis pelas atividades biológicas de plantas consumidas pela população, são compostos oriundos do metabolismo secundário vegetal e conferem às plantas proteção contra herbivoria e resistência a estresses, sendo esses ativos de grande relevância para produção de fitoterápicos e desenvolvimento de novos fármacos.

Os metabólitos secundários são sintetizados por enzimas específicas e podem variar quali e quantitativamente em grupos vitais distintos ou dentro de uma mesma espécie. Essas variações ocorrem, por exemplo, devido variações genéticas e ambientais, estádio fenológico e condições de cultivo, que atuam como moduladores das vias biossintéticas desses compostos.

O teor de metabólitos secundários em plantas usadas na medicina tradicional ou na produção de fármacos interfere diretamente sobre as atividades biológicas exibidas por eles, tais como as atividades antioxidante, antimicrobiana e anticâncer. Desta forma, para assegurar tanto a eficácia quanto a qualidade de plantas medicinais e derivados, há a constante necessidade de se buscar espécies e/ou variedade vegetais que exibam melhores atividades biológicas.

Nesta perspectiva, os estudos fitoquímicos em conjunto aos ensaios de atividade biológica fornecem uma gama de informações indispensáveis à descoberta de novos ativos vegetais, triagem de plantas com potencial interesse farmacêutico e para trazer novas perspectivas de uso/aplicação para metabólitos vegetais já conhecidos.

REVISÃO BIBLIOGRÁFICA

1.1. PLANTAS MEDICINAIS

1.1.1. Uso tradicional de plantas e fitoterápicos

O uso de plantas para o tratamento de doenças é uma prática antiga e o conhecimento da aplicação medicinal de espécies vegetais configura-se, muitas vezes, como o único recurso terapêutico disponível para determinadas comunidades e/ou grupos étnicos. Desta forma, devido à aplicação terapêutica de certas espécies vegetais, muitas plantas medicinais são comumente comercializadas em feiras livres e mercados ou são cultivadas por populares (MACIEL, PINTO, VEIGA, 2012).

Em decorrência do desenvolvimento da medicina ocidental, houve acentuado declínio do uso de plantas medicinais, principalmente, na Europa e na América do Norte (EVANS, 1997). Contudo, mesmo com a expansão da medicina alopática a partir da segunda metade do século XX, o uso de plantas medicinais voltou a crescer (JÚNIOR; PINTO; MACIEL, 2005).

Nos últimos anos, aplicação de plantas para promoção de saúde tem evoluído, com usos que incluem desde formas mais simples, como tratamento tópico, até aqueles que envolvem o beneficiamento do material vegetal por indústrias (LORENZI; MATOS, 2002). Em contrapartida, o uso de plantas com finalidade terapêutica sem respaldo científico configura-se como uma prática perigosa e um entrave a implantação da fitoterapia, recurso terapêutico eficaz usado para o atendimento de algumas necessidades básicas de serviços de saúde (BERTOLUCCI; CAPPELLE; PINHEIRO, 2001).

O aumento da procura por drogas vegetais está relacionado à maior facilidade de acesso e a crença popular de que produtos naturais não promovam danos à saúde (RATES, 2001). Somado a isso, discute-se sobre os benefícios advindos do consumo de drogas vegetais ao invés de medicamentos sintéticos, como a

existência de poucas contraindicações e reduzidos efeitos colaterais e prejuízos à saúde (LORENZI; MATOS, 2002).

A Organização Mundial de Saúde (OMS) estima que aproximadamente 80% da população mundial fazem ou já fizeram uso de plantas medicinais para tratamento de doenças, sendo a maior parcela desta população composta por pessoas que acreditam na ausência de efeitos colaterais/tóxicos (CARNEIRO et al., 2014). A OMS também estima que aproximadamente 80% das pessoas de países em desenvolvimento utilizam medicamentos à base de produtos naturais para o tratamento de doenças (FONSECA; PEREIRA, 2013), o que demonstra a relevância dos produtos naturais para promoção de saúde das populações de baixa renda.

A ANVISA (Agência Nacional de Vigilância Sanitária) define droga vegetal como sendo a própria planta medicinal ou partes dela após os processos de coleta, estabilização e secagem, disponibilizada para uso na forma pulverizada, triturada, rasurada ou íntegra. Já o fitoterápico é definido como medicamento produzido exclusivamente com derivados de drogas vegetais, sem que haja em sua composição a adição de substâncias isoladas. Além disso, os medicamentos fitoterápicos são caracterizados quanto à eficácia de uso, riscos advindos do consumo e apresentam reprodutibilidade e constância de sua qualidade (BRASIL, 2010 – RDC 10, OLIVEIRA, et al., 2016).

A crescente aceitação da fitoterapia pelos profissionais de saúde da atenção básica é, em parte, fruto de incentivos realizados pela OMS desde 1978, por meio do investimento em políticas públicas para o desenvolvimento de fármacos à base de plantas medicinais (HOMAR, 2005). Mesmo com os investimentos já realizados para produção de plantas medicinais e derivados, o uso de plantas medicinais no Brasil baseia-se majoritariamente na coleta de plantas em seu local de ocorrência natural, o que pode levar à grande variabilidade química, comprometendo sua

eficácia, como também pode conduzir ao desaparecimento de espécies vegetais de uma determinada região (MING et al., 2003).

Frente ao aumento da comercialização e a utilização de fármacos à base de plantas medicinais, nos últimos anos, tem sido observado o gradual desenvolvimento da indústria de fitoterápicos e sua distribuição pelo Sistema único de Saúde (SUS). Na 8ª Conferência Nacional de Saúde, que ocorreu em Brasília em 1986, foram colocados em pauta temas relacionados à saúde da população, como o uso de plantas medicinais na atenção básica e a necessidade de introduzir tais práticas ao tratamento público prestado pelo SUS, e assim aprimorar e valorizar as práticas tradicionais de tratamento de doenças (ROSA; CÂMARA; BÉRIA, 2011).

Desta forma, com o crescente uso das práticas tradicionais para promoção de saúde, foi implementada a Política Nacional de Plantas Medicinais e Fitoterápicos (PNPMF), que entrou em vigor com o decreto Nº 5.813, de 22 de junho de 2006. Tal decreto versa, dentre outros pontos, acerca da necessidade de uniformizar a qualidade química e eficácia de plantas e medicamentos fitoterápicos utilizados pela população, o que exige o controle desde o cultivo das plantas usadas como matéria prima até à sua dispensação, ou seja, versa sobre a necessidade de desenvolver métodos adequados para produção de fitoterápicos e seu controle de qualidade (BRASIL, 2006; MATSUCHITA; MATSUCHITA, 2015). Além disso, a PNPMF objetiva garantir aos brasileiros o uso seguro e racional de plantas medicinais e fitoterápicos, de modo que estes sejam obtidos e produzidos de modo sustentável, reduzindo impactos à biodiversidade (ANTONIO, 2013).

1.1.2. Seleção de plantas medicinais

O aumento do uso de plantas para o tratamento de doenças está muitas vezes atrelado a fatores sociais e econômicos, como o custo relativamente alto de

fármacos industrializados e a ausência de assistência médica para população (BELTRÁN et al., 2014).

A qualidade e eficácia dos medicamentos fitoterápicos estão diretamente relacionadas aos compostos produzidos pelas plantas usadas como matéria prima. Somado a isso, as variações nos teores e nos tipos de ativos encontrados nos vegetais podem ocorrer devido oscilações nas condições ambientais em que as plantas são crescidas, como temperatura, precipitação, sazonalidade, solo, assim como podem oscilar devido ao estádio de desenvolvimento em que as plantas são coletadas (TAIZ e ZEIGER, 2017).

A divulgação das aplicações terapêuticas de vegetais ocorre, principalmente, a partir das observações do uso e eficácia de tratamento, sendo prescritas pelos efeitos medicinais que apresentam, mesmo sem serem conhecidos os constituintes químicos destas plantas e seus derivados. Então, frente à eficácia dos produtos naturais para o tratamento de doenças, usuários de plantas medicinais e derivados mantém em voga a prática de consumo de fitoterápicos, além de incitar, de maneira indireta, o interesse de pesquisadores sobre os aspectos botânicos, farmacológicos e fitoquímicos dos recursos vegetais (MACIEL, PINTO, VEIGA, 2012).

Estudos etnobotânicos sugerem diversos possíveis meios de seleção de espécies vegetais usadas medicinalmente (ELISABETSKY, MORAES, 1988; CORDELL, 1990; CORDELL, BEECHER, PEZZUTO, 1991; CORDELL, 1993, CORDELL ET AL., 1993; COX, BALICK, 1994; HOLLAND, 1994; BRUHNS, 1994). Dentre os possíveis meios de seleção destacam-se:

1. Abordagem randômica: em que a escolha da planta ocorreria sem qualquer critério e que apresenta como fator determinante a disponibilidade do recurso vegetal;

2. Abordagem quimiotaxonômica ou filogenética: na qual a seleção das plantas estaria relacionada à ocorrência de uma classe química específica;
3. Abordagem etnofarmacológica: em que a seleção das ervas medicinais ocorreria devido à constatação do uso terapêutico por determinado grupo étnico.

No que tange a descoberta de novas substâncias bioativas de interesse farmacêutico, a abordagem de seleção etnofarmacológica é a mais promissora, pois o histórico de usos e eficiência terapêutica das espécies vegetais gera economia de tempo e dinheiro para seleção de plantas com potencial para o desenvolvimento de novos fármacos (MACIEL, PINTO, VEIGA, 2012). A etnofarmacologia, área da ciência que investiga as interações das pessoas com as plantas medicinais, pode auxiliar na triagem de recursos vegetais com aplicação terapêutica e potencialmente usados para desenvolvimento de novos medicamentos (YEUNG; HEINRICH; ATANASOV, 2018).

Esta área do conhecimento, ao gerar informações acerca de vegetais usados para fins medicinais, pode auxiliar na promoção de melhores condições de saúde para população e no desenvolvimento práticas de uso racional de plantas medicinais (CHELLAPPANDIAN et al., 2018). Do mesmo modo que, estudos multidisciplinares, que interliguem à etnofarmacologia à áreas como etnobotânica, química e farmacologia, auxiliam na validação do uso de uma planta como medicinal ou não (MACIEL, PINTO, VEIGA, 2012).

A priori, antes de definir os locais de coleta e aspectos a serem avaliados, se faz necessária extensa revisão de literatura. Esta deve ser conduzida de maneira objetiva, por meio da coleta de dados de investigações completas ou parciais já realizadas com a planta de interesse, que reforçarão, além das aplicações terapêuticas, a necessidade de novas investigações científicas. Subsequentemente, será definida a origem do material vegetal a ser investigado, o qual pode ser oriundo de viveiros ou de coletas conduzidas em ambientes de

ocorrência natural, como floresta amazônica, cerrado, mata atlântica, pantanal, caatinga e manguezal. Cabe ressaltar que, durante o processo de revisão de literatura sobre a planta alvo, recomenda-se que as buscas sejam sistematizadas, de maneira a obter informações acerca do gênero, família e classes de substâncias químicas predominantes (MACIEL, PINTO, VEIGA, 2012).

No que tange à seleção de plantas medicinais e aos compostos por elas sintetizados, já é reportado em literatura que flutuações nos teores de metabólitos secundários podem ocorrer devido a fatores genéticos e ambientais, assim como devido ao estádio fenológico e ao órgão vegetal estudado, sendo, portanto, fatores relevantes para investigações científicas (OLIVEIRA et al., 2018; PRETTI et al., 2018; SANTOS et al., 2018). Mesmo dentro de um mesmo país, plantas crescidas em diferentes regiões podem apresentar modificações em sua composição química em decorrência de variações ambientais, como fertilidade do solo, umidade, radiação solar, vento, temperatura, herbivoria, poluição atmosférica e poluição do solo. Assim como, a idade da planta e período em que ocorre a coleta podem ser fatores em que conduzem a modificações nas rotas biossintéticas dos metabólitos secundários, e, por conseguinte, sejam capazes de alterar a qualidade e eficácia de produtos naturais de origem vegetal (MACIEL, PINTO, VEIGA, 2012).

1.1.3. Etapas da coleta de material vegetal

De acordo com Maciel e colaboradores (2012), ao final do levantamento bibliográfico, estabelecimento da planta alvo e local de coleta, deve ser elaborado um projeto de pesquisa que contemple, dentre outros pontos, os principais objetivos, metodologias e metas a serem alcançadas, e somente após isso é iniciado o processo de coleta de material vegetal.

Recomenda-se que a coleta da planta alvo seja conduzida em duas etapas. A primeira etapa consiste em uma coleta prévia, em que a planta alvo é coletada e

seguramente identificada, sendo indispensável a participação de pessoal especializado, como taxonomistas vegetais (botânicos ou técnicos especializados). Subsequentemente,é realizada a segunda etapa de coleta, a coleta definitiva do material vegetal, em que é coleto material vegetal em grandes quantidades, destinado a estudos fitoquímicos e/ou farmacológicos (MACIEL, PINTO, VEIGA, 2012).

Ainda segundo Maciel et al. (2012), a coleta de plantas destinada aos estudos fitoquímicos e/ou farmacológicos pode ser feita em uma única ou várias coletas; optando-se por mais de uma coleta quando há a necessidade de coletas em diferentes épocas do ano ou diferentes localidades. Nesta etapa de estudo, por meio de triagem fitoquímica e de atividades biológicas, selecionam-se quais órgãos da planta serão investigados, como por exemplo, raiz, caule, galhos, folhas, flores e frutos, assim como é determinada a quantidade de material vegetal necessária para condução de experimentos. O processo de seleção da planta medicinal e das partes do vegetal a serem estudados deve, por sua vez, favorecer a integração dos estudos fitoquímicos e atividades farmacológicas, considerando, sempre que possível, as informações disponíveis sobre o uso tradicional da planta alvo e informações etnofarmacológicas.

1.1.4. Desenvolvimento de novos fármacos

As plantas medicinais, além de serem usadas na medicina popular para o tratamento de doenças, têm sido usadas ao longo dos anos para obtenção de diversos fármacos que são amplamente utilizados em tratamentos clínicos, tais como a emetina, vincristina, colchicina e rutina (CECHINEL FILHO, 1998). Somado a isso, mesmo com o desenvolvimento da síntese de moléculas orgânicas e dos processos biotecnológicos, em países industrializados mais de 90 espécies vegetais são usadas para produção de fármacos e aproximadamente

25% dos medicamentos prescritos são derivados do beneficiamento de plantas (LOZOYA, 1997).

No que diz respeito à terapia anticâncer, é comum aliar tratamento radioterápico e a quimioterapia às práticas cirúrgicas de retirada de tumores (COSTA-LOTUFO et al., 2010). Contudo, muitos tumores respondem de forma moderada aos protocolos clínicos já estabelecidos, o que limita tanto a indicação quanto a eficácia do tratamento adjuvante para tumores primários e metástases (Kummar et al., 2004). Sendo assim, faz-se necessário encontrar, desenvolver e introduzir alternativas terapêuticas mais eficientes, que auxiliem no controle e eliminação de células neoplásicas (COSTA-LOTUFO et al., 2010).

No século XX foram observados grandes avanços na pesquisa de produtos naturais, o que culminou na descoberta de vários compostos usados principalmente na terapia antineoplásica (COSTA-LOTUFO et al., 2010). Tendo em vista a eficácia do uso de produtos naturais como alternativas clínicas para o combate ao câncer, cerca de 60% dos fármacos usados na clínica são originários de produtos naturais (NEWMAN, CRAGG, 2007; BUTLER, 2008; HARVEY, Alan, 2008) e movimentam anualmente um mercado aproximadamente 60 bilhões de dólares (PINTO et al., 2002). Dentre os compostos mais comumente usados na terapia antineoplásica pode-se destacar a vimblastina, vincristina, vindesina, vinorelbina, paclitaxel, docetaxel, podofilotoxina, etoposídeo, teniposídeo, camptotecina, topotecano e irinotecano (PINTO et al., 2002).

Ensaios preliminares utilizando extratos vegetais viabilizam conhecer melhor a composição química da planta alvo e podem indicar a natureza química dos fitocompostos, facilitando a escolha de possíveis técnicas de isolamento de compostos e a seleção de técnicas para avaliar as atividades biológicas. Por intermédio de testes analíticos, pode-se detectar em extratos vegetais compostos como ácidos graxos, terpenóides, esteroides, fenóis, alcaloides, cumarinas e

flavonoides, que devem ser posteriormente analisados e catalogados (MACIEL, PINTO, VEIGA, 2012).

Mesmo com os inúmeros avanços biotecnológicos e técnicas de análises químicas, o isolamento de ativos vegetais e a compreensão de seus mecanismos de ação são considerados grandes desafios da química farmacêutica, bioquímica e farmacologia (GEBHARDT, 2000). Os extratos vegetais são formados por diversos compostos, de classes ou estruturas diferentes, que podem contribuir para uma mesma atividade biológica, e assim levar a efeitos sinérgicos. Assim, considerando a complexidade química dos extratos vegetais, se faz necessário selecionar bioensaios adequados para detecção de efeitos específicos, de maneira que as técnicas adotadas sejam de simples execução, sensíveis e reprodutíveis.

1.1.5. Uso de plantas medicinais como agentes anticâncer

Apesar de o câncer causar mais mortes do que todas as doenças coronarianas ou todos os tipos de acidentes vasculares cerebrais, registrando 14,1 milhões de novos casos e 8,2 milhões de mortes, em 2012 (FERLAY, SOERJOMATARAM, 2015). Além disso, as taxas de incidência e morte de vários tipos de câncer estão aumentando, como é o caso dos cânceres fígado e pâncreas (SIEGEL, MILLER, JEMAL, 2016). Em países de baixa e média renda per capta a incidência do câncer é mais acentuada, de modo que, aproximadamente, 70% das mortes são em decorrência de câncer, e apenas um em cada cinco países de baixa renda possui infraestrutura necessária para impulsionar a política de combate e prevenção ao câncer (WHO, 2017; ADELOYE et al., 2016).

Desta forma, o avanço da luta contra o câncer requer um aumento do investimento em pesquisa acerca da patologia do câncer e o desenvolvimento de alternativas terapêuticas, como a utilização de novos agentes anticâncer seguros, eficazes,

baratos e de efeito colateral mínimo (SECA, PINTO, 2018). As plantas, por sua vez, emergem como uma alternativa comum para o tratamento do câncer em muitos países, já que mais de 3000 plantas em todo o mundo têm propriedades anticancerígenas e, normalmente. apresentam efeitos colaterais mais brandos. Entre os produtos naturais usados clinicamente no tratamento de neoplasias estão compostos como vinblastina e vincristina, taxol e podofilotoxinas ou epipodofilotoxinas (KANTARJIAN, O'BRIEN, CORTES, 2013; CHANG, MENG ET AL., 2017; LÜ, WANG, 2014).

1.2. FISIOLOGIA MOLECULAR DE PLANTAS

A Fisiologia Vegetal é um ramo da Botânica que investiga os *processos* vegetais, relacionados às funções vitais das plantas, como por exemplo, o crescimento, desenvolvimento e mecanismos de resposta às interações dos vegetais com ambiente. Somado a isso, entende-se que os processos fisiológicos de uma planta abarcam todas as transformações fisico-químicas que ocorrem no interior da célula ou organismo (CORRÊA, 2009; TAIZ et al., 2017) e, nesta perspectiva, a Fisiologia Molecular, por meio do uso de ferramentas para análise genômica, metabolômica, genética molecular e bioquímica, configura-se como área de estudo da Botânica que visa identificar genes que participam do metabolismo primário e secundário vegetal, como também visa caracterizar a composição química dos vegetais, relacionando-a aos aspectos fisiológicos.

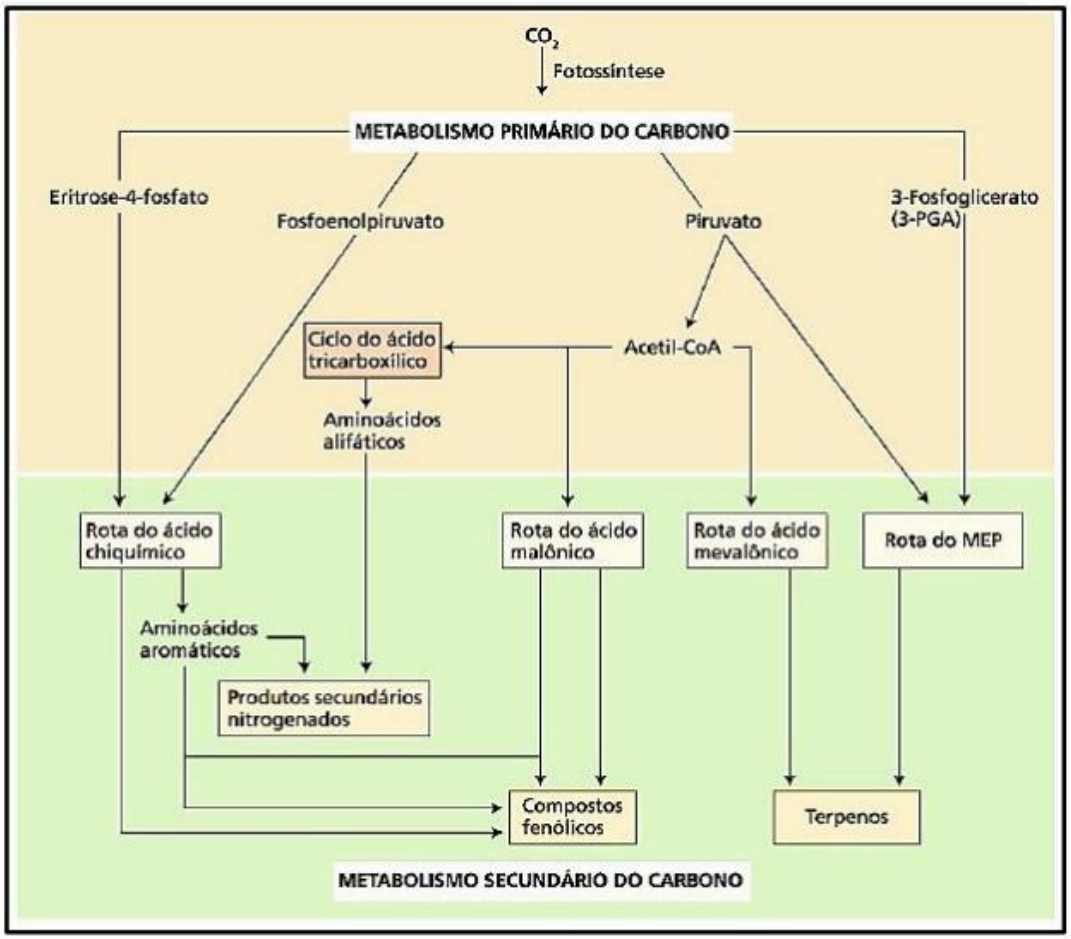
1.2.1. Metabolismo primário e secundário de plantas

As reações químicas que ocorrem no interior de uma célula ou organismo constituem o metabolismo. Grande parcela do carbono, nitrogênio e energia disponíveis na planta são combinados e usados para síntese de compostos comuns a todas as células, os metabólitos primários, como aminoácidos,

nucleotídeos, açúcares e lipídios, que são necessários para que a célula ou organismo mantenha seu funcionamento (GARCÍA, CARRIL, 2011).

Diferente de outros organismos, nas plantas, grande parte do carbono e energia são alocados para síntese de moléculas que não apresentam relação direta com os processos fotossintéticos, respiratórios, assimilação de nutrientes, transporte de solutos ou síntese de proteínas, carboidratos ou lipídios; tais moléculas são chamadas de metabólitos secundários (GARCÍA, CARRIL, 2011) (figura 1).

Figura 1. Principais rotas do metabolismo secundário a partir do metabolismo primário.



Fonte: Taiz et al. (2017).

1.2.2. Metabólitos secundários

A produção de metabólitos secundários em vegetais é relativamente alta e diversa, e ultrapassa o total de 100.000 compostos identificados. As plantas produzem uma gama de metabólitos secundários que formam misturas complexas, que diferem de órgão para órgão ou entre as espécies (WINK, 2018). Esses compostos podem apresentar nitrogênio, como é o caso dos alcaloóides, aminas, glicosídeos cianogênicos, aminoácidos não proteicos e glucosinolatos, ou podem ser isentos de nitrogênio, como os terpenos, policetídeos, saponinas e poliacetilenos.

Os metabólitos secundários são sintetizados em tecidos e órgãos específicos, por enzimas biossintéticas próprias, e os genes responsáveis pela síntese desses compostos são controlados pelos complexos mecanismos de regulação gênica que codificam enzimas do metabolismo primário. Essas substâncias, por sua vez, são produzidas e armazenadas em grandes quantidades, alcançando entre 1 e 3% do peso seco das plantas que os sintetizam (ROBBERS; SPEEDIE; TYLER, 1996; WINK, 1990; WINK, 2018). Além disso, deve-se considerar que os locais de síntese (tecidos ou órgãos) não são necessariamente os mesmo em que essas substâncias são armazenadas, e o transporte desses compostos a longas distâncias podem ocorrer via xilema, floema ou apoplasto (WINK, 2018).

Alguns produtos do metabolismo secundário apresentam funções ecológicas, são capazes de atrair ou repelir animais, como também podem estar relacionados às cores e sabores de flores e frutos, atraindo animais que atuarão como polinizadores ou dispersores de sementes. Esses compostos são usados há milhares de anos pela humanidade para diversos fins, tais como: corantes (índigo e shikonin), flavorizantes (vanilina, capsaicina e óleos de mostarda), fragrâncias (óleo de rosas, lavanda e outros óleos essenciais), estimulantes (cafeína, nicotina e efedrina), alucinógenos (morfina, cocaína, escopolamina e tetraidrocanabinol), inseticidas (nicotina, piperina e piretrina), venenos (estricnina e aconitina), e como

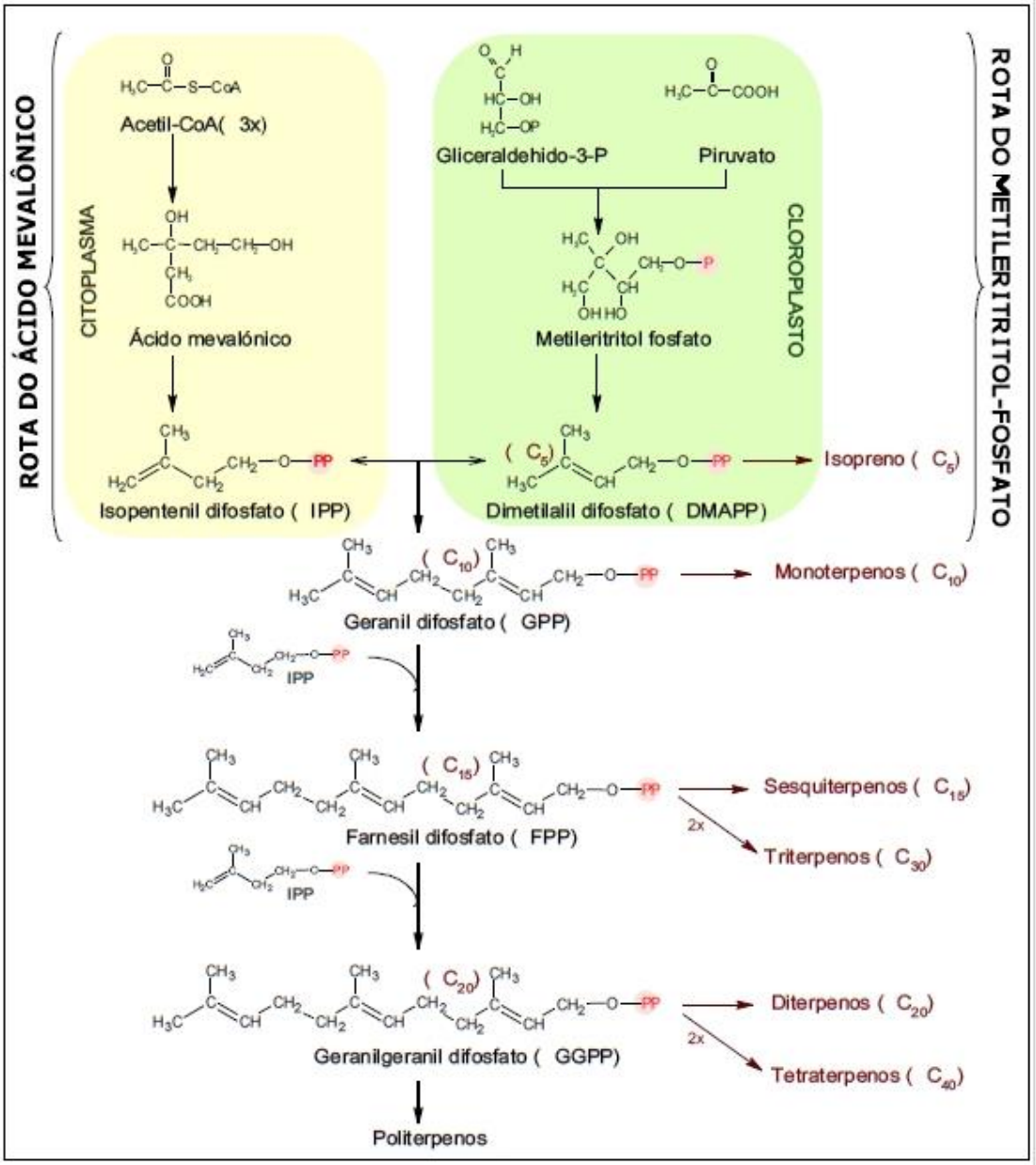
agentes terapêuticos (atropina, quinina, cardenolidos, codeína e outros), contudo, algumas dessas aplicações tem sido contestadas (MANN, 1992; ROBERTS, WINK, 1998).

1.2.2.1. Terpenos

Terpenos, ou terpenoides, apresentam mais de 40.000 moléculas diferentes e são considerados por alguns autores o grupo mais numeroso dos metabólitos secundários. As rotas biossintéticas destas substâncias dão origem a metabólitos primários e secundários necessários ao crescimento e sobrevivência das plantas. Entre os metabolitos primários produzidos por estas vias estão os hormônios vegetais giberelina, ácido abscísico e citocininas, carotenoides, clorofilas e plastoquinonas, ambos relacionados ao processo fotossintético, ubiquinonas, substâncias que participam do processo respiratório, e esteróis, compostos de grande importância para estrutura da membrana celular (WINK, 2018, TAIZ et al., 2017).

Os terpenóides são geralmente insolúveis em água, formados por unidades de isopreno (5 átomos de carbono) e classificados de acordo com o número de unidades de isopreno (C5) que contêm (figura 2): monoterpenos apresentam 10 carbonos (duas unidades de isopreno); sesquiterpenos apresentam 15 carbonos (três unidades de isopreno); diterpenos apresentam 20 carbonos (quatro unidades de isopreno); triterpenos e tetraterpenos apresentam 30 e 40 carbonos, respectivamente e; os politerpenos, substâncias formadas por mais de 8 unidades de isopreno (WINK, 2018, TAIZ et al., 2017).

Figura 2. Vias de biosíntese de terpenos e sua classificação.



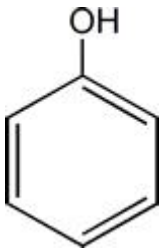
Adaptado de: Wink (2018).

1.2.2.2. Compostos fenólicos

Os aminoácidos aromáticos podem ser direcionados à biossíntese tanto de compostos do metabolismo primário quanto do metabolismo secundário. O grupo fenol apresenta um anel aromático com um grupo hidroxila (figura 3) e está presente em grande quantidade de metabólitos secundários sintetizados pelas

plantas, formando compostos fenólicos, polifenóis ou fenilpropanóides (ANGELO, JORGE, 2007).

Figura 3. Estrutura química do fenol.

