UNIVERSIDADE FEDERAL DE PERNAMBUCO
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TESE DE DOUTORADO

INVESTIGAÇÃO DO POTENCIAL ANTIOXIDANTE, IMUNOMODULATÓRIO E TOXICOLÓGICO DE FRAÇÃO RICA EM COMPOSTOS FENÓLICOS E DE LIGNINA E PECTINA ISOLADAS DE *Conocarpus erectus* L. (COMBRETACEAE)

DAYANE KELLY DIAS DO NASCIMENTO SANTOS

Orientador: Prof. Dr. Thiago Henrique Napoleão
Coorientadora: Profª. Drª. Cristiane Moutinho Lagos de Melo

RECIFE
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Tese de Doutorado apresentado como um dos requisitos para o cumprimento parcial das exigências para obtenção do título de Doutora em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

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RESUMO

Conocarpus erectus é uma planta de mangue amplamente distribuída no mundo, utilizada pela população para fins terapêuticos. Alguns estudos utilizando folhas de *C. erectus* em diferentes formulações (chá, infusão, macerado) relatam potencial antioxidante e imunomodulador. No presente trabalho, frações enriquecidas em compostos fenólicos e lignina e pectina isoladas de folhas de *C. erectus* foram avaliadas quanto às atividades antioxidante e imunomoduladora. Frações ricas em compostos fenólicos foram obtidas a partir do extrato aquoso das folhas e caracterizadas quimicamente por cromatografia líquida de alta eficiência (CLAE) e métodos colorimétricos. A lignina e a pectina foram caracterizadas por espectroscopia no infravermelho (FITR) e ressonância magnética nuclear de prótons (1H RMN); ainda, a lignina também foi caracterizada pela determinação da análise espectroeletrônica na região ultravioleta e a pectina avaliada quanto ao peso molecular e à estabilidade térmica. A atividade antioxidante foi avaliada pelos métodos de varredura dos radicais DPPH e ABTS, atividade antioxidante total e inibição da peroxidação lipídica. Os efeitos imunomoduladores da lignina e pectina foram investigados em células mononucleares do sangue periférico humano (PBMCs) através de ensaios de viabilidade e proliferação celular, avaliação de estresse oxidativo, imunofenotipagem e quantificação da liberação de citocinas e NO. Já a ação imunomoduladora das frações foi avaliada em esplenócitos de camundongo. As frações apresentaram principalmente flavonoides em sua composição e atividade antioxidante foi semelhante ou maior que a dos padrões antioxidantes sintético (BHT) e natural (ácido ascórbico). As frações F01 e F03 apresentaram perfil imunossupressor sobre os esplenócitos. O isolamento da pectina foi confirmado pela presença de ácidos galacturônicos esterificados apresentando um peso molecular de 27,0±0,2 kDa e estabilidade térmica de aproximadamente 95% a 100 °C. A lignina de folhas de *C. erectus* foi caracterizada como sendo do tipoguaiacil-siringyl-p-hidroxifenil (G-S-H) apresentando um conteúdo fenólico de 465,90 ± 1,07 mg/g GAE. A lignina e pectina apresentaram alta atividade antioxidante contra os radicais DPPH (IC50 = 231,16 e 189,21 µg/mL, respectivamente) e ABTS (IC50 = 356,03 e 248,25 µg/mL, respectivamente). Ainda, a pectina também promoveu uma inibição moderada da peroxidação lipídica (41,81±0,5 %). A lignina e a pectina promoveram aumento nos níveis de espécies reativas de oxigênio (EROs) mitocondrial e de Ca2+ citosólico, bem como diminuição do potencial de membrana de mitocôndria (ΔΨm) em PBMCs, porém sem provocar toxicidade às células. A lignina ainda promoveu o aumento da produção de EROs citosólicas e estimulou a proliferação e ativação de linfócitos T CD8+ e inibição de linfócitos T CD4+ (CTLA4+). A pectina, por outro lado, estimulou a proliferação e ativação de linfócitosT CD4+ e inibição de linfócitos T CD8+ (CTLA4+). As duas moléculas promoveram uma maior liberação de citocinas envolvidas na resposta Th1, IL-2, TNF-α e IFN-γ, assim como aumento da produção de óxido nítrico. Esses resultados destacam o potencial uso de compostos fenólicos, lignina e pectina de *C. erectus* como compostos imunomoduladores em estudos futuros.

Palavras Chave: Combretaceae; Mangue de Botão; Imunomodulador; Produtos naturais.

ABSTRACT

Conocarpus erectus is a mangrove plant widely distributed around the world, used by the population for therapeutic purposes. Some studies using *C. erectus* leaves in different formulations (tea, infusion, macerate) report antioxidant and immunomodulatory potential. In the present work, fractions enriched in phenolic compounds and lignin and pectin composed of *C. erectus* leaves were evaluated for antioxidant and immunomodulatory activities. Fractions rich in phenolic compounds were eliminated from the aqueous extract of the leaves and chemically characterized by high performance liquid chromatography (HPLC) and colorimetric methods. Lignin and pectin were characterized by infrared spectroscopy (FITR) and proton nuclear magnetic resonance (1H NMR); yet, lignin was also characterized by the determination of spectroelectronic analysis in the ultraviolet region and pectin was evaluated for molecular weight and thermal stability. Antioxidant activity was evaluated by DPPH and ABTS radical scanning methods, total antioxidant activity and inhibition of lipid peroxidation. The immunomodulatory effects of lignin and pectin were investigated in human peripheral blood mononuclear cells (PBMCs) through cell viability and proliferation assays, oxidative stress assessment, immunophenotyping and quantification of cytokine and NO release. The immunomodulatory action of the fractions was evaluated in mouse splenocytes. The main flavonoid fractions in their composition and antioxidant activity similar to or greater than that of synthetic (BHT) and natural (ascorbic acid) antioxidant patterns. Fractions F01 and F03 immunosuppressive profile on splenocytes. The isolation of pectin was confirmed by the presence of esterified galacturonic acids with a molecular weight of 27.0 ± 0.2 kDa and thermal stability of approximately 95% at 100 °C. The lignin from leaves of C. erectus was characterized as being of the type guaiacyl-syringyl-p-hydroxyphenyl (GSH) which has a phenolic content of 465.90 ± 1.07 mg/g GAE. Lignin and pectin complement high antioxidant activity against DPPH radicals (IC50 = 231.16 and 189.21 µg / mL, respectively) and ABTS (IC50 = 356.03 and 248.25 µg / mL, respectively). Furthermore, pectin also promoted a moderate inhibition of lipid peroxidation (41.81 ± 0.5%). Lignin and pectin increased the levels of mitochondrial reactive oxygen species (ROS) and cytosolic Ca2+, as well as decreased mitochondrial membrane potential ($\Delta\Psi m$) in PBMCs, but without causing toxicity to the cells. Lignin also promotes increased production of cytosolic ROS and stimulates the proliferation and activation of CD8+ T lymphocytes and inhibition of CD4+ T lymphocytes (CTLA4+). Pectin, on the other hand, stimulated the proliferation and activation of CD4+ T lymphocytes and inhibition of CD8+ T lymphocytes (CTLA4+). The two molecules promoteda greater release of cytokines involved in the Th1, IL-2, TNF-α and IFN-γ response, as well as increased production of nitric oxide. These results highlight the potential use of phenolic compounds, lignin and pectin from *C. erectus* as immunomodulating compounds in future studies.

Keywords: Combretaceae; Button Mangrove; Immunomodulator; Natural products.

LISTA DE ILUSTRAÇÕES

FUNDAMENTAÇÃO TEÓRICA

ARTIGO 1

ARTIGO 3

LISTA DE TABELAS

FUNDAMENTAÇÃO TEÓRICA

ARTIGO 1

ARTIGO 2

ARTIGO 3

SUMÁRIO

1 INTRODUÇÃO

A cultura humana tem sido profundamente influenciada pela diversidade de espécies de plantas que são utilizadas para diversos fins, inclusive terapêuticos. Essa relação foi estabelecida por um longo processo de seleção realizado por populações antigas em todo o planeta (FLOR; BARBOSA, 2015). o conhecimento sobre as propriedades medicinais das plantas tem sido o foco de diversas investigações científicas em todo o mundo, com objetivo de se desenvolver terapias alternativas eficientes para doenças que afetam a população (POMPERMAIER et al., 2018; KAMBLE; GACCHE, 2019).