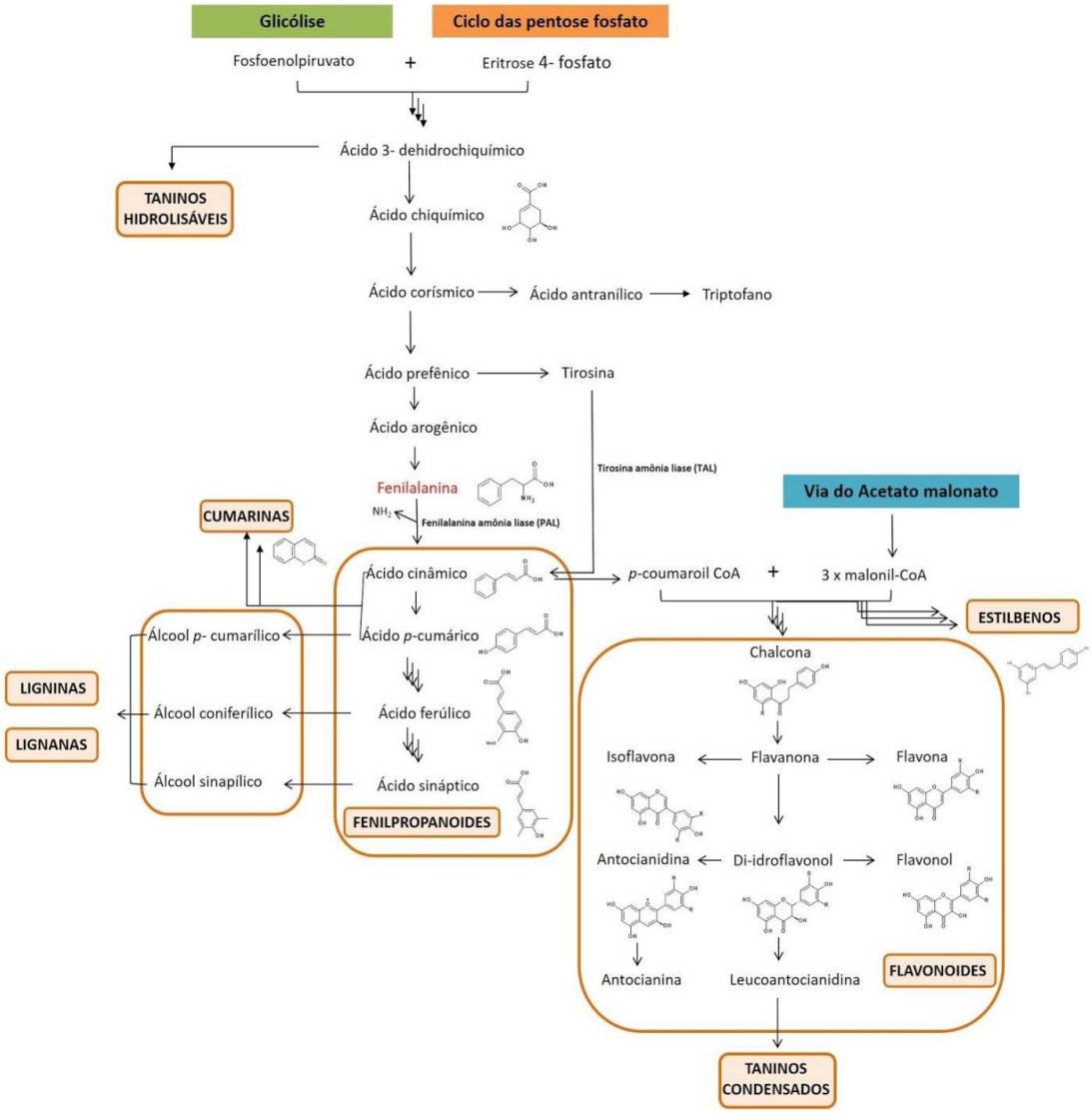


Fonte: Wink (2018).

Esses compostos possuem estruturas químicas diversificadas, variando de ácidos fenólicos, moléculas relativamente simples, até compostos como taninos e lignina, que são polímeros complexos. Os flavonoides também estão incluídos neste grupo e, frequentemente, estão relacionados às interações planta-herbívoro (TAIZ et al., 2017).

São descritas na literatura duas rotas biossintéticas relacionadas à produção dos compostos fenólicos, a via do ácido chiquímico e a via do ácido malônico (figura 4). Fungos e bactérias normalmente sintetizam fenóis a partir da via do ácido malônico, fenômeno raramente observado em plantas superiores. Em contrapartida, a via do ácido chiquímico é a responsável biossíntese da maioria dos compostos fenólicos das plantas. A partir da eritrose 4-fosfato e do fosfoenolpiruvato (ácido fosfoenolpirúvico) inicia-se uma série de reações que leva à síntese do ácido chiquímico e derivados, como os aminoácidos aromáticos fenilalanina, triptofano e tirosina (DE REZENDE, 2016).

Figura 4. Vias de síntese de compostos fenólicos.

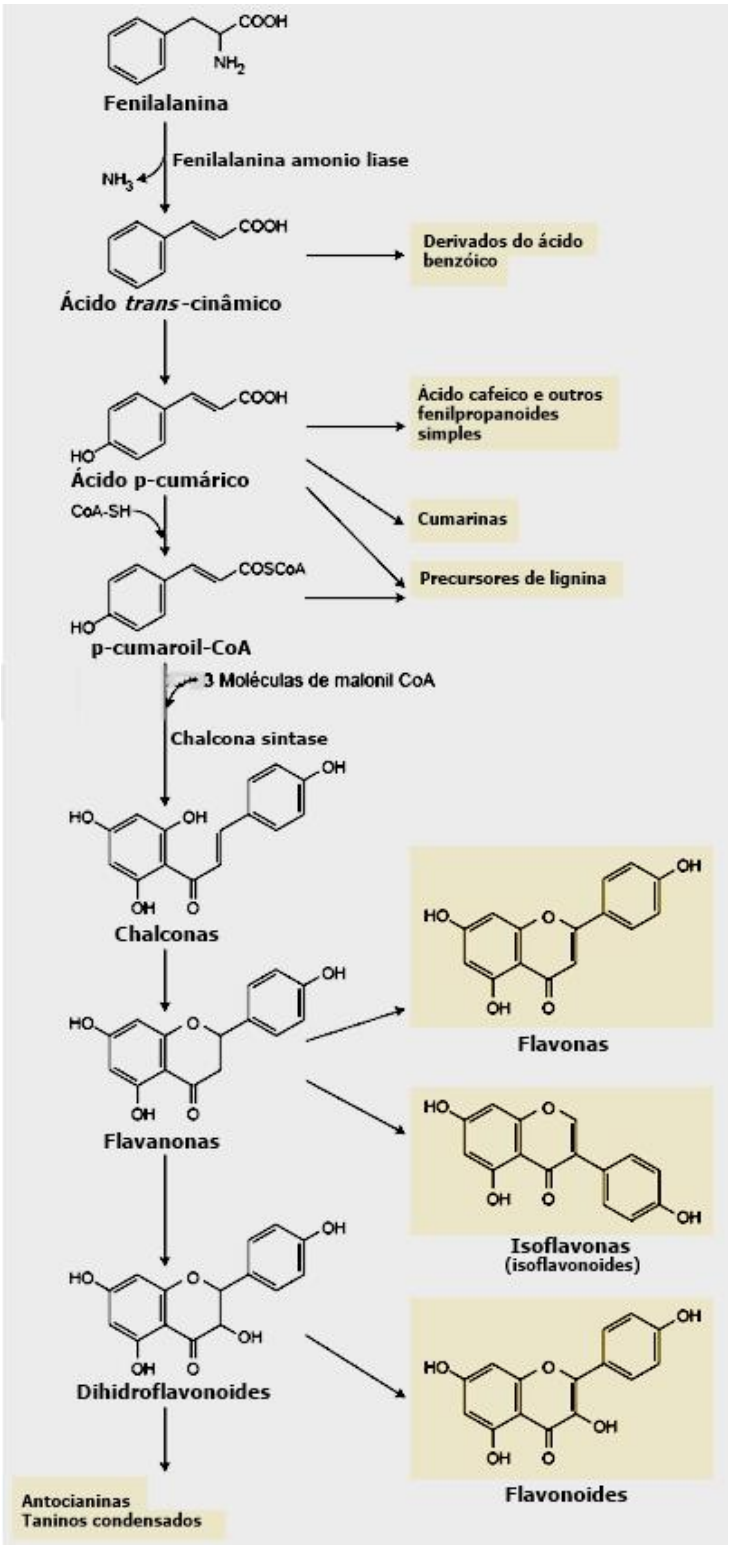


Fonte: De Rezende (2016).

A maior parte dos compostos fenólicos deriva da fenilalanina, rota biossintética presente em plantas, fungos e bactérias. Tanto a fenilalanina quanto o triptofano são classificados como aminoácidos essenciais para os animais, e precisam ser incorporados à dieta; já a tirosina não é classificada como um aminoácido essencial, tendo em vista que pode ser sintetizada pela hidroxilação da fenilalanina (DE REZENDE, 2016).

A enzima fenilalanina amônia liase (PAL) está localizada em um ponto de ramificação entre o metabolismo primário e o secundário, catalisa a formação de ácido cinâmico pela eliminação de uma molécula de amônio da fenilalanina e regula a síntese de muitos compostos fenólicos (Figura 5). Após reações catalisadas pela PAL ocorre a adição de mais grupos hidroxila e de outros substituintes. Os ácidos ferúlico e cafeico são formados a partir da metabolização dos ácidos trans-cinâmico e p-cumárico e precursores de derivados mais complexos, como cumarinas, lignina, taninos, flavonoides e isoflavonoides (WINK, 2018).

Figura 5. Rota do ácido chiquímico: desaminação da fenilalanina catalisada pela PAL e formação dos ácidos cinâmico e cumárico, precursores da lignina, flaconas, isoflavonas e flavonoides.



Adaptado de: Wink (2018).

Entre os compostos fenólicos, as cumarinas destacam-se por terem sido identificadas em mais de 800 espécies de plantas, apresentarem mais de 1500 formas e atuarem como antimicrobianos e inibidores da germinação. A cumarina de estrutura química mais simples conhecida é encontrada no óleo de bergamota (óleo essencial que fornece aroma ao tabaco, chá e outros produtos) e as cumarinas mais tóxicas são produzidas por fungos, como a aflatoxina sintetizada pelo *Aspergillus flavus*, um dos mais potentes carcinógenos das toxinas naturais. Dentre os fenólicos também estão os derivados do ácido benzoico, formados pelos fenilpropanóides que perderam um fragmento de dois carbonos da cadeia lateral, vanilina e o ácido salicílico, que atua como regulador do crescimento das plantas e na resistência da planta contra patógenos (ANGELO, JORGE, 2007; WINK, 2018).

Os flavonoides, substâncias que integram o grupo dos compostos fenólicos, apresentam esqueleto que contêm 15 carbonos dispostos em dois anéis aromáticos unidos por uma ponte de três carbonos e são classificados de acordo com seu grau de oxidação, destacando-se como principais compostos as antocianinas (pigmentos), flavonas, flavonóis e isoflavonas. Entre suas funções está a defesa e a pigmentação. A primeira etapa da biossíntese destes compostos implica na condensação de 3 moléculas de malonil-CoA com uma molécula de p-coumaroil-CoA, catalisada pela chalcona sintase, formando a naringerina chalcona, precursora de flavonóis e antocianinas (ANGELO, JORGE, 2007; Taiz et al., 2017; WINK, 2018).

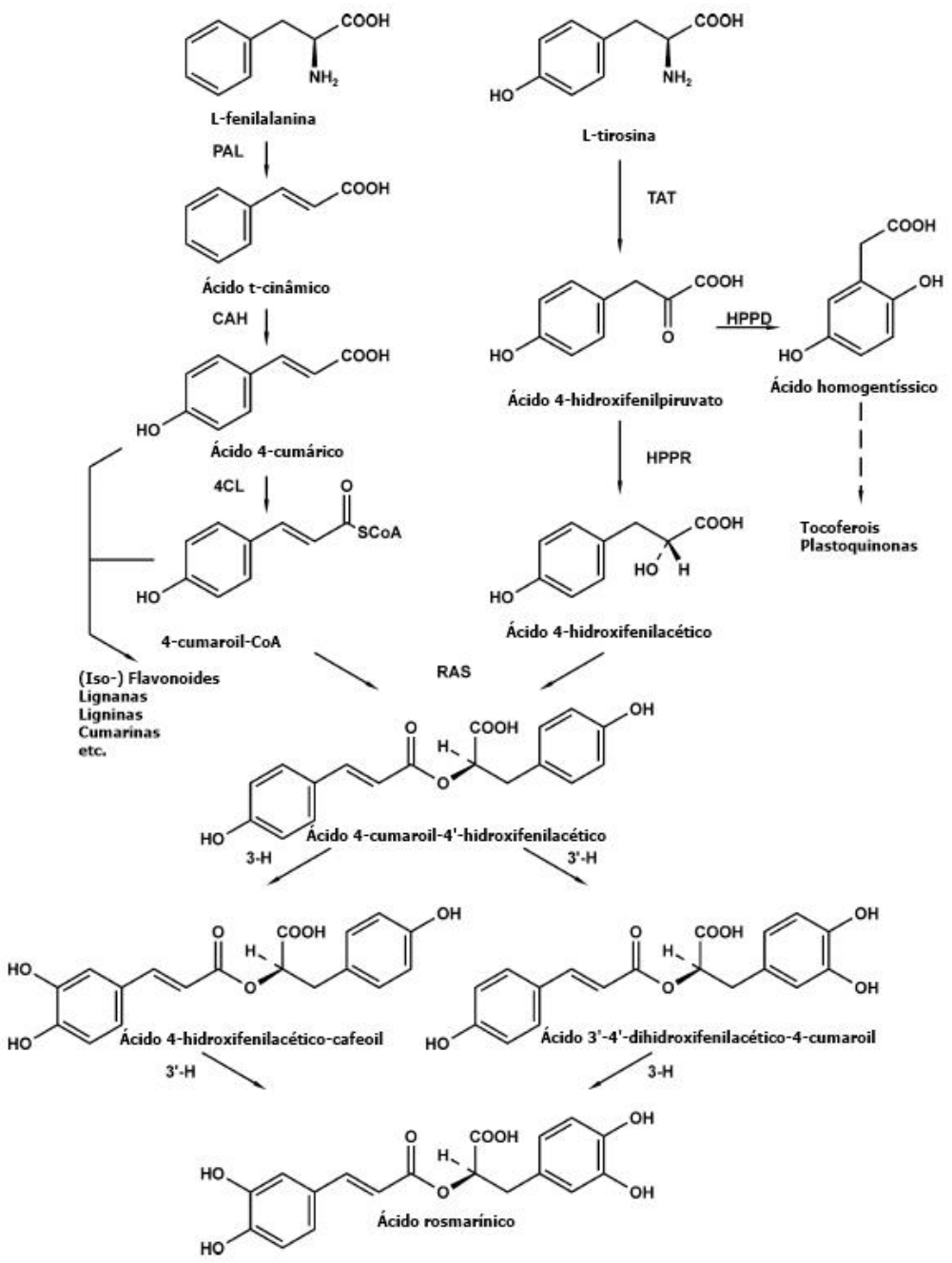
As antocianinas, flavonoides com cor, são responsáveis pela pigmentação da maioria das flores e frutos, desempenhando importante papel na polinização e dispersão de sementes. Essas substâncias apresentam um açúcar na posição 3 e a cor que apresentam depende do número de grupos hidroxila e metoxila no anel B e do pH dos vacúolos nos quais eles são armazenados (LÓPEZ et al., 2000).

Os taninos são compostos fenólicos poliméricos divididos em duas classes: taninos condensados e taninos hidrolisáveis. Taninos condensados são polímeros formados pela união de flavonoides ligados C-C, não são hidrolisáveis, mas podem ser oxidados por um ácido forte, gerando antocianinas. Em contrapartida, taninos hidrolisáveis são polímeros heterogêneos que contém ácidos fenólicos, geralmente são menores do que os taninos condensados e mais facilmente hidrolisados (WINK, 2018).

O ácido rosmarínico é um composto encontrado em grandes quantidades nas plantas da família Lamiaceae, sendo considerado um marcador químico para este grupo vegetal, é um bioativo não volátil (ZGÓRKA, GŁOWNIAK, 2001) e promove atividades biológicas, como atividades antioxidantes, antiinflamatórias e anticâncer (LIMA, FERNANDES-FERREIRA, PEREIRA-WILSON, 2006; LIU, SHEN, ONG, 2000; XAVIER, LIMA, FERNANDES-FERREIRA, 2009).

A síntese do ácido rosmarínico é mediada por enzimas da via geral dos fenilpropanoides (Figura 6), relacionadas à biossíntese de flavonoides e ligninas (Petersen, Simmonds, 2003). A biossíntese do ácido rosmarínico envolve a transformação da L-fenilalanina em ácido t-cinâmico pela ação da PAL, ocorrendo de forma coordenada à atividade da PAL e o acúmulo de ácido rosmarínico no tecido vegetal (RAZZAQUE, ELLIS, 1977).

Figura 6. Via biossintética do ácido rosmarínico.



PAL = fenilalanina amônia-liase, CAH = ácido cinâmico 4-hidroxilase, 4CL = hidroxicinamato: coenzima A ligase, TAT = tirosina aminotransferase, HPPR = hidroxifenilpiruvato redutase, HPPD = hidroxifenilpiruvato dioxigenase, RAS = hidroxicinamoil-CoA: hidroxifenilactato hidroxicinamoil transferase.

Adaptado de: Petersen, Simmonds (2003).

1.2.2.3. Alcaloides

Os alcaloides são metabólitos secundários que solúveis em água, contêm pelo menos um átomo de nitrogênio e exibem atividade biológica. São encontrados em aproximadamente 20% das plantas vasculares, sendo a maioria heterocíclico (WINK, 2018; KHALIL, 2017).

A síntese de aminas pode ocorrer por meio da hidrólise de compostos nitrogenados, decomposição térmica ou descarboxilação de aminoácidos (Figura 7), sendo esta ultima a principal via biossintética (BARDÓCZ, 1995). De modo geral, a síntese de aminas biogênicas é mediada pela descarboxilação de aminoácidos, como por exemplo, a descarboxilação da tirosina para formação da tiramina (GLORIA, 2005), um alcaloide primário encontrado em grande quantidade em plantas da família Cactaceae (DAVET et al., 2009).

Figura 7. Formação de aminas por descarboxilação de aminoácidos.

Fonte: Gloria (2005).

Nos seres humanos, os alcaloides podem interagir com os neurotransmissores e gerar respostas fisiológicas e psicológicas. Doses elevadas de alcaloides são reportadas como muito tóxicas, mas doses baixas podem apresentar alto valor terapêutico, e serem usadas como relaxantes musculares, tranquilizantes ou analgésicos.

1.3. ATIVIDADES BIOLÓGICAS DE PLANTAS MEDICINAIS

1.3.1. Atividade antioxidante

Substâncias antioxidantes são conhecidas por retardarem a velocidade da oxidação, como por exemplo, em processos patofisiológicos ou por fatores ambientais que desencadeiam a produção excessiva de radicais de oxigênio (HUANG, OU, PRIOR, 2005). Os radicais livres podem produzidos endogenamente ou adquiridos de forma exógena, por meio da dieta, e seu excesso pode levar ao estresse oxidativo (NÚÑEZ-SELLÉS, 2005).

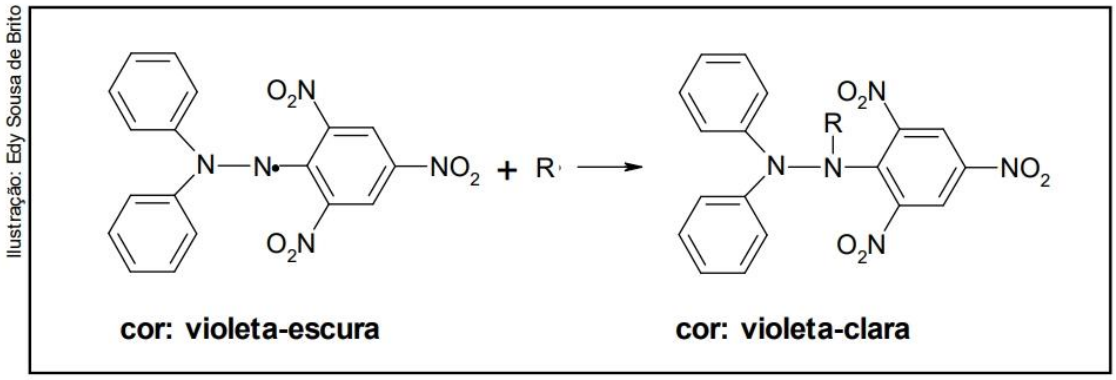
A redução da oxidação promovida por compostos antioxidante pode ocorrer, por exemplo, por meio de mecanismos de inibição de radicais livres ou complexação de metais (DUARTE-ALMEIDA, SANTOS, 2006). Os compostos antioxidantes e suas atividades biológicas têm sido cada vez mais conhecidos e difundidos, o que insita a um maior consumo e interesse para seu uso em produtos alimentícios, cosméticos e farmacêuticos, bem como a substituição de antioxidantes sintéticos, suspeitos de induzir câncer, por antioxidantes naturais (SASAKI et al., 2002; DJERIDANE et al., 2006).

Desta forma, devido à menor toxicidade e reduzidos efeitos colaterais, observa-se nos últimos anos grande interesse pelos antioxidantes naturais, como os provenientes dos extratos de plantas aromáticas e medicinais, consideradas fontes de antioxidantes naturais (WOLFE, WU, LIU, 2003). Nessa perspectiva, muitos metabólitos secundários têm sido reportados como compostos antioxidantes, inibindo de modo eficaz a formação de radicais livres (SINGER, CROWLEY, THOMPSON, 2003; LAPORNIK, PROŠEK, WONDRA, 2005), promovendo efeitos biológicos benéficos à saúde, o que contribui para sua melhor aceitação para fins terapêuticos (BATISH, SINGH, 2008).

1.3.1.1. Ensaio DPPH

O ensaio DPPH baseia-se na captura do radical 2,2-difenil-1- picril-hidrazil (DPPH) por agentes antioxidantes, conduzindo ao decréscimo da absorbância do sistema (BRAND-WILLIAMS et al., 1995). O radical DPPH pode ser facilmente obtido por meio da dissolução do reagente em meio orgânico (Figura 8).

Figura8. Estabilização do radical livre DPPH.

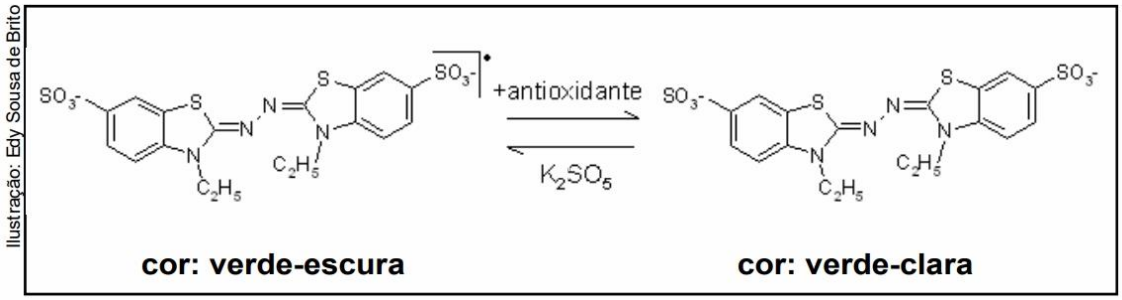


Fonte: Rufino et al. (2007).

1.3.1.2. Ensaio ABTS

O ensaio ABTS, assim como o ensaio DPPH, é um dos métodos mais utilizados para medir a atividade antioxidante. Esse método se baseia na captura do radical 2,2´- azinobis(3-etilbenzotiazolina-6-ácido sulfônico) (ABTS), gerado pela reação química, eletroquímica ou enzimática (Figura 9), e permite avaliar a atividade antioxidantes de compostos tanto de natureza hidrofílica quanto lipofílica (KUSKOSKI et al., 2005).

Figura 9. Estabilização do radical $ABTS^{\bullet+}$ por um antioxidante e sua formação pelo persulfato de potássio.



Fonte: Rufino et al. (2007).

1.3.1.3. Atividade quelante do íon Fe^{2+}

Neste ensaio, a ferrozina pode gerar um complexo de cor vermelha a partir da formação de quelatos com Fe^{2+}. Tal reação é restrita na presença de outros agentes quelantes, e leva a diminuição da cor vermelha dos complexos ferrozina-Fe^{2+}. Assim, a detecção da redução da coloração é usada para determina a atividade quelante de diferentes compostos (SOLER-RIVAS et al., 2000).

1.3.1.4. Ensaio β-caroteno/ácido linoleico

Metabólitos secundários podem agir como redutores de espécies reativas de oxigênio, como oxigênio singleto, e assim atuarem sobre reações de oxidação lipídica e na quelação de metais (SATUÉ-GARCIA, HEINONEN, FRANKEL, 1997; HOPIA, HEINONEM, 1999). O ensaio da oxidação do β-caroteno/ácido linoléico objetiva avaliar o potencial de compostos químicos inibirem a ação de radicais livres gerados durante a peroxidação do ácido linoleico, que, por meio degradação oxidativa do ácido linoléico e oxidação do β-caroteno, geram a descoloração do sistema (MARCO, 1968; MILLER, 1971).

1.3.2. Toxicidade *in vitro* e *in vivo*

Plantas com aplicações medicinais e usadas para produção de fitoterápico são de suma importância para o desenvolvimento de novos fármacos (YUNES; CALIXTO, 2001) e sua correta utilização pode trazer grandes benefícios à saúde. Entretanto, considerando que compostos presentes em produtos naturais podem ser tóxicos, há a necessidade de se estudar seus efeitos tóxicos dessas substâncias para os organismos vivos (NUNES; ARAUJO, 2003), como avaliação da citotxicidade, mutagenicidade, genotoxicidade, assegurando seu uso pela população.

A avaliação da citotoxicidade e mutagenicidade de substâncias presentes em produtos naturais ou semi-sintéticos tem sido feita utilizando ensaios *in vitro* e *in vivo* (DE ALENCAR, ISLAM et al., 2016; BELCAVELLO et al., 2012; HOLDEN, MAJESKA, STUDWELL, 1997). Ensaios *in vitro* podem fornecer medidas múltiplas e complementares de citotoxicidade (FENECH, 2000), bem como são úteis na avaliação dos efeitos do contato direto de produtos naturais e quimioterápicos com as células (MARULLO et al., 2013; KATALINIC et al., 2005) e seu potencial uso na prevenção de danos citotóxicos (OLIVEIRA et al., 2018; 2018; SANTOS et al., 2018). Somado a isso, os ensaios *in vivo* complementam os estudos *in vitro*, uma vez que podem fornecer informações que ajudam na compreensão dos efeitos da metabolização sobre uma dada substância e, por conseguinte, seus efeitos toxicológicos (BELCAVELLO et al., 2012).

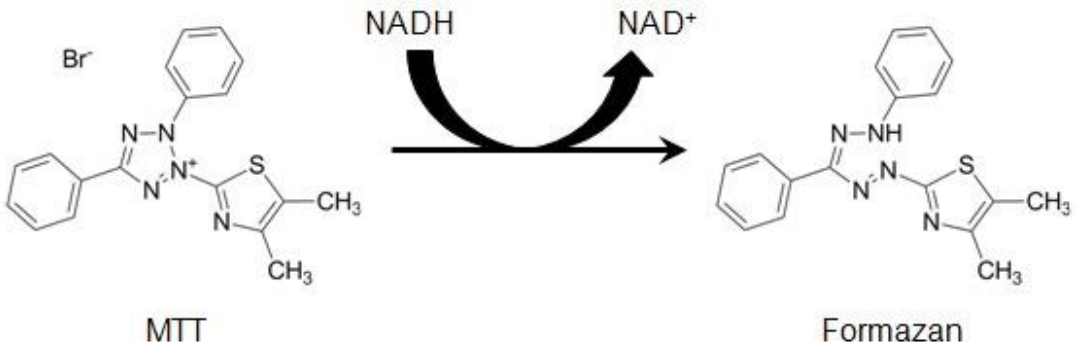
No que tange a seleção de plantas medicinais para o desenvolvimento de fármacos, a avaliação das atividades biológicas de extratos vegetais *in vitro* faz parte do processo inicial de triagem dos materiais vegetais mais promissores. Neste processo, o uso de células humanas sadias, como linfócitos humanos, e de células tumorais de roedores, como as do sarcoma 180, são relevantes, pois ajudam a verificar efeitos citotóxicos e possíveis aplicações das substâncias avaliadas para o tratamento de cânceres (OLIVEIRA et al., 2018; 2018; SANTOS et al., 2018).

1.3.2.1. Viabilidade celular

O ensaio do MTT (brometo de 3- [4-dimetiltiazol-2-il] -2,5-difeniltetrazólio), vermelho neutro, lactato desidrogenase (LDH) e ensaio com proteínas são teste usados para verificar a viabilidade celular. Fotakis e Timbrell (2006) propõem em suas análises que tanto o ensaio do vermelho neutro quanto do MTT sejão os ensaios de citotoxicidade mais sensíveis e recomendados para detecção de toxicidade precoce (FOTAKIS, TIMBRELL, 2006)

O MTT é um sal de tetrazólio amarelo, solúvel em água, que é convertido em formazan, cristais de cor púrpura insolúveis em água (Figura 10). Essa conversão é mediada pela clivagem do anel de tetrazólio pela succinato desidrogenase na mitocôndria, que pode ser mediada polo NADH ou NADPH no interior da mitocondria (FOTAKIS, TIMBRELL, 2006; BERRIDGE, TAN, 1993).

Figura 10. Estruturas do MTT e do produto formazan.



Fonte: Sittampalam et al. (2004)

Algumas substâncias podem inibir a respiração mitocondrial e induzir a produção de espécies reativas de oxigênio relacionadas à morte celular, promovendo danos aos componentes mitocondriais. Considerando que o ensaio MTT permite inferir

sobre a citotoxicidade por meio da avaliação da atividade respiratória mitocondrial, este teste é recomendado para detecção de toxicidade precoce após exposição a um tóxico mitocondrial (FOTAKIS, TIMBRELL, 2006).

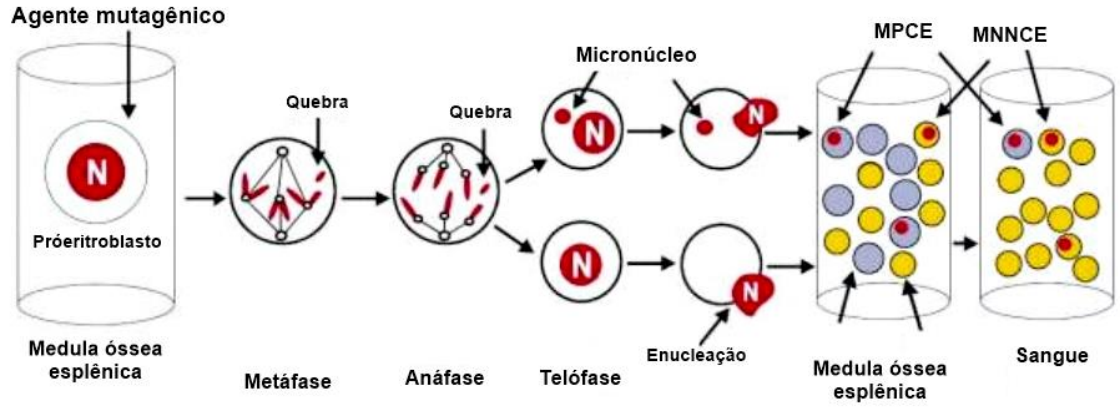
1.3.2.2. Mutagenicidade

A mutação corresponde ao primeiro evento que desencadeia a carcinogênese, contudo a maior parte das mutações é corrigida pelos mecanismos de reparo células (HAVSTEEN, 2002). Todavia, se agentes genotóxicos estiverem presentes eles podem interagir com DNA e causar danos a regiões do genoma relacionadas ao ciclo celular, conduzindo a apoptose e acelerando processo neoplásico (SANTOS, 2016). Sendo assim, são indispensáveis avaliações toxicológicas dos compostos terapêuticos utilizando uma abordagem genotóxica.

Os micronúcleos são formados a partir de quebras ou de cromossomos inteiros, que não são incorporados no núcleo principal das células filhas em decorrência do atraso na anáfase. Eles podem ser analisados em eritrócitos, células da mucosa oral ou linfócitos e permitem estimar a frequência do dano genético (HAYASHI, 1983).

Os eritroblastos, no decorrer do processo de divisão celular na medula óssea, passam por duplicação final dos cromossomos diferenciando-se em eritrócitos policromáticos (Figura 11). Os eritrócitos jovens possuem grande quantidade de ribossomos, são facilmente corados e diferenciados dos eritrócitos maduros, eritrócitos normocromaticos, em que ribossomos são praticamente ausentes. Na presença de agentes mutagênicos, os fragmentos cromossômicos formados podem não ser incorporados no núcleo principal das células filhas após a divisão celular, dando origem o micronúcleo. Depois de formado, o micronúcleo permanece no citoplasma e pode ser visualizado com facilidade (SANTOS, 2016).

Figura 11. Mecanismo de formação de eritrócitos policromáticos, normocromáticos e micronúcleos.



PCE- Eritrócitos policromáticos; NCEEritrócitos normocromáticos; MNPCE- Eritrócitos policromáticos micronucleados; MNCEEritrócitos normocromáticos micronucleados.

Adaptado de: Krishna e Hayashi, (2000).

1.4. FAMÍLIAS BOTÂNICAS DE IMPORTÂNCIA MEDICINAL

O Brasil é reconhecido mundialmente pela sua rica biodiversidade, sendo que o país apresenta cerca de 55 mil dessas espécies, das mais de 500 mil espécies vegetais descritas. Contudo, mesmo com esta vasta diversidade, estima-se que menos de 15% das espécies vegetais tenham sido investigadas quanto a sua aplicação medicinal (CONSERVATION INTERNATIONAL, 2010), fato que reforça a necessidade pesquisas com diferentes espécies vegetais para buscar novas substâncias/princípios ativos que possam constituir novos medicamentos (ZAGO, DE MOURA, 2018), assim como para atribuir novas aplicações terapêuticas para ativos vegetais já conhecidos.