As plantas produzem uma grande variedade de metabólitos primários e secundários que desempenham um papel fundamental na sua sobrevivência, bem como na interação das mesmas com o meio ambiente (ASHRAF et al., 2018; MISHRA; FATIMA; ARORA, 2018). Estes compostos desempenham funções importantes como resistência contra pragas e doenças, atração de polinizadores e interação com microrganismos simbiontes (ZAYNAB et al., 2018). Sendo assim, os compostos naturais de plantas são candidatos importantes para a descoberta de novas moléculas bioativas (POMPERMAIER et al., 2018).

As pectinas e ligninas, por exemplo, são compostos originários das vias de biossíntese do metabolismo primário e secundário, respectivamente. Ambos compõem a parede celular vegetal e desempam importantes papeis de sustentação, firmeza, resistência mecânica do tecido (SCHEER; WOSIACKI; MORENO; 2012 CRUZ FILHO, 2016).

Mundialmente, as florestas de manguezais são consideradas como um dos ecossistemas de maior produtividade biológica. Suas espécies possuem adaptações fisiológicas e morfológicas que as permitem sobreviver em condições extremas como alta salinidade, variação de marés, ventos fortes, temperaturas elevadas e terras barrentas (LEE et al., 2014; VINOTH; KUMARAVEL; RANGANATHAN, 2019). Dessa forma, podem constituir fontes de compostos bioativos com características peculiares.

Conocarpus erectus L., pertencente à família Combretaceae e conhecida popularmente como mangue de botão, é uma planta que cresce sobre linhas costeiras nas regiões tropicais e subtropicais de todo o mundo (BAILEY, 1976). Na medicina popular, é utilizada no tratamento da anemia, hemorragia, diabetes, diarreia, febre, dor de cabeça, inchaços, brotoeja, além de ser utilizada para algumas infecções como gonorreia, conjuntivite, orquite e sífilis (ABDEL-HAMEED et al., 2012; ABDEL-HAMEED; BAZAID; SABRA, 2013). Em suas folhas, caules, frutos e flores, foi identificada uma variedade de compostos fenólicos, os quais têm sido relacionados com suas propriedades terapêuticas (BANDEIRA, 2003; ABDEL-

HAMEED et al., 2012; NASCIMENTO et al., 2016). Um exemplo é a miricetina 3-O-β-glucupiranuronida, isolada do extrato hidroalcóolico de folhas frescas de *C. erectus*, que apresentou capacidade antioxidante pelo ensaio da hipoxantina/xantina oxidase (AYOUB, 2010). Estudos científicos comprovam as propriedades bioativas dessa espécie, tais como antioxidante, antimicrobiana, antifúngica e anticancerígena *in vitro* e anti-hiperglicêmica e hepatoprotetora *in vivo* (AYOUB, 2010; RAZA et al., 2016; SILVA, 2004; ABDEL-HAMEED et al., 2012; ABDEL-HAMEED; BAZAID; SABRA, 2013; SHOHAYEB et al., 2013; RAZA et al., 2018; ABDEL-HAMEED et al., 2014).

A investigação de compostos biologicamente ativos nesta planta nos permite explorar diferentes atividades biológicas de recursos naturais manipulados pela população para uma perspectiva de produção farmacêutica. Em trabalho prévio realizado com esta planta, os extratos etanólico, aquoso e em acetato de etila de folhas de *C. erectus* apresentaram promissora atividade antioxidante e imunomoduladora (SANTOS et al., 2018a, 2018b). Neste sentido, o presente trabalho visou identificar, isolar e caracterizar macromoléculas presentes nas folhas desta planta com uma possível ação antioxidante e potencial imunomodulador.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 PLANTAS MEDICINAIS

O uso de plantas para o tratamento e alívio de doenças tem sido relatado há muitos anos, tratando-se de um conjunto de conhecimentos populares passados de gerações a gerações (SHI et al., 2010). Essas plantas, nas mais diversas formulações, como chás, extratos, decocções, infusões, tintura ou pó, ajudam na prevenção e cura de doenças e distúrbios devido à presença de numerosos fitoquímicos (EZZAT et al., 2019). Registros fósseis indicam que no período do Paleolítico médio, há cerca de 60.000 anos atrás, já existia o uso humano de plantas como remédios (PARASURAMAN, 2018). Acredita-se que, em busca de alimento, os humanos primitivos tenham enfrentado condições adversas ao consumir plantas venenosas, o que resultou em valiosos conhecimentos e tecnologias tradicionais sobre materiais comestíveis e medicamentos de fontes naturais (YUAN et al., 2016). O uso eficaz e seguro dessas plantas e de seus produtos derivados representa, até os dias atuais, um grande desafio para o homem considerando a falta de comprovação científica de segurança e eficácia de muitos desses produtos (NASCIMENTO et al, 2016).

De acordo com a Organização Mundial da Saúde (OMS), cerca de 85% da população mundial faz uso de medicamentos à base de plantas para atender suas necessidades básicas de saúde. Além disso, a maior parte de populações de países em desenvolvimento depende da medicina tradicional e/ou complementar para fins profiláticos ou curativos, por serem mais acessíveis e de baixo custo. As plantas constituem 80% da matéria-prima utilizada no sistema médico tradicional e podem ser tão ou mais eficazes que algumas terapias alternativas modernas (FENALTI et al., 2016; NASCIMENTO, 2017; BODEKER; GRAZ, 2020).

Os vegetais apresentam em sua composição grande diversidade de biomoléculas importantes para a manutenção da saúde. Sendo assim, despertam grande interesse, não só por suas atividades biológicas exercidas em resposta aos estímulos do meio ambiente, mas também por sua importância comercial, tanto na área farmacêutica, quanto nas áreas alimentar, agronômica e perfumaria (SILVA; LIMA, 2016; NASCIMENTO, 2017). Papéis farmacológicos como antioxidante, antineoplasica, antimicrobiano, anti-inflamatório, cicatrizante, imunomodulador, entre outros, são descritos para formulações à base de plantas (STOHS; HARTMAN, 2015; AGUIAR et al., 2018; SANTOS et al., 2018a; BODEKER e GRAZ, 2020).

Friedrich Serturner, em 1806, foi um dos pioneiros em extrair princípios ativos de vegetais, ao isolar a morfina de papoula (*Papaver somniferum*), o que alavancou a busca por outros medicamentos derivados de plantas (DUTRA et al., 2016). Recentemente, a forskolina, isolada da planta *Coleus forskohlii* utilizada na medicina tradicional indiana para o tratamento de doenças cardíacas, foi validada empiricamente pela medicina moderna e atualmente é comercializada para o tratamento da hipertensão, glaucoma (diminui a pressão intraocular) e asma, além de auxiliar a perda de peso por acelerar o metabolismo (GURUNG, 2019). Notavelmente, produtos naturais bioativos têm sido empregados para o desenvolvimento de novos medicamentos, pois além de desempenharem um papel importante na terapia moderna, suas estruturas químicas têm sido utilizadas como modelos valiosos para a concepção de novos análogos com otimizado potencial biotecnológico (Tabela 1) (COSTA, 2015; MONTEIRO; BRANDELLI, 2017). Estima-se que cerca de 30% dos medicamentos terapêuticos empregados na medicina moderna sejam derivados de fontes naturais, principalmente plantas e microrganismos (DUTRA et al., 2016). Em Oncologia por exemplo, medicamentos derivados de plantas corresponde a 60% das terapias anticâncer desenvolvidas (MISHRA; TIWARI, 2011; ROY, JAUHARI; BHARADVAJA, 2018).

Tabela 1. Compostos naturais isolados de plantas medicinais para a produção de compostos análogos estruturais bioativos.