Em relação às famílias botânicas de uso medicinal no Brasil, Zago e De Moura (2018), em um levantamento cienciométrico, descrevem que as famílias Fabaceae, Asteraceae, Lamiaceae e Euphorbiaceae foram as que tiveram maior destaque e geraram maior número de estudos científicos nos últimos 22 anos. Cabe ressaltar que, mesmo com número de estudos menos expressivos,

investigações com outras famílias vegetais têm apresentando atividades biológicas promissoras para o desenvolvimento de novos fármacos, como aquelas conduzidas com plantas da família Cactaceae (CHOI et al., 2002; QIU et al., 2002).

1.4.1. Cactaceae

Dentre as Angiospermas, a família Cactaceae representa o segundo grupo vegetal mais numeroso da região neotropical. No Brasil, as plantas que compõem esta família podem ser encontradas em diferentes climas e solos, com maior ocorrência nos ecossistemas Caatinga, Florestas Tropicais, Cerrado, Campos rupestres e Restingas (CRUZ, 2011; ARRUDA, 2010).

Este grupo botânico é formado por, aproximadamente, 127 gêneros e 1500 espécies (LUCENA et al., 2015), subdivididas em quatro Subfamílias (Maihuenoideae, Pereskeoideae, Opuntioideae e Cactoideae) (CRUZ, ; REIS, 2009; V. DE LOS A. PÁEZ, A. R. ANDRADA et al., 2012; DA SILVA SANTOS et al., 2013), e o Brasil, com o registro de 39 gêneros e mais de 260 espécimes, ocupa o ranking de terceiro maior centro mundial de diversidade de cactáceas (TAYLOR et al., 2015).

As cactáceas estão entre os vegetais mais usados pelo homem, principalmente em regiões áridas do Brasil (DE LUCENA et al., 2012). Nessas áreas observa-se estreita relação entre a população e os cactos, expressas por meio de manifestações culturais, como na música “Xote das Meninas”, de Luiz Gonzaga, e de sua utilização como bioindicadores de chuvas, usos místico-culturais, na ornamentação de residências, praças e jardins, componentes de telhados verdes, na produção artesanal de canos para cachimbos, na fabricação de portas, janelas, caibros e ripas, como enchimento para almofadas e cangalhas (BRAVO FILHO, 2014; LUCENA et al., 2015; MENEZES; RIBEIRO-SILVA, 2015).

1.4.1.1. *Cereus jamacaru* D.C.

Cereus jamacaru D.C., mandacaru, é uma Cactaceae bem adaptada às condições semiáridas do bioma Caatinga e é amplamente distribuída no Brasil, formando uma paisagem típica na Região Nordeste (DE ALMEIDA et al., 2011; KAVAMURA et al., 2013; DE AZEVEDO FERNANDES et al., 2016) (Figura 12).

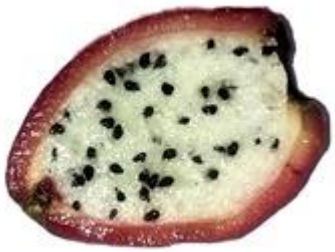
Figura 12. Exemplar de *Cereus jamacaru* no Agreste de Alagoas-Brasil.

Fonte: acervo pessoal.

As investigações etnobotânicas demonstram que há um rico reconhecimento tradicional sobre os usos de *C. jamacaru*. Todos os órgãos desta planta são usados: os cladódios servem como fonte de alimento e para fins terapêuticos; caules e raízes são usados para produzir infusões, uma das formas de preparo mais comumente utilizada para tratar doenças (MORS; RIZZINI; PEREIRA, 2000; LORENZI; MATOS, 2002; CASTRO; CAVALCANTE, 2010).

Os frutos de *C. jamacaru* (figura 13) são normalmente consumidos frescos e têm sido estudados devido ao seu grande potencial na indústria alimentar, como para a produção de sorvetes à base de polpa de fruta (FARIAS et al., 2015). O seu elevado teor de sólidos solúveis totais e de açúcares totais também têm sido objetos de investigação, uma vez que apresentam aspectos relevantes para produção de bebidas fermentadas e destiladas (DE ALMEIDA et al., 2011).

Figura 131. Fruto de *C. jamacaru* partido ao meio (corte sagital).



Fonte: acervo pessoal.

1.4.2. Lamiaceae

A família Lamiaceae é uma das maiores famílias botânicas do planeta, formada por aproximadamente 236 gêneros e cerca de 6900 a 7200 espécies (AGHAKHANI; KHARAZIAN; LORI GOOINI, 2018). Os numerosos estudos já conduzidos com este grupo vegetal demonstraram que, além de serem comumente usadas como condimentos, as espécies que compõem este grupo botânico apresentam grande capacidade de eliminar radicais livres (TZIMA; BRUNTON; RAI, 2018) e são relevantes comercialmente, como gêneros *Plectranthus*, *Salvia*, *Ocimum* e *Mentha*, aos quais são atribuídos com uma rica diversidade de benefícios etnobotânicos (ARUMUGAM; SWAMY; SINNIAH, 2016).

1.4.2.1. Melissa officinalis

Melissa officinalis L., popularmente conhecida como melissa, erva-cidreira verdadeira ou cidreira (GRANDI, 2014), é uma erva medicinal da família Lamiaceae, nativa do Mediterrâneo e cultivada em toda a Europa, América do Norte e Ásia (Figura 14). Em seu uso tradicional, é comumente administrada como infusão usada para tratar doenças gastrointestinais, dor de cabeça e febre (DERMARDEROSIAN; BEUTLER, 2002; SHAKERI; SAHEBKAR; JAVADI, 2016).

Figura 14. Ilustração de botânica de *M. officinalis*.

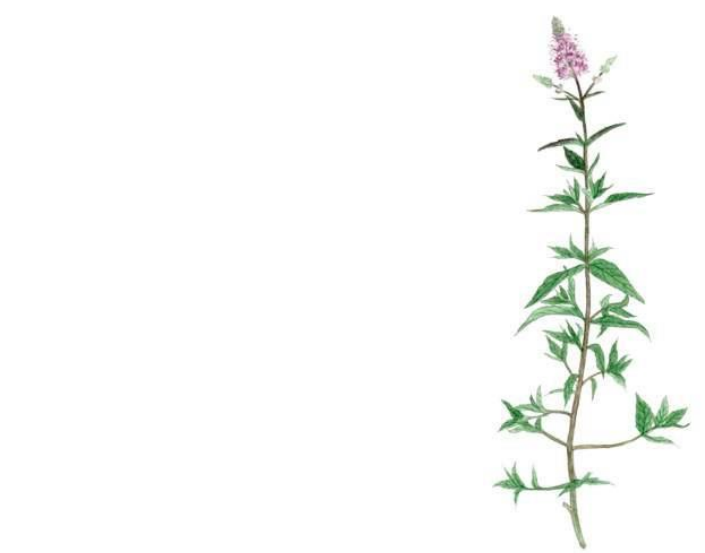
Fonte: Grandi (2014).

Similar às outras Lamiaceae, *M. officinalis* apresenta altos teores de ácidos fenólicos, particularmente os derivados do ácido cafeico, como o ácido rosmarínico (FECKA; TUREK, 2007; BARROS et al., 2013; MILEVSKAYA et al., 2017), que têm sido associados à alta capacidade antioxidante desta erva (PROESTOS et al., 2005; DASTMALCHI et al., 2008; SKOTTI et al., 2014), e também às suas atividades antiproliferativas (LIN et al., 2012) e antiprotozoárias (CUNHA et al., 2016).

1.4.2.2. *Mentha piperita*

Mentha piperita L. (Lamiaceae), conhecida por populares como hortelã-pimenta, sândalo, ou hortelã-inglesa, é uma das ervas mais populares e usadas como ingrediente de chás (Figura 15). Seus usos tradicionais são variados, sendo principalmente usada para tratar distúrbios biliares, dispepsia, enterite, flatulência, gastrite, cólica intestinal e espasmos do ducto biliar, da vesícula biliar e gastrointestinal. Entre os compostos fenólicos encontrados nas folhas destacam-se o ácido rosmarínico e flavonoides como eriocitrina, luteolina e hesperidina, sendo os extratos dessa planta conhecidos por suas atividades antimicrobianas e antivirais e fortes ações antioxidantes e antitumorais (MCKAY; BLUMBERG, 2006; GRANDI, 2014).

Figura 15. Ilustração de botânica de *Mentha piperita.*



Fonte: Grandi (2014).

1.4.2.3. *Ocimum basilicum*

Ocimum basilicum L. é uma Lamiaceae conhecida como manjericão, manjericão grande, manjericão de folha larga (Figura 16). É uma erva popular valorizada pelo seu sabor, levemente apimentado, com um toque de hortelã e cravo, sendo

amplamente utilizada na culinária e como planta ornamental (JAVANMARDI et al., 2002; CHANG; ALDERSON; WRIGHT, 2009; GRANDI, 2014). Estudos realizados sugerem que *Ocimum basilicum* L. apresenta atividade analgésica, antiinflamatória, antimicrobiana, antioxidante, antiulcerogênica, estimulante cardíaco, quimiomoduladora, depressora do sistema nervoso central, hepatoprotetora, hipoglicêmica, hipolipidêmica, imunomoduladora e larvicida, sendo usada pela população para fins medicinais (BILAL et al., 2012).

Figura16. Ilustração de botânica de *O. basilicum.*

Fonte: Grandi (2014).

1.4.2.4. Ocimum selloi

Ocimum selloi Benth, elixir-paregórico, alfavaquinha ou atroveran, é uma erva medicinal da Família Lamiaceae, tradicionalmente usada no tratamento da diarréia, dor e inflamação e como repelente de insetos (LORENZI; MATOS, 2002; DE PAULA; GOMES-CARNEIRO; PAUMGARTTEN, 2003) (Figura 17). Entre os compostos químicos mais comuns encontrados neste vegetal estão o metil

chavicol (estragol) e metil eugenol e elemicina (SOBTI et al., 1981; MARTINS et al., 1997), substâncias químicas relacionadas as atividades biológicas exibidas por seus extratos e relevância para as indústrias farmacêutica, cosmética e alimentícia (COSTA et al., 2010)

Figura17. Ilustração de botânica de *Ocimum selloi* Benth.

Fonte: Grandi (2014).

1.4.2.5. Plectranthus amboinicus

Plectranthus amboinicus (Lour) Spreng, popularmente intitulada hortelã grossa, hortelã grande ou hortelã de quibe, é uma das espécies mais documentadas da família Lamiaceae (figura 18). Essa erva da Família Lamiaceae apresenta folhas carnudas e suculentas, conhecida por seu sabor e odor, sendo usada tanto na culinária quanto na medicina tradicional (LUKHOBA; SIMMONDS; PATON, 2006; GRANDI, 2014). Em contrapartida, a versão variegada desta planta, com folhas de

bordas brancas, *Plectranthus amboinicus* “variegata” Ehrh, é usada normalmente como planta ornamental (Figura 18).

Figura 18. *Plectranthus amboinicus* e *P. amboinicus* “variegata” em condições de cultivo.

A

B

A = *Plectranthus amboinicus*; B = *Plectranthus amboinicus* “variegata”.

Fonte: acervo pessoal.

As propriedades medicinais descritas desta espécie correspondem a 68% de todas as aplicações habituais deste gênero (LUKHOBA; SIMMONDS; PATON, 2006). As principais classes de fitoquímicos descritas neste grupo vegetal incluem os monoterpenoides, diterpenoides, triterpenoides, sesquiterpenoides, fenólicos, flavonoides, ésteres, álcoois e aldeídos. Em relação às atividades biológicas já descritas estão as atividades antimicrobiana, antiinflamatória, antitumoral, cicatrizante, antiepilética, larvicida, antioxidante e analgésica (ARUMUGAM; SWAMY; SINNIAH, 2016).

1.4.2.6. Plectranthus ornatus Codd

Plectranthus ornatus Codd (Lamiaceae) é conhecido como boldinho, boldo chinês, boldo gambá, boldo miúdo ou boldo rasteiro (MAURO et al., 2008) (Figura 19), é indicado na medicina tradicional para tratar problemas no fígado, má digestão, controle da gastrite, na dispepsia, azia e mal estar gástrico, e o seu sabor amargo é considerado estimulante da digestão e do apetite (MAURO et al., 2008; GRANDI, 2014). Estudos conduzidos com esta espécie sugerem que as atividades biológicas exibidas por essa espécie estão relacionadas aos fitoquímicos presente nesta espécie, como ornantina, barbatusina, labdano e forskolina, alquilfenois e flavonoides (OLIVEIRA et al., 2005, OLIVEIRA et al. 2011).

Figura 19. Ilustração de botânica de *P. ornatus.*

Fonte: Grandi (2014).

2. OBJETIVO GERAL

Avaliar a composição química de extratos vegetais de 08 plantas medicinais pertencentes às famílias Lamiaceae e Cactaceae e relacioná-las às suas atividades antioxidantes, citotóxicas e anti-citotóxicas *in vitro* e às suas atividades antitumorais e mutagênicas *in vivo*.

2.1. OBJETIVOS ESPECÍFICOS

Realizar a prospecção fitoquímica das classes de metabólitos secundários presentes nos extratos vegetais produzidos a partir de todas as espécies em estudo;

Determinar os teores totais de flavonoides dos extratos hidroalcoólicos das espécies vegetais avaliadas nesta pesquisa;

Quantificar o teor total de ácido rosmarínico dos extratos das plantas selecionadas da família Lamiaceae;

Avaliar as atividades antioxidantes dos extratos vegetais pelos ensaios DPPH, ABTS, quelante de Fe^{2+} em todas as espécies em estudo;

Verificar os efeitos citotóxicos e anti-citotóxicos dos extratos vegetais, das espécies estudadas, em linfócitos humanos, *in vitro*;

Avaliar os efeitos antiproliferativos de todos os extratos vegetais em análise, em células de sarcoma 180, *in vitro*;

Relacionar os resultados obtidos na avaliação fitoquímica com as atividades biológicas testadas;

Verificar a influência da variegação foliar presente em *Plectranthus amboinicus* sobre as atividades biológicas desta espécie;

Comparar as atividades biológicas da espécie *Cereus jamacaru* em duas diferentes condições fenológicas, estádio vegetativo e de frutificação;

Verificar a mutagenicidade do extrato vegetal de *Cereus jamacaru*, em sangue periférico de camundongos, pelo ensaio de micronúcleos;

Estabelecer, dentre as espécies estudadas, as mais promissoras para o desenvolvimento de fitoterápicos.

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CAPITULO 1 – *Cereus jamacaru* D.C. Hydroalcoholic Extract Promotes Anti-Cytotoxic and Antitumor Activity

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RESUMO

Cereus jamacaru D.C. (mandacaru) é um cacto usado como alimento e na medicina tradicional. No presente estudo, o extrato hidroalcoólico de *C. jamacaru* foi avaliado por sua composição química, atividade antioxidante, efeitos citotóxicos e anti-citotóxicos em linfócitos humanos e células do sarcoma 180 *in vitro* pelo ensaio MTT e efeitos antitumorais, mutagênicos e citotóxicos em camundongos sarcoma- induzido *in vivo*. A caracterização fitoquímica mostrou reações positivas para cumarina, flavanol e tiramina e teor de flavonoides totais de 0,51 µg/mL. *C. jamacaru* apresentou atividade antioxidante após ensaios com DPPH (EC_{50} = 427,74 µg/mL), ABTS (EC_{50} = 270,57 µg/mL) e atividade quelante de íons Fe^{2+} (EC_{50} = 41,18 µg/mL). *C. jamacaru* induziu diminuição significativa da viabilidade do sarcoma 180 em 24 horas e 48 horas de tratamento, não induziu citotoxicidade em linfócitos humanos e inibiu a citotoxicidade da cisplatina *in vitro*. Após ensaios *in vivo*, *C. jamacaru* promoveu redução tumoral (86,07% de inibição tumoral), sem induzir dano mutagênico ou citotóxico nas células sanguíneas dos camundongos. Propomos que compostos fenólicos e alcalóides presentes no extrato estejam relacionados à atividade antioxidante, aumentando sua capacidade de atividade quelante de metais e promovendo atividade anti-citotóxica contra a cisplatina, assim como estes compostos podem atuar sobre o ciclo celular das células tumorais *in vitro* e *in vivo*, levando a efeitos anticancerígenos e redução tumoral.

Palavras-chave: mandacaru; fitoquímica; atividade antioxidante; ensaio MTT; sarcoma 180 antitumor.

ABSTRACT

Cereus jamacaru D.C. (mandacaru) is a cactus used as food and in the traditional medicine. In the present study, hydroalcoholic extract of *C. jamacaru* was evaluated for its chemical composition, antioxidant activity, cytotoxic and anti-cytotoxic effects in human lymphocytes and sarcoma 180 cells *in vitro* by MTT assay and antitumoral, mutagenic and cytotoxic effects on mice sarcoma-induced *in vivo*. Phytochemical characterization showed positive reactions for coumarin, flavanol and tyramine and total flavonoid content of 0.51 µg/mL. *C. jamacaru* showed antioxidant activity following DPPH (EC_{50} = 427.74 µg/mL), ABTS (EC_{50} = 270.57 µg/mL) and Fe^{2+} chelating ions assays (EC_{50} = 41.18 µg/mL). *C. jamacaru* induced significant decrease of sarcoma 180 viability at 24 h and 48 h of treatment, did not induce cytotoxicity in human lymphocytes and inhibits the cytotoxicity of cisplatin *in vitro*. Following in vivo assays, *C. jamacaru* promoted tumor reduction (86.07% of tumor inhibition), without inducing mutagenic or cytotoxic damage on mice blood cells. We propose that phenolic and alkaloid compounds in the extract are related to antioxidant activity, increasing its ability in metal chelating activity and promoting anti-cytotoxic activity against cisplatin, as well as these compounds may act on the cell cycle of the tumor cells *in vitro* and *in vivo*, leading to anticancer effects and tumor reduction.

Keywords: mandacaru; phytochemistry; antioxidant activity; MTT assay; sarcoma 180 antitumor.

1. INTRODUCTION

Brazil presents the major flora diversity of the world, exclusives biomes and a great number of vegetal species adapted to arid regions [1], such as cacti. *Cereus jamacaru* D.C., commonly known as “mandacaru,” is a native Brazilian cactaceae that occur naturally in Caatinga biome and is described as a source of food and medicinal products used in the treatment of urinary infection, kidney inflammation and rheumatism [2].

Natural products may contain non-fully identified compounds that present biological activities, inducing minimal side effects in biological systems and purchased at a relatively low cost [3]. Additionally, medicinal plants used as food, such as cacti, may have compounds such as flavonoids and alkaloids and exhibit strong antioxidant and anticancer effects [4–6]. In that way, cactus plants used for humans may exhibit wide variety of phytocompounds, such as phenolic and nitrogen compounds, which have been related to their biological activities [7–9].

In vitro and *in vivo* assays using human and rodent cells have been used to access the cytotoxicity and mutagenicity of many substances, such as natural or semi-synthetic products, as well as their effects on DNA [10–12]. *In vitro* techniques provide multiple and complementary measures of cytotoxicity [13] and can be used to evaluate the effect of natural products and chemotherapeutic compounds on direct contact with cells [14,15]. In addition, *in vivo* studies, such as those using rodents, have been used to complement *in vitro* studies and may help to understand the effect of metabolization and the toxicological effects of various compounds [11], as well as their potential use in the treatment of cancers.

To our knowledge, the effects of *C. jamacaru* consumption and its possible antioxidant, anti-cytotoxic and anticancer activities are unclear and not well documented in the literature. Thus, the aim of this study was to evaluate the *C. jamacaru* extract chemical composition, antioxidant activity, cytotoxic and anti-cytotoxic effects in human lymphocytes and sarcoma 180 cells *in vitro* and antitumoral, mutagenic and cytotoxic effects *in vivo* mice sarcoma-induced.

2. MATERIAL AND METHODS

2.1. Chemicals

Ficoll® Paque Plus (Sigma–Aldrich, Missouri, United States); RPMI 1640 culture medium (Cultilab, Campinas, Brazil); fetal calf serum (Gibco, Miami, United States); Cisplatin (Fauldcispla®, Libbs, São Paulo, Brazil); 2,2-diphenyl-1-picrylhydrazyl (Sigma–Aldrich, Missouri, United States); h); 2,2′-azino-bis-(-3-ethylbenzothiazoline-6-sulfonic acid) (Sigma–Aldrich, Missouri, United States); 3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (Sigma–Aldrich, Missouri, United States).

2.2. Plant Material

C. jamacaru cladodes were collected at Alagoas state, Northeast of Brazil (9°40′44.7″ S, 36°41′21.9″ W), in September 2016. A voucher specimen was deposited in the Herbarium of Universidade Federal do Espírito Santo—VIES. The thorns of plant material were removed with a blade, the cladodes were sliced and 485 g of the fragmented material was oven dried at 50 °C for 24 h.

2.3. Hydroalcoholic Extract

Dried plant material was powdered, macerated in EtOH/H_2O (70:30 *v*/*v*) solution (dry plant: EtOH/H_2O—1:5 *w*/*v*) at room temperature (25–30 °C), protected from the light, for five days, filtered and evaporated under reduced pressure at 60 °C to obtain the crude extract of *C. jamacaru*. The extract was stored at 6–10 °C and protected from the light until use. The yield of the extract was calculated by the formula:

$$\text{Total extract yield (\%)} = (FM/IM) \times 100$$

where “FM” = final mass of dry extract (g); “IM” = initial mass of dry plant (g).

2.4. Phytochemistry Analysis

2.4.1. Preliminary Phytochemistry

In order to identify secondary metabolites, preliminary phytochemical prospection was performed using *C. jamacaru* crude extract. Tests for coumarins,

flavonoids, alkaloids, naphthoquinones, saponin, steroids, tannins and triterpenoids were conducted according to the procedures described in the literature [16].

2.4.2. Thin Layer Chromatography

Identification of tyramine in *C. jamacaru* extract was performed by thin layer chromatography (TLC), as described by Davet et al. [7].

2.4.3. Flavonoid Content

Total flavonoid content of *C. jamacaru* extract was measured by the colorimetric method described for Zhishen et al. [17], with minor modifications. The absorbance at 430 nm was detected by ELISA reader and the experiment was performed in triplicate. Methanolic dilutions series of rutin were prepared and assayed. The amount of flavonoid in extract was expressed in milligram of flavonoid equivalent to rutin per gram of dry matter of extract.

2.5. Antioxidant Activity

2.5.1. DPPH

Antioxidant activity of *C. jamacaru* extract was evaluated by the radical reduction method, DPPH• (2,2-diphenyl-1-picrylhydrazyl), which fixing an H• leads to a decrease in absorbance [18]. The absorbance was taken by ELISA reader at 517 nm and the test was performed in triplicate. The reduction percentage of the DPPH radical was calculated by the following formula:

$$\% \text{ inhibition} = [(\text{AbsControl} - \text{AbsSample})/\text{AbsControl}] \text{ X } 100$$

where “% inhibition” is the percentage of inhibition capacity of DPPH•; “AbsControl” is the absorbance of DPPH• reaction control; and “AbsSample” is the absorbance of the sample.

2.5.2. ABTS

Total antioxidant activity of *C. jamacaru* extract was measured by capturing method 2,2′- azino-bis-(-3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), $ABTS^{\bullet+}$ radical [19]. The absorbance was taken by ELISA reader at 734 nm and experiment was performed in triplicate. The percentage of scavenging inhibition capacity of $ABTS^{\bullet+}$ of *C. jamacaru* extract was calculated by the following equation:

$$\% \text{ inhibition} = [(\text{AbsControl} - \text{AbsSample})/\text{AbsControl}] \text{ X } 100$$

where “% inhibition” is the percentage of scavenging inhibition capacity of ABTS•$^{+}$; “AbsControl” is the absorbance of ABTS•$^{+}$ reaction control; and “AbsSample” is the absorbance of the sample.

2.5.3. Fe^{2+} Chelation Ions

Ferrous ions (Fe^{2+}) chelating activity was measured by the inhibition of ferrous–ferrozine complex formation after treatment with *C. jamacaru* extract [20]. The absorbance was measured by the ELISA reader at 562 nm. The percentage of ferrous ion chelating effect was calculated using the following equation:

$$\% \text{ chelating effect} = [(\text{AbsControl} - \text{AbsSample})/\text{AbsControl}] \text{ X } 100$$

where “% chelating effect” is the percentage of ferrous ions (Fe^{2+}) chelating effect; “AbsControl” is the absorbance of Fe^{2+} reaction control; and “AbsSample” is the absorbance of the sample.

2.6. *In vitro* Cell Assays

2.6.1. Human Lymphocytes

Human lymphocytes were obtained from the peripheral blood sample of a healthy non-smoking volunteer with informed consent, aged between 20 and 30 years, with no history of recent disease, exposure to radiation or drug use and no alcohol ingestion thirty days before blood donating. The lymphocytes were isolated by the traditional method on Ficoll® Paque Plus gradient, as recommended by manufacturer, with minimal modifications. All protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 2.333.879). Human lymphocytes were plated in 96-well plates with 2.10^5 cells in each well. Cells from the control group were not treated and cisplatin group cells received cisplatin at 50.0 µg/mL. To evaluate cytotoxicity, human lymphocytes cells received *C. jamacaru* extract diluted with water at 10.0, 50.0 or 100.0 µg/mL. The cells were cultured with *C. jamacaru* extract for 24 h or 48 h. In order to allow the evaluation of the anti-cytotoxicity, human lymphocytes were treated with *C. jamacaru* extract more cisplatin following the protocols of pre-treatment, simultaneous treatment and post-treatment. In the pre-treatment protocol, the cells

were previously treated with *C. jamacaru* extract diluted with water at 10.0, 50.0 or 100 µg/mL and 24 h after was added cisplatin at 50.0 µg/mL. Cells in the simultaneous protocol received *C. jamacaru* extract at 10.0, 50.0 or 100.0 µg/mL and cisplatin at 50.0 µg/mL simultaneously. In the post-treatment protocol the cells previously received cisplatin at 50.0 µg/mL and 24 h after the cell were treated with *C. jamacaru* extract at 10.0, 50.0 or 100.0 µg/mL.

The percentage of cytotoxic damage reduction was calculated using the adapted formula [21]:

$$\% \text{ Reduction} = \frac{(\% \text{ cell viability in A} - \% \text{ cell viability in B})}{(\% \text{ cell viability in A} - \% \text{ cell viability in C})} \text{ X } 100$$

where “A” is the cell group treated with cisplatin; “B” is the cell group treated with *C. jamacaru* extract more cisplatin; and “C” is the control group of cells.

2.6.2. Sarcoma 180

Sarcoma 180 cells (murine sarcoma) were acquired from Banco de Células do Rio de Janeiro and all protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 89/2015). Sarcoma 180 cells were plated in 96-well plates with 2.10^5 cells in each well. Control group cells were untreated and cisplatin group cells were treated with cisplatin at 50.0 µg/mL. Treated cells received *C. jamacaru* extract diluted with water at 10.0, 50.0 or 100.0 µg/mL. Cells were cultured with *C. jamacaru* extract for 24 h or 48 h to evaluate its antiproliferative effect.

2.6.3. Cell Culturing Methods

Cells were cultured with RPMI 1640 culture medium, supplemented with antibiotic gentamicin (50.0 mg/L) and antimycotic amphotericin B (2.0 mg/L) and 10% of fetal calf serum at 37 °C, 5% of CO_2 saturation and humid atmosphere. Cells were cultured under these conditions 24 h before starting the treatments. 24 h after the last treatment, MTT assay was used to determine cell viability.

2.6.4. MTT Assay

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate cell viability. After treatment, the plates were centrifuged at 860 rcf for 10 min, the supernatant discarded and 20 µL of MTT at 5

mg/mL were added to each well. 3 h later, the plates were centrifuged at 860 rcf for 5 min, the supernatant was discarded, 100 µL of DMSO was added and absorbance was detected in ELISA reader at 590 nm. The experiment was performed in triplicate and the results were expressed as relative percentage of cell viability in comparison to control.

2.7. *In vivo* Mice Antitumor

2.7.1. Animals and Sarcoma Induction

Twenty albino male mice *Swiss* strain (*Mus musculus*) (*n* = 20), aged 6 to 8 weeks and approximately 40 g of body weight (b.w.) were supplied by the biotery of Univerdidade Federal do Espírito Santo. The animals were placed in polypropylene cages with metal bars and wood shavings and they passed an acclimatization period of 7 days before the start of the experiments, with free access to standard commercial feed and water and they were kept under light/dark cycles of 12 h.

For the induction of solid tumors and evaluation of the antitumor activity of *C. jamacaru* extract, 200 µL of sarcoma 180 cells diluted in NaCl (0.9%) (5.10^5 cells/mL) were injected, subcutaneously, in the dorsal region of the animals between the neck and the shoulder girdle. This procedure was conducted with 16 animals (*n* = 16). After the tumor induction protocol, the 16 animals were randomly separated into four treatment groups with four animals each (*n* = 4). A group of four animals that did not receive the sarcoma 180 cells (*n* = 4) was used as a healthy control.

All protocols involving animals were conducted according to the ethical principles of animal experimentation established by the Research Ethical Committee on Animal Use Univerdidade Federal do Espírito Santo (CEUA/UFES, certificate 89/2015).

2.7.2. Selection of Doses and Treatment Groups

The treatment of the animals was started three days after tumor induction. The dosages chosen for the experiments were based on the LD_{50} of hydroalcoholic extract of *C. jamacaru* cladodes [22]. Three experimental groups of mice with sarcoma tumor received daily *C. jamacaru* extract i.p. at the dose of 5.0, 10.0 or

20.0 mg/kg b.w. (*C. jamacaru* treatment groups); a group of mice with sarcoma tumor received daily NaCl (0.9%) i.p (sarcoma group); and the group of healthy animals (without sarcoma tumor induction) received daily NaCl (0.9%) i.p (healthy group). The animals received the doses of *C. jamacaru* extract or NaCl (0.9%) for 20 consecutive days and 24 h after the last treatment the animals were euthanized by cervical dislocation.

2.7.3. Tumor Inhibition

At the end of the experiment period, the tumors of the mice of each experimental group were removed. The tumors were weighed and the mean tumor weight of each experimental group was used to calculate the percentage of tumor inhibition by the formula:

$$(\%)\ \text{Tumor inhibition} = \frac{(\text{Tumor weight in A} - \text{Tumor weight in B})}{\text{Tumor weight in A}} \text{ X } 100$$

where “A” is the mean of the tumor weight of sarcoma group; “B” is the mean of the tumor weight of the *C. jamacaru* treatment groups.

2.7.4. Macroscopic Analysis of Organs

Kidneys, liver, spleen and heart of animals from each experimental group were removed. The organs were evaluated for possible macroscopic abnormalities and subsequently weighed for comparison between the experimental groups.

2.7.5. Micronucleus Test in Mice Peripheral Blood Cells

Three days after tumor induction, to assess the mutagenicity and cytotoxicity, peripheral blood was collected from mice prior to initiation of treatment (0 day of treatment) and at the end of treatment with NaCl (0.9%) or doses of *C. jamacaru* extract (20 days of treatment). The peripheral blood was obtained from the caudal artery of each animal of experimental groups using a sterile needle and the smears of whole blood were prepared on clean microscope slides, air dried. For each peripheral blood collection performed, two slides were prepared per animal, the cells were fixed in methanol (100%) and stained with Leishman twice in two different concentrations (100% for three minutes and 1 Leishman: 6 distilled water, for fifteen minutes) to differentiate immature polychromatic erythrocytes (PCE) and mature normochromatic erythrocytes (NCE), following the criteria described by

Krishna and Hayashi [23]. The slides were analyzed under an optical microscope (Nikon Eclipse E200) with an increase of 1000 times. For the evaluation of mutagenicity, 2000 NCE were recorded per animal, 1000 NCE per slide, considering the micronucleated normochromatic erythrocytes (MNNCEs) [23]. The frequency of PCE in 2000 (PCE) per animal, 1000 NCE per slide, was used as a parameter of cytotoxicity [12].

2.8. Statistical Analysis

Data were evaluated a priori by normality test and the results were expressed as the mean ± standard deviation or median (Percentile 25—Percentile 75). *C. jamacaru* extract concentration required to reduce 50% of the DPPH, ABTS or Fe^{2+} chelating ions (EC_{50}) and R^2 were obtained by the linear curve, relating the antioxidant capacity of the extract and its concentrations. To evaluate the cytotoxicity of *C. jamacaru* extract *in vitro*, the cell viability of lymphocytes and sarcoma 180 were compared to the respective control cells by ANOVA *post hoc* Dunnett's test ($p < 0.05$). The comparison between human lymphocytes and sarcoma 180 cells was performed by multiple *t* test ($p < 0.05$). For the evaluation of the anti-cytotoxicity of *C. jamacaru* extract *in vitro*, the lymphocytes cell viability was compared to the cisplatin treated cells by ANOVA *post hoc* Dunnett's test ($p < 0.05$). To analyze the antitumor effect, mutagenicity, cytotoxicity and organs weight alterations on *in vivo* mice treatments, it was performed a comparison between the sarcoma group and experimental groups by Mann Whitney test ($p < 0.05$). Wilcoxon's test ($p < 0.05$) was performed to evaluate mutagenicity and cytotoxicity *in vivo* prior to treatment initiation (0 day of treatment) and after treatment (20 days of treatment with NaCl (0.9%) or doses of *C. jamacaru* extract).