Compostos naturais	**Uso tradicional**	**Plantas Medicinais**	**Análogos**	**Referência**
Anabasina	Relaxante muscular esquelético.	*Anabasis aphylla* L.	Isômeros estruturais da nicotina.	Maridass e John (2008)
Artemisinina	Antimalárico.	*Artemisia annua*	Artesunato, Arteméter, Dihidroartemisinina, Ácido artelínico.	Dondorp et al. (2005)
Atropina	Anticolinérgico, usado para tratar envenenamentos.	*Atropa belladonna* L.	Homatropina, Atropina Metonitrato, Adifenina, Ciclopentolato.	Ainsworth (2014)
Citisina	Parar de fumar.	*Cytisus laburnum L.*	Vareniclina	Walker et al. (2014)
Cocaína	Anestésico local.	*Erythroxylum coca*	Othococaine, Niravanina, Amilocaína, Procaína.	Salim et al. (2008)
Codeína	Usado para tratar	*Papaver*	Nicocodeína, isocodeína e seus	Prommer

	dor, tosse e diarréia.	*somniferum*	derivados.	(2011)
Curcuminóides	Antioxidante, colerético.	*Curcuma longa*	Curcumina, Desmetoxicurcumina, Bisdemetoxicurcumina.	Jayaprakasha et al. (2006)
Digitoxina	Cardiotônico, anti-câncer.	*Digitalis purpurea L.*	Digitoxigenina.	Elbaz et al. (2012)
Efedrina	Estimulante, pressão arterial baixa.	*Ephedra sinica*	Anfetamina, Metilfenidato, Dexanfetamina.	Bagchi e Preuss (2013)
Enoxolona	Ulceração esofágica e oral e inflamação.	*Glycyrrhiza glabra*	Carbenoxolona sódica.	Connors (2012)
Esesculetina	Anti-disenteria.	*Fraxinus rhynchophylla*	Escopoletina, Isoscopoletina.	Maridass e John (2008)
Indirubin	Leucemia mielóide crônica.	Indigo naturalis	Meisoindigo.	Nirmala et al. (2011)
Khellin	Doença coronariana, asma brônquica, vitiligo e psoríase.	*Ammi visnaga*	Benzarone, Amiodarona, Benziodarona, Nedocromil.	Shinde e Laddha (2014)
Lobelina	Estimulante respiratório.	Lobelia *spp*.	Lobelanidina, Lobelanina.	Stead et al. (2012)
Morfina	Analgésico	*Papaver somniferum*	250 derivativos. Heroína, Oximorfona, Nalorfina Diprenorfina.	Courtwright (2009)
Taxol	Anticâncer	*Taxus brevifolia*	Docetaxel, Bacatina III.	Fischer e Ganellin (2006)
Protopanaxadiol	Anticâncer.	*Panax ginseng*	Protopanachatril, Panachatril.	Nirmala et al. (2011)
Quinina	Tratamento de malária e babesiose.	Cinchona *spp*.	Cloroquina, primaquina, mefloquina, bissulfato de quinina.	Achan et al. (2009)
Salvinorin A	Enteógeno.	*Salvia divinorum*	Salvinorina B, C, D, E, F.	MacLean et al. (2013)
Δ9-trans-tetra-hidrocanabinol	Agente psicoativo, estimulante do apetite e antiemético.	*Cannabis sativa* L.	113 canabinóides.	Salim et al. (2008)

2.2 FITOQUÍMICOS

As diversas reações que ocorrem nas células vegetais são responsáveis pela degradação e síntese de substâncias essenciais aos processos fisiológicos envolvidos diretamente na adaptação e sobrevivência do organismo (ASHRAF et al., 2018; MISHRA; FATIMA; ARORA, 2018). Nos processos metabólicos, o produto de uma reação serve de reagente às reações subsequentes e seus produtos intermediários podem ser redirecionados a outras vias metabólicas estabelecendo um ciclo de reações químicas (ALMEIDA, 2017). Os compostos resultantes desses processos bioquímicos vão executar distintas funções de sobrevivência e de adaptações a estresse bióticos e abióticos, sendo classificados como metabólitos primários ou secundários (ZANGUEU et al., 2018).

O metabolismo primário é responsável pela síntese de compostos presentes em ciclos de reações essenciais à sobrevivência da planta, contribuindo diretamente para o crescimento, desenvolvimento e reprodução da planta (OLIVEIRA et al., 2011; DWIVEDI et al., 2020). A ele estão associados processos que irão resultar na síntese de ácidos carboxílicos, aminoácidos, carboidratos, lipídios, proteínas e ácidos nucléicos (MOREIRA, 2015; ALMEIDA, 2017). Os metabólitos primários, por estarem presentes em todas as espécies vegetais, são considerados de distribuição universal e são produzidos em grandes quantidades (REZENDE et al., 2016; DWIVEDI et al., 2020). Estas moléculas desempenham funções estrutural, enzimática, de transporte, sinalização, energética e de defesa (NELSON; COX, 2011); atuam diretamente na fotossíntese, glicólise, no ciclo do ácido cítrico, na síntese de aminoácidos, proteínas, enzimas e coenzimas, síntese de materiais estruturais, duplicação do material genético e absorção de nutrientes (GROENIGEN, 2015). As pectinas, por exemplo, são polissacarídeos estruturais encontrados na parede celular primária das células vegetais e nas camadas intercelulares, sendo responsáveis pela adesão entre as células, firmeza e resistência mecânica do tecido (SCHEER; WOSIACKI; MORENO, 2012).

O metabolismo secundário envolve vias responsáveis por mecanismos de adaptação e sobrevivência ligados à interação da planta com o ambiente ao seu redor (ALMEIDA, 2017). Os metabólitos secundários não são essenciais à vida da planta, sendo encontrados em menores concentrações nos tecidos vegetais e apresentando particularidades em sua distribuição, sendo restritas a grupos diferenciados taxonomicamente (O‘CONNOR, 2015; SILVA; LIMA, 2016). Compostos provenientes do metabolismo primário são precursores desses metabólitos, cuja síntese ocorre pelas vias do metileritritol fosfato, do ácido

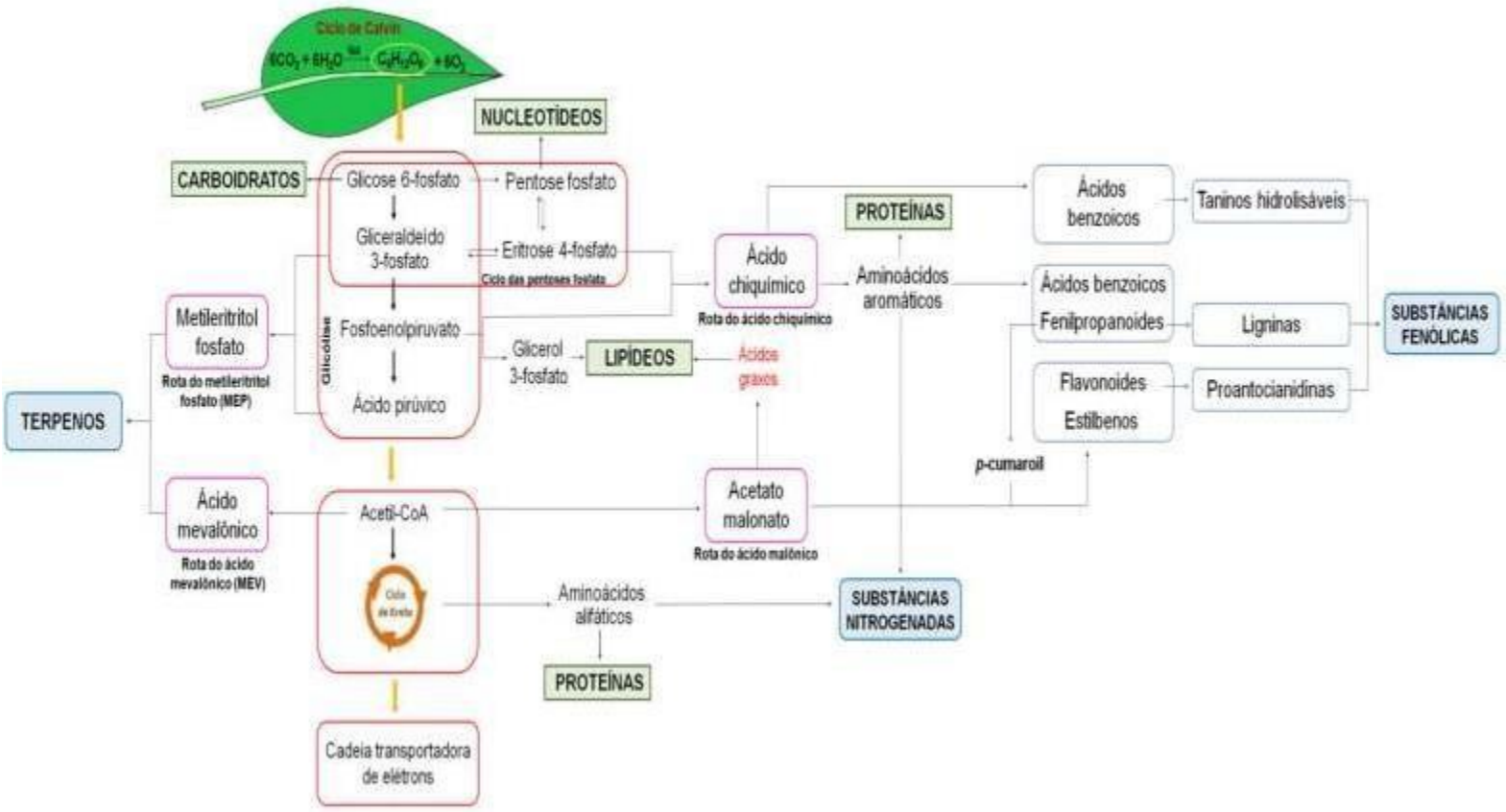
mevalônico, do ácido chiquímico e do ácido malônico (Figura 1) (CARRINGTON et al., 2018).

Os metabólitos secundários são classificados biossinteticamente, em numerosos grupos, entre eles, alcaloides (compostos que contêm pelo menos um nitrogênio básico em um anel heterocíclico), compostos fenólicos (que possuem em sua estrutura um grupo hidroxila ligado a um grupo hidrocarboneto aromático), e terpenoides (compostos que apresentam um esqueleto de hidrocarboneto formado a partir de unidades de isopreno) (TIWARI; SANGWAN; SANGWAN, 2016; AMORIM et al., 2016; EGBUNA et al., 2019).

Os produtos do metabolismo vegetal exibem diversas variações estruturais e com isso uma ampla variedade de atividades biológicas (O‘CONNOR, 2015). O isolamento de moléculas naturais levou à descoberta de medicamentos promissores, utilizados na medicina moderna até hoje. Alguns exemplos são a lovastatina, usada no controle lipídico, ciclosporina e rapamicinas, usadas como imunossupressores em transplante de órgãos, e os conhecidos paclitaxel e irinotecan, medicamentos anticâncer (Tabela 2) (EZZAT et al., 2019). Tanto metabólitos primários quanto secundários têm despertado grande interesse da comunidade científica por suas potenciais aplicações nas áreas farmacêutica, alimentar, agronômica e cosmética (TIWARI; SANGWAN; SANGWAN, 2016; DO NASCIMENTO et al., 2017).

Segundo Ezzat e colaboradores (2019), há três etapas essenciais para a descoberta de novas moléculas bioativas a partir de plantas medicinais. Inicia-se com a coleta e autenticação da planta selecionada, feita por um profissional especializado. O segundo passo consiste na preparação de diferentes extratos vegetais e sua triagem biológica através de ensaios biológicos e toxicológicos. E o terceiro passo trata-se da purificação e caracterização química de compostos ativos por meio do fracionamento guiado por bioensaio, para identificar as moléculas específicas responsáveis pela atividade biológica.

Figura 1.: Esquema geral das vias de biossíntese do metabolismo vegetal primário e secundário. Em vermelho: vias de biossíntese do metabolismo primário. Retângulos em verde: metabólitos primários. Delimitadas em rosa: vias de biossíntese do metabolismo secundário. Retângulos azuis: metabólitos secundários.



Fonte: MOREIRA (2015)

Após identificar o potencial biológico da substância, é essencial determinar sua biodisponibilidade. Antes de atingir o seu alvo biológico, essas substâncias interagem com um grande número de moléculas no lúmen gastrointestinal, enterócitos e fígado. Essas interações, por sua vez, podem resultar em atrasos ou diminuição de absorção, os quais podem afetar as funcionalidades da molécula (BRIGUGLIO et al., 2018; LIU et al., 2019). Sendo assim, é essencial determinar as diferentes fases do processo farmacocinético, tais como: absorção, distribuição, metabolismo e eliminação, desta molécula, os quais irão determinar o seu nível de biodisponibilidade (DOMÍNGUEZ-AVILA et al., 2016; BRIGUGLIO et al., 2018). Os parâmetros farmacocinéticos são variáveis de acordo com o tamanho da molécula, complexidade estrutural, carga, matriz alimentar e presença de outros compostos ou drogas (DOMÍNGUEZ-AVILA et al., 2016).

Tabela 2. Algumas classes importantes de fitoquímicos bioativos precursores ou protótipos de medicamentos.

Classe	Fitocompostos	Referência
Alcaloides	Atropina, Berberina, Cafeína, Cocaína, Efedrina, Galantamina, Morfina, Nicotina, Quinidina, Quinina, Reserpina, Vincamina, Vincristina.	EGBUNA et al., 2019.
Aminoácidos não proteicos	Azatirosina, Canavanina.	EGBUNA et al., 2019.
Glicosídeos cianogênicos	Amygdalin, Dhurrin, Linamarin, Lotaustralin, Prunasin.	EGBUNA et al., 2019.
Pectinas	Pectina cítrica, Pectina de bagaço de maçã	CHEN et al., 2021; DRANCA; VARGAS; OROIAN, 2019.
Lectinas	Concanavalin A, Fitohemaglutinina.	EGBUNA et al., 2019.
Terpenos	Taxol, Azadirachtina, Artemisinina, Tetra-Hidrocanabinol, Farnesol, Digitogenina, Caroteno, Cicloartenol (esteróide).	EGBUNA et al., 2019.
Flavonóides	Resveratrol, Luteolina, Quercetina.	EGBUNA et al., 2019; REFAT et al., 2021.
Taninos	Ácido tânico, *Punicalin*, *Tellimagrandin*.	EGBUNA et al., 2019; FRAGA-CORRAL et al., 2021.
Lignina	Podophyllotoxin.	EGBUNA et al., 2019.

A biodisponibilidade dos polifenóis é altamente variável. Aproximadamente 5 a 10% dos polifenóis são absorvidos no intestino delgado e os polifenóis restantes são metabolizados

por bactérias residentes no intestino grosso e excretados nas fezes (PATURI et al., 2021). Ao atingirem o trato gastrointestinal, os polifenóis são identificados como xenobióticos pelo corpo, o qual desenvolve mecanismos para excretá-los rapidamente, o que leva a níveis muito baixos de absorção intestinal (<2% para muitas classes de polifenóis). A maioria dos polifenóis ingeridos por via oral atinge o intestino delgado com sua estrutura intacta, onde são catabolizados pela microbiota intestinal. Os metabólitos microbianos então são absorvidos em maior extensão (até 10 vezes maior) do que suas formas originais e podem permanecer em circulação sistêmica por um período mais longo (CLADIS; WEAVER; FERRUZZI, 2020). Alguns flavonoides, por exemplo são diretamente metabolizados no intestino delgado e no fígado, enquanto outros, para serem absorvidos, sofrem fissão do anel mediada pela microbiota, produzindo ácidos fenólicos que são absorvidos, entrando na circulação sanguínea e em órgãos periféricos, contribuindo para as funções fisiológicas, e finalmente excretados na urina (LUCA et al., 2019).

Os polissacarídeos, por sua vez, apresentam boa estabilidade, segurança, baixa toxicidade, hidrofilicidade, capacidade de gelificação e biodegradabilidade, que fundamenta sua aplicabilidade para entrega de sistemas alvo específico (TIWARI et al., 2019). A maioria dos polifenóis, por exemplo, exibe biodisponibilidade limitada, no entanto, estudos demonstram que ao adicionar pectina à dieta, esta, pode melhorar a absorção de polifenóis, atrasando o esvaziamento gástrico, estendendo a absorção no intestino delgado e consequentemente aumentando a biodisponibilidade dos polifenóis (PATURI et al., 2021).

2.3 LIGNINAS

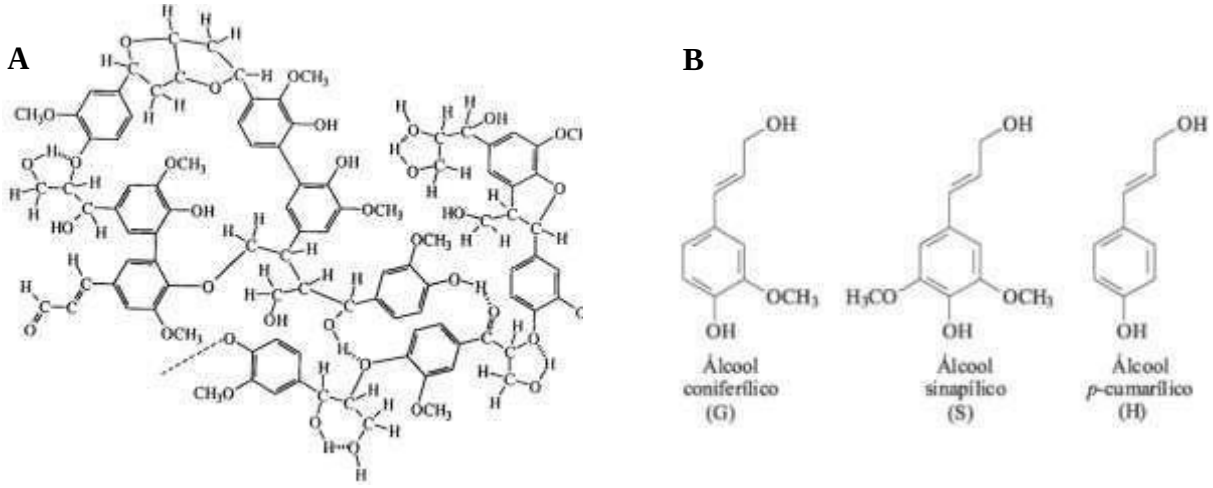
A palavra lignina é derivada do latim *lignum*, cujo significado é madeira. Essas moléculas foram descobertas em 1838, quando Anselme Payen submeteu a madeira a tratamento químico com ácido sulfúrico concentrado (SHIMIZU, 2018). A lignina é considerada a terceira macromolécula orgânica mais abundante na natureza, sendo encontrada em todos os vegetais, ficando atrás apenas da celulose e hemicelulose (SOUTO; CALADO; PEREIRA Jr., 2015).