3. RESULTS

3.1. Extract Yield and Phytochemistry

After 24 h in an oven dried at 50 °C were obtained 82.0 g of dried plant material. A total of 6.8 g of *C. jamacaru* hydroalcoholic crude extract was obtained, which corresponds to 8.29% of total extract yield. Preliminary phytochemistry of *C. jamacaru* extract showed positive reactions to coumarins, flavonoids and flavanol (cyanidin reaction) and TLC showed the presence of the alkaloid tyramine. The total flavonoid content was assayed by $AlCl_3$ colorimetric method and determined that the crude extract of *C. jamacaru* cladodes contained 0.51 ± 0.14 µg/mL (rutin equivalent).

3.2. Evaluation of Antioxidant Activity

The results of the antioxidant activity of *C. jamacaru* extract are summarized in the Figure 1. In the DPPH assay, *C. Jamacaru* extract reached 9.43–57.36% of antioxidant activity and the standard trolox reached 94.42–94.60% (Figure 1A). Following the ABTS assay, the extract reached 20.25–65.76% of antioxidant activity and the standard ascorbic acid reached 87.66–92.86% (Figure 1B). In the chelating activity on Fe^{2+} ions, *C. jamacaru* extract reached 59.21–76.06% of antioxidant activity and the standard EDTA reached 96.47–96.92% (Figure 1C). The antioxidant activity of *C. jamacaru* extract expressed in half-maximal effective concentration (EC_{50}) was: DPPH - EC_{50} = 427.74 ± 5.80 µg/mL, ABTS - EC_{50} = 270.57 ± 4.99 µg/mL, Fe^{2+} chelating ions - EC_{50} = 41.18 ± 7.59 µg/mL.

3.3. Cytotoxicity and Antiproliferative Activity *in vitro*

In Table 1 the results of *C. jamacaru* extract cytotoxicity in human lymphocytes and sarcoma 180 cells are summarized.

C. jamacaru extract was able to reduce human lymphocyte cell viability after 24 h of exposure at 10.0 and 50.0 µg/mL and promote lymphocytes proliferation at 100.0 µg/mL. After 48 h of exposure, the lymphocytes cell viability at 10.0 µg/mL was reduced, at 50.0 µg/mL lymphocytes viability was statistically similar to the control and at 100.0 µg/mL *C. jamacaru* extract continued to promote lymphocytes

proliferation. On the other hand, *C. jamacaru* extract reduced significantly the viability of sarcoma 180 cells after 24 h and after 48 h of exposure at all doses tested. When cytotoxicity of healthy and tumor cells was compared by multiple *t* test ($p < 0.05$), it was possible to infer that *C. jamacaru* extract was more cytotoxic to sarcoma 180 cells than to human lymphocytes at 24 h and 48 h.

3.4. Anti-Cytotoxic Activity *in vitro*

Table 2 summarize the results of anti-cytotoxicity in the protocols of pre-treatment, simultaneous treatment and post-treatment with *C. jamacaru* extract. Following the pre-treatment protocol, compared to cisplatin-treated cells, it was observed that *C. jamacaru* extract increased cell viability of human lymphocytes at all tested doses. For the simultaneous treatment, it was observed statistical reduction of cisplatin induced-damage in cell viability at the treatment dose of 50.0 and 100.0 µg/mL. In the post-treatment protocol the treatment dose of 50.0 and 100.0 µg/mL reduced the damage induced for cisplatin. In all treatment protocol the dose of 100.0 µg/mL was the most effectively in cytotoxic damage inhibition.

3.5. Antitumor Activity *in vivo*

Table 3 shows the results of antitumor activity induced by tested doses of *C. jamacaru* extract in mice with sarcoma. In comparison to sarcoma group, animals receiving *C. jamacaru* extract at the treatment dose of 20.0 mg/kg b.w. showed tumor reduction (86.07% of tumor inhibition). The treatment doses of 5.0 and 10.0 mg/kg b.w. were not able to induce a decrease in tumor weight.

3.6. Weight and Macroscopic Analysis of Organs

Macroscopic morphological abnormalities were not observed in the animal organs of the experimental groups. The weight of mice organs of each group is presented in Table 4. It was observed the reduction of the weight of the kidneys and heart of the animals treated with 5.0 and 10.0 mg/kg b.w. of *C. jamacaru* extract. The liver weight of the animals did not change and the spleen weight was increased in all animals with sarcoma compared to the healthy group of animals.

3.7. Mutagenicity and Cytotoxicity *in vivo*

MNNCE and PCE frequency related to PCE in peripheral blood cells of rodents is summarized in Table 5. After tumor induction, at 0 day of treatment, MNNCE frequency before the start of treatments was increased in animals with sarcoma, indicating that the sarcoma tumor induction promotes mutagenic effects. However, at the end of treatment time (20 days of treatment), the frequency of MNNCE was not statistically different between the sarcoma group and the treatment groups, suggesting the absence of mutagenic damage. For the comparison over time (0 day vs. 20 days of treatment), no statistical difference was observed in the frequency of MNNCE.

Prior to initiation of treatment, at 0 day of treatment, the frequency of PCE per 1000 NCE was not significantly different between the experimental groups and the sarcoma group. After 20 days of treatment, in comparison to the group of healthy mice, there was no significant decrease in the frequency of PCE per 1000 NCE, suggesting the absence of cytotoxic damage. On the other hand, the frequency of PCE per 1000 NCE was increased in animals with sarcoma (sarcoma group) and treated with 5.0 mg/kg b.w. of *C. jamacaru* extract, suggesting alterations on mitotic cycle. Along treatment time, the comparison between 0 and 20 days of treatment showed a significant increase in PCE frequency in sarcoma group after 20 days of treatment (p = 0.0078). For the other treatment groups, no significant alterations were observed over time for the conditions tested.

4. DISCUSSION

Biological active constituents present in natural products are not fully identified but due to their effectiveness, presumably minimal side effects and relatively low cost, their uses have increased [3]. A variety of bioactive compounds are found in natural products included in a healthy diet and they have been associated to the promotion of human health in the last decade [24]. In that way, plants of cactaceae family have been characterized as a feed source, as well as some studies evaluating cacti have demonstrated their uses as antioxidants and anticancer [25–27]. *Opuntia*, *Pereskia* and *Pilosocereus* are cacti genus that have been extensively studied and well documented for their antioxidant capacity and biological applications, however, there are few studies with *C. jamacaru*.

Phytochemicals are non-nutritive chemicals that occur naturally in plants and can be used in the plant defense mechanisms [28]. In the phytochemical analysis of *C. jamacaru* we detected coumarins, flavonoids (flavanol) and the alkaloid tyramine. Coumarin and flavonoids are phenolic compounds present in many plant foods and among polyphenolic substances, flavonoids are the class of compounds most commonly found in plants and can be classified into flavanols, flavanones, flavones, isoflavones, catechins, anthocyanins, proanthocyanidins and others [5]. On the other hand, alkaloids are nitrogen compounds found in medicinal plants that have been extensively studied and reported as antioxidants [29,30].

In our study, the total flavonoid content in hydroalcoholic extract of *C. jamacaru* cladodes was 0.51 µg/mL, similar to the observed in the study of De Sousa Araújo et al. [8], using methanolic extract of *C. jamacaru* cladodes (0.59 µg/mL). Both plants were collected in arid regions of Brazil and probably grown under similar light, water and nutrient availability. Figueroa-Cares et al. [31], in a study with cultivars, verified that flavonoid content may vary and suggests that the differences between flavonoid content in the cultivars can be related to the water scarcity and soil nutrients.

Alkaloids tyramine and N-methyltyramine are compounds produced by *C. jamacaru* and they are chemical markers of this specie [32]. Preliminary phytochemistry showed negative reaction to alkaloids by Dragendorff reagent and presented a positive reaction to the tyramine alkaloid by thin layer chromatography. Alkaloids are chemical compounds that have nitrogen atom in their structures. This

group of chemicals is divided in true alkaloids, protoalkaloid, polyamine alkaloids, peptide and cyclopeptide alkaloids and pseudoalkaloids [33]. Dragendorff reagent has been used to detect tertiary and quaternary alkaloids [34–36]. Tyramine, as well as N-methylthyramine, are classified as primary alkaloids and were detected in our study by the specific protocol developed by Davet et al. [7]. The analysis suggests that the amount of tertiary and quaternary alkaloids in the extract of *C. jamacaru* was relatively low and not detected.

C. jamacaru extract showed antioxidant activity in all protocols tested (Figure 1). DPPH and ABTS assays are methods used to measure the ability of antioxidant to scavenging free radicals, which are the major factor in biological damages caused by oxidative stress and both radicals used in these assays are reduced to their stable or less reactive derivatives by the antioxidant compounds [37]. *C. jamacaru* hydroalcoholic extract was able to inhibit the activity of DPPH radicals (Figure 1A), EC_{50} = 427.74 µg/mL. The concentration of *C. jamacaru* extract required to achieve a 50% reduction in DPPH radicals was lower than the observed in MeOH and n-hexane fractions of cladodes extracts of the cactaceae *Opuntia monacantha*, EC_{50} = 833.0 and 469.0 µg/mL, respectively and larger than the observed in OM–EtOAc and OM–n-BuOH fractions of the same cactus extract, EC_{50} = 53.2 and 278.0 µg/mL, respectively [25]. For other cacti of the *Opuntia* genus, such as *O. dillenii*, the concentration required to reduce 50% of DPPH radicals was 48.0 µg/mL—EtOH 80% stem extract [38] and to the ethanolic extract of *O. ficus-indica* stem extract the EC_{50} was equivalent to 9.3 µg/mL [39].

ABTS assay showed better result than the observed in DPPH assay (Figure 1A,B). In a similar assay, *Pereskia bleo* aqueous extract showed a low antioxidant effect when compared to the ascorbic acid standard [40], different to that observed in our study. Added to this, the EC_{50} of *C. jamacaru* extract in ABTS test was 270.7 µg/mL, higher than the observed in *Pilosocereus gounellei* stem extract, EC_{50} = 62.4 µg/mL [9].

Ethanolic extracts of *Rumex vesicarius* leaves produced by three different extraction methods showed good antioxidant activity on chelating activity on Fe^{2+} ions assay, presenting EC_{50} between 157.4–185.3 µg/mL [41]. For the antioxidant assays performed in this study, the *C. jamacaru* extract presented better antioxidant activity in the chelating activity on Fe^{2+} ions assay, EC_{50} = 41.18 µg/mL. *C. jamacaru* extract presented in its composition alkaloids and phenolic

compounds. In a study conducted by Klimaczewski et al. [30], the boldine alkaloid—commonly found in *Peumus boldus*—was able to induce good antioxidant activity in the DPPH assay but was not effective in promoting ion chelation in the Fe^{2+} ions. On the other hand, natural products rich in phenolics compounds have exhibited good ability to chelate ferrous ions, which has been related to the hydroxyl group on flavonoids [42]. Added to this, a study conducted with the alkaloid tyramine, compound detected in *C. jamacaru* extract, showed strong scavenging activity in DPPH assay and reducing power, reaching 86.34% of DPPH radical inhibition [43], which may have contributed to overall antioxidant activity.

The 3-[4-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is a yellow tetrazolium salt, water soluble, that is converted to formazan, purple and insoluble in water. The conversion of MTT to formazan is mediated by the cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria, as well as, it can be mediated by NADH or NADPH within the cells and out of mitochondria [44,45]. MTT assay is a cell viability assay used to determine cytotoxicity upon exposure to toxic substances and has been used to verify the viability of human lymphocytes [46,47] and sarcoma 180 cells exposed to natural products [48,49].

Neutral red, LDH leakage and the protein assays are also used to evaluate cell viability. However, neutral red and the MTT assay have been described as the most sensitive cytotoxicity assays and appear to be more sensitive in detecting early toxicity [44]. In this way, some compounds may inhibit mitochondrial respiration and induces active oxygen related cell death, generating reactive oxygen species within the mitochondria that promotes the damage mitochondrial components and therefore a cytotoxicity assay based on mitochondrial respiratory activity might be used to detect early toxicity following exposure to a mitochondrial toxicant [44].

We observed that *C. jamacaru* extract effectively reduced the viability of sarcoma cells 180 and that the extract did not induce significant reduction in the cellular viability of human lymphocytes, preventing and repairing cytotoxic damages (Tables 1 and 2). These results suggest that *C. jamacaru* extract may promotes descytotoxic activity, acting directly on the cisplatin, inducing chemical or enzymatic inactivation of cytotoxic compounds (pre-treatment and simultaneous treatment protocols), as well as promoting bioanticytotoxic activity, inducing the

repair process or acting on the processes that induce the cytotoxic damage (post-treatment protocol).

Cisplatin is a highly reactive molecule used for the treatment of cancers due to its ability to binds to RNA, DNA and proteins, forming different types of adducts and thus generate cytotoxic effects [50,51]. In particular, the adducts formed with nuclear DNA have been reported as key lesions that mediates the cytotoxic effect of cisplatin and the repair of these lesions may occur by intracellular DNA damage management pathways, such as nucleotide excision repair pathway, which plays a major role in removing cisplatin-nuclear DNA adducts [14,52].

Nuclear DNA damage is not sufficient to explain its use as effectiveness as an anticancer agent, since cisplatin toxicity does not depend only on the amount of drug accumulation in normal tissues, suggesting that nuclear DNA transcription blockage may not be the unique mechanism to determinate the toxic effect of cisplatin in non-replicating cells [14,53]. Cisplatin may accumulates into mitochondria, form adducts with mitochondrial DNA and proteins and increase intracellular ROS in normal cells [54–58].

The impairment of electron transport chain protein synthesis, as well as cisplatin-induced ROS generation, occur as consequence of its direct effect on mitochondrial DNA. Mitochondrial redox status, DNA integrity and bioenergetic functionality are also reported as key modulators of the cellular response to cisplatin-induced mitochondrial impairment and may be factors determining resistance to its cytotoxic effect [14]. Cisplatin exposure induces a mitochondria-dependent ROS response, which significantly contributes to cell death by enhancing the cytotoxic effect exerted through the formation of nuclear DNA damage [14]. Additionally, cisplatin promotes mitochondrial injury, energy imbalance and oxidative damage [14,59,60] and antioxidant compounds treatment may ameliorates the toxic effects promoted to cisplatin by increasing mitochondrial ROS scavenging.

Iron is related to tissue injury, ROS synthesis and reported as mediator of cisplatin tissue injury in cisplatin-induced nephrotoxicity [61,62]. This metal is reversibly oxidized and may generate powerful oxidant species, such as the hydroxyl radical (Haber-Weiss reaction), or generates highly reactive iron-oxygen complexes such as ferryl or perferryl ions [61], increasing the damage induced by cisplatin. On the other hand, the treatment with both iron chelators and hydroxyl

radical scavengers may prevent cisplatin cytotoxicity and renal failure [62], which suggests that compounds capable of chelating iron ions may reduce cisplatin-induced damage.

In vivo anticancer activity of *C. jamacaru* extract has already been reported in the literature. In the study conducted by Souza et al. [22], after the induction of sarcoma, male mice received daily doses of hydroethanolic extract of *C. jamacaru* cladodes at dose of 40 mg/kg b.w. and exhibited 65.61% tumor inhibition. Similar to the study of Souza et al., we demonstrated in our study the ability of the *C. jamacaru* hydroalcoholic extract to promote antitumor activity against sarcoma *in vivo* (86.07% tumor inhibition).

In our study, no macroscopic abnormalities were observed, kidney and heart weight decreased in animals receiving *C. jamacaru* extract doses of 5.0 and 10.0 mg/kg b.w. and, compared with the healthy group of animals, the weight of the spleen was increased in all animals with sarcoma. No macroscopic or histopathological abnormalities have been described in healthy rats receiving doses of ethanolic extract of cladodes of *C. jamacaru* for 30 days, as well as alteration in kidney, liver, spleen and heart weight in relation to body [63].

Micronucleus test in peripheral blood can be performed in different time points along treatment time and allows to verify, through the differentiation between polychromatic and normochromatic erythrocytes, both mutagenicity and cytotoxicity [12]. In an experiment conducted with pregnant rats treated with of methanolic extract of *C. jamacaru*, Messias et al. [64] report that the extract of this cactus did not induce cytotoxic and mutagenic damages and was able to promote mild antimutagenic effect. Our findings suggest that sarcoma induction promotes mutagenic damage, detected three days after the induction of solid tumors (0 day of treatment). However, at the end of the treatment time (20 days of treatment) this effect is not observed, suggesting the absence of mutagenic damage over time. In addition, at the end of the treatment time, it was observed that the presence of solid tumors of sarcoma led to the increase of PCE frequency, indicating changes in the mitotic cycle that may lead to increase of PCE proliferation.

There are several mechanisms involved in the evolution of a normal cell into a potentially malignant cell and most of them interfere in cell division [65]. In this way, knowledge of the cell cycle and its mechanisms are important tools for understanding the etiology of cancer [66]. The drugs used in cancer treatment may

have action on tumor cells that are in the cell cycle (specific cell cycle drugs) or act on tumor cells regardless of whether they are traversing the cycle or being resting in the G_0 compartment (non-specific cell cycle drugs) [67–69].

Natural products with specific cell cycle action are used as antineoplastic agents. Among the natural cytotoxic products used clinically in the treatment of neoplasias are plant alkaloids, nitrogenous compounds biosynthesized from amino acids, such as vinblastine and vincristine (promote inhibition of mitotic spindle, binding to the microtubular proteins and interrupting cell division in metaphase) [70]; taxol (inhibits mitotic spindle, induces tubulin dimerization and stabilization of tubules, protecting them from depolymerization, leading to blocking of multiplication and loss of cell viability) [70]; and podophyllotoxins or epipodophyllotoxins (block the cells in the S and G_2 phases and inhibit the action of the enzyme topoisomerase II, leading to DNA damage) [67,69,70].

5. CONCLUSIONS

C. jamacaru extract presented in its chemical composition phenolic and nitrogen compounds and good antioxidant ability to chelate metallic ions. We suggest that the increased ability of *C. jamacaru* extract in chelating metals may be related to anti-cytotoxic activity against cisplatin *in vitro*. In addition, we suggest that *C. jamacaru* extract selectively acts on tumor cells, probably blocking metabolic processes to the survival of the cells, acting on cell cycle of the tumor cells *in vitro* and *in vivo*, leading to anticancer effects and tumor reduction. However, additional studies are necessary to determine the mechanisms involved in the anti-cytotoxic and anticancer effects. These findings reinforce that this cactus used in human food also presents great therapeutic potential and can be used for drug discovery.

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Tables and figures

Table 1. *In vitro* cytotoxicity of *C. jamacaru* extract (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes and sarcoma180 cells by MTT assay.

Treatment	Cell Viability (%) ± SD							
	24 h of Treatment				48 h of Treatment			
	Lymphocyte	*p*	Sarcoma-180	*P*	Lymphocyte	*p*	Sarcoma-180	*p*
Control	100.00 ± 2.41	–	100.00 ± 0.99	–	100.00 ± 4.83	–	100.00 ± 3.48	–
C. jamacaru 10.0 µg/mL	81.08 ± 1.16 ****†	<0.0001	30.53 ± 3.51 ####†	<0.0001	84.40 ± 1.58 ***†	0.0003	16.09 ± 0.17 ####†	<0.0001
C. jamacaru 50.0 µg/mL	88.80 ± 1.34 **†	0.0043	29.84 ± 1.18 ####†	<0.0001	95.41 ± 0.80 †	0.4243	17.54 ± 0.44 ####†	<0.0001
C. jamacaru 100.0 µg/mL	122.39 ± 5.47 ****†	<0.0001	19.82 ± 1.60 ####†	<0.0001	133.95 ± 7.07 ****†	<0.0001	22.29 ± 1.10 ####†	<0.0001

The values are the means ± SD. Cell viability was compared to its respective control cells by ANOVA *post hoc* Dunnett's test—** $p < 0.01$, *** $p < 0.001$ or **** $p < 0.0001$ vs. human lymphocytes control; #### $p < 0.0001$ vs. sarcoma 180 control. The comparison between the human lymphocytes and sarcoma 180 cells was performed by multiple *t* test—† $p<0.05$.

Table 2. *In vitro* anti-cytotoxicity of *C. jamacaru* extract (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes cells by MTT assay.

Treatment	Cell Viability (%) ± SD								
	Pre-Treatment	***p***	**% Reduction**	**Simultaneous Treatment**	***P***	**% Reduction**	**Post-Treatment**	***p***	**% Reduction**
Control	100.00 ± 4.83*	0.0182	–	100.00 ± 2.4***	0.0006	–	100.00 ± 4.83***	0.0008	–
Cisplatin	73.85 ± 6.50	–	–	74.52 ± 2.68	–	–	73.85 ± 6.50	–	–
C. jamacaru 10.0 µg/mL + Cisplatin	89.44 ± 5.84*	0.0201	59.62	79.15 ± 2.41	0.6822	20.27	76.15 ± 4.42	0.9635	8.80
C. jamacaru 50.0 µg/mL + Cisplatin	96.79 ± 2.11*	0.0367	87.72	87.65 ± 3.54*	0.0421	52.77	90.82 ± 2.75*	0.0147	64.89
C. jamacaru 100.0 µg/mL + Cisplatin	127.06 ± 4.83***	0.0001	>100.00	106.57 ± 10.62***	0.0001	>100.00	115.60 ± 8.38****	<0.0001	>100.00

The values are the means ± SD. Cell viability was compared to the cisplatin treated cells in each treatment protocol by ANOVA *post hoc* Dunnett's test—**p* < 0.05, ***p* < 0.01, ****p* < 0.001 or *****p* < 0.0001 vs. human lymphocytes control.

Table 3. Tumor weight of Swiss albino mice sarcoma induced treated with *C. jamacaru* extract (5.0, 10.0 or 20.0 mg/kg b.w.).

Treatment	Tumor Weight (P_{25}–P_{75})	*P*	% Tumor Inhibition
Sarcoma + NaCl (0.9%)	0.070 (0.038–0.210)	–	–
Sarcoma + *C. jamacaru* 5.0 mg/kg b.w.	0.130 (0.010–0.610)	0.8571	–
Sarcoma + *C. jamacaru* 10.0 mg/kg b.w.	0.130 (0.048–0.430)	0.5606	–
Sarcoma + *C. jamacaru* 20.0 mg/kg b.w.	0.015 (0.012–0.023) *	0.0238	86.07

The values are median (Percentile 25—Percentile 75). P_{25} = Percentile 25; P_{75} = Percentile 75. Tumor weight of *C. jamacaru* treatment groups were compared to the sarcoma group by Mann Whitney test ($p < 0.05$)—* $p < 0.05$ vs. tumor weight of sarcoma group.

Table 4. Organs weight of *Swiss* albino mice sarcoma induced treated with *C. jamacaru* extract (5.0, 10.0 or 20.0 mg/kg b.w.).

Treatment	Weight (g) (P_{25}–P_{75})							
	Kidney	***p***	**Liver**	***P***	**Spleen**	***p***	**Heart**	***p***
Sarcoma + NaCl (0.9%)	0.690 (0.628–0.713)	–	2.510 (2.460–3.043)	–	0.270 (0.233–0.415)	–	0.245 (0.228–0.300)	–
Sarcoma + *C. jamacaru* 5.0 mg/kg b.w.	0.575 (0.443–0.655) *	0.0429	2.495 (1.978–2.653)	0.5619	0.260 (0.228–0.353)	0.8048	0.205 (0.140–0.225) *	0.0286
Sarcoma + *C. jamacaru* 10.0 mg/kg b.w.	0.530 (0.480–0.570) *	0.0047	2.400 (1.910–2.580)	0.3625	0.250 (0.200–0.310)	0.4219	0.190 (0.150–0.220) **	0.0047
Sarcoma + *C. jamacaru* 20.0 mg/kg b.w.	0.630 (0.535–0.688)	0.1810	2.715 (2.405–2.905)	>0.9999	0.285 (0.240–0.335)	0.8048	0.205 (0.190–0.235)	0.0524
Health + NaCl (0.9%)	0.560 (0.505–0.668)	0.0762	2.290 (1.895–2.663)	0.1238	0.170 (0.108–0.225) *	0.0381	0.170 (0.145–0.240)	0.1095

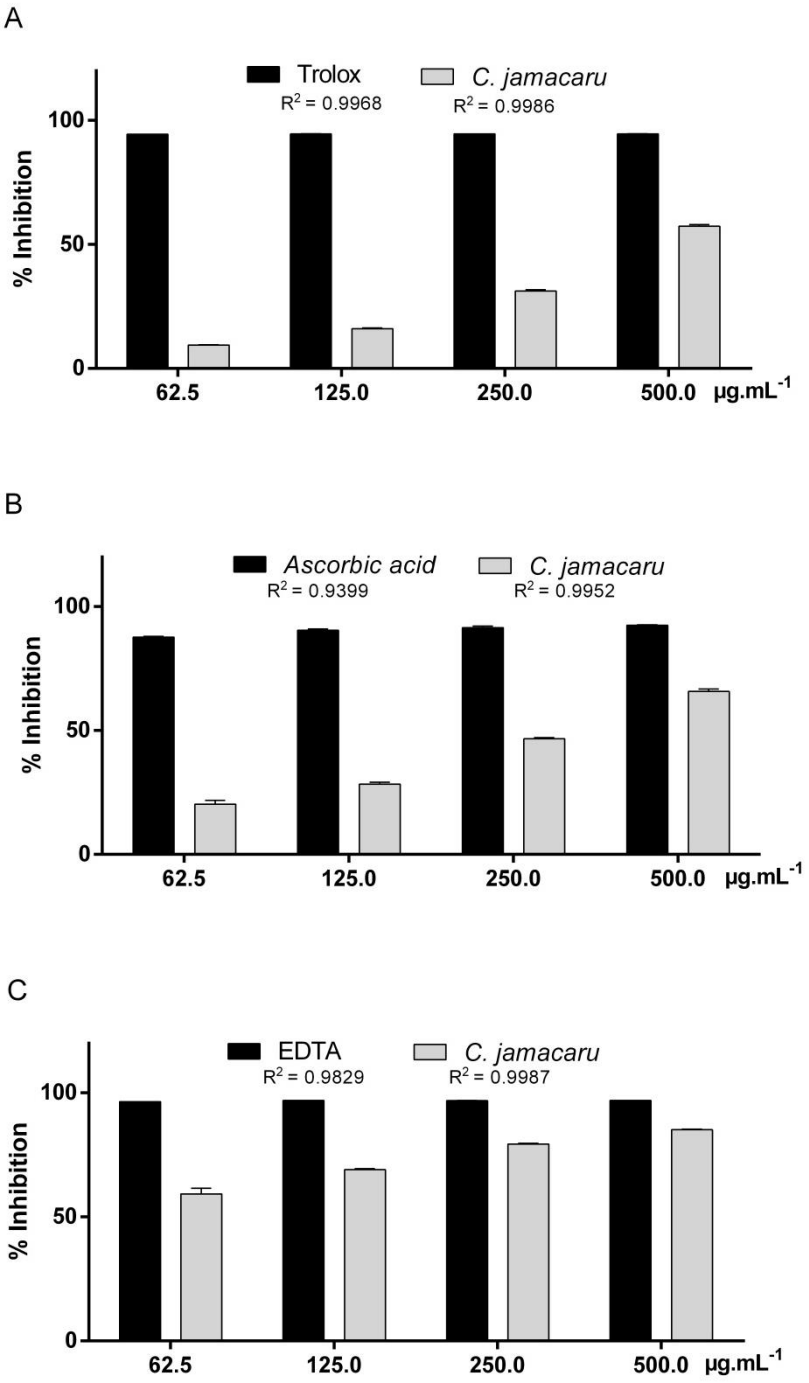
The values are median (Percentile 25—Percentile 75). P_{25} = Percentile 25; P_{75} = Percentile 75. Organs weight of experimental groups were compared to the sarcoma group by Mann Whitney test ($p < 0.05$)—* $p < 0.05$ or ** $p < 0.01$ vs. organ weight of sarcoma group.

Table 5. Frequency of micronucleated normochromatic erythrocytes (MNNCE) and polychromatic erythrocytes (PCE) in 1000 NCE in whole peripheral blood of *Swiss* albino mice sarcoma induced treated with *C. jamacaru* extract (5.0, 10.0 or 20.0 mg/kg b.w.).

Treatment	MNNCE/1000 NCE (P_{25}–P_{75})				PCE/1000 NCE (P_{25}–P_{75})			
	0 Day of Treatment	*p*	20 Days of Treatment	*p*	0 Day of Treatment	*p*	20 Days of Treatment	*p*
Sarcoma + NaCl (0.9%)	5.50 (3.25–6.75)	–	3.00 (0.25–4.75)	–	7.50 (4.00–21.75) †	–	23.50 (16.00–38.00) †	–
Sarcoma + *C. jamacaru* 5.0 mg/kg b.w.	5.50 (3.25–6.75)	>0.9999	1.50 (0.25–3.50)	0.6246	7.50 (4.00–21.75)	>0.9999	16.50 (4.75–21.75)	0.0870
Sarcoma + *C. jamacaru* 10.0 mg/kg b.w.	5.50 (3.25–6.75)	>0.9999	4.50 (2.25–5.25)	0.4272	7.50 (4.00–21.75)	>0.9999	8.50 (6.25–17.00) #	0.0263
Sarcoma + *C. jamacaru* 20.0 mg/kg b.w.	5.50 (3.25–6.75)	>0.9999	2.50 (2.00–3.00)	0.9459	7.50 (4.00–21.75)	>0.9999	10.00 (4.50–14.25) ##	0.0093
Health + NaCl (0.9%)	0.00 (0.00–1.75) **	0.0031	2.50 (1.25–3.75)	0.7120	12.00 (7.75–27.75)	0.2890	9.50 (5.00–13.50) ##	0.0093

The values are median (Percentile 25—Percentile 75). P_{25} = Percentile 25; P_{75} = Percentile 75. MNNCE or PCE frequency in 1000 NCE were compared to the sarcoma group by Mann Whitney test—** $p < 0.01$ vs. MNNCE frequency per 1000 NCE of sarcoma group; # $p < 0.05$ or ## $p < 0.01$ vs. PCE frequency per 1000 NCE of sarcoma group. The comparison of MNNCE or PCE frequency per 1000 NCE at 0 and 20 days of treatment was performed by Wilcoxon test—† $p < 0.01$

Figure 1. Antioxidant activity of *C. jamacaru* extract following DPPH, ABTS and Fe^{2+} chelation ions**.**



(**A**) Antioxidant activity of *C. jamacaru* extract and of the Trolox standard shown by the percentage of inhibition of DPPH. (**B**) Antioxidant activity of *C. jamacaru* extract and of the ascorbic acid standard shown by the percentage of inhibition of ABTS. (**C**) Antioxidant activity of *C. jamacaru* extract and of the EDTA standard shown by the percentage in chelating activity on Fe^{2+} ions.

CAPITULO 2 – Fruiting increases total content of flavonoids and *in vitro* antiproliferative effects of *Cereus jamacaru* D.C. hydroalcoholic extract

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RESUMO

Objetivo: Avaliar a influência do estádio fenológico de frutificação no teor de flavonoides totais, na atividade antioxidante e nos efeitos antiproliferativos de cladódios e frutos de *Cereus jamacaru* (*C. jamacaru*) (mandacaru). **Métodos**: O fruto e cladódios no estádio vegetativo e frutificação de *C. jamacaru* foram coletados. O fruto foi dissecado e a casca, polpa e sementes foram separadas. Cladódios vegetativos e frutificantes, juntamente com a casca, polpa e sementes foram utilizados para obtenção de cinco extratos hidroalcoólicos. Os extratos foram investigados quanto ao teor de flavonoides totais, utilizando o método colorimétrico de $AlCl_3$, atividade antioxidante pela capacidade de eliminação de radicais 2,2-difenil-1-picrilhidrazil e 2,2'-azino-bis (3-etilbenzotiazolino-6-sulfônico) e atividade quelante de íons Fe^{2+} e efeitos antiproliferativos *in vitro* (células do sarcoma 180) pelo ensaio de brometo de 3- (4,5-dimetil-2-tiazolil) -2,5-difenil-2H-tetrazólio. **Resultados**: O extrato de cladódios de *C. jamacaru* no estádio de frutificação apresentou maior teor de flavonoides em relação aos demais extratos. O extrato das sementes apresentou maior atividade antioxidante nos ensaios 2,2-difenil-1-picrilhidrazil e 2,2'-azino-bis (3-etilbenzotiazolin-6-sulfônico), e o extrato de cladódios no estádio vegetativo apresentou melhor atividade antioxidante na atividade quelante de íons Fe^{2+}. O extrato dos cladódios promoveu maior efeito antiproliferativo em relação aos demais extratos. **Conclusões**: Estes achados sugerem que a frutificação aumenta o teor de flavonoides e os efeitos antiproliferativos de cladódios de *C. jamacaru*. Esses dados reforçam o potencial uso de cladódios de *C. jamacaru* e seus frutos como antioxidantes naturais e potentes agentes anticâncer.

Palavras-chave: mandacaru, teor de flavonoides, DPPH, ABTS, atividade quelante de íons Fe^{2+}, ensaio MTT, células de sarcoma 180.