A lignina (Figura 2A) é uma macromolécula de natureza polifenólica tridimensional, derivada da via de biossíntese do ácido chiquímico. É um dos principais componentes das paredes celulares de plantas superiores (LAURICHESSE; AVÉROUS, 2014). Liga-se à hemicelulose e envolve de forma parcial, polissacarídeos e microfibrilas de celulose, desempenhando papel de suporte mecânico e elástico à estrutura da biomassa (CRUZ FILHO,

2016). Ainda, auxilia no transporte de água e nutrientes na planta, atua como barreira física, protegendo a madeira da degradação enzimática e dificulta a entrada de patógenos microbianos (CAI et al., 2017; VANHOLME et al., 2019).

A lignina é formada por combinações de três unidades de fenilpropanóides: guaiacil (G), siringil (S) e *p*-hidroxifenil (H) e estes são derivados dos álcoois, coniferílico, sinapílico e *p*-cumarílico, respectivamente (Figura 2B**)**. O volume molar dessas unidades de fenilpropanóides, G, S e H, varia de acordo com o tipo do vegetal (ARAÚJO et al., 2016) e sua biossíntese pode ser influenciada por várias condições de estresse biótico e abiótico (VANHOLME et al., 2010). Suas subunidades estão ligadas por ligações do tipo éter (C-O-C) e carbono-carbono (C-C), distribuídas de forma aleatória (GAMBARATO, 2014).

Figura 2. Estrutura química da lignina **(A)** e dos álcoois precursores das unidades fenilpropanóides guaiacila (G), siringila (S) e p-hidroxifenila (H) **(B)**.



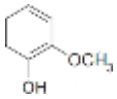
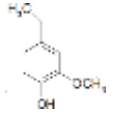
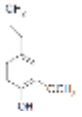
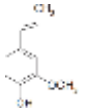
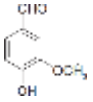
Fonte: Figueiroa (2017) e Lopes (2018), respectivamente.

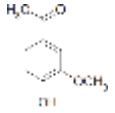
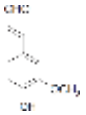
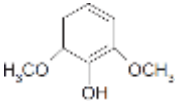
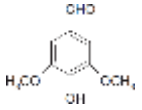
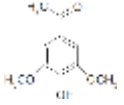
Por ser um produto secundário disponível em grandes quantidades além de ser um produto de extração relativamente fácil, de baixo custo e alto rendimento, a utilização de uma lignina com elevada bioatividade resultaria em produto farmacológico promissor (KUN; PUKÁNSZKY, 2017). Estudos têm sido realizados com o objetivo de aprimorar o manuseio desse polímero natural para o desenvolvimento de novas tecnologias voltadas para aplicações industriais (SEN; PATIL; ARGYROPOULOS, 2015; LOPES et al., 2018). A relação estrutura-atividade que existe entre os grupos farmacofóricos das ligninas (Tabela 3) é crucial para identificar seu potencial biotecnológico e determinar as direções mais promissoras para

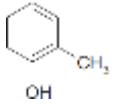
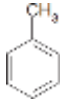
sua atualização, a fim de produzir compostos bioativos mais eficazes direcionados à vários sistemas de aplicação (PONOMARENKO et al., 2015).

Ligninas isoladas de diferentes espécies vegetais têm apresentado atividades farmacológicas promissoras, como antioxidante (NIU et al., 2016; TREVISAN; REZENDE, 2020), imunomoduladora (CAI et al., 2017; MORGANTI et al., 2017), antitumoral (SOLOVYEV et al., 2017), antiviral (MATSUHISA et al., 2015; OEYEN et al., 2019), antibacteriana (ALZAGAMEEM et al., 2019; DOMÍNGUEZ-ROBLES et al., 2020) e cicatrizante (KAI et al., 2015). Neste sentido, ligninas isoladas de plantas utilizadas tradicionalmente para o tratamento de doenças podem ser fortes candidatas a medicamentos fitoterápicos com finalidade profilática, curativa ou paliativa.

Tabela 3. Grupos farmacofóricos de ligninas do tipo GSH (Adaptado de PONOMARENKO et al., 2015).

Grupo Farmacofórico	Estrutura Química	Atividade Biológica
G-fenóis		
Guaiacol (2-metoxifenol)		Antioxidante (AZADFAR et al., 2015), inibidor da COX-2 e NF-κB (MURAKAMI et al., 2007).
4-etilguaiacol (4-etil-2-metoxi-fenol)		Antioxidante (BORTOLOMEAZZI et al., 2007).
Eugenol (4-alil-2-metoxifenol)		Anti-inflamatório (DEWHIRST, 1980), inibidor da COX-2 e NF-κB (MURAKAMI et al., 2007) e agregação plaquetária (DEWHIRST, 1980), fotoprotetor (LEE; JUNG, 2017).
Iso-eugenol (2-metoxi-4- (prop-1-en-1-il) fenol)		Antioxidante (BORTOLOMEAZZI et al., 2010; CHEN et al., 2021), anti-inflamatória, antiartrítica, analgésica (KAUR; SULTANA, 2012), fotoprotetor (LEE; JUNG, 2017), antimicrobiano (DAMBOLENA et al., 2012).
Vanilina (4-hidroxi-3-metoxibenzaldeído)		Inibidor da COX-2 e NF-κB (MURAKAMI et al., 2007), anti-inflamatório (WU et al., 2009) antimetastático (LIRDPRAPAMONGKOL et al., 2005), antiangiogênico (LIRDPRAPAMONGKOL et al., 2009), antitumoral (LIRDPRAPAMONGKOL et al., 2010), antioxidante (CHEN et al., 2021).

Acetovanilona (1- (4-hidroxi-3-metoxifenil) etanona)		Inibidor de p38-MAPK e caspase-3 (LIU et al., 2019), inibidor da NADPH oxidase (IMPELLIZZERI et al., 2010; FRANCIS et al., 2016).
Aldeído coniferílico (3-(4-hidroxi-3-metoxifenil) prop-2-enal)		Fotoprotetor (LEE; JUNG, 2017), antioxidante (KARAMAC et al., 2017), anti-inflamatório (AKRAM et al., 2016).
S-fenóis		
Siringol (1,3-dimetoxi-2-hidroxibenzeno)		Antioxidante (BORTOLOMEAZZI et al., 2007).
Seringaldeído (4-hidroxi-3,5-dimetoxibenzaldeído)		Antioxidante (BORTOLOMEAZZI et al., 2007; BOUNTAGKIDOU et al., 2010), antimicrobiano (IBRAHIM et al., 2012), antitumoral (IBRAHIM et al., 2012).
Acetoseringona (4-hidroxi-3, 5-dimetoxiacetofenona)		Antioxidante (BORTOLOMEAZZI et al., 2007).
H-fenóis		

Fenol		Antioxidante (KILANI-JAZIRI et al., 2016; BHUTTO et al., 2018; CHEN et al., 2021), anti-inflamatória (DEWHIRST, 1980), inibidor da ciclooxigenase (DEWHIRST, 1980), anti-angiogênese (SUN; HEILMANN; KÖNIG, 2015), imunomodulador (KILANI-JAZIRI et al., 2016).
p-cresol (4-metilfenol)		Inibidor da ciclooxigenase (DEWHIRST, 1980)
o-cresol (2-metilfenol)		Inibidor da ciclooxigenase (DEWHIRST, 1980)
Toluol		Antimicrobiano (GALLUCCI et al., 2014; DAMBOLENA et al., 2012; WALSH et al., 2019), atividade antifumonisina (DAMBOLENA; ZYGADLO; RUBINSTEIN, 2011).

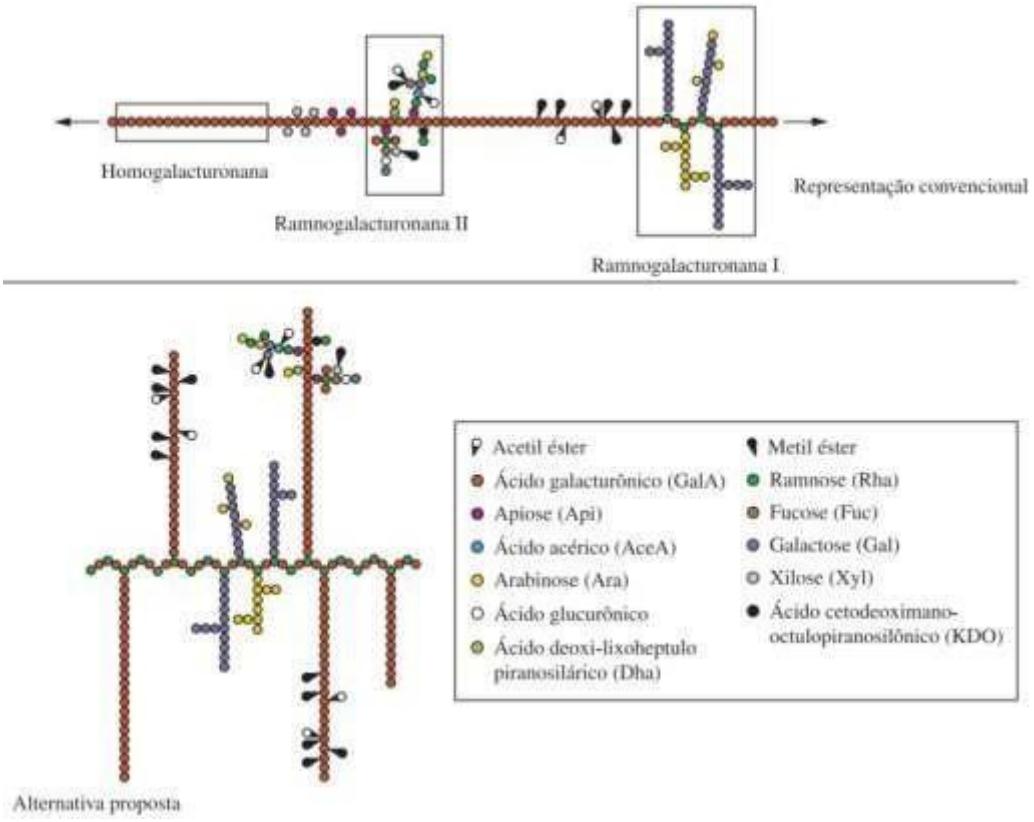
COX-2: Ciclo-oxigenase-2; **NF-κB:** fator nuclear kappa B, do inglês, *nuclear factor kappa B*; **MAPK:** proteína quinase ativada por mitógeno do inglês, *mitogen-activated protein kinase*; **NADPH:** nicotinamida adenina dinucleótido fosfato.

2.4 PECTINAS

As pectinas são polissacarídeos encontrados principalmente na parede celular primária das células vegetais, atuando como agente cimentante, contribuindo para firmeza, resistência mecânica e coesividade do tecido (PAIVA; LIMA; PAIXÃO, 2009). Tratam-se de hidrocoloides naturais que formam um grupo heteromolecular de polissacarídeos. O termo pectina é normalmente usado de forma genérica para designar preparações de galacturoglicanas hidrossolúveis. A estrutura consiste em unidades de ácido galacturônico (Figura 3) unidos por ligações glicosídicas α (1→4) com grau variável de grupos carboxilas metil esterificados (CANTERI; WOSIACKI; SCHEER, 2012; CHALIHA, 2018). A homogalacturonana (HG), homopolímero linear de ácido galacturônico ligado a α (1→4), é o polissacarídeo péctico mais abundante e compreende 65% da pectina. Os outros polissacarídeos pécticos são consideravelmente mais complexos e incluem as Hás substituídas, como a ramnogalacturonana I (RG-I), que representa 20-35% da pectina e é o polissacarídeo péctico estruturalmente mais variável, com alto grau de especialização celular dependente do tipo e número de açúcares simples e oligossacarídeos ligados a sua cadeia, geralmente responsável pela funcionalidade da molécula; e a ramnogalacturonana II (RG-II); estrutura mais complexa, quase invariante entre as espécies, que corresponde a aproximadamente 10% da pectina, consiste de um esqueleto de homogalacturonana de no mínimo oito unidades monoméricas, contendo cadeias laterais de até 12 diferentes tipos de açúcares (Figura 3) (MOHNEN, 2008; CANTERI; WOSIACKI; SCHEER, 2012).

A síntese de pectina ocorre no lúmen do complexo de Golgi pela ação de glicosiltransferases (GTs), que transferem resíduos de glicosil de nucleotídeos de açúcar para oligossacarídeos ou receptores de polissacarídeos; após sintetizados, os polímeros são transportados para a parede do Golgi em vesículas de membrana através do movimento ao longo dos filamentos de actina que possuem motores de miosina (ABDEL-MASSIH; BAYDOUN; BRETT, 2003; IBAR; ORELLANA, 2007; MOHNEN, 2008). A pectina é constituída de, no mínimo, 65% de ácido galacturônico, com graus variáveis de éster metílico e de neutralização (VORAGEN et al., 2009). Alguns dos grupos carboxila da pectina podem se apresentar metilados, na forma livre ou forma de sais de sódio, potássio ou amônio, sendo mais frequentemente encontrado na forma de sódio (LEFSIH et al., 2018).

Figura 3. Representação esquemática convencional da pectina mostrando os três polissacarídeos pécticos homogalacturonano (HG), ramnogalacturonano I (RG-I) e ramnogalacturonano II (RG-II) ligados um ao outro e estrutura hipotética de uma pectina com modificações de acordo com novas evidências científicas.



Fonte: CANTERI; WOSIACKI; SCHEER, 2012

O uso de pectinas tem se consolidado na indústria alimentícia. Pectinas comerciais são obtidas da casca de frutas cítricas e da maçã para uso como gelificantes, espessantes ou estabilizadores de alimentos (compotas, conservas, geleias, queijos, sorvetes e molhos) (DO NASCIMENTO et al., 2017). Autoridades que regulamentam os aditivos alimentícios, como o *Codex Committee on Food Additives* (CCFA) reconhecem a pectina como um valioso aditivo, seguro para a saúde (CCFA, 1979). No Brasil, a Secretaria de Vigilância Sanitária do Ministério da Saúde, também reconhece a utilização desse polissacarídeo como coadjuvante de tecnologias voltados para fabricação de produtos (BRASIL, 2018).

No entanto, o potencial das pectinas vai além da sua aplicação na indústria alimentícia, sendo também utilizadas na indústria farmacêutica, para modificar a viscosidade de seus produtos, na indústria de plásticos e na produção de vinhos, como aglutinantes no processo de

clarificação do vinho, que é uma das etapas de afinamento (acabamento) do vinho (ALVAREZ-PÉREZ et al., 2015). A baixa toxicidade, capacidade de gelificação e presença de grupos funcionais em sua estrutura, que são facilmente modificáveis (-COOH, -OH), permite sua aplicação como excipiente em sistemas de administração de medicamentos (ZHANG; XU; ZHANG, 2015). A pectina também possui benefícios como fibra dietética natural, como nutracêutico, é um potencial prebiótico e demonstrou ter efeitos favoráveis na saúde humana (WICKER et al., 2014; AMORIM et al., 2016). A pectina, como fontes de fibras alimentares, também está associada à saúde gastrointestinal, tolerância à glicose, digestão lipídica e consequente controle de peso (CIRIMINNA et al., 2016; LEFSIH et al., 2018).

Alguns estudos mostraram que as pectinas podem ser aplicadas, como agentes imunomoduladores (CAFFALL; MOHNEN et al., 2009; MERHEB et al., 2019), antioxidantes (ENCALADA et al., 2019) e anti-inflamatórios (RAMACHANDRAN et al., 2017). Além disso, há relatos da possibilidade de aplicação da pectina na terapia do câncer através da inibição do crescimento e metástase do tumor, sensibilização das células tumorais à quimioterapia e regulação de células imunológicas (ZHANG; XU; ZHANG, 2015; NAQASH et al., 2017).

Os grupos farmacofóricos da pectina encontram-se no domínio ramnogalacturana I (RG-I) e são formados geralmente durante a eliminação de β e a hidrólise ácida (MORRIS et al., 2011). Em pacientes com câncer, por exemplo, a pectina liga-se por meio desses farmacóforos à galectina 3 (Gal-3) (molécula ocasionalmente supressora da função imune que apresenta superexpressão em alguns cânceres metastáticos) através de um domínio de reconhecimento de carboidratos. A ligação ao Gal-3 pró-metastático bloqueia sua interação com outros peptídeos, interrompendo as vias que envolvem a adesão celular, migração e apoptose, além de inibir a apoptose direta das células T mediada pela Gal-3 livre (ZHANG; XU; ZHANG, 2015; NAQASH et al., 2017).