ABSTRACT

Objective: To evaluate the influence of fruiting phenological stage on total flavonoid content, antioxidant activity, and antiproliferative effects of *Cereus jamacaru* (*C. jamacaru*) (mandacaru) cladodes and fruit. **Methods**: Fruit and cladodes at vegetative and fruiting stage of *C. jamacaru* were collected. The fruit was dissected and bark, pulp, and seeds were separated. Vegetative and fruiting cladodes, together with bark, pulp, and seeds were used to obtain five hydroalcoholic extracts. The extracts were investigated for total flavonoid content, using $AlCl_3$ colorimetric method, antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl and 2,2′- azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging capacity and Fe^{2+} ion chelating activity, and *in vitro* antiproliferative effects (sarcoma 180 cells) by 3-(4,5-dimethyl2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. **Results**: The extract of *C. jamacaru* cladodes at the fruiting stage showed higher flavonoid content compared to the other extracts. Seed extracts showed the highest antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) assays, and the extract of cladodes at vegetative stage showed better antioxidant activity in Fe^{2+} ion chelating activity. The extract of fruiting cladodes promoted higher antiproliferative effects compared to the other extracts. **Conclusions**: These findings suggest that fruiting increases the content of flavonoids and antiproliferative effects of *C. jamacaru* cladodes. Data reinforce the potential use of *C. jamacaru* cladodes and fruits as natural antioxidants and potent anticancer agent.

Keywords: mandacaru, flavonoid content, dpph, abts, Fe^{2+} ion chelating activity, MTT assay. sarcoma 180 cells.

1. INTRODUCTION

Cereus jamacaru (*C. jamacaru*) D.C. (mandacaru) is a Cactaceae native from Brazil, found mainly on arid areas and is used as food and, in traditional medicine, to treat urinary infection, kidney inflammation, and rheumatism[1]. The antioxidant and antiproliferative actions of *C. jamacaru* have already been reported in the literature, which motivated the conduction of more researches on the effective use of this plant as a natural source of antioxidants and for the development of new drugs[2-4].

Optimization of the uses of natural products is not restricted to the identification of the vegetal actives and their biological effects. Many studies have aimed to evaluate the influence of genetic and environmental factors, crop management, and phenology on the production of phytochemicals[5-8]. The influence of phenology on the frugivory, dispersion, and germination of seeds of *C. jamacaru* has been reported[9]. However, there are no studies demonstrating the influence of phenology on the biological activities of this cactus, such as the antioxidant and antiproliferative activities.

In recent years, there has been a growing use of *in vitro* techniques to evaluate the antioxidant and antiproliferative potential of natural products. These techniques have been used for multiple and complementary measures of antioxidant and cytotoxicity activities[5,6,10,11]. In this context, antioxidant assays, such as 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Fe^{2+} ion chelating activity, and *in vitro* antiproliferative assays using cell lines, such as sarcoma 180 (murine cancer), have been efficiently used as screening tools for natural products of plant origin with potential use for health promotion and development of new drugs[5-8].

Therefore, the aim of this study was to evaluate the influence of fruiting on the total flavonoids content of extracts of *C. jamacaru* cladodes at the vegetative stage and fruiting, as well as their fruits, and to relate these results to the *in vitro* antioxidant activities and antiproliferative effects.

2. MATERIAL AND METHODS

2.1. Chemicals

The chemicals used in this study included Ficoll® Paque Plus (Sigma-Aldrich); RPMI 1640 culture medium (Cultilab); fetal calf serum (Gibco); Cisplatin (Fauldcispla®, Libbs); 2,2-diphenyl-1-picryl-hydrazyl (Sigma-Aldrich);2,2′-azinobis-(-3-ethylbenzothiazoline-6-sulfononic acid) (Sigma-Aldrich); potassium persulfate (Sigma-Aldrich); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Sigma-Aldrich); 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4,4'-disulfonic acid Na-salt (Sigma-Aldrich); iron chloride Ⅱ (Sigma-Aldrich); 3-(4,5- dimethyl2- thiazolyl)-2,5- diphenyl-2H- tetrazolium bromide (Sigma-Aldrich).

2.2. Plant material

The fruit and cladodes of *C. jamacaru* in the vegetative stage (without flowers or fruits) and fruiting stage (with fruit) were collected in the state of Alagoas, northeastern Brazil (9° 40' 44.7" S, 36° 41' 21.9" W), in July 2018. The thorns of cladodes were removed and the cladodes were later sliced with a blade. The fruit was collected together with cladodes in the fruiting stage, dissected and the bark, pulp, and seeds were obtained. The plant material was oven-dried at 50 ℃ for 24 h and vegetative and fruiting cladodes, bark, pulp, and seeds were used to obtain five hydroalcoholic extracts.

2.3. Hydroalcoholic extract

Dried plant material was powdered, macerated in EtOH/H_2O (70:30 v/v) solution (dry plant: EtOH/H_2O - 1:5 w/v) at room temperature (25-30 ℃), protected from the light for 3 d, filtered and stored. Vegetal material was submitted to the same conditions of maceration (plus three days of maceration) for exhaustion of the compound extraction, totaling six days of maceration. The solvent was evaporated under reduced pressure at 60 ℃ to obtain the crude extracts of *C. jamacaru*. The extracts were stored at 6-10 ℃ and protected from the light until its utilization. The yield of the extracts were calculated by the formula[12]:

Total extract yield (%) = FM/IM × 100

Where “FM” = final mass of dry extract (g); “IM” = initial mass of dry plant (g).

2.4. Total flavonoid content

The colorimetric method described by Zhishen *et al.*[13] was used to determine the total flavonoid content of *C. jamacaru* extracts. The experiment was performed in triplicate and the absorbance was detected at 430 nm, using an ELISA reader. Rutin was used as a standard for flavonoids and for this purpose a series of methanolic dilutions of rutin (500.0, 250.0, 125.0, 62.5, 31.3, 15.6, and 7.8 µg/mL) were prepared and assayed. The total flavonoid content in the extracts was expressed as rutin equivalent (µg/mL).

2.5. Antioxidant activity

2.5.1. DPPH

DPPH radical reduction method was used to evaluate the antioxidant activity of *C. jamacaru* extracts and ascorbic acid standard[14]. The absorbance was taken at 517 nm by ELISA reader. The test was performed in triplicate and the percentage of reduction of $DPPH^{\bullet}$ radical was calculated by the equation:

$$\% \text{ inhibition} = [(Abs_{Control} - Abs_{Sample}) / Abs_{Control}] \times 100$$

Where “% inhibition” is the percentage of inhibition capacity of $DPPH^{\bullet}$; “$Abs_{Control}$” is the absorbance of $DPPH^{\bullet}$ reaction of the control; and “Abs_{Sample}” is the absorbance of the sample.

2.5.2. ABTS

ABTS capturing method was performed to evaluate the total antioxidant activity of *C. jamacaru* extracts and ascorbic acid standard [15]. The absorbance was taken at 734 nm by ELISA reader. The experiment was performed in triplicate and the

percentage of scavenging inhibition capacity of $ABTS^{+\bullet}$ of the *C. jamacaru* extract was calculated by the following equation:

$$\% \text{ inhibition} = [(Abs_{Control} - Abs_{Sample} / Abs_{Control})] \times 100$$

Where "% inhibition" is the percentage of scavenging inhibition capacity of $ABTS^{+\bullet}$; "$Abs_{Control}$" is the absorbance of $ABTS^{+\bullet}$ reaction of the control; and "Abs_{Sample}" is the absorbance of the sample.

2.5.3. Fe^{2+} chelation ions

Chelating activity of ferrous ions (Fe^{2+}) was assayed by inhibition of ferrous-ferrozine complex formation after treatment with *C. jamacaru* extracts and EDTA standard[16]. The absorbance was taken at 562 nm by ELISA reader. The experiment was performed in triplicate and the percentage of ferrous ion chelating effect was calculated using the following equation:

$$\% \text{ chelating effect} = [(Abs_{Control} - Abs_{Sample})/Abs_{Control}] \times 100$$

Where "% chelating effect" is the percentage of ferrous ions (Fe^{2+}) chelating effect; "$Abs_{Control}$" is the absorbance of Fe^{2+} reaction of the control; and "Abs_{Sample}" is the absorbance of the sample.

2.6. Cytotoxicity *in vitro*

2.6.1. Sarcoma 180 cells

Sarcoma 180 cells (murine sarcoma) were acquired from Banco de Células do Rio de Janeiro. In 96-well plates, sarcoma 180 cells were plated with 2×10^5 cells per well. The cells that received *C. jamacaru* extracts were diluted with water at 10.0, 50.0, 100.0, 200.0, 300.0, 400.0, and 500.0 µg/mL and untreated cells (0.0 µg/mL of extract) were used as a control. Sarcoma 180 cells were cultured for 24 h, 48 h, or 72 h to evaluate the anticancer effect of *C. jamacaru* extracts. All protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 89/2015).

2.6.2. Cell culturing methods

Culture medium RPMI 1640, supplemented with gentamicin (50.0 mg/L), amphotericin B (2.0 mg/L), and 10% of fetal calf serum at 37 ºC, 5% of CO_2 saturation and humid atmosphere was used to grow the sarcoma 180 cells. The cells were cultured under these conditions 24 h before starting the treatments. At the end of the treatment, the cell viability was evaluated.

2.6.3. 3-(4,5- dimethyl-2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

MTT assay was used to determine cell viability of sarcoma 180. At the end of the treatment, the plates were centrifuged at 860 rcf for 10 min, the supernatant was discarded and 20 µL of MTT at 5 mg/mL were added to each well. 3 h later, 100 µL of DMSO were added in each well and the absorbance was detected at 590 nm by ELISA reader. The results were expressed as a relative percentage of cell viability in comparison to the untreated cells. The experiment was performed in triplicate.

2.7. Statistical analysis

Normality of the data was evaluated a priori and results were expressed as mean ± standard deviation. To quantify the total content of flavonoid in *C. jamacaru* extracts, a linear curve relating rutin concentrations and absorbances was performed and the equation and R^2 were obtained. The total flavonoid content in *C. jamacaru* extracts was compared by multiple *t* test ($P<0.05$). The antioxidant capacity of the extracts at different concentrations was compared by ANOVA *post hoc* Tukey test ($P<0.05$). To evaluate the anticancer activity of *C. jamacaru* extracts *in vitro*, the cell viability of sarcoma 180 treated with extract concentrations was compared to untreated cells (0.0 µg/mL of extract) by ANOVA *post hoc* Dunnett's test ($P<0.05$). Principal component analysis (PCA) and Pearson correlation were performed to visualize relationships between the total flavonoid content, antioxidant activities, and anticancer effects of *C. jamacaru* extracts. In the PCA analysis and Pearson

correlation, the values of antioxidant activities and anticancer effects at the concentration of 500.0 µg/mL, concentration that presents the best results for the biological activities tested.

3. RESULTS

3.1. Flavonoid content

Table 1 summarizes the total yield and total flavonoid content of *C. jamacaru* extracts. The total flavonoid content was calculated using the equation y = 0.003 345x + 0.121 2 (R^2 = 0.994 8; P<0.000 1). The highest total yield was observed in the pulp extract (70.34%) and *C. jamacaru* cladode extract at the fruiting stage presented the highest total content of flavonoids [(201.45 ± 16.54) μg/mL] among all extracts, approximately 10 times higher than the *C. jamacaru* cladode extract at the vegetative stage [(20.85 ± 3.78) μg/mL].

3.2. Evaluation of antioxidant activity

The results of the antioxidant evaluation of extracts are presented in Table 2. In the DPPH test, *C. jamacaru* seeds extract presented better antioxidant activity when compared to the other extracts, which was similar to the results of the ascorbic acid as a standard in some tested concentrations. In comparison to the other extracts, the *C. jamacaru* fruit pulp extract showed lower antioxidant activity in DPPH assay and no antioxidant activity was detected at concentrations of 62.5 and 125.0 μg/mL.

For the ABTS assay, *C. jamacaru* cladode extract at the fruiting stage was the one with the better antioxidant activity when compared to the other extracts. The extract of seeds showed antioxidant activity similar to the standard ascorbic acid at the concentration of 500.0 μg/mL. Moreover, similar to the results observed in the DPPH assay, the *C. jamacaru* fruit pulp extract presented the lowest antioxidant power in the ABTS assay and no antioxidant activity was detected at concentrations of 62.5 and 125.0 μg/mL.

At the evaluation of the chelating activity of Fe^{2+} ions, the *C. jamacaru* cladode extract at the vegetative stage presented the highest antioxidant power in comparison to the other extracts. At concentration of 62.5 and 125.0 μg/mL, no antioxidant activity was detected in the *C. jamacaru* fruit bark extract. However, at all tested concentrations of *C. jamacaru* seeds extract, no antioxidant activity was

detected. The values of DPPH, ABTS, and Fe^{2+} chelation, for fruiting cladodes, are higher for 250.00 µg/mL compared to 500.00 µg/mL concentration (Table 2).

3.3. Anticancer activity *in vitro*

Figure 1 shows the cytotoxicity of *C. jamacaru* extracts in sarcoma cells 180 *in vitro*. Compared to the group of untreated cells (0.0 µg/mL of extract), it was observed that all *C. jamacaru* extracts significantly reduced the viability of tumor cells after 24 h (Figure 1A), 48 h (Figure 1B), and 72 h of treatment in all concentrations (Figure 1C). At the end of 72 hours of treatment (Figure 1C), it was found that the *C. jamacaru* cladode extract at the fruiting stage was able to promote a more pronounced reduction in the viability of the tumor cells at the concentrations of 200.0, 300.0, 400.0, and 500.0 µg/mL. Likewise, the seed extract was able to promote a more pronounced reduction in the viability of tumor cells at concentrations of 300.0, 400.0, and 500.0 µg/mL.

3.4. Explorative analyses: Correlations between total flavonoid content and antioxidant and anticancer assays

PCA and Pearson correlation coefficient were used to correlate the results obtained in different assays. In PCA, it was accounted 52.23% and 25.92% in the first and second principal components, respectively, and total variance of 78.16% (Figure 2). PC1 was dominated by the following variables: total flavonoid content, DPPH, ABTS, and MTT assay after 48 h and 72 h of treatment. PC2 was dominated by the variables: total flavonoid content, Fe^{2+} chelating activity, and MTT assay after 24 h of treatment. These results suggested that flavonoid content, DPPH, and ABTS antioxidant assays were correlated with anticancer effects after 48 h and 72 h of treatment, while Fe^{2+} chelating activity was correlated with anticancer effect after 24 h of treatment. *C. jamacaru* cladode extract at fruiting stage presented the highest total flavonoid content level in comparison to the other extracts and it seemed to be the main factor related to its antioxidant activity (DPPH and ABTS assays) and anticancer effects. DPPH and ABTS assays were the main factor related to the anticancer activity exhibited for seeds extract. Added to this, Fe^{2+} chelating activity

seemed to be the main factor related to the anticancer effects exhibited for *C. jamacaru* cladode extract at vegetative stage and fruit pulp extract.

Table 3 presents the results of Pearson correlation analysis. Values followed by negative sign indicate an inversely proportional relation between the factors. Thus, considering that MTT assay was used to verify the viability of tumor cells after treatment with *C. jamacaru* extracts, negative correlations between the MTT assay and the total flavonoid content or antioxidant activities suggest increasing anticancer effect. A moderate negative correlation between anticancer effect after 24 h of treatment (MTT 24 h) and total flavonoid content level was demonstrated. Moreover, a moderate negative correlation between anticancer effect after 48 h of treatment (MTT 48 h) and total flavonoid content, DPPH, and ABTS activities was observed. The anticancer effect after 72 h of treatment (MTT 72 h) showed a moderate negative correlation with the DPPH activity and moderate positive correlation with Fe^{2+} chelating activity.

4. DISCUSSION

Phytochemicals production and accumulation in plants may differ due to genetic, environmental, and phenological factors[5,17]. The phytochemicals can accumulate in different parts of the plants[18], while the variety, processing, cooking, and growing conditions may determine the level of phytochemicals accumulated in plant tissues[19].

Studies on the phenological aspects of cacti are scarce and usually address factors related to ecology or increase in fruit and plant biomass production, as the studies of Gomes *et al.*[9] and Arba *et al.*[20]. Gomes *et al.*[9] performed a study relating phenology, frugivory, dispersion and germination of *C. jamacaru* seeds, and Arba *et al*.[20] investigated the effect of irrigation on plant biomass at different phenological stages in cactus of the genus *Opuntia* produced for commercial purposes.

C. jamacaru is a succulent plant adapted to arid regions and the fruiting stage seems to play an important modulating role in the production of flavonoids. The total flavonoid content in cladodes extract of *C. jamacaru* at vegetative stage (20.85 µg/mL) was higher than that observed by de Sousa Araújo *et al*.[21] in methanolic extract (0.59 µg/mL) and Dutra *et al*.[4] in hydroalcoholic extract (0.51 µg/mL). For the content of flavonoids in the bark and pulp, the values found in our study differ from those observed by Lima[22], evaluating the total yellow flavonoid in *C. jamacaru* fresh fruits, 3.20 and 2.35 mg/100g in bark and pulp, respectively.

The plant material used in our study was collected in July 2018, after the fructification peak of June 2018[9]. Considering that plants grown in natural environments may show heterogenous flowering and fruiting, it was possible to find and collect in the same population *C. jamacaru* plants in vegetative and fruiting stage.

Compared to previous studies conducted with *C. jamacaru* cladodes at the vegetative stage[4,21], our study found that there is an increase in the total production of flavonoids during the fruiting stage. Our study also showed that the extract of *C. jamacaru* cladodes in the vegetative stage had relatively high total flavonoid content (20.85 µg/mL) compared to what Dutra *et al.* (0.51 µg/mL) described using *C. jamacaru* cladodes collected at the same region and phenological

stage in September 2016[4]. Fluctuations in flavonoid content may occur due to environmental changes, as suggested by Pretti *et al.*[5]. However, we believe that fruiting increases plant flavonoid production and that the relatively high level of flavonoids in cladodes at the vegetative stage may have occurred due to post-fructification stage.

Several methods can be employed to assess the antioxidant capacity of vegetable extracts *in vitro*[23,24]. The use of a single method of antioxidant evaluation provides basic information about antioxidant properties of a sample. Therefore, the use of different methods of antioxidant evaluation is recommended, since it allows a better understanding of antioxidant properties and mechanism of action of a sample[23,25].

DPPH, ABTS, and Fe^{2+} chelation ions assays were used to evaluate the antioxidant activity of *C. jamacaru* extracts in this work. A low antioxidant activity in pulp and bark extracts was observed, and in some concentrations the antioxidant activity was not detected (62.5 and 125.0 µg/mL for DPPH and ABTS assays using pulp extract and for Fe^{2+} chelation assay using bark extract. The studies with fruits showed that plant phenology and physiological responses to biotic and abiotic factors were able to modify the properties of potential antioxidant, such as under climate scenarios[7,26,27]. *C. jamacaru* is a cactus adapted to semi-arid conditions, surviving in high temperatures[28]. However, the high temperatures in which this cactus develops may degrade the phytotochemicals found in the fruits and reduce their antioxidant capacity[26], as observed in our study.

Bark extract presented higher flavonoid content and better antioxidant activity in DPPH and ABTS than pulp extract, similar to that observed by Lima[22], evaluating the flavonoid content and ABTS antioxidant activity of fresh fruits of *C. jamacaru* collected in the same biome. The flavonoids are phenolic compounds reported as the main factor responsible for the chelating activity[29]. The extract of *C. jamacaru* cladodes at the fruiting stage presented higher flavonoid content, but it did not show the best results in chelating activity. Therefore, no direct relationship was observed between total flavonoid content and chelating activity, suggesting that, as observed by dos Santos *et al*.[6], the antioxidant ability of plant extracts may be related to the chemical structure of flavonoids.

For the conditions tested in our study, all extracts of *C. jamacaru* were able to promote *in vitro* antiproliferative effects, demonstrating that the cladodes and the fruit of this cactus present potential use for the prevention/treatment of cancer. *In vivo* antiproliferative effects of cladodes extract of *C. jamacaru* at vegetative stage is reported in the literature[2-4]. The aqueous extract of the cladodes induces antiproliferative effects and increases cellular aberrations in *Allium cepa* test system cells[3] and hydroethanolic extract from the cladodes promotes antiproliferative effect on sarcoma 180 tumor cells in rodents[2,4].

Phenological stage, as well as the variation of climate, soil, and time conditions, seem to exert an important influence on the secondary metabolism, modifying the quality and properties of plant compounds, inducing changes in chemical composition[30], and interfering with the production of phenolic compounds, such as flavonoids[5,6].

Secondary metabolism products provide plant protection against herbivors, pathogen attack, protection of abiotic stresses, and can act as modulators of gene expression and signal transduction, influencing the content/availability of phytochemicals[31,32]. In addition, flowering and fruiting are also able to modulate the secondary metabolism of plants and alter production of these compounds and the biological activities related to them[6,18].

Among more than 200 000 different types of compounds produced by plants[33], there are many colored substances, pigments, such as flavonoids found in different plant organs. These secondary metabolites belong to the class of phenylpropanoids and present a wide color range, from pale-yellow to blue. The color development in fruits is considered an important evolutionary trait and is a factor that interfere in fruit quality and market value[34].

The fruits of *C. jamacaru* present reddish-brown barks when ripe. The pigmentation of the bark is related to compounds such as anthocyanins, a class of flavonoids responsible for the colors ranging from orange to blue found in many flowers, leaves, fruits, seeds, and other matrices[35]. Anthocyanins are used as food dyes mainly in the beverage industry and in recent years the consumption of these substances has

increased due to the public concern about the possible adverse effects of synthetic food dyes[36].

Following the comparison of *C. jamacaru* extracts at different developing stages, we suggest that the differences between the flavonoid contents and biological activities observed are related to the modulations of the primary and secondary metabolic pathways in the plants. In addition, the diversity of phenolic compounds in plants is a result of derivations in the shikimic acid pathway, a process mediated by the enzyme L-phenylalanine ammonia-lyase (PAL), an enzyme that acts on a branch point between the primary and secondary metabolism, shikimic acid and phenylpropanoid production pathways, respectively[37].

PAL acts on a limited set of molecular structures, producing several phenolic compounds, such as flavonoids, the most abundant class of plant polyphenols synthesized by the combination of the shikimic acid and acyl polylate pathways[37]. The enzymatic action of PAL madiates the synthesis of L-phenylalanine, a compound used in the production of trans-cinnamic acid, a molecule from which most of the phenolic compounds derive[38,39]. Thus, modulations in the pathway of secondary plant metabolism may induce both the production of phytochemicals and the increase in the content and/or accumulation of these substances.

5. CONCLUSIONS

Our findings indicate that during the fruiting stage there is modulation of the secondary metabolic pathway of *C. jamacaru*, leading to an increase in total flavonoid content. The extracts of the cladodes and the fruit of *C. jamacaru* presented antioxidant activity and antiproliferative effects, however no direct relationship between the flavonoid content and the biological activities was observed. This study also suggests the potential use of *C. jamacaru* cladodes and fruits as a source of natural antioxidants and as potent anticancer agents. These data reinforce the effectiveness of the biological activities of natural products, such as those observed in our study, favor the extensive use of *C. jamacaru* in cooking, industry, drug development and also assist in understanding the role of secondary metabolite pathway in metabolites synthesis.

Conflict of interest statement

The authors declare no conflict of interest.

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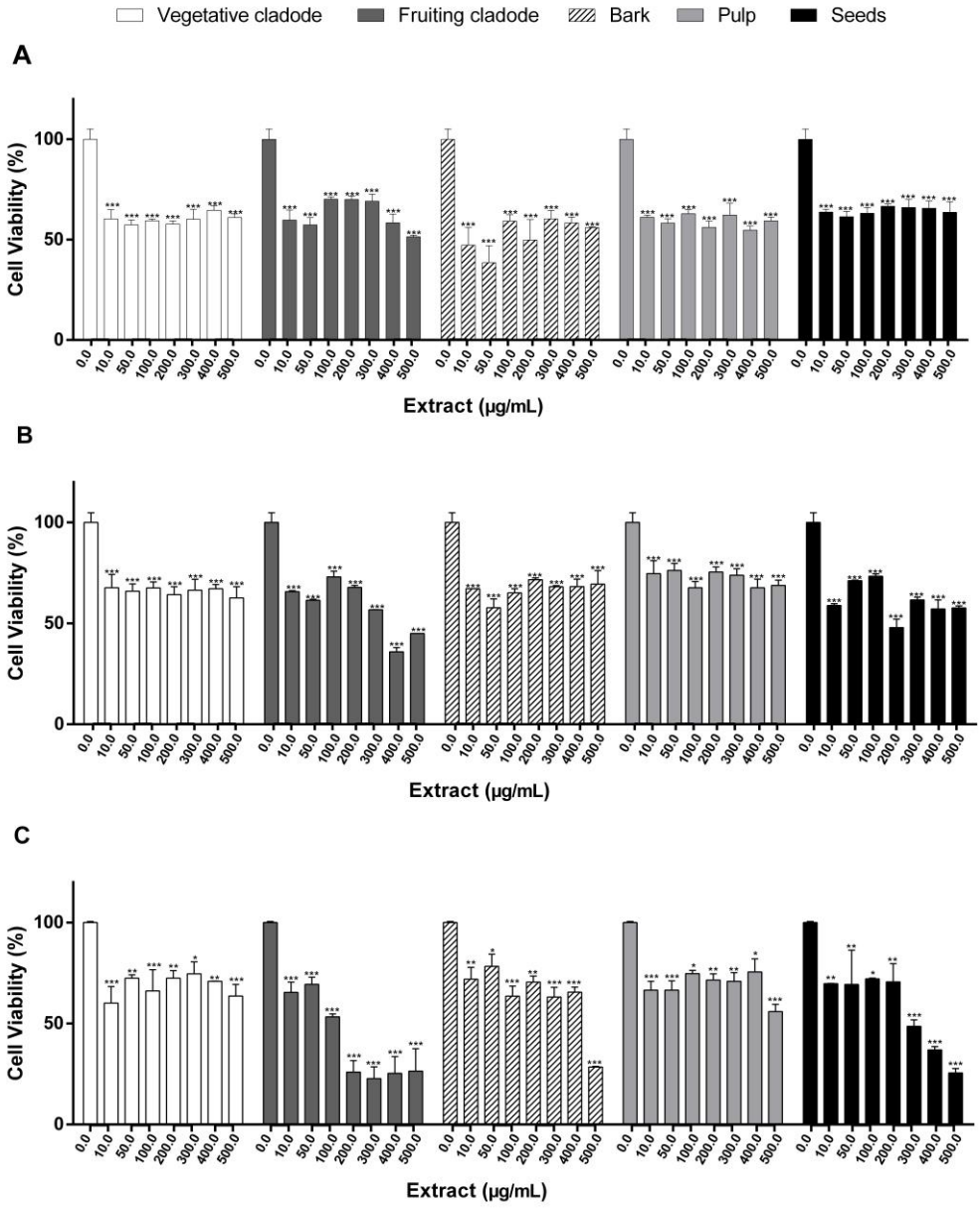
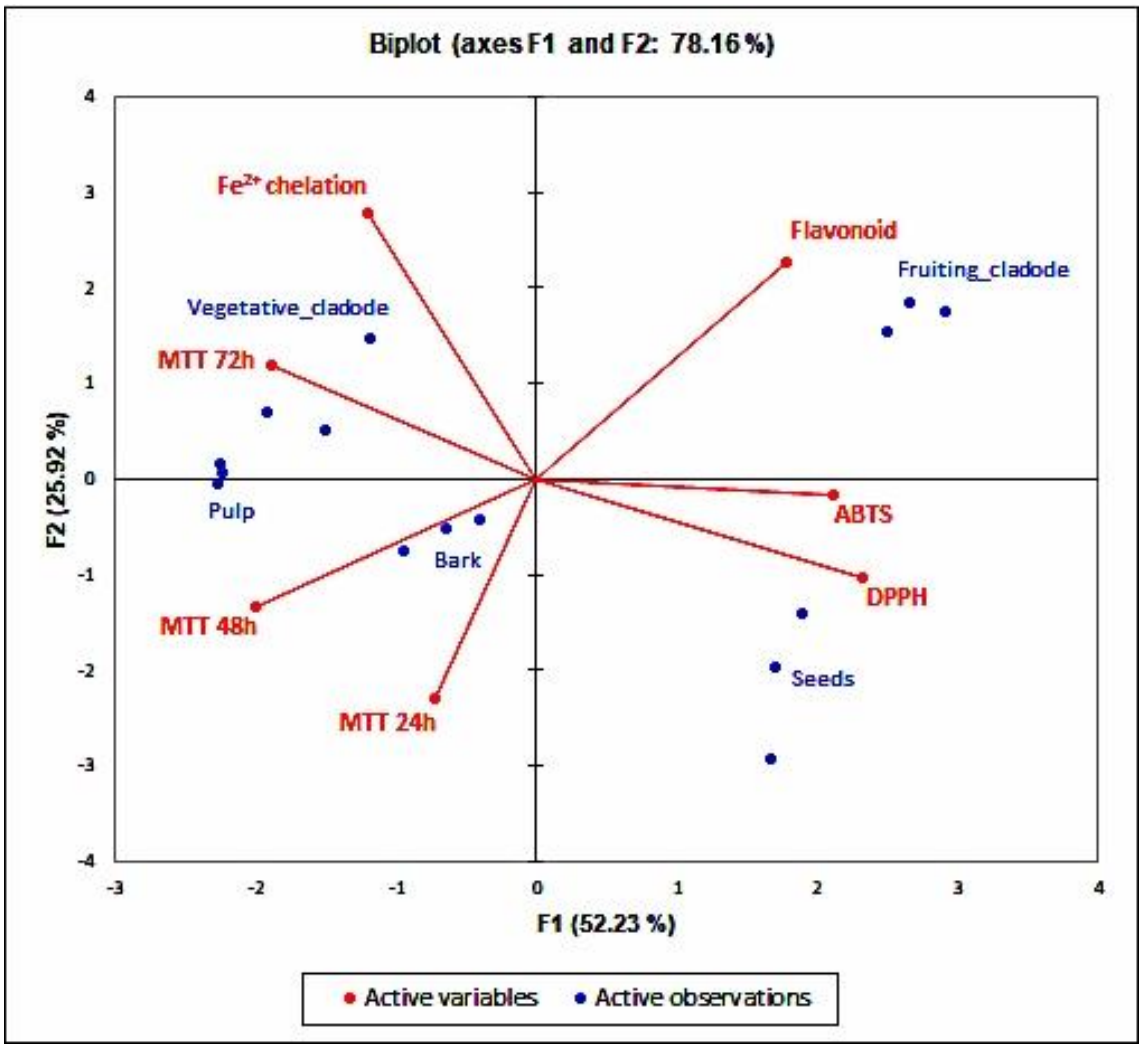
Figures and tables

Figure 1. Cytotoxicity of *C. jamacaru* extracts in sarcoma 180 cells *in vitro* by MTT assay.



Percentage of viable cells is presented as mean ± standard deviation (SD). Sarcoma 180 cells were treated with *C. jamacaru* extracts for 24 h (A), 48 h (B), or 72 h (C). The comparison between untreated cells (0.0 µg/mL) and cells treated with *C. jamacaru* extracts was performed by ANOVA *post hoc* Dunnett's test - $^{*}P<0.01$, $^{**}P<0.001$, or $^{***}P<0.000\ 1$ *vs.* untreated cells.

Figure 2. Principal component analysis (scores and loading plots, biplot) based on total flavonoid content analyzed in *C. jamacaru* extracts (vegetative and fruiting cladodes, bark, pulp, and seed fruit extracts) and their antioxidant activities (DPPH, ABTS, and Fe^{2+} chelating activity) and anticancer effects (MTT assay).



To evaluate anticancer effects, MTT assay was performed after 24 h of treatment (MTT 24 h), 48 h of treatment (MTT 48 h), and 72 h of treatment (MTT 72 h). Values of antioxidant activity and anticancer effect at the concentration of 500.0 µg/mL were used to perform PCA analysis.

Table 1. Total extract yield and total flavonoid content of *C. jamacaru* extracts.

Extract	Initial plant dry mass (g)	Final extract dry mass (g)	Total extract yield (%)	Flavonoid content (µg/mL) (mean± SD)
Vegetative cladode	43.44	5.25	12.08	20.85 ± 3.78[b]
Fruiting cladode	53.04	8.28	15.61	201.45 ± 16.54 [a]
Bark	4.92	1.29	26.27	9.54 ± 1.43 [c]
Pulp	2.1	1.48	70.34	4.69 ± 0.17 [d]
Seeds	1.29	0.09	6.98	15.09 ± 2.10 [b]

The values of plant and extract mass are the observed weight. The total flavonoid content is presented as mean ± standard deviation (SD). The comparison between the total content of flavonoids in *C. jamacaru* extracts was performed by the *t* test ($P<0.05$) - means followed by different lowercase letters in the column [(a, b, c, d)] differ statistically.

Table 2. Antioxidant activity of *C. jamacaru* extracts and standards by DPPH, ABTS, and Fe^{2+} chelation ions assays (mean±SD).