A pectina é uma molécula de extração relativamente fácil e econômica. Estudos tem demonstrado que a fonte da qual a molécula é extraída, o método de isolamento, o conteúdo de ácido galacturônico, o peso molecular, a viscosidade, influenciam diretamente as características estruturais da pectina e consequentemente suas propriedades biológicas, neste sentido a pectina pode ser moldada para aplicações específicas com a sua melhor eficácia (ZHANG et al., 2012; NAQASH et al., 2017; ZHU et al., 2019) (Tabela 4).

Tabela 4. Características químicas e propriedade biofuncionais de pectinas extraídas de diferentes plantas.

Pectina	Teor de Ac.Gal. (%)	Peso Molecular (kDa)	Composição de Monossacarídeos	Principais ligações estruturais	Atividade	Mecanismos envolvidos	Referências
Casca de *Ficus carica*	75,79	6910	Glc, Fuc, Ara, Gal, Ram, Man	C=O, -OH, -CH, -CH_2, -CH_3	Antioxidante	Ação sequestradora dos radicais DPPH e $ABTS^+$.	GHARIBZAHEDI et al., 2019
Folhas de *Fragaria vesca*	11,5	-	Gal, Ara, Glc, Ram, Xil, Man, Fuc	-OH, -CH, C=O, -COO-, C=C, C-OC, C-C	Anticoagulante	Inibidor da antitrombina em relação ao fator Xa em plasma humano.	PAWLACZYK-GRAJA et al., 2019
Casca de jaca	-	51,5	Glc, Ara, Gal, Ram	-CH, -OH, C-OC, C=O	Antioxidante	Ação sequestradora dos radicais DPPH e $ABTS^+$.	XU et al., 2018
Cucurbita maxima	75,1	26	Ara, Ram, Fuc, Glc, Man, Gal	OH, -CH, C=O, -COO	Citoprotetora e Antioxidante	Ação citoprotetora e antioxidante das linhagens celulares HT-29 e MDCK1 contra o estresse oxidativo e a citotoxicidade induzida por íons de metais pesados.	TORKOVA et al., 2018
Opuntia ficus-indica	31,2	7890	Ara, Xil, Gal, Ram	-CH, C-O-H, C-OC, C-O, C=O, C-OS e S=O	Anticoagulante	Prolonga o tempo parcial de tromboplastina ativado (TTPA) e tempo de trombina (TT) em sangue humano.	CHAOUCH et al., 2018
Folhas de *Suaeda fruticosa*	47,5	229	Ara, Glc, Ram, Gal, Man, Xil	- OH, C=O, C-OC, -COO	Antioxidante, anti-inflamatória e antinociceptiva	Ação sequestradora dos radicais DPPH e $ABTS^+$, inibição da peroxidação lipídica e poder de redução férrica. Efeito anti-inflamatório em edema de pata de ratos wistar. Ação antinociceptiva em contorção induzida por ácido acético e teste de placa quente em camundongos albinos suíços.	MZOUGHI et al., 2018

Prunus avium	77,18	139	Glc, Fuc, Ara, Gal, Ram, Man	-CH, C-O, C=O	Imunoestimulante	Estimulou células RAW264.7 ativadas com LPS a liberar ON, TNFα, IL-6 e IL-10 e expressar os genes de TNFα, IL-6, IL-10, GCSF e COX-2.	CAO et al., 2018
Pólen de abelha de *Nelumbo nucifera*	12,1	380	Ram, Gal, Ara, Glc,	-	Imunomoduladora	Estimula a fagocitose de macrófagos e inibe a	LI et al., 2018
Raiz de *Ulmus pumila*	33,74	2.697	Gal, Ram, Glc	C=O, C-H, -CH, -CH_2, -CH_3	Anti-inflamatória	Efeito inibitório na produção de ON, expressão de iNOS e fosforilação de p38, induzida por LPS nas células RAW 264.7.	LEE et al., 2018
Pimenta doce	67	367	Ara, Gal, Ram, Glc, Xyl	-CH_3, -COO-	Imunomoduladora	Modulou a secreção de TNF-α, IL-1β e IL-10 por macrófagos THP-1 estimulados com LPS.	DO NASCIMENTO et al., 2017
Fruta de *Ficus pumila*	99,83	34,69	Gal	-CH, C-O-H, C-O, C=O, CH_2, -CH_3	Hipoglicêmica	Diminuiu a hiperglicemia e melhorou o metabolismo hepático do glicogênio em camundongos C57BL/KsJ db /db, através da via de sinalização da insulina IRS-1/ PI3K/Akt/GSK3β/GS, da via AMPK/GSK3β/GS e da regulação da expressão de glucocinase, fosfoenolpiruvato carboxiquinase e glicose-6-fosfatase.	WU et al., 2017
Folhas de caqui	27	-	Gal, Ara, Ram	-	Antitumoral	Ação tumoricida mediada por células NK e inibição de metástase do tumor em camundongos BALB/c e aumento da produção de IL-6 e IL-12 em macrófagos peritoneais murinos.	PARK et al., 2017
Casca	56	251.900	Glc, Fuc, Ara, Gal,	-	Pro-inflamatória	Promoveu proliferação de macrófagos peritoneais	AMORIM et al.,

Theobroma cacao		($g \times mol^{-1}$)	Ram, Man			ativados e desencadeou a secreção de mediadores pro-inflamatórios (NO, TNF- e IL-12) e IL-10 por essas células.	2016
Flores de *Lonicera japonica*	7.88	54	Gal, Ara, Ram	-	Antitumoral	Inibiu o crescimento de células cancerígenas pancreáticas BxPC-3 e PANC-1	LIN et al., 2016

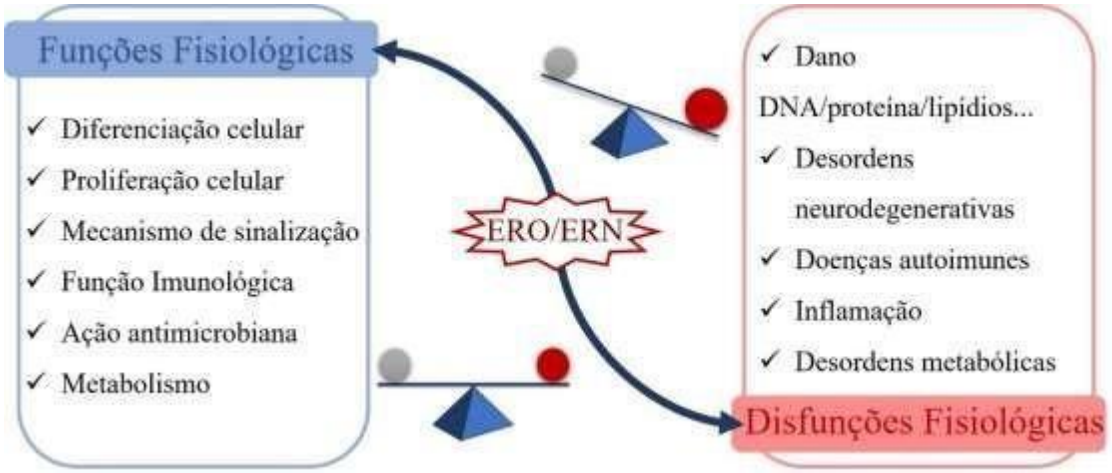
Ac.Gal.: Ácido Galacturônico. **Ara**: Arabnose. **Fuc:** Fucose. **Gal**: Galactose. **Glc**: Glicose. **Man**: Manose. **Ram**: Rmnose. **Xil**: Xilose. **DPPH**: 2,2-difenil-1-picril-hidrazil. **ABTS^{+}**: 2,2′-azinobis-(3-etilbenzotiazolina-6-sulfonato). **HT-29**: linhagem celular de câncer de cólon humano. **MDCK1**: células renais caninas Madin-Darby. **RAW264.7**: Linhagem celular de macrófagos. **LPS**: Lipopolissacarídeo. **ON**: Óxido nítrico. **iNOS**: Óxido nítrico-sintase induzida. **THP-1**: Linhagem celular monocítica humana. **NK**: *natural killer*. **BxPC-3**: Linhagem celular de câncer pancreático humano. **PANC-1**: Linhagem celular de câncer pancreático humano.