Assay	Concentration (µg/mL)	% inhibition ± SD						
		Vegetative cladode	Fruiting cladode	Bark	Pulp	Seeds	Ascorbic acid	EDTA
DPPH	62.50	9.36 ± 4.26^{dB}	45.25 ± 5.08^{cB}	2.16 ±0.60^{eC}	nd	58.29 ± 2.12^{bB}	89.01 ±0.17^{aA}	–
	125.00	8.41 ± 1.62^{cB}	58.67 ± 3.68^{bB}	6.06 ± 0.23^{cC}	nd	87.78 ± 0.19^{aA}	90.09 ± 0.23^{aA}	–
	250.00	13.71 ± 0.14^{cAB}	71.64 ± 2.97^{bA}	15.00 ± 0.60^{cB}	2.66 ± 0.14^{dA}	87.27 ± 0.13^{aA}	91.85 ± 0.17^{aA}	–
	500.00	20.88 ± 1.80^{cA}	69.83 ± 9.69^{bA}	31.18 ± 2.34^{cA}	2.45 ± 0.92^{dA}	86.34 ± 0.54^{aA}	92.29 ±1.13^{aA}	–
ABTS	62.50	7.36 ± 1.94^{dD}	43.90 ± 1.32^{bC}	2.47 ± 1.85^{eC}	nd	25.50 ± 1.44^{cD}	89.48 ± 0.44^{aA}	–
	125.00	20.82 ± 0.78^{dC}	68.92 ± 1.06^{bB}	3.70 ±1.40^{eC}	nd	43.85 ± 2.57^{cC}	91.89 ± 0.60^{aA}	–
	250.00	37.33 ± 3.44^{dB}	83.24 ± 1.00^{bA}	8.33 ± 1.89^{eB}	0.36 ± 0.04^{fA}	52.86 ± 0.77^{cB}	92.73 ± 0.94^{aA}	–
	500.00	55.15 ± 1.03^{cA}	83.20 ± 3.65^{bA}	22.55 ± 1.53^{dA}	0.63 ±0.02^{eA}	91.19 ± 1.23^{aA}	93.57 ± 0.35^{aA}	–
Fe^{2+} chelation	62.50	14.48 ± 4.20^{cD}	46.02 ± 0.60^{bB}	nd	2.70 ± 0.71^{dD}	Nd	–	96.47 ± 0.05^{aA}
	125.00	35.01 ± 1.71^{cC}	56.83 ± 0.61^{bA}	nd	10.97 ± 1.00^{dC}	Nd	–	96.97 ± 0.05^{aA}
	250.00	59.48 ± 3.76^{bB}	60.70 ± 2.64^{bA}	11.93 ± 5.44^{dB}	25.94 ± 1.02^{cB}	Nd	–	96.89 ± 0.12^{aA}
	500.00	87.64 ± 1.38^{bA}	59.86 ± 3.21^{cA}	36.27 ± 3.58^{dA}	51.32 ± 1.70^{cA}	Nd	–	96.92 ± 0.08^{aA}

The percentage of inhibition in each antioxidant assay is presented as mean ± standard deviation (SD). The comparison between the percentage of inhibition exhibited for each concentration of *C. jamacaru* extracts was performed by ANOVA *post hoc* Tukey test (P<0.05) - values followed by different lowercase letters in the row $^{(a, b, c, d, e, f)}$ or followed by different capital letters $^{(A, B, C, D)}$ in the column differ statistically. nd = not detected.

Table 3. Pearson correlation analysis between total flavonoid content, antioxidant activities (DPPH, ABTS, and Fe^{2+} chelation), and anticancer effects (MTT assay) of *C. jamacaru* extracts.

	Flavonoid	DPPH	ABTS	Fe^{2+} chelation	MTT 24 h	MTT 48 h	MTT 72 h
Flavonoid	1						
DPPH	0.4530	1					
ABTS	0.5131	0.8768	1				
Fe^{2+} chelation	0.1474	-0.6566	-0.2920	1			
MTT 24 h	-0.6025	-0.0527	0.0132	-0.1837	1		
MTT 48 h	-0.7999	-0.6536	-0.7320	0.0466	0.2533	1	
MTT 72 h	-0.3885	-0.7421	-0.4516	0.7097	0.2470	0.3046	1

To evaluate anticancer effects, MTT assay was performed after 24 h of treatment (MTT 24 h), 48 h of treatment (MTT 48 h), and 72 h of treatment (MTT 72 h). Values of antioxidant activities and anticancer effects at the concentration of 500.0 µg/mL were used to perform Pearson Correlation.

CAPITULO 3 – Variegation improves in biological activities and chemical composition of *Plectranthus amboinicus* (Lamiaceae)

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RESUMO

Objetivo: Avaliar a influência da variegação foliar na composição química e nas atividades biológicas de *P. amboinicus* e *P. amboinicus* “Variegata”. **Métodos:** Os extratos hidroalcoólicos de *P. amboinicus* e *P. amboinicus* “Variegata” foram avaliados quanto à sua fitoquímica preliminar; teor de flavonoides totais e ácido rosmarínico por métodos espectrofotométricos e de HPLC, respectivamente; atividade antioxidante nos ensaios DPPH, ABTS, atividade quelante do íon Fe^{2+} e sistema β-caroteno/ácido linoléico; e efeitos citotóxicos e anti-citotóxicos em linfócitos humanos e efeitos antiproliferativos em células de sarcomas 180 *in vitro* pelo ensaio MTT. **Resultados**: A fitoquímica preliminar mostrou que ambas as plantas apresentam flavonoides, flavonas e cumarinas. *P. amboinicus* "Variegata" apresentou maiores teores de flavonoides e ácido rosmarínico e maior atividade antioxidante em ensaios de DPPH e ABTS, assim como o extrato de *P. amboinicus* apresentou melhor atividade antioxidante na atividade quelante e inibição da oxidação do β-caroteno. Ambos os extratos induziram um efeito citotóxico pronunciado nas células do sarcoma 180 (efeito anticâncer) e foram capazes de inibir o dano citotóxico induzido pela cisplatina (atividade anti-citotóxica). **Conclusões:** Supomos que a variegação promova modulações no metabolismo secundário, o que leva ao aumento dos flavonoides totais e à biossíntese de ácido rosmarínico, interferindo nas atividades biológicas exibidas pelos extratos de *P. amboinicus*.

Palavras-chave: Variegação foliar, fitoquímica, atividade antioxidante, anti-citotoxicidade sarcoma 180, anti-citotoxicidade, ensaio MTT.

ABSTRACT

Objetive: To evaluate the influence leaf variegation on chemical composition and biological activities of *P. amboinicus* and *P. amboinicus* “Variegata”. **Methods:** *P. amboinicus* and *P. amboinicus* “Variegata” hydroalcoholic extracts were evaluated for their preliminary phytochemistry; total flavonoid and rosmarinic acid content by spectrophotometry and HPLC methods, respectively; antioxidant activity on DPPH, ABTS, Fe^{2+} chelation ions and β-carotene/linoleic acid assays; and *in vitro* cytotoxic and anti-cytotoxic effects in human lymphocytes and antiproliferative effects in saroma 180 cells by MTT assay. **Results:** Preliminary phytochemistry showed that both plants present flavonoids, flavone and coumarins. *P. amboinicus* “Variegata” extract presented higher levels of flavonoid and rosmarinic acid and higher antioxidant activity in DPPH and ABTS assays, as well as *P. amboinicus* extract showed better antioxidant activity in the Fe^{2+} chelating activity and inhibition of β-carotene oxidation. Both extracts induced pronounced cytotoxic effect on sarcoma 180 cells (anticancer effect) and were able to inhibit cytotoxic damage induced by cisplatin (anti-cytotoxic activity). **Conclusions:** We hypothesize that variegation promotes modulations in secondary metabolism, which leads to the increase of total flavonoids and rosmarin acid biosynthesis, interfering in the biological activities exhibited for *P. amboinicus* extracts.

Keywords: leaf variegation, phytochemistry, antioxidant activity, sarcoma 180 anticancer, anti-cytotoxicity, MTT assay.

1. INTRODUCTION

World Health Organization estimates that 80% of people around the world depends of medicinal plants to treat diseases, and due to the low cost, easy accessibility and low side effects, the use of medicinal plants to treat health disorders has increased in the last years[1,2]. On this hand, *Plectranthus amboinicus* (Lour) Spreng, a member of Lamiaceae family, has been traditionaly used both in folk medicine and culinary.

P. amboinicus shows an attractive heart-shaped foliage, fresh aroma when touched[3] and can be used to enhance the taste and aroma of foods[4]. *Plectranthus amboinicus* “Variegata” Ehrh, the variegated version of *P. amboinicus*, has also been used for the same purpuses and differ morphologically fom *P. amboinicus* due to the variegation in the leaves – *P. amboinicus* “Variegata” presents white-edged leaves[4].

Chemical composition and antioxidant activity of *P. amboinicus* is well described in the literature. This plant is considered a source of potent antioxidants, with great ability to scavenge free radicals and chelate metal ions, promote anti-inflammatory effects and is a promising anticancer[4–6]. However, studies on chemical composition and biological activities of *P. amboinicus* "Variegata" are scarce.

Differences on chemical constitution and antioxidant activities of *P. amboinicus* and *P. amboinicus* "Variegata" have already been reported[6], but there are few studies studies correlating the chemical composition and biological activities of these plants. Hence, considering the potential use of these plants as antioxidants and for drug development, and the possible interference of variegation on plant secondary metabolites biosynthesis, the present study aimed to evaluate the chemical composition, antioxidant activity, cytotoxic, anti-cytotoxic and anticancer effects of *P. amboinicus* and *P. amboinicus* "Variegata" extracts.

2. MATERIAL AND METHODS

2.1. Chemicals

RPMI 1640 culture medium (Cultilab); fetal calf serum (Gibco); Ficoll® Paque Plus (Sigma–Aldrich); Cisplatin (Fauldcispla®, Libbs); 2,2-diphenyl-1-picryl-hydrazyl (Sigma–Aldrich); 2,2′-azinobis-(-3-ethylbenzothiazoline-6-sulfononic acid) (Sigma–Aldrich); iron chloride II (Sigma–Aldrich); β-carotene (Sigma–Aldrich); linoleic acid (Sigma–Aldrich); 3-(4,5- dimethyl2- thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide (Sigma–Aldrich).

2.2. Plant material

P. amboinicus and *P. amboinicus* “Variegata” were grown under organic cultivation at Jucuruaba Experimental Farm of the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural [Incaper] (20º25'23.0"S, 40°28'37.4"W). Composition of soil was analyzed by the AgroLab - Analysis and Control of Quality LTDA (Vila Velha / ES), methodology described by EMBRAPA (Claessen 1997). Total aerial part of the plants was collected in February 2016 and dried at room temperature (32–34 ºC). A voucher specimen of each variety was deposited in the Herbarium of Universidade Federal do Espírito Santo – VIES (registration number: *P. amboinicus* – 38107, *P. amboinicus* “Variegata” – 38110).

2.3. Hydralcoholic extract

Total aerial part of plants was powdered and macerated in EtOH/H_2O (70:30 v/v; plant : solvent - 1:5 w/v) for four days and filtered. The resulting liquid from the filtration was stored and protected from light. Maceration process was repeated twice using the same plant material, totaling twelve days of maceration. To obtain the crude extracts of each plant, the solvent was evaporated under reduced pressure at 65-70 °C. The two extracts obtained were stored protected from the light at 6–10 ºC until its utilization.

2.4. Phytochemistry analysis

2.4.1. Preliminary phytochemistry

Preliminary phytochemistry prospection was performed to identify secondary metabolites in *P. amboinicus* and *P. amboinicus* “Variegata” extracts. Extracts were tested for coumarins, flavonoids, alkaloids, naphthoquinones, saponins, steroids, tannins and triterpenoids, according to described in the literature[8].

2.4.2. Flavonoid content

The method described for Zhishen et al.[9] was used to measure total flavonoid content of *P. amboinicus* and *P. amboinicus* “Variegata” extracts. A serie of methanolic dilutions of rutin was prepared and assayed (15.63, 31.25, 62.50, 125.00, 250.00, 500.00, and 1000.00 µg/mL). Absorbance was detected at 430 nm by ELISA reader and the amount of total flavonoid was expressed in milligram of rutin equivalent flavonoid per gram of dry matter of extract. The experiments were performed in triplicate.

2.4.3. Rosmarinic acid content

Determination of rosmarinic acid concentration on hidroalcoholic extracts was performed according to described in literature[10]. After preparation of the samples, 20µL of methanolic solutions of *P. amboinicus* and *P. amboinicus* “Variegata” extracts were injected on a high performance liquid chromatograph (HPLC) using a Waters Breeze System equipped with Waters 1525 binary pump and UV/VIS Waters 2489 detection system. Supelco Nucleosil® C18 (250 x 3.2 mm, 5 µm) column was employed using a low rate of 0.5mL/min, maximum wavelength at 332 nm and isocratic elution (H_2O-MeOH 53:47). A serie of methanolic dilutions of rosmarinic acid was prepared and assayed (15.63, 31.25, 62.50, 125.00, 250.00, 500.00, and 1000.00 µg/mL). The amount of rosmarinic acid on extracts was expressed in microgram of rosmarinic acid equivalent per gram of dry matter of extract. The experiments were performed in triplicate.

2.5. Antioxidant activity

2.5.1. DPPH assay

Antioxidant activities of *P. amboinicus* and *P. amboinicus* “Variegata” extracts were evaluated by the radical reduction method, DPPH• (2,2-diphenyl-1-picryl-hydrazyl), where the H• fixation leads to the decrease in absorbance[11]. Extracts and standard ascorbic acid were used at concentration of 62.50, 125.00, 250.00 and 500.00 µg/mL. Absorbance was detected at 517nm by ELISA reader and the test was performed in triplicate. The percentage of inhibition of DPPH radical was calculated by the following formula:

$$\% \text{ inhibition} = [(\text{AbsControl} - \text{AbsSample}) / \text{AbsControl}] \times 100$$

where “% inhibition” is the percentage of inhibition capacity of DPPH•; “AbsControl” is the absorbance of DPPH• reaction control; and “AbsSample” is the absorbance of the sample.

2.5.2. ABTS assay

P. amboinicus and *P. amboinicus* “Variegata” extracts antioxidant activity was also evaluated by capturing method 2,2′- azinobis-(-3-ethylbenzothiazoline-6-sulfononic acid) (ABTS), using $ABTS^{\bullet+}$ radical[12]. Extracts and standard ascorbic acid were used at concentration of 62.50, 125.00, 250.00 and 500.00 µg/mL. Absorbance was detected at 734 nm by ELISA reader and the test was performed in triplicate. Percentage of scavenging inhibition of $ABTS^{\bullet+}$ was calculated by following equation:

$$\% \text{ inhibition} = [(\text{AbsControl} - \text{AbsSample}) / \text{AbsControl}] \times 100$$

where “% inhibition” is the percentage of scavenging inhibition capacity of $ABTS^{\bullet+}$; “AbsControl” is the absorbance of $ABTS^{\bullet+}$ reaction control; and “AbsSample” is the absorbance of the sample.

2.5.3. Fe^{2+} chelation ions assay

Inhibition of ferrous–ferrozine complex formation was used to measure the chelating activity of the extracts on ferrous ions (Fe^{2+})[13]. Extracts and standard EDTA were used at concentration of 62.50, 125.00, 250.00 and 500.00 µg/mL.Absorbance was detected at 562 nm by ELISA reader and the test was performed in triplicate. Percentage of ferrous ion chelating effect was calculated by following equation:

$$\% \text{ chelating effect} = [(\text{AbsControl} - \text{AbsSample}) / \text{AbsControl}] \times 100$$

where “% chelating effect” is the percentage of ferrous ions (Fe^{2+}) chelating effect; “AbsControl” is the absorbance of Fe^{2+} reaction control; and “AbsSample” is the absorbance of the sample.

2.5.4. β-carotene/linoleic acid assay

The antioxidant activity of the extracts was also evaluated by β-carotene/linoleic acid assay[14]. In this test the oxidation of β-carotene, induced by oxidative degradation products of linoleic acid, leads to discoloration of the system. Absorbance was detected at 470 nm by ELISA reader immediately (t = 0 min) and after 120 min of incubation (t = 120 min) at 50 °C. Test was performed in triplicate and the percentage inhibition of oxidation was calculated by following equation:

$$\% \text{ Inhibition of oxidation} = [(\Delta \text{ Abs}_0 – \Delta \text{ Abs}_1) / \Delta \text{ Abs}_0] \text{ x } 100$$

where “Δ Abs_0” is the initial absorbance − final absorbance of control; and “Δ Abs_1” is the initial absorbance – final absorbance of sample. Extracts and standard Trolox were used at concentration of 500.00 µg/mL. Percentage of inhibition of oxidation was expressed as percentage at the concentration of 500 µg/mL.

2.6. *In vitro* cell assays

2.6.1. Human lymphocytes

Peripheral blood sample was obtained from a healthy nonsmoking volunteer with informed consent, aged between 20-30 years, without any history of recent disease, exposures to radiation or drug use and without alcohol ingestion thirty days prior blood donating. Human lymphocytes were isolated from peripheral blood by Ficoll® Paque Plus gradient. Cells were plated in 96-well plates ($2x10^5$ cells / well) and used to evaluate *in vitro* cytotoxicity and anti-cytotoxicity of *P. amboinicus* and *P. amboinicus* "Variegata" extracts. The extracts were diluted with water at 10.0, 50.0 or 100.0 µg/mL and cells were cultured with extract doses for 24h or 48h to evaluate the cytotoxicity or cultured with extract doses more cisplatin (50.0 µg/mL) to evaluate the anti-cytotoxicity in the pre, simultaneous and post-treatment protocols, as described by Dutra et al.[15]. Untreated cells were used as negative control and cisplatin treated cells were used as positive control. All protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 2.333.879)

Percentage of cytotoxic damage reduction was calculated by the adapted formula[16]:

$$\% \text{ Reduction} = \frac{(\% \text{ cell viability in A} - \% \text{ cell viability in B})}{(\% \text{ cell viability in A} - \% \text{ cell viability in C})} \times 100$$

where ''A'' is the cell group treated with cisplatin; ''B'' is the cell group treated with *P. amboinicus* or *P. amboinicus* "Variegata" extract more cisplatin; and ''C'' is the control group of cells.

2.6.2. Sarcoma 180

Sarcoma 180 (murine sarcoma cells) was acquired from Banco de Células do Rio de Janeiro. Sarcoma 180 cells were plated in 96-well plates ($2x10^5$ cells / well) and the cytotoxicity evaluation of *P. amboinicus* and *P. amboinicus* "Variegata" extracts was tested using extract doses diluted with

water (10.0, 50.0 or 100.0 µg/mL). Antiproliferative effects of extracts were observed in sacoma 180 cells cultured with extract for 24h or 48h. All protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 89/2015).

2.6.3. Cell culturing methods

Human lymphocytes and sarcoma 180 cells were cultured with RPMI 1640 culture medium (gentamicin (50.0 mg/L) and amphotericin B (2.0 mg/L)), and 10% of fetal calf serum at 37 ºC, 5% of CO_2 saturation and humid atmosphere. Cells were cultured under these conditions twenty-four hours before starting the treatments. MTT assay was performed twenty-four hours after the last treatment to determine cell viability.

2.6.4. MTT assay

Cell viability was evaluated by MTT assay (3-(4,5- dimethyl-2- thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide). Twenty-four hours after the last treatment, the plates were centrifuged (860 rcf) for 10 minutes, the supernatant was discarded and 20 µL of MTT (5 mg/mL) were added to each well. After 3h, the plates were centrifuged (860 rcf) for 5 minutes, supernatant was discarded, 100 µL of DMSO were added. Absorbance was detected at 590 nm by ELISA reader, the test was performed in triplicate and the results were expressed as relative percentage of cell viability in comparison to the negative control.

2.7. Statistical analysis

Results were expressed as the mean ± standard deviation or mean ± standard error. Normality of the data was evaluated a priori by Shapiro-Wilk test (p<0.05). Flavonoids and rosmarinic acid contents in the extracts were evaluated by the *t* test (p<0.05). Linear curve was used to determine R^2 on DPPH, ABTS or Fe^{2+} ion chelating assays. Antioxidant capacity of extrats was compared by multiple *t* test (p<0.05). *In vitro* cytotoxicity of extracts in human

lymphocytes and sarcoma 180 cells were compared to their respective negative control cells by ANOVA *post hoc* Dunnett's test (p<0.05). Anti-cytotoxicity of extracts *in vitro* was performed by the comparison between the cells treated with doses of extract and the group of cells treated with cisplatin, using ANOVA *post hoc* Dunnett's test (p<0.05). Comparison between plants was performed by the *t* test (p<0.05). Principal component analysis (PCA) and Pearson correlation were used to determine relationships between total flavonoid and rosmarinic acid content, antioxidant activities (DPPH, ABTS, Fe^{2+} chelation, and β-carotene/ linoleic acid assays), cytotoxic and anti-cytotoxic effects (MTT assay) of *P. amboinicus* and *P. amboinicus* "Variegata" extracts. The highest concentrations tested, 100.0 and 500.0 µg/mL, showed better results in anti-cytotoxic and anticancer activities and antioxidant assays, respectively, and were used to perform PCA and Pearson's Correlation.

3. RESULTS

3.1. Soil analysis

The soil in which *P. amboinicus* and *P. amboinicus* “Variegata” were grown showed: pH (H_2O) – 6.49, phosphorus (P) – 98.40 mg/dm^3, potassium (K) – 139.00 mg/dm^3, calcium (Ca) – 7.50 cmol$_c$/dm^3, magnesium (Mg) – 3.98 cmol$_c$/dm^3, aluminum (Al) - < 0.10 cmol$_c$/dm^3, iron (Fe) – 69.30 mg/dm^3, zinc (Zn) – 14.30 mg/dm^3, cuprum (Cu) – 1.40 mg/dm^3, manganese (Mn) – 59.50 mg/dm^3, boron (B) – 0.70 mg/dm^3, súlfur (S) – 18.40 mg/dm^3, potential acidity (H + Al) – 3.95 cmol$_c$/dm^3, sum of bases – 11.84 cmol$_c$/dm^3, cations exchangeability – 15.79 cmol$_c$/dm^3, base saturation – 74.98 %, organic matter 6.80 dag/dm^3.

Considering the reference values[17], the soil used was characterized as weak acidity soil, with hight sum of bases, cations exchangeability and base saturation capacity, medium potential acidity, hight content of P, K, Ca, Mg, B and S, medium content of Fe, Zn and Mn and low content of Al and Cu.

3.2. Phytochemistry

In the preliminary phytochemical tests, both extracts of the plants showed positive reactions to flavonoids, flavone (cyanidin reaction) and coumarins; only the extract of *P. amboinicus* “Variegata” showed a positive reaction for tannins.

Figure 1 summarizes the total content of flavonoids and rosmarinic acid in *P. amboinicus* and *P. amboinicus* “Variegata” extracts. The extract of *P. amboinicus* “Variegata” presented higher total flavonoid (Figure 1A) and rosmarinic acid content (Figure 1B) than *P. amboinicus*.

3.3. Evaluation of antioxidant activity

Antioxidant activity of *P. amboinicus* and *P. amboinicus* “Variegata” extracts are summarized in the Figure 2 and 3. In the DPPH and ABTS assay, *P. amboinicus* “Variegata” showed higher antioxidant activity than *P. amboinicus*, reaching 9.79–66.76% and 5.62–90.12% of antioxidante activity in

DPPH and ABTS, respectively (Figure 2A and B), while *P. amboinicus* reached 6.90–42.94% and 1.65–27.70% of antioxidante activity in DPPH and ABTS, respectively (Figure 2A and B). On the other hand, *P. amboinicus* presented higher antioxidante activity in Fe^{2+} ion chelation and β-carotene/linoleic acid assays, reaching 76.56–90.47% of antioxidante activity on chelation ion (Figure 2C) and 39.37 ± 1.30% of inhibiton of oxidation (Figure 3), while *P. amboinicus* "Variegata" exhibited in the same assays 57.92–90.18% of antioxidante activity on chelation ion (Figure 2C) and 30.48 ± 2.83% of inhibiton of oxidation (Figure 3).

3.3.1. Cytotoxicity *in vitro*

Results of the cytotoxic activity of the extracts of *P. amboinicus* and *P. amboinicus* "Variegata" are summarized in table 1. After 24 hours of treatment, both extracts did not induce cytotoxic damage to human lymphocytes and *P. amboinicus* extract at all doses tested and *P. amboinicus* "Variegata" extract at 50.0 and 100.0 µg/mL were cytotoxic to sarcoma 180 cells. At 48 hours of treatment, the extracts were cytotoxic for both human lymphocytes and sarcoma 180 cells, being more cytotoxic for sarcoma.

3.3.2. Anti-cytotoxic activity *in vitro*

Anti-cytotoxicity of *P. amboinicus* and *P. amboinicus* "Variegata" extracts in the protocols of pre-treatment, simultaneous treatment and post-treatment are summarized in table 2.

For the pre-treatment protocol, we verified that the extract of *P. amboinicus* at 50.0 and 100.0 µg/mL were able to reduce the cytotoxic damage induced by cisplatin, reaching 18.39–39.46% of damage reduction; however the extract of *P. amboinicus* "Variegata" was more effective in promoting the reduction of cisplatin- induced damage. All tested doses of *P. amboinicus* "Variegata" extract promoted reduction of cytotoxic damage, reaching 25.11–72.21% of damage reduction.

Following the simultaneous treatment protocol, all tested doses of *P. amboinicus* extract induced a reduction in cytotoxicity, reaching 2.97–53.17% reduction in cytotoxic damage. In this treatment protocol, the extract of *P. amboinicus* “Variegata” at 50.0 and 100.0 µg/mL induced reduction of cisplatin cytotoxicity and reached 13.08–48.10% of damage reduction.

In the post-treatment protocol, *P. amboinicus* and *P. amboinicus* “Variegata” were able to reduce cisplatin-induced cytotoxic damage at 50.0 and 100.0 µg/mL. At the concentrations tested, the extract of *P. amboinicus* reached 3.59–5.38% of damage reduction and the extract of *P. amboinicus* “Variegata” reached 8.09–23.32% of reduction of cisplatin-induced damage.

3.4. Explorative analyses: chemical content *vs.* biological activities

Data obtained were correlated by PCA and Pearson correlation, and results are summarized in figure 4 and table 3, respectively.

A total variance of 77.42 % was observed in PCA, reaching 59.96 and 17.46 % in the first and second principal components (PC1 and PC2), respectively (figure 4). Variables total flavonoid and rosmarinic acid content, DPPH and ABTS antioxidant activities and extract cytotoxicity on sarcoma 180 cells after 48h of treatment dominated PC1. Inhibiton of β-carotene oxidation and extract cytotoxicity on human lymphocytes after 48h of treatment were variables that dominated PC2. These finds suggest that total flavonoid and rosmarinic acid content and DPPH and ABTS antioxidant activities are correlated with cytotoxic effects of extracts on sarcoma 180 cells after 48h of treatment, promoting anticancer effects. Similarly, these analyzes suggest that inhibiton of β-carotene oxidation are correlated with cytotoxicity observed on human lymphocytes after 48h of treatment with extracts.

P. amboinicus “Variegata” extract presents the highest total flavonoid and rosmarinic acid content levels in relation to *P. amboinicus* extract and it seems to be the main factor related to DPPH and ABTS antioxidant activities, cytotoxic effects in human lymphocytes after 24h and 48h of treatment, anti-cytotoxic

effects in pre and post-treatment protocols and anticancer effect 48h of treatment. Likewise, inhibiton of β-carotene oxidation seems to be the main factor related to the anticancer activity after 24h of treatment exhibited for *P. amboinicus* extract.

Positive values in Pearson correlation indicate a directly proportional relation between the factors. Following the Pearson correlation analysis (table 3), flavonoid and rosmarinic acid contents are strongly correlated to antioxidant activity in the DPPH and ABTS assays and strongly related to anti-cytotoxic activity in the post-treatment protocol and anticancer effect after 48h of treatment. Total content of flavonoids seems to exert a moderate correlation with the anti-cytotoxic activity in the pre-treatment protocol, while the rosmarinic acid content presents a strong correlation with this biological activity. Antioxidant activities in the DPPH and ABTS assays were moderately correlated to the cytotoxic effects in human lymphocytes after 24h of treatment, anti-cytotoxic activity in the pre and post treatment protocols and strongly correlated to the anticancer activity after 48h of treatment. The inhibition of β-carotene oxidation was moderately related to anticancer activity after 24h of treatment.

4. DISCUSSION

Plants may be used as a source of nutritive compounds, enhancing the taste and prolong the shelf life of food products, as well as for medicinal purposes. The medicinal properties exhibited by plants are related to their chemical composition and may vary due to growing conditions, genetic and environmental factors[18,19].

Plant growth conditions, such as the type and availability of nutrients in the soil, may interfere in the production of flavonoids, coumarins and tannins[20]. Maia et al.[21] consider that organic fertilization provides lower nutrient content than mineral fertilization, however, the authors emphasizes that organic fertilization can provide greater variety of nutrientes, interfering with the secondary metabolism of plants. Different from that proposed by Maia et al.[21], organic fertilization applied to the growth of *P. amboinicus* and *P. amboinicus* "Variegata" used in our study provided the necessary nutrients for plants development at high and medium levels.

P. amboinicus and *P. amboinicus* "Variegata" are related species that differ morphologically due to the variegation in the leaves, which interfere in the production of secondary metabolites and other plant bioactives[6]. Phytochemical studies demonstrated that *P. amboinicus* presents several phenolic compounds, such as tannins, rosmarinic acid, chrysoerythol, caffeic acid and ρ-coumaric acid, eryodyctiol, luteolin and quercetin [22]. Added to this, the study performed by Lee et al.[6] has demonstrated that *P. amboinicus* and *P. amboinicus* "Variegata" contain in their chemical composition polyphenols, coumarins and flavone and that the content of these compounds differs. These findings reinforce those found in our study, where we verified the presence of the same compounds and significative differences in flavonoid and rosmarinic acid contente present in these plants.

Rosmarinic acid is a non-volatile bioactive compound, found in large quantities in plants of the family Lamiaceae (chemical marker)[23] and promotes antioxidant, anti-inflammatory and anticancer activities[24–26]. Synthesis of rosmarinic acid is mediated by enzymes of the general pathway of phenylpropanoids, related to biosynthesis of flavonoids and lignins[27]. The

biosynthesis of rosmarinic acid involves the transformation of l-phenylalanine to t-cinnamic acid by action of phenylalanine ammonia-lyase (PAL), occurring coordinately the PAL activity and rosmarinic acid accumulation[28].

Phenylpropanoid pathway can be influenced by sucrose and phenylalanine, and they are capable of altering the production of plant metabolites[29]. Furthermore, phosphate, nitrate, and calcium have been related to the increase of rosmarinic acid content in *Anchusa officinalis* cells *in vitro*[30], as well as manganese has been related to the activation of enzymes involved in the biosynthesis of phenylpropanoids, such as in tricarboxylic acid cycle, shikimic acid and isoprenoids pathway[31]. Hence, we suppose that the intake of nutrients in *P. amboinicus* “Variegata” is higher, in comparison to *P. amboinicus*, interfering with PAL activity, and consequently promotes the increase of flavonoids and rosmarinic acid biosynthesis and accumulation.

Solvents used to obtain plant extracts are responsible for the extraction of specific groups of bioactive compounds, which may interfere in the biological activities exhibited by them. The aqueous leaf extract of *P. amboinicus* is reported to present high superoxide-scavenging, nitric oxide-scavenging and ferrous ion-chelating capacity[32], as well as aqueous leaf extract is reported to present higher content of total flavonoids and total phenolics and antioxidant activity than ethanolic extract[33].

Hydroalcoholic extracts used in our study showed a strong metallic chelating capacity in the highest concentrations tested. In comparison to *P. amboinicus*, *P. amboinicus* “Variegata” extract presented better antioxidant activity in chelation and inhibition of oxidation of β-carotene. On the other hand, *P. amboinicus* exhibited better antioxidant activity in DPPH and ABTS. Different to the observed by Lee et al.[6], using 70% acetone for preparation of the extracts, in comparison to *P. amboinicus*, *P. amboinicus* “Variegata” presented higher antioxidant power in the DPPH assay.

Gurgel et al.[5] reports the antiproliferative activity of *P. amboinicus* extract on sarcoma 180 and Erlich ascites carcinoma cells *in vivo*. In this way, our findings reinforce those described by Gurgel et al.[5] and suggest that both *P.*

amboinicus and *P. amboinicus* “Variegata” extracts are capable to induce antiproliferative effects. Monoterpenoids (carvacrol), sesquiterpenoids, diterpenoids, terpenoids (β-sitosterol) and phenolic compounds, such as quercetin, luteolin, caffeic acid and cinnamic derivates have been related to the anticancer activity of *P. amboinicus*[34–37] and may help to understand the anticancer effect exhibited by these extracts.

In our study, both extracts of *P. amboinicus* and *P. amboinicus* “Variegata” were able to inhibit cytotoxic damages induced by cisplatin. Cisplatin is a molecule used in the treatment of cancer due to its high ability to bind to RNA, DNA and proteins and to generate adducts that lead to cytotoxic effects[38,39]. Following the pre-treatment and simultaneous treatment protocols, both extracts were able to act directly on cisplatin and promote descytotoxic activity, inducing the chemical or enzymatic inactivation of this compound, as well as, following the post-treatment protocol, it was verified that *P. amboinicus* “Variegata” extract was more efficient than *P. amboinicus* extract in inducing repair mechanisms or acting on processes that induce cytotoxic damage, acting as a bioanticytotoxic agent. As suggested by Dutra et al.[15], the antioxidant capacity of plant extracts is related to its ability to inhibit cytotoxic damage, however, the author emphasizes that inhibition of cisplatin-induced damage may be related to the ability of extracts to chelate metal ions.

5. CONCLUSION

Our data suggests that variegation alter secondary metabolism pathway of *P. amboinicus* "Variegata" and leads to the increase of biosynthesis and accumulation of phenolic compounds. Total flavonoid and rosmarin acid content were positively correlated to the antioxidant activities and promotion of anti-cytotoxic and anticancer effects. Further studies are required to understand the mechanisms related to the increased production of these compounds and the biological activities observed. These findings corroborate the traditional use of this condimentar plants and also suggest that variegation is a factor to be considered for the selection of plants used for therapeutic purposes and drug development.

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Figures and tables

Table 1. *In vitro* cytotoxicity of *Plectranthus amboinicus* and *Plectranthus amboinicus* "Variegata" extracts (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes and sarcoma 180 cells by MTT assay.

	Treatment	Cell viability (%) ± SD							
		24 hours of treatment				48 hours of treatment			
		P. amboinicus	*p*	*P. amboinicus* "Variegata"	*P*	*P. amboinicus*	*P*	*P. amboinicus* "Variegata"	*p*
Human lymphocytes	Control	100.00 ± 6.13	–	100.00 ± 6.13	–	100.00 ± 8.59	–	100.00 ± 8.59	–
	Extract 10.0µg/mL	96.27 ± 7.23	0.8835	101.30 ± 14.70	0.9932	76.54 ± 0.95****	< 0.0001	73.07 ± 3.04****	< 0.0001
	Extract 50.0µg/mL	97.17 ± 2.25	0.9429	99.55 ± 13.03	0.9997	76.85 ± 2.38****	< 0.0001	76.85 ± 3.82****	< 0.0001
	Extract 100.0µg/mL	96.57 ± 0.55	0.9058	101.60 ± 5.46	0.9877	84.41 ± 0.55**	0.0027	85.98 ± 5.67**	0.0070
Sarcoma 180	Control	100.00 ± 7.38	–	100.00 ± 7.38	–	100.00 ± 3.49	–	100.00 ± 3.49	–
	Extract 10.0µg/mL	82.81 ± 3.52*	0.0248	85.25 ± 13.99	0.0598	59.54 ± 4.38****	< 0.0001	70.92 ± 10.35****	< 0.0001
	Extract 50.0µg/mL	71.73 ± 3.27***	0.0002	76.61 ± 9.98**	0.0020	65.53 ± 4.23****	< 0.0001	69.93 ± 14.88****	< 0.0001
	Extract 100.0µg/mL	66.56 ± 3.56****	< 0.0001	59.95 ± 0.16****	< 0.0001	58.07 ± 2.90****	< 0.0001	65.75 ± 2.55****	< 0.0001

The values are the mean ± SD. Cell viability was compared to its respectively control cells by ANOVA *post hoc* Dunnett's test - $^{**}p$<0.01, $^{***}p$<0.001 or $^{****}p$<0.0001 *vs*. human lymphocytes control; $^{\#\#\#\#}p$<0.0001 *vs*. sarcoma 180 control. Results of *P. amboinicus* and *P. amboinicus* "Variegata" were compared by *t* test.

Table 2. *In vitro* anti-cytotoxicity of *Plectranthus amboinicus* and *Plectranthus amboinicus* "Variegata" extracts (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes cells by MTT assay.

	Treatment	Cell viability (%) ± SD					
		P. amboinicus	*p*	% Reduction	*P. amboinicus* "Variegata"	*p*	% Reduction
Pre-treatment	Control	100.00 ± 8.59****	< 0.0001	–	100.00 ± 8.59****	< 0.0001	–
	Cisplatin	64.88 ± 0.723	–	–	64.88 ± 0.723	–	–
	Extract 10.0µg/mL + Cisplatin	63.94 ± 0.54	0.9965	–	73.70 ± 5.26	0.0788	25.11
	Extract 50.0µg/mL + Cisplatin	71.34 ± 0.67	0.3389	18.39	73.70 ± 0.83	0.1278	25.11
	Extract 100.0µg/mL + Cisplatin	78.74 ± 1.96**	0.0044	39.46	90.24 ± 11.36****	< 0.0001	72.21
Simultaneous treatment	Control	100.00 ± 6.13****	< 0.0001	–	100.00 ± 6.13****	< 0.0001	–
	Cisplatin	64.68 ± 6.09	–	–	64.68 ± 6.09	–	–
	Extract 10.0µg/mL + Cisplatin	65.73 ± 5.69	0.9991	2.97	64.08 ± 1.86	0.9998	–
	Extract 50.0µg/mL + Cisplatin	74.51 ± 8.31	0.2432	27.83	69.30 ± 0.64	0.8583	13.08
	Extract 100.0µg/mL + Cisplatin	83.46 ± 7.60**	0.0094	53.17	81.67 ± 8.07*	0.0191	48.10
Post-treatment	Control	100.00 ± 8.59****	< 0.0001	–	100.00 ± 8.59****	< 0.0001	
	Cisplatin	64.88 ± 0.723	–	–	64.88 ± 0.723	–	
	Extract 10.0µg/mL + Cisplatin	63.62 ± 1.97	0.9661	–	62.05 ± 1.09	0.6487	–
	Extract 50.0µg/mL + Cisplatin	66.14 ± 2.50	0.9655	3.59	67.72 ± 1.45	0.6469	8.09
	Extract 100.0µg/mL + Cisplatin	66.77 ± 1.97	0.8735	5.38	73.07 ± 3.82*	0.0161	23.32

The values are the mean ± SD. Cell viability was compared to the cisplatin treated cells in each treatment protocol by ANOVA *post hoc* Dunnett's test - $^{‡}p<0.05$, $^{‡‡}p<0.01$, $^{‡‡‡}p<0.001$ or $^{‡‡‡‡}p<0.0001$ *vs*. human lymphocytes control. Results of *P. amboinicus* and *P. amboinicus* "Variegata" were compared by *t* test.

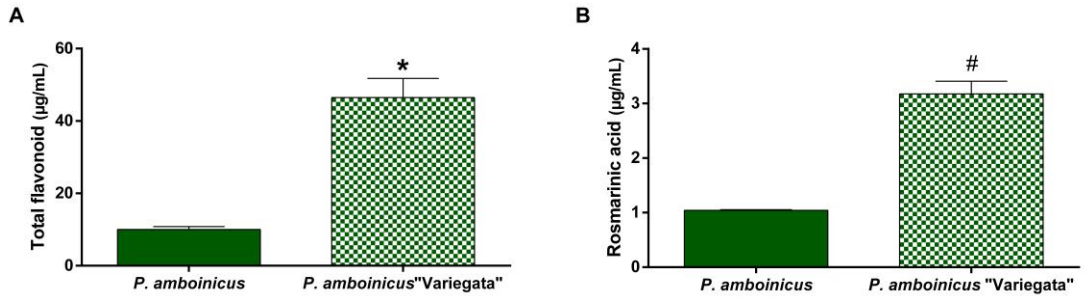
Table 3. Pearson correlation analysis between total flavonoid and rosmarinic acid content, antioxidant activities (DPPH, ABTS, Fe^{2+} chelation and β-carotene/ linoleic acid assays), cytotoxic and anti-cytotoxic effects (MTT assay) of *P. amboinicus* and *P. amboinicus* "Variegata" extracts.

	Flavonoid	Rosmarinic acid	DPPH	ABTS	Fe^{2+} chelation	β-carotene	MTT 24h Cit. Lymp.	MTT 24h Cit. S-180	MTT 48h Cit. Lymp.	MTT 48h Cit. S-180	MTT Pre-treat.	MTT Sim. treat.	MTT Post-treat.
Flavonoid	**1**												
Rosmarinic acid	0.9911	**1**											
DPPH	0.9491	0.9460	**1**										
ABTS	0.9801	0.9843	0.9846	**1**									
Fe^{2+} chelation	-0.1090	-0.0169	-0.0720	-0.0636	**1**								
β-carotene	-0.7394	-0.8161	-0.7906	-0.8240	-0.3776	**1**							
MTT 24h Cit. Lymp.	0.5089	0.5117	0.7199	0.6277	-0.2708	-0.5234	**1**						
MTT 24h Cit. S-180	-0.8483	-0.8393	-0.8647	-0.8426	-0.2576	0.6741	-0.4021	**1**					
MTT 48h Cit. Lymp.	0.0927	0.1139	0.3899	0.2587	-0.0380	-0.3361	0.8760	-0.1585	**1**				
MTT 48h Cit. S-180	0.8030	0.8197	0.8727	0.8630	-0.2660	-0.7325	0.8588	-0.5398	0.5362	**1**			
MTT Pre-treat.	0.7516	0.8222	0.6405	0.7408	0.3128	-0.8499	0.1929	-0.5742	-0.1176	0.6145	**1**		
MTT Simult. treat.	-0.2400	-0.1474	-0.1978	-0.1815	0.1885	-0.1606	0.1860	0.4004	0.2748	0.2121	0.2396	**1**	
MTT Post-treat.	0.8174	0.8489	0.6409	0.7473	0.0710	-0.6684	0.1276	-0.5816	-0.2854	0.6018	0.9363	0.1033	**1**

Flavonoid = total flavonoid content; rosmarinic acid = rosmarinic acid content; DPPH = inhibition of DPPH radical; ABTS = inhibition of ABTS radical; Fe^{2+} chelation: chelation activity; β-carotene = inhibition of oxidation of β-carotene; MTT 24h Cit. Lymp. = extracts cytotoxicity in human lymphocytes after 24h of treatement; MTT 24h Cit. S-180 = extracts cytotoxicity in sarcoma 180 cells after 24h of treatement; MTT 48h Cit. Lymp. = extracts cytotoxicity in human lymphocytes after 48h of treatement; MTT 48h Cit. S-180 = extracts cytotoxicity in sarcoma 180 cells after 48h of treatement; MTT Pre-treat. = extracts anti-cytotoxicity in human lymphocytes in pre-treatement protocol; MTT Sim. treat. = extracts anti-cytotoxicity in human lymphocytes in simultaneous treatement protocol; MTT Post-treat. = extracts anti-cytotoxicity in human lymphocytes in post-treatement protocol. Values of antioxidant activities at 500.0 µg/mL of extract and cytotoxic and anti-cytotoxic effects (MTT assay) at 100.0 µg/mL of extract were used to perform Pearson Correlation.

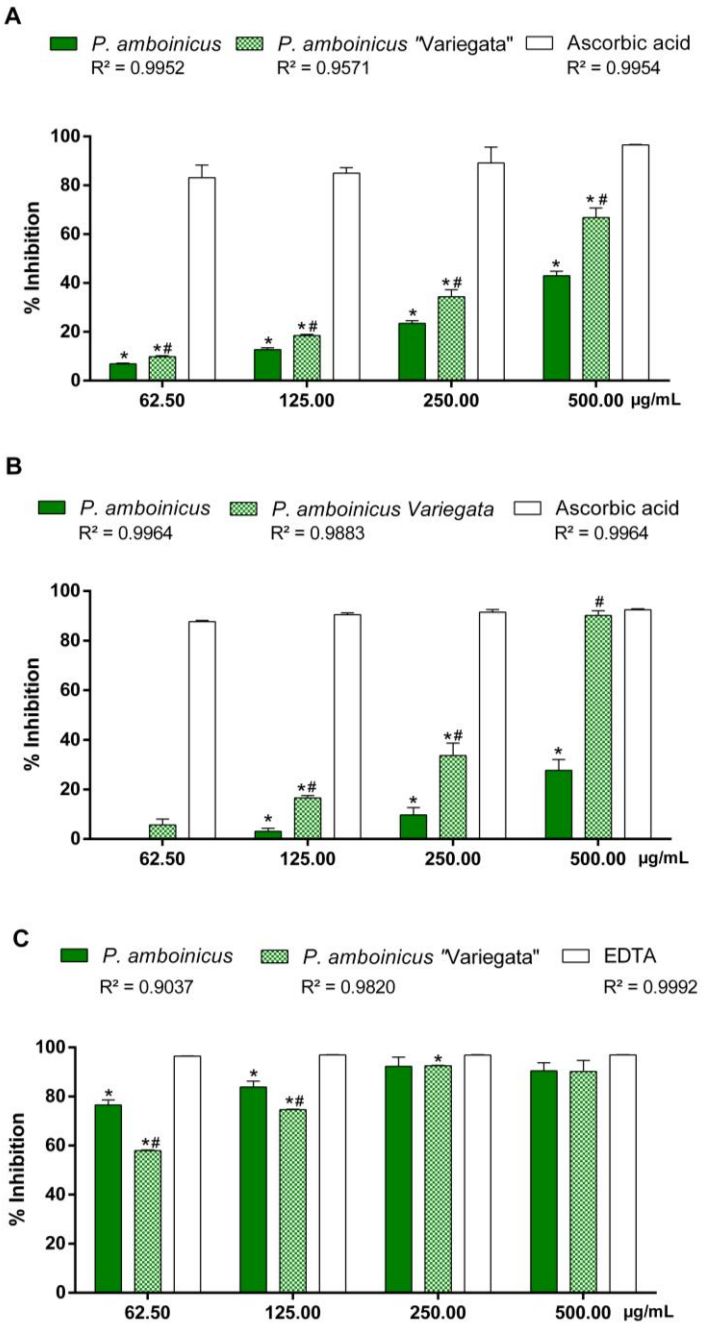
Figure legends

Figure 1. **Total flavonoid and rosmarinic acid content of *P. amboinicus* and *P. amboinicus* "Variegata" extracts.**



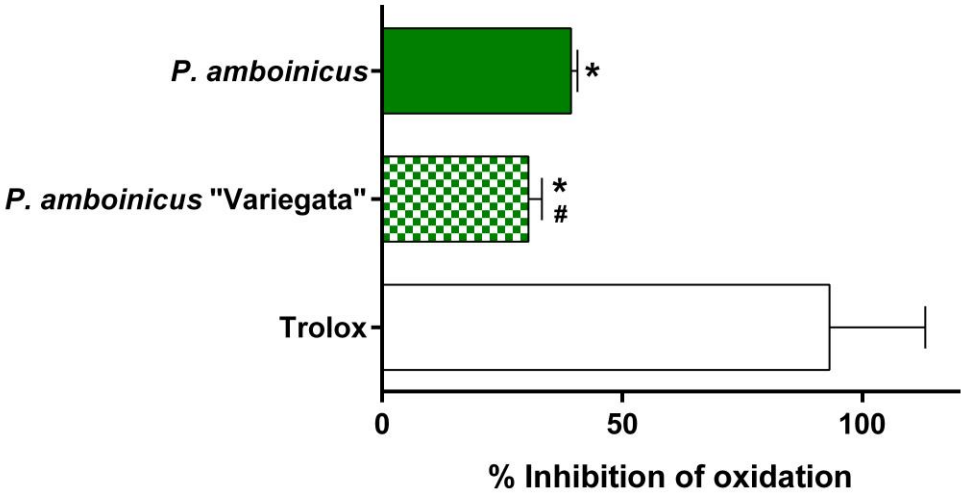
The values are the mean ± SD. Comparisons between flavonoid or romarinic acid contents in extracts were performed by the *t* test ($p<0.05$) – $^{*}p = 0.0003$; $^{\#}p<0.0001$.

Figure 2. Antioxidant activity of *P. amboinicus* and *P. amboinicus* “Variegata” extracts following DPPH, ABTS and Fe^{2+} ion chelation assays.



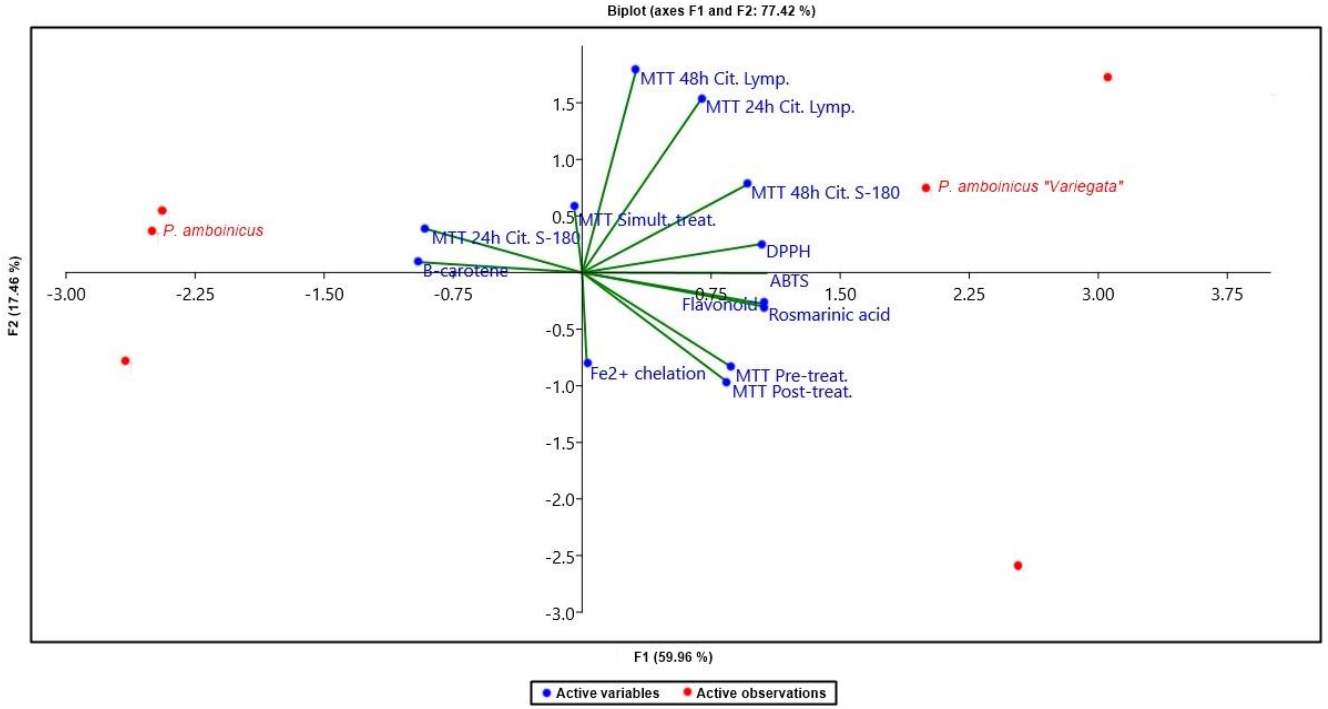
The values are the mean ± SD. Antioxidant activity of extracts and of the standards shown by the percentage of inhibition in (A) DPPH, (B) ABTS and (C) in chelating activity on Fe^{2+} ions. Inhibition of DPPH and ABTS or Fe^{2+} chelating of *P. amboinicus, P. amboinicus* “Variegata” extracts and standards was compared by multiple *t* test ($p<0.05$) - *standard vs. *P. amboinicus* or *P. amboinicus* “Variegata” ($p<0.05$); $^{\#}$*P. amboinicus* vs. *P. amboinicus* “Variegata”.

Figure 3. Antioxidant activity of *P. amboinicus* and *P. amboinicus* “Variegata” extracts following β-carotene/linoleic acid assay.



The values are the mean ± SE. Comparisons between extracts and Trolox were performed by the *t* test ($p<0.05$) – *Trolox vs. *P. amboinicus* or *P. amboinicus* “Variegata” ($p<0.05$); $^{\#}$*P. amboinicus* vs. *P. amboinicus* “Variegata” ($p<0.05$).

Figure 4. Principal component analysis (scores and loading plots, biplot) based on total flavonoide and rosmarinic acid content analyzed in *P. amboinicus* and *P. amboinicus* “Variegata” extracts and their antioxidant activities and cytotoxic and anti-cytotoxic effects of *P. amboinicus* and *P. amboinicus* “Variegata” extracts.



MTT 24h Cit. Lymp.: extracts cytotoxicity in human lymphocytes after 24h of treatement; MTT 24h Cit. S-180: extracts cytotoxicity in sarcoma 180 cells after 24h of treatement; MTT 48h Cit. Lymp.: extracts cytotoxicity in human lymphocytes after 48h of treatement; MTT 48h Cit. S-180: extracts cytotoxicity in sarcoma 180 cells after 48h of treatement; MTT Pre-treat.: extracts anti-cytotoxicity in human lymphocytes in pre-treatement protocol; MTT Sim. treat.: extracts anti-cytotoxicity in human lymphocytes in simultaneous treatement protocol; MTT Post-treat.: extracts anti-cytotoxicity in human lymphocytes in post-treatement protocol. Values of antioxidant activities at 500.0 µg/mL of extract and cytotoxic and anti-cytotoxic effects (MTT assay) at 100.0 µg/mL of extract were used to perform PCA.

CAPITULO 4 – Phytochemical, antioxidant, anti-cytotoxic and anticancer screening of Lamiaceae medicinal plants

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RESUMO

Objetivo: Lamiaceae é uma família botânica formada por plantas usadas na alimentação e na medicina tradicional. O objetivo deste trabalho foi avaliar o teor total de flavonóides e ácido rosmarínico, atividade antioxidante, citotoxicidade, efeito anti-citotoxicidade e anticancerígeno utilizando extratos de cinco plantas da Família Lamiaceae. Extratos hidroalcoólicos de *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Ocimum selloi* e *Plectranthus* ornatus foram preparados e os teores totais de flavonóides e ácido rosmarínico foram determinados por protocolos de espectrofotometria e HPLC, respectivamente; a atividade antioxidante foi avaliada pelos ensaios DPPH, ABTS e atividade quelante de íons Fe^{2+}; a citotoxicidade e anti-citotoxicidade contra cisplatina foi avaliada em linfócitos humanos e a atividade anticancerígena em células do sarcoma 180 *in vitro* (ensaio MTT). Entre as plantas avaliadas, o extrato de *Melissa officinalis* foi o que apresentou maior teor de ácido rosmarínico e menor concentração de flavonoides totais. *Ocimum basilicum* apresentou maior atividade quelante de metais e *Melissa* officinalis e *Mentha piperita* apresentaram melhor atividade antioxidante nos ensaios de DPPH e ABTS, além de reduzirem acentuadamente o dano citotóxico induzido pela cisplatina. Esses achados reforçam o uso de plantas da Família Lamiaceae na alimentação e sugere que as espécies vegetais em estudo apresentam potencial para o desenvolvimento de drogas que previnam danos genômicos e capazes de combater cânceres.

Palavras-chave: Lamiaceae, compostos fenólicos, atividade antioxidante, sarcoma 180 anticâncer, teste MTT.

ABSTRACT

Objetive: Lamiaceae is a botanical family formed by plants used in food and traditional medicine. The objective of this work was to evaluate the total content of flavonoids and rosmarinic acid, antioxidant activity, cytotoxicity, anti-cytotoxicity and anticacer effects of five Lamiaceae plants. Hydroalcoholic extracts of *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Ocimum selloi* and *Plectranthus ornatus* were prepared and the total content of flavonoids and rosmarinic acid were determined by spectrophotometric and HPLC protocols, respectively; antioxidant activity was evaluated by DPPH, ABTS and Fe^{2+} chelation activity; cytotoxic and anti-cytotoxic activity against cisplatin were evaluated in human lymphocytes and anticancer activity in sarcoma 180 cells *in vitro* (MTT assay). Comparing the evaluated plants, the extract of *Melissa officinalis* showed highest content of rosmarinic acid; *Ocimum basilicum* shows higher metal chelation activity; *Melissa officinalis* and *Mentha piperita* showed better antioxidant activity in the DPPH and ABTS assays and effectively reduce the cytotoxic damage induced by cisplatin. These findings reinforce the use of these plants as food and for drug development.

Keywords: Lamiaceae plants, phenolic compounds, antioxidant activity, sarcoma 180 anticancer, MTT assay.

1. INTRODUCTION

World Health Organization estimates that approximately 80% of world population uses medicinal plants to treat diseases [1]. Along the tima, plants have been widely used for humans to treat health disorders without previous studies, and the majority of people that uses plants for this purpose believe in the absence of side effects of natural products [1].

Lamiaceae Family, one of the largest botanical families on the world, consistes of approximately 236 genera and about 6900-7200 species [2] that are widely used in food, such as spice plants, and have been used for health promotion due to their great antioxidant ability [3].

Medicinal uses of Lamiaceae plants are related to the secondary metabolites synthesized by these vegetables, such as flavonoids and rosmarinic acid [4,5]. Rosmarinic acid, a non-volatile bioactive, is widely found in Lamiaceae plants, is considered a chemical marker for this plant group [6] and is known to promote biological activities such as antioxidant, antiinflammatory activities and anticancer effects [7–9].

Different genera of the Lamiaceae plants are reported for their biological activities and are considered promising as antioxidants and against cancer, such as *Melissa* [10], *Mentha* [11], *Orthosiphon* [12], *Ocimum* [13], *Plectrantus* [5], *Rosmarinus* [14] and others. Thus, considering the promising antioxidant and anticancer activities of Lamiaceae plants, the aim of this work was to evaluate the total flavonoid and rosmarinic acid content, antioxidant activity, cytotoxicity, anti-cytotoxicity and anticancer activity *in vitro* of *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum*, *Ocimum selloi* and *Plectranthus ornatus*, correlating their chemical composition with their biological activities.

2. MATERIAL AND METHODS

2.1. Chemicals

RPMI 1640 culture medium (Cultilab); fetal calf serum (Gibco); Ficoll® Paque Plus (Sigma–Aldrich); Cisplatin (Fauldcispla®, Libbs); 2,2-diphenyl-1-picryl-hydrazyl (Sigma–Aldrich); 2,2′-azinobis-(-3-ethylbenzothiazoline-6-sulfononic acid) (Sigma–Aldrich); iron chloride II (Sigma–Aldrich); 3-(4,5- dimethyl2- thiazolyl)-2,5- diphenyl-2H- tetrazolium bromide (Sigma–Aldrich).

2.2. Plant material

Melissa officinalis L., *Mentha piperita* L., *Ocimum basilicum* L., *Ocimum selloi* Benth and *Plectranthus ornatus* Codd were obtained from Jucuruaba Experimental Farm of the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural [Incaper] (20º25'23.0"S, 40°28'37.4"W). Plants were grown under organic cultivation and total aerial part of the plants was collected in February 2016. Plant material was dried at room temperature (32–34 ºC) and a voucher specimen of each variety was deposited in the Herbarium of Universidade Federal do Espírito Santo – VIES (registration number: *Melissa officinalis* L.–38104; *Mentha piperita* L.–38111; *Ocimum basilicum* L.–38106; *Ocimum selloi* Benth–38105; *Plectranthus ornatus* Codd–38108).

2.3. Hydralcoholic extract

Dried material was powdered and macerated in EtOH/H_2O (70:30 v/v; plant : solvent - 1:5 w/v) for four days and filtered. This process was repeated twice using the same plant material, performing a total of twelve days of maceration. Resulting liquid from maceration was stored protected from light and the solvent was evaporated under reduced pressure, at 65-70 °C, to obtain the crude extracts of each plant, totaling five extracts. Extracts were stored protected from the light at 6–10 ºC until its utilization.

2.4. Phytochemistry analysis

2.4.1. Preliminary phytochemistry

Coumarins, flavonoids, alkaloids, naphthoquinones, saponins, steroids, tannins and triterpenoids on Lamiaceae plants were evaluated by preliminary phytochemistry prospection, as described in the literature [15].

2.4.2. Flavonoid content

Flavonoid content on extract plants was measured by spectrophotometric protocol [16] A serie of methanolic dilutions of rutin was prepared and assayed (15.63, 31.25, 62.50, 125.00, 250.00, 500.00, and 1000.00 µg/mL). Using an ELISA reader (Epoch – BioTech®), the absorbance of samples was detected at 430 nm. The amount of total flavonoid was expressed in milligram of rutin equivalent flavonoid per gram of dry matter of extract. Experiments were performed in triplicate.

2.4.3. Rosmarinic acid content

Rosmarinic acid concentration on extracts was determined as described in literature [17]. Samples were prepared and 20µL of methanolic solutions of extracts were injected on a high performance liquid chromatograph (HPLC) using a Waters Breeze System equipped with Waters 1525 binary pump and UV/VIS Waters 2489 detection system. Column Supelco Nucleosil® C18 (250 x 3.2 mm, 5 µm) was employed performing a low rate of 0.5mL/min, maximum wavelength at 332 nm and isocratic elution with H_2O-MeOH (53:47 v/v). Methanolic dilutions of rosmarinic acid at 15.63, 31.25, 62.50, 125.00, 250.00, 500.00, and 1000.00 µg/mL was prepared and assayed. Rosmarinic acid concentration on samples was expressed in microgram of rosmarinic acid equivalent per gram of dry matter of extract. Experiments were performed in triplicate.

2.5. Antioxidant activity

2.5.1. DPPH assay

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical reduction method was used to evaluate the antioxidant activity of extracts [18]. Absorbance was detected at 517nm by ELISA reader. Experiment was performed in triplicate and the percentage of inhibition of DPPH radical was calculated by the following formula:

$$\% \text{ inhibition} = [(\text{AbsControl} - \text{AbsSample}) / \text{AbsControl}] \times 100$$

where "% inhibition" is the percentage of inhibition capacity of DPPH•; "AbsControl" is the absorbance of DPPH• reaction control; and "AbsSample" is the absorbance of the sample. Ascorbic acid was used as standard.

2.5.2. ABTS assay

Capturing method 2,2′- azinobis-(-3-ethylbenzothiazoline-6-sulfononic acid) (ABTS) was also used to measure antioxidant activity of extracts [19]. Absorbance was detected at 734 nm by ELISA reader. Experiment was performed in triplicate and the percentage of scavenging inhibition of $ABTS^{\bullet+}$ was calculated by following formula:

$$\% \text{ inhibition} = [(\text{AbsControl} - \text{AbsSample}) / \text{AbsControl}] \times 100$$

where "% inhibition" is the percentage of scavenging inhibition capacity of $ABTS^{\bullet+}$; "AbsControl" is the absorbance of $ABTS^{\bullet+}$ reaction control; and "AbsSample" is the absorbance of the sample. Ascorbic acid was used as standard.

2.5.3. Fe^{2+} chelation ions assay

Chelating activity of the extracts on ferrous ions (Fe^{2+}) was measured by the inhibition of ferrous–ferrozine complex formation [20]. Absorbance was detected at 562 nm by ELISA reader. Experiment was performed in triplicate and percentage of ferrous ion chelating effect was calculated by following equation:

$$\% \text{ chelating effect} = [(\text{AbsControl} - \text{AbsSample}) / \text{AbsControl}] \times 100$$

where “% chelating effect” is the percentage of ferrous ions (Fe^{2+}) chelating effect; “AbsControl” is the absorbance of Fe^{2+} reaction control; and “AbsSample” is the absorbance of the sample. EDTA was used as standard.

2.6. *In vitro* cytotoxicity assays

2.6.1. Human lymphocytes

Human lymphocytes were obtained from peripheral blood sample of a healthy nonsmoking volunteer with informed consent, aged between 20-30 years, without any history of recent disease, exposures to radiation or drug use and without alcohol ingestion thirty days prior blood donating. Ficoll® Paque Plus gradient was used to isolate human lymphocytes. To evaluate *in vitro* cytotoxicity and anti-cytotoxicity of extracts, cells were plated in 96-well plates, $2x10^5$ cells/well. Extracts were diluted at 10.0, 50.0 or 100.0 µg/mL using water. Cells received extract concentrations for 24h or 48h to evaluate the cytotoxicity or cultured with extract concentrations more cisplatin (50.0 µg/mL) to evaluate the anti-cytotoxicity in the pre, simultaneous and post-treatment protocols[21]. Untreated group of cells were used as negative control and cisplatin treated cells were used as positive control for cytotoxicity. All protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 2.333.879)

Percentage of cytotoxic damage reduction was calculated by the adapted equation [22]:

$$\% \text{ Reduction} = \frac{(\% \text{ cell viability in A} - \% \text{ cell viability in B})}{(\% \text{ cell viability in A} - \% \text{ cell viability in C})} \times 100$$

where ‘‘A’’ is the cell group treated with cisplatin; ‘‘B’’ is the cell group treated with Lamiaceae plant extracts more cisplatin; and ‘‘C’’ is the control group of cells.

2.6.2. Sarcoma 180

Sarcoma 180 cells were plated in 96-well plates, $2x10^5$ cells/well and the cytotoxicity of extracts was assayed using extract at tested using extract at 10.0,

50.0 or 100.0 µg/mL, diluted with water. Cells were cultured with extract doses for 24h or 48h.

Sarcoma 180 cells were acquired from Banco de Células do Rio de Janeiro and all protocols were approved by the Research Ethical Committee of Universidade Federal do Espírito Santo (certificate 89/2015).

2.6.3. Cell culturing methods

Cells were cultured with RPMI 1640 culture medium containing gentamicin (50.0 mg/L) and amphotericin B (2.0 mg/L). Culture medium was supplemented with 10% of fetal calf serum and cells were maintained at 37 ºC, 5% of CO_2 saturation and humid atmosphere. Cells were previously maintained under these conditions twenty-four hours before starting the treatments.

2.6.4. MTT assay

Twenty-four hours after the last treatment, assay (3-(4,5- dimethyl-2-thiazolyl)-2,5- diphenyl-2H- tetrazolium bromide) (MTT) assay was conducted to determine cell viability. Plates were centrifuged (860 rcf) for 10 minutes, the supernatant was discarded, 20 µL of MTT (5 mg/mL) were added to each well and the cells were maintained under similar culture conditions for 3h. The plates were centrifuged (860 rcf) for 5 minutes, supernatant was discarded, 100 µL of DMSO were added. Using an ELISA reader, absorbance was detected at 590 nm and relative cell viability was calculated using negative control cells. Experiment was performed in triplicate.

2.7. Statistical analysis

Results were expressed as the mean ± standard deviation. Shapiro-Wilk test (p<0.05) was used to evaluate normality of the data. Flavonoids and rosmarinic acid contents and antioxidant activity of extracts were evaluated by by ANOVA *post hoc* Tukey test (p<0.05). Following protocols of cytotoxicity and Anti-cytotoxicity, cell

viability of treated cells was compared to their respective control groups by ANOVA *post hoc* Dunnett's test ($p<0.05$). Pearson correlation was used to determine relationships between total flavonoid and rosmarinic acid content, antioxidant activities (DPPH, ABTS and Fe^{2+} chelation assays), cytotoxic and anti-cytotoxic effects (MTT assay) of extracts. Results of extract on cytotoxicity and anti-cytotoxicity protocols at 100.0 µg/mL and results of antioxidant activity at 500.0 µg/mL presented higher biological activities and were used to perform Pearson's Correlation.

3. RESULTS

3.1. Phytochemistry

Preliminary phytochemistry showed that all extracts presented positive reaction for flavonoids, flavone (cyanidin reaction), steroids and coumarin. All extracts presented positive reaction for saponin, except the extract of *Plectranthus ornatus*, as well as, the extract of *Plectranthus ornatus* was the only one that presented positive reaction for triterpenes.

Total content of flavonoids and rosmarinic acid in Lamiaceae extracts are summarized in Table 1. Extracts of *Ocimum selloi* and *Plectranthus ornatus* showed the highest total flavonoid content. On the other hand, *Melissa officinalis* presented higher and *Plectranthus ornatus* presented the lowest content of rosmarinic acid.

3.2. Antioxidant activity

Antioxidant activity of the Lamiaceae plants is summarized in Table 2. Among extracts evaluated in the DPPH assay, extracts of *Mentha piperita* and *Ocimum basilicum* presented the highest antioxidant capacity, reaching 76.03 - 89.69% and 66.53 - 91.70% inhibition of the radical, respectively. Extract of *Mentha piperita* also present higher antioxidant capacity in the ABTS assay compared to the other extracts, reaching 93.51 - 93.70% of radical inhibition. Comparing the chelating activity of metal ions, *Plectranthus ornatus* presented the highest chelation power, reaching 48.30 - 87.99% inhibition.

3.3. Cytotoxic effects

Cytotoxic effects promoted by the extracts in human lymphocytes and sarcoma 180 cells are summarized in Figure 1. After 24 hours of treatment, all extracts at all concentrations tested were non-cytotoxic to human lymphocytes (Figure 1A) and induced cytotoxic damage to sarcoma 180 cells (Figure 1B). Extracts of *Melissa officinalis*, *Ocimum basilicum* and *Ocimum selloi* induced cell proliferation of human lymphocytes.

After 48 hours of treatment, extracts tested were also able to induce cytotoxic damage in human lymphocytes after 48 hours of exposure (Figure 1C), as well as, all extracts at all concentrations tested promoted cytotoxic effects on sarcoma 180 cells (Figure 1D). *Melissa officinalis*, *Mentha piperita* and *Ocimum basilicum* at concentrations of 50.0 and 100.0 µg/mL did not induce cytotoxic damage in human lymphocytes, and *Ocimum selloi* at 100.0 µg/mL did not induce cytotoxic damage in human lymphocytes.

3.4. Anti-cytotoxic effects

Anti-cytotoxicity of Lamiaceae extracts in the protocols of pre, simultaneous and post-treatment are summarized in Figure 2, and Table 1 summarize the percentage of cytotoxic damage reduction against cisplatin.

Following the pre-treatment protocol, all tested concentrations of *Ocimum selloi* were able to inhibit cytotoxic damage induced by cisplatin (Figure 2A), but the highest percentages of cytotoxic damage reduction were promoted by *Melissa officinalis*, *Mentha piperita* and *Ocimum basilicum* (Table 3).

In the simultaneous treatment protocol, all tested concentrations of *Ocimum basilicum* were able to inhibit the cytotoxic damage induced by cisplatin (Figure 2B). Both *Ocimum basilicum* and *Melissa officinalis* presented higher percentages of reduction of cytotoxic damage (Table 3).

For the post-treatment protocol, only the extracts of *Melissa officinalis*, *Mentha piperita*, *Ocimum basilicum* and *Ocimum selloi* at 50.0 and 100 µg/mL concentration inhibited cytotoxic damage induced by cisplatin (Figure 2C), and the extracts of *Melissa officinalis* and *Mentha piperita* presented higher percentages of cytotoxic damage reduction (Table 3).

3.5. Phytochemistry and biological activities correlations

Correlations between secondary metabolites and biological activities exhibited by Lamiaceae extracts were performed by Pearson correlation analysis and are presented in Table 4.

Positive correlation values indicate a directly proportional relation between the factors. Pearson's correlation analysis showed that total flavonoid content was strongly correlated with iron ion chelating activity and that rosmarinic acid was moderately correlated to cytotoxicity in human lymphocytes and sarcoma 180 after 24 hours of treatment, and strongly correlated with cytotoxicity in lymphocytes after 48 hours of treatment. Rosmarinic acid also showed a strong correlation with anti-cytotoxic activities in pre and post treatment protocols and very strong correlation with the anti-cytotoxic effect in the simultaneous treatment.

4. DISCUSSION

Lamiaceae family plants are well known for their use as a condiment. In addition to its application in cooking, some studies suggest that this group of plants has a great ability to eliminate free radicals [3], which adds commercial value to these plants, making them commercially relevant [23].

In our study, it was observed that all the evaluated species had in their chemical constitution flavonoids, flavone and coumarin (phenolic compounds) and steroids, saponins and triterpenes (terpenoids). These group of substances are also found in other species of the Lamiaceae family, such as phenolic compounds found in *Salvia miltiorrhiza* [24] and *Micromeria fruticosa* [25] and terpenoids found in *Clerodendrum polycephalum* [26] and *Cunila menthoides* [27].

Studies as conducted by Trueba and Sanchez [28] have shown that there is a direct relationship between antioxidant activity and flavonoid concentration. In our study, the extract of *Plectranthus ornatos* presented higher flavonoid content and, in comparison to the other extracts, better antioxidant activity on iron chelaton. Our find reinforces the role of flavonoids in metal chelation, as described in literature [29]. However, in the DPPH and ABTS assays, no direct relationship was observed between flavonoid concentration and antioxidant activities. Our data suggests that highest rosmarinic acid concentrations appear to be related to these antioxidant activities. Rosmarinic acid is already described as an antioxidant compound in the DPPH and ABTS assays [30], and our data support these findings.

In the cytotoxicity assays we observed that all the extracts tested induce the reduction of the cellular viability of sarcoma 180 cells, promoting anticancer effect. Likewise, the extracts tested were able to prevent and repair damage induced by cisplatin. Mesquita et al. [31] suggests that plants of Lamiaceae Family presents phytochemicals with good perspective for the development of new alternatives for the treatment and prevention of diseases. In our analyses, rosmarinic acid was correlated with chemoprotective activity and this finding corroborate those already described in literature, which describes this substance as a promoter of biological activities, such as antioxidant, anti-inflammatory and anticancer activities [7–9].

Extracts of *Melissa officilanis* and *Mentha piperita*, in comparison to the other extracts, were those that presented higher rosmaric acid content and stood out in the antioxidant activities, reduction of cytotoxic damages and induction of anticancer activity. Rosmarinic acid found in these plants is synthesized by enzymes of the general pathway of phenylpropanoids, related to the biosynthesis of flavonoids [32]. The biosynthesis of rosmarinic acid involves the transformation of L-phenylalanine into t-cinnamic acid by the action of PAL, occurring in a coordinated way to the activity of PAL and the accumulation of rosmarinic acid in the plant tissue [33]. Considering biosynthesis of rosmarinic acid, we propose that the low total content of flavonoids in these plants may occur due to modulation in the action of phenylalanine ammonia-lyase (PAL) that lead to the increase biosynthesis of rosmarinic acid in detriment of biosynthesis of flavonoids.

5. CONCLUSION

Extracts of Lamiaceae plants presented antioxidant activity in different protocols, reduced damages induced by cisplatin and promoted anticancer effect *in vitro*. Total content of flavonoids was correlated with the ability to chelate metal ions and rosmarinic acid content was correlated with anti-cytotoxic activities. Our findings also suggest that increased biosynthesis of rosmarinic acid occurs in the detriment of flavonoid biosynthesis, however further studies are needed to understand this process. Our results reinforce the use of these plants in feeding and suggest that among the investigated plants, *Melissa officinais* and *Mentha piperita* are the most promising to obtain rosmarinic acid and promotion of biological activities relevant to the development of new therapeutic practices, treatment and prevention of diseases, and drug development.

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Conflicts of Interest: The authors declare no conflict of interest.

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Figures and tables

Table 1. Total flavonoid and rosmarinic acid content of Lamiaceae plants extracts.

Plant	Phenolic Compound Concentration (µg/mL)	
	Flavonoid	Rosmarinic Acid
Melissa officinalis	5.74 ± 2.36^{c}	16.91 ± 2.34^{a}
Mentha piperita	40.60 ± 4.52^{b}	12.33 ± 0.97^{b}
Ocimum basilicum	47.59 ± 3.54^{b}	7.81 ± 0.29^{c}
Ocimum selloi	55.05 ± 6.88^{ab}	6.52 ± 0.28^{c}
Plectranthus ornatus	69.17 ± 6.58^{a}	2.35 ± 0.11^{d}

The values are the mean ± SD. Comparisons between flavonoid or romarinic acid contents in extracts were performed by ANOVA *post hoc* Tukey test (p<0.05). Different lowercase letters in the column $^{(a, b, c \text{ or } d)}$ mean statistical difference.

Table 2. Antioxidant activity of Lamiaceae plants extracts following DPPH, ABTS and Fe^{2+} ion chelation assays.

Assay	Concentration (µg/mL)	% of inhibition					
		Melissa officinalis	*Mentha piperita*	*Ocimum basilicum*	*Ocimum selloi*	*Plectranthus ornatus*	Standard
DPPH	62.5	9.48 ± 3.90^{c}	41.97 ±1.62^{c}	0.20 ± 0.28^{d}	34.21 ±1.05^{c}	8.93 ± 3.54^{c}	79.42 ± 5.53^{b}
	125.0	37.43 ± 3.68^{b}	76.03 ± 4.50^{b}	7.84 ± 7.70^{c}	66.53 ± 0.97^{b}	15.96 ± 3.19^{c}	81.41 ± 2.37^{b}
	250.0	82.97 ± 3.88^{a}	91.68 ± 0.72^{a}	30.79 ± 4.52^{b}	91.70 ±0.08^{a}	34.36 ± 0.85^{b}	85.88 ± 7.05^{b}
	500.0	89.71 ± 0.57^{a}	89.69 ± 0.06^{a}	78.87 ± 3.54^{a}	91.01 ± 0.09^{a}	65.91 ± 0.25^{a}	93.92 ± 0.20^{a}
ABTS	62.5	27.75 ± 9.26^{c}	93.70 ± 0.08^{a}	12.35 ± 1.25^{d}	0.34 ± 0.21^{d}	9.30 ± 3.53^{d}	92.88 ± 0.82^{a}
	125.0	58.21 ± 9.19^{b}	93.75 ± 0. 05^{a}	24.94 ± 6.65^{c}	3.24 ± 1.06^{c}	20.77 ± 6.95^{c}	92.85 ± 0.80^{a}
	250.0	88.96 ± 6.94^{a}	93.51 ± 0.42^{a}	49.83 ± 4.88^{b}	9.88 ± 3.21^{b}	45.76 ± 2.12^{b}	92.01 ± 2.56^{a}
	500.0	92.74 ± 1.32^{a}	93.61 ± 0.25^{a}	79.28 ± 6.46^{a}	19.03 ± 1.48^{a}	82.42 ± 0.15^{a}	93.70 ± 0.30^{a}
Fe^{2+} chelation	62.5	15.47 ± 0.63^{d}	7.29 ± 0.80^{d}	15.79 ± 5.80^{d}	3.81 ± 4.54^{d}	48.31 ± 0.32^{c}	96.47 ± 0.04^{a}
	125.0	24.11 ± 1.21^{c}	14.68 ± 3.63^{c}	25.50 ± 10.43^{c}	25.93 ± 5.89^{c}	67.82 ± 0.40^{b}	96.97 ± 0.05^{a}
	250.0	35.88 ± 4.56^{b}	46.28 ± 1.31^{b}	48.28 ± 0.72^{b}	51.34 ± 5.46^{b}	84.28 ± 0.14^{a}	96.89 ± 0.12^{a}
	500.0	58.52 ± 6.71^{a}	78.28 ± 1.18^{a}	71.85 ± 0.53^{a}	67.51 ± 3.52^{a}	87.86 ± 0.37^{a}	96.92 ± 0.80^{a}

The values are the mean ± SD. Antioxidant activity of extracts and of the standards shown by the percentage of inhibition in (A) DPPH, (B) ABTS and (C) in chelating activity on Fe^{2+} ions. Inhibition of DPPH and ABTS or Fe^{2+} chelating of Lamiaceae plats extracts and standards was compared by ANOVA *post hoc* Tukey test (p<0.05). Different lowercase letters in the column $^{(a, b, c, d, e)}$ mean statistical difference.

Table 3. Percentage of cytotoxic damage reduction induced by Lamiaceae extracts against cisplatin following anti-cytotoxic protocols in human lymphocyties *in vitro*.

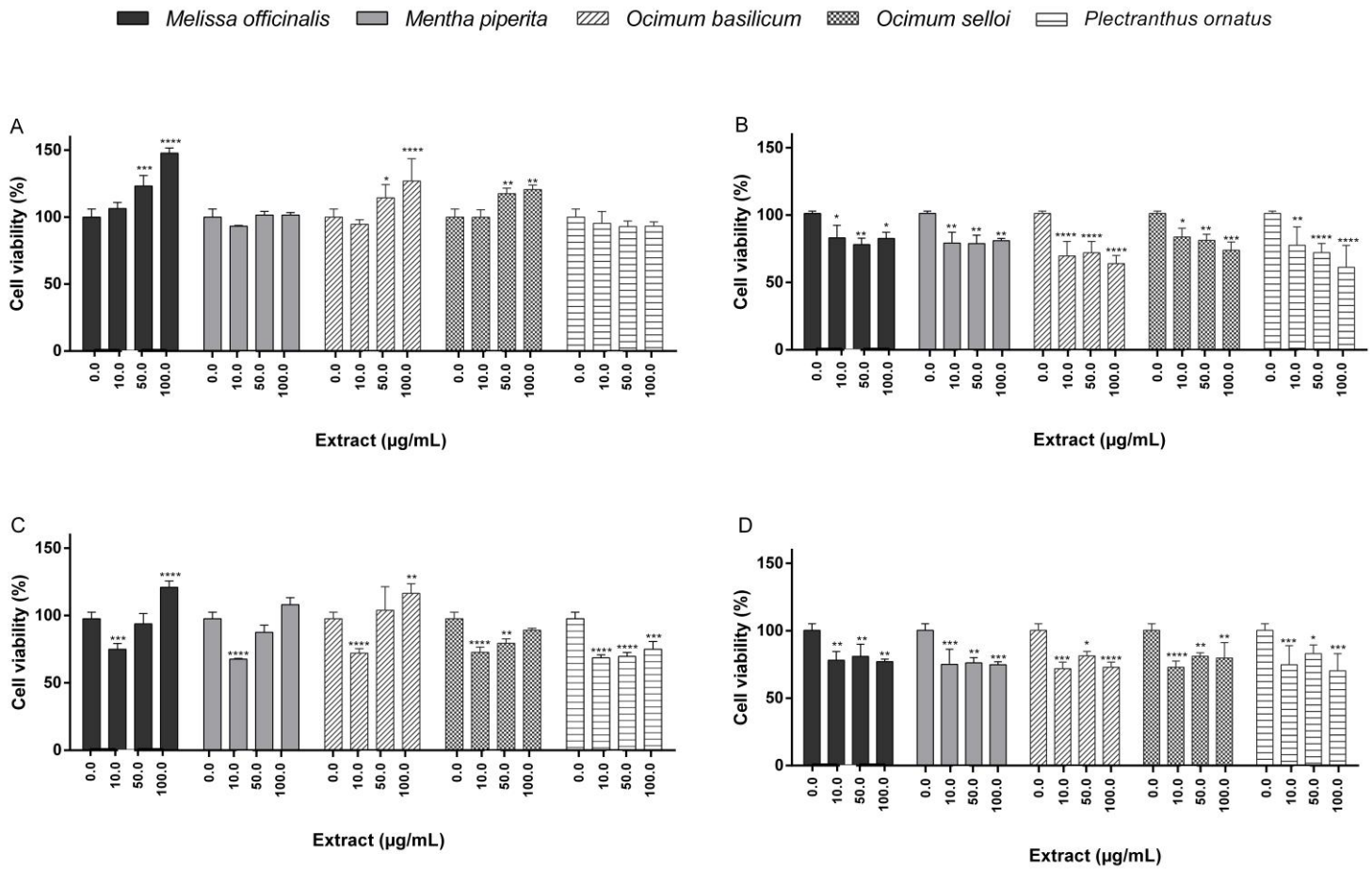
Anti-cytotoxic protocol	Extract	% Cytotoxic Damage Reduction				
		Melissa officinalis	*Mentha piperita*	*Ocimum basilicum*	*Ocimum selloi*	*Plectranthus ornatus*
Pre treatment	10.0 µg/mL	30.10	7.77	21.36	50.48	10.68
	50.0 µg/mL	88.36	68.93	> 100.00	38.84	13.59
	100.0 µg/mL	> 100.00	> 100.00	> 100.00	51.46	30.10
Simultaneous treatment	10.0 µg/mL	29.54	19.41	43.88	25.31	5.07
	50.0 µg/mL	96.21	71.73	96.20	68.36	19.42
	100.0 µg/mL	> 100.00	> 100.00	> 100.00	> 100.00	42.20
Post treatment	10.0 µg/mL	30.11	34.96	5.83	33.01	8.73
	50.0 µg/mL	> 100.00	> 100.00	42.73	72.81	20.40
	100.0 µg/mL	> 100.00	> 100.00	70.87	> 100.00	33.01

Table 4. Pearson correlation analysis between total flavonoid and rosmarinic acid content, antioxidant activities (DPPH, ABTS and Fe^{2+} chelation), cytotoxic and anti-cytotoxic effects (MTT assay) of Lamiaceae extracts.

	Flavonoid	Rosmarinic acid	DPPH	ABTS	Iron chelation	MTT 24h Cit. Lymp.	MTT 48h Cit. Lymp.	MTT 24h Cit. S-180	MTT 48h Cit. S-180	MTT Pre-treat.	MTT Sim. treat.	MTT Post-treat.
Flavonoid	1											
Rosmarinic acid	-0,9390	1										
DPPH	0,3009	-0,5308	1									
ABTS	-0,2718	0,2699	-0,0542	1								
Iron chelation	0,7618	-0,8000	0,5570	-0,4147	1							
MTT 24h Cit. Lymp.	-0,7654	0,6189	0,0054	-0,0613	-0,6212	1						
MTT 48h Cit. Lym	-0,7741	0,7993	-0,5109	0,3678	-0,9481	0,7006	1					
MTT 24h Cit. S-180p.	-0,6738	0,6935	-0,4850	-0,0496	-0,4330	0,3128	0,3786	1				
MTT 48h Cit. S-180	-0,1917	0,2251	-0,1693	-0,3542	0,0212	0,2228	0,0959	-0,0174	1			
MTT Pre-treat.	-0,7487	0,7771	-0,4785	0,4908	-0,9134	0,6286	0,9800	0,3274	0,1267	1		
MTT Simult. treat.	-0,9344	0,9166	-0,2938	0,1558	-0,7635	0,7857	0,7782	0,5432	0,3099	0,7454	1	
MTT Post-treat.	-0,6540	0,7724	-0,8012	0,1012	-0,5524	0,2581	0,5198	0,7123	0,2384	0,4869	0,5849	1

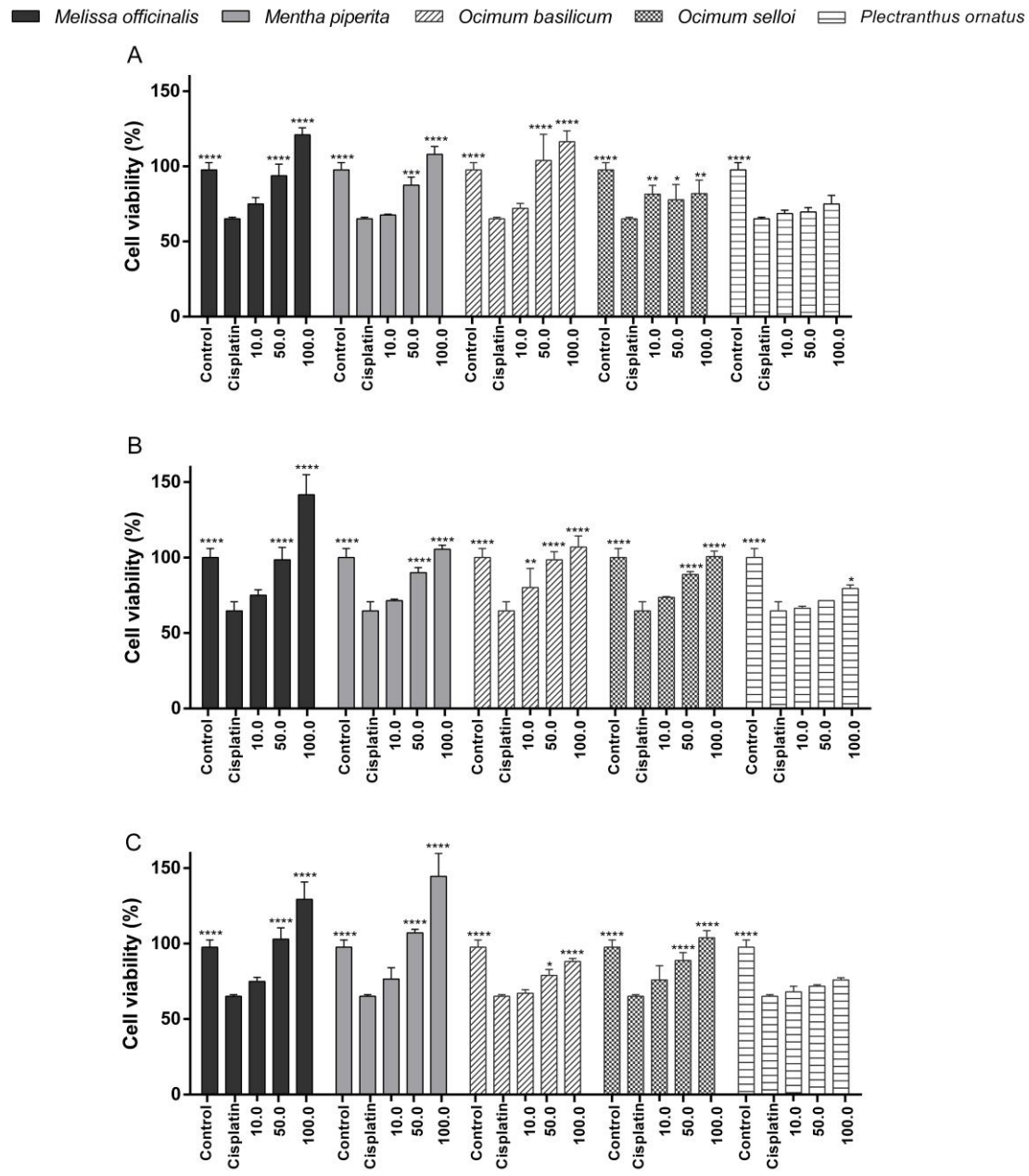
Flavonoid = total flavonoid content; rosmarinic acid = rosmarinic acid content; DPPH = inhibition of DPPH radical; ABTS = inhibition of ABTS radical; Fe^{2+} chelation: chelation activity; MTT 24h Cit. Lymp. = extracts cytotoxicity in human lymphocytes after 24h of treatement; MTT 24h Cit. S-180 = extracts cytotoxicity in sarcoma 180 cells after 24h of treatement; MTT 48h Cit. Lymp. = extracts cytotoxicity in human lymphocytes after 48h of treatement; MTT 48h Cit. S-180 = extracts cytotoxicity in sarcoma 180 cells after 48h of treatement; MTT Pre-treat. = extracts anti-cytotoxicity in human lymphocytes in pre-treatement protocol; MTT Sim. treat. = extracts anti-cytotoxicity in human lymphocytes in simultaneous treatement protocol; MTT Post-treat. = extracts anti-cytotoxicity in human lymphocytes in post-treatement protocol. Values of antioxidant activities at 500.0 µg/mL of extract and cytotoxic and anti-cytotoxic effects (MTT assay) at 100.0 µg/mL of extract were used to perform Pearson Correlation (p<0.05).

Figure 1. *In vitro* cytotoxicity of Lamiaceae plants extracts (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes and sarcoma 180 cells by MTT assay.



The values are the mean ± SD. Cell viability was compared to its respectively control cells by ANOVA *post hoc* Dunnett's test ($p<0.05$) - $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ or $^{****}p<0.0001$.

Figure 2. *In vitro* anti-cytotoxicity of Lamiaceae plants extracts (10.0, 50.0 or 100.0 µg/mL) in human lymphocytes cells by MTT assay.



The values are the mean ± SD. Cell viability was compared to cisplatin treatedlls by ANOVA *post hoc* Dunnett's test ($p<0.05$) - $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ or $^{****}p<0.0001$.

ANEXO I

CERTIFICADO EMITIDO PELA A COMISSÃO DE ÉTICA NO USO DE ANIMAIS.

UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO
CENTRO DE CIÊNCIAS DA SAÚDE
COMISSÃO DE ÉTICA NO USO DE ANIMAIS - CEUA

CERTIFICADO

Certificamos que o Projeto intitulado"Caracterização Fisiológica, Fitoquímica e de Atividades Biológicas de Plantas Medicinais com Potencial Econômico para Produção de Fitoterápicos.",Protocolo nº.89/2015, sob a responsabilidade de Maria do Carmo Pimentel Batitucci que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata(exceto o homem), para fins de pesquisa científica(ou ensino)encontra-se de acordo com os preceitos da Lei 11.794, de 8 de outubro de 2008, do Decreto nº6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal(CONCEA),e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS(CEUA) DO(A) Centro de Ciências da Saúde-Maruípe-Vitória-ES em 04-03-2016

Vigência do Projeto	Início: Abril/2016 Término:Fevereiro/2019
Espécie/Linhagem	Camundongos Swiss(Mus musculus)
Nº de Animais	Experimento Piloto:20 Protocolo Experimental:256 Total:276
Peso/Idade	Peso: 30-40g Idade:6-8 semanas
Sexo	Macho
Origem	Mamíferos

Vitória (ES), 04 de março de 2016.

ANEXO II

PARECER EMITIDO PELO COMITÊ DE ÉTICA EM PESQUISA COM SERES HUMANOS

UFES - UNIVERSIDADE FEDERAL DO ESPÍRITO SANTO - CAMPUS GOIABEIRA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Análise do potencial mutagênico, antimutagênico e citotóxico de plantas medicinais utilizadas pela população capixaba em linfócitos humanos in vitro.

Pesquisador: Maria do Carmo Pimentel Batitucci

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 2

CAAE: 71093016.6.0000.5542

Instituição Proponente: Centro de Ciências Humanas e Naturais da Universidade Federal do Espírito

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.333.879

Apresentação do Projeto:

O projeto de pesquisa "Análise do potencial mutagênico, antimutagênico e citotóxico de plantas medicinais utilizadas pela população capixaba em linfócitos humanos in vitro" de Maria do Carmo Pimentel Batitucci, tem por objetivo avaliar o potencial mutagênico, antimutagênico e citotóxico do banco de extratos (e/ou de seus derivados) do Laboratório de Genética Vegetal e Toxicológica da UFES, produzidos a partir de espécies vegetais usadas como medicinais por meio de testes em células humanas (linfócitos) in vitro. Tem como hipóteses que plantas medicinais utilizadas pela população do Espírito Santo possuem efeito citotóxico em linfócitos humanos;-Plantas medicinais utilizadas no estado do Espírito Santo possuem efeito mutagênico em linfócitos humanos;-Plantas medicinais utilizadas no estado do Espírito Santo que não possuem efeito citotóxico em linfócitos, apresentam efeito antimutagênico.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar o potencial mutagênico, antimutagênico e citotóxico do banco de extratos (e/ou de seus derivados) do Laboratório de Genética Vegetal e Toxicológica da UFES, produzidos a partir de espécies vegetais usadas como medicinais por meio de testes em células humanas (linfócitos) in vitro.

Objetivo Secundário:

1- Avaliar possível ação mutagênica e antimutagênica dos extratos produzidos a partir de diferentes espécies vegetais utilizadas pela população do ES, em células sanguíneas humanas;2- Avaliar a atividade citotóxica dos extratos produzidos a partir de diferentes espécies vegetais utilizadas pela população do ES, através da análise de células sanguíneas humanas;3- Avaliar a genotoxicidade de extratos produzidos a partir de diferentes espécies vegetais utilizadas pela população do ES, em células sanguíneas humanas;4- Fornecer subsídios para o uso medicinal seguro de diferentes espécies vegetais utilizadas pela população.

Avaliação dos Riscos e Benefícios:

A pesquisadora esclarece que o Projeto oferece riscos mínimos ao doador de sangue, por considerar a eventual possibilidade de hematoma (roxo ou vermelhidão) e/ou dor mínima na região onde ocorrerá a coleta. Esclarece, ainda, que para minimizar os riscos todos os procedimentos serão realizados por profissional da área e, em caso de algum mal estar, será prestado atendimento aos participantes. Após recomendação do Comitê de Ética, outros riscos foram incluídos como alergia derivada do processo de coleta de sangue, pergunta inserida no Questionário de Saúde Pessoal. Destaco, ainda, que foram esclarecidos na metodologia as dúvida sobre como será o processo de coleta de dados, com as devidas explicações sobre a pesquisa será in vitro.

Em relação aos benefícios a pesquisa o trará à população em geral, pois indicará se os extratos de vegetais usados pela população como medicinais têm ação citotóxica, genotóxica e/ou mutagênica sobre células humanas, e ainda se esses extratos podem apresentar alguma atividade que possa prevenir mutações no DNA humano. Como desfecho primário, identificará a citotoxicidade, a genotoxicidade e a antimutagenicidade de extratos/derivados de espécies de plantas medicinais utilizadas no Estado do Espírito Santo.

Comentários e Considerações sobre a Pesquisa:

O projeto é relevante cientificamente e socialmente, e está bem delimitado com sólida argumentação teórica e metodológica. Destaco, ainda, que atendeu todas as recomendações apresentadas no Parecer anterior.

Considerações sobre os Termos de apresentação obrigatória:

No TCLE, apresenta todas as informações necessárias em conformidade com a Resolução 466, de dezembro de 2012, do Conselho Nacional de Saúde, tais como: objetivos, procedimentos, riscos,

benefícios, indenização, ressarcimento. Além disso, esclarece os procedimentos da pesquisa e esclarece sobre o significado da pesquisa em in vitro.

Recomendações:

Levando em consideração que foram atendidas no Projeto as demandas apresentadas no parecer anterior sobre: 1) Critério de Inclusão; 2) Riscos; 3) Benefícios; 4) Metodologia, recomendo aprovação do Comitê.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.

Considerações Finais a critério do CEP:

Projeto aprovado por esse comitê, estando autorizado a ser iniciado.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_553693.pdf	05/09/2017 20:57:07		Aceito
Outros	Anexo1_05_09_2017.doc	05/09/2017 20:56:12	Maria do Carmo Pimentel Battucci	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_05_09_2017.doc	05/09/2017 20:52:54	Maria do Carmo Pimentel Battucci	Aceito
Projeto Detalhado / Brochura Investigador	projetocomitehumano_05_09_2017.doc	05/09/2017 20:52:37	Maria do Carmo Pimentel Battucci	Aceito
Folha de Rosto	Folha_Rosto.pdf	30/05/2017 10:54:18	Maria do Carmo Pimentel Battucci	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

VITORIA, 17 de Outubro de 2017

Assinado por:
Fabiana Pinheiro Ramos
(Coordenador)

Endereço: Av. Fernando Ferrari,514-Campus Universitário, Prédio Administrativo do CCHN
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