2.5 ATIVIDADE ANTIOXIDANTE

Os radicais livres podem ser formados durante processos fisiológicos naturais, processos patológicos, assim como podem ser derivados de fontes externas (PHANIENDRA; JESTADI; PERIYASAMY, 2015). O radical livre é um átomo ou molécula que possui um ou mais elétrons não emparelhados na última camada de valência. O número ímpar de elétrons de um radical livre o torna mais instável e altamente reativo. Nas células, a formação destes radicais ocorre principalmente por fissão de ligações química homolítica, fotólise, radiólise, e reações redox (CHANDRA; SHARMA; ARORA, 2019; MUSTAFA et al., 2020).

Os radicais livres podem atuar como moléculas sinalizadoras em importantes processos fisiológicos, mas também são consideradas subprodutos tóxicos do metabolismo aeróbico, podendo causar danos oxidativos em lipídios, proteínas, DNA e RNA (figura 4) (SURESH et al., 2013; WANG et al., 2015; POPRAC et al., 2017). Diversos tipos de radicais livres podem ser formados, os mais comuns, geralmente envolvidos na fisiologia humana, são as Espécies Reativas de Oxigênio (EROs) e as Espécies Reativas de Nitrogênio (ERNs) (CHANDRA; SHARMA; ARORA, 2019; HOU et al., 2020).

Figura 4. Funções e disfunções fisiológicas promovidas por radicais livres.



Fonte: Adaptado de Hou et al. (2020).

Em um organismo vivo, durante o metabolismo, uma molécula de oxigênio (O_2) sofre uma redução de quatro elétrons, podendo dar origem a espécies reativas de oxigênio (EROs),

espécies químicas que incluem pelo menos um átomo de oxigênio em sua molécula, são formas parcialmente reduzidas ou excitadas do oxigênio, que exibem uma reatividade mais forte que o oxigênio molecular (RECZEK; CHANDEL, 2015; MITTLER et al., 2017). Entre as EROs mais importantes, estão os radicais, hidroxil ($OH^{\bullet}$), ânion superóxido ($O_2^{\bullet -}$) e o peroxil ($ROO^{\bullet}$), além das espécies não radicais, como o peróxido de hidrogênio (H_2O_2), o oxigênio singlete ($^1O_2$) e o ácido hipocloroso (HOCl) (KARDEH; ESFAHANI; ALIZADEH, 2014). As ERNs compreendem principalmente o óxido nítrico (ON) e moléculas originadas do ON que apresentam átomos de nitrogênio oxidativo em sua composição, como o peroxinitrito ($ONOO^-$) e nitroxil (HNO) (HOU et al., 2020).

Os processos de oxidação são correlacionados com o gerenciamento de energia do organismo, consequentemente, são mantidos sob controle rigoroso por vários mecanismos celulares (HOSSAIN; SHAH, 2015). Existe um controle da geração de EROs desempenhado por agentes não enzimáticos (α-cetoglutarato, oxalacetato) e por enzimas como superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx) voltados para a manutenção da homeostase redox (ARAÚJO, 2018).

O desequilíbrio entre os agentes oxidantes e antioxidantes, seja ele por produção excessiva do primeiro ou síntese ineficiente do segundo é chamado de estresse oxidativo (MADAMANCHI; RUNGE, 2013; BELIKOV; SCHRAVEN; SIMEONI, 2015). O acúmulo de EROs, por exemplo, pode levar à uma ativação prolongada de componentes da cascata de sinalização de estresse, como as c-Jun N-terminal quinases (JNK), provocando inflamação, apoptose e necrose de células hepáticas e aumento da proliferação de adipócitos (GRIFFIN et al., 2020). Neste sentido, como mostra a Figura 4, os radicais livres estão envolvidos em diversas disfunções celulares, induzindo e estando presente em vários processos patológicos, bem como a manutenção dessas doenças, como em casos de diabetes, doenças neurodegenerativas, doenças cardiovasculares, catarata, asma, artrite reumatoide, inflamação, queimaduras, doenças gastrointestinais, câncer e patologias isquêmicas (LEE et al., 2012; DZOYEM; ELOFF, 2014; POPRAC et al., 2017).

Considerando as poucas terapias curativas voltadas à algumas dessas doenças, o interesse em avaliar os benefícios terapêuticos dos agentes antioxidantes tem aumentado, baseado na possibilidade dessas substâncias de prevenir e retardar a evolução dessas doenças por meio dos seus múltiplos efeitos, como anti-apoptóticos, anti-inflamatórios e estabilizadores de membrana (GHEORGHE et al., 2019).

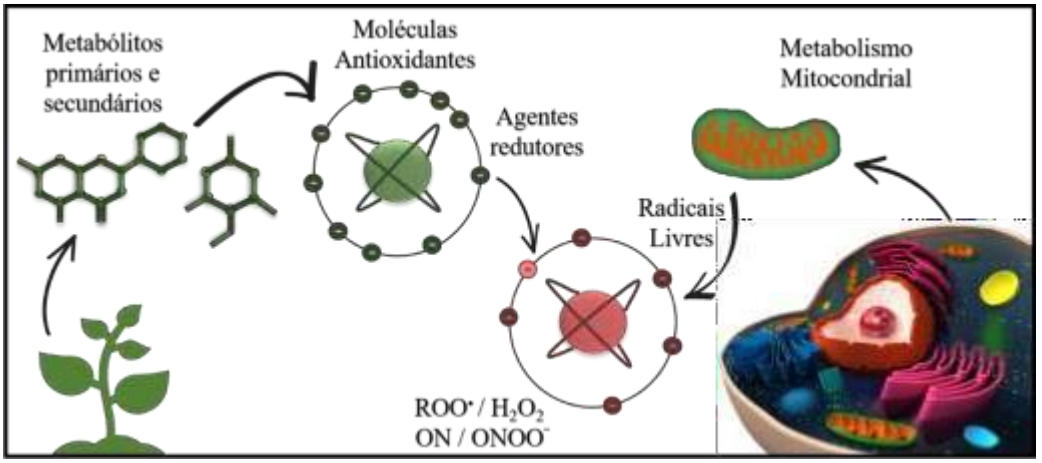
Embora o organismo humano seja munido de sistema de controle de dano oxidativo, quando há ocorrência de alta produção de EROs, existe a necessidade da ação de agentes

antioxidantes exógenos (FARHAT et al., 2013; ARAÚJO, 2018). Estudos têm sugerido a suplementação com antioxidantes (vitamina C, vitamina E, α-tocoferol, β-caroteno e vários compostos fenólicos) como adjuvantes no tratamento de doenças inflamatórias, neurodegenerativas e câncer (PHANIENDRA; JESTADI; PERIYASAMY, 2015; POPRAC et al., 2017; CHANDRA; SHARMA; ARORA, 2019).

Atualmente, antioxidantes sintéticos, como o hidroxitolueno butilado (BHT), hidroxianisol butilado (BHA), o galato de propila (PG) e a terc butil hidroquinona (TBHQ), são utilizados como aditivos alimentares para prevenção da peroxidação lipídica. No entanto, alguns componentes tóxicos e cancerígenos são formados durante a degradação desses compostos, limitando suas aplicações, principalmente para alimentos. Neste sentido, a procura por antioxidantes naturais mais seguros, que apresentem papel potencial na saúde e nutrição humana, tem sido alvo de diversas pesquisas científicas (LI et al. 2018; ARAÚJO, 2018; POPRAC et al., 2017; AMARAL et al., 2019).

Uma variedade de plantas (plantas medicinais, legumes, frutas) estão documentadas como fontes ricas de antioxidantes naturais potencialmente mais seguros. Compostos polifenólicos, vitaminas, proteínas, óleos essenciais, são relatados acerca de sua capacidade de capturar e eliminar radicais livres (Figura 5) (IQBAL; SALIM; LIM, 2015; AMARAL et al., 2019).

Figura 5. Ação antioxidante de moléculas provenientes de plantas.



Os compostos antioxidantes de natureza fenólica são bem conhecidos por serem doadores eficazes de hidrogênio, apresentando elevada capacidade de impedir a peroxidação lipídica e inibir a decomposição dos hidroperóxidos em radicais livres. Seu potencial antioxidante está relacionado diretamente ao número de grupos ativos, OH ou NH_2. O grupo

O-H em particular é ainda mais ativo, devido às energias de dissociação das ligações, que são mais baixas. Além disso, a posição desses grupos funcionais também desempenha um papel importante na atividade; a posição orto, por exemplo, é considerada mais ativa, por sua capacidade de formar ligações de hidrogênio intramoleculares (IQBAL; SALIM; LIM, 2015; BABA; MALIK, 2015; WOJTUNIK-KULESZA et al., 2020).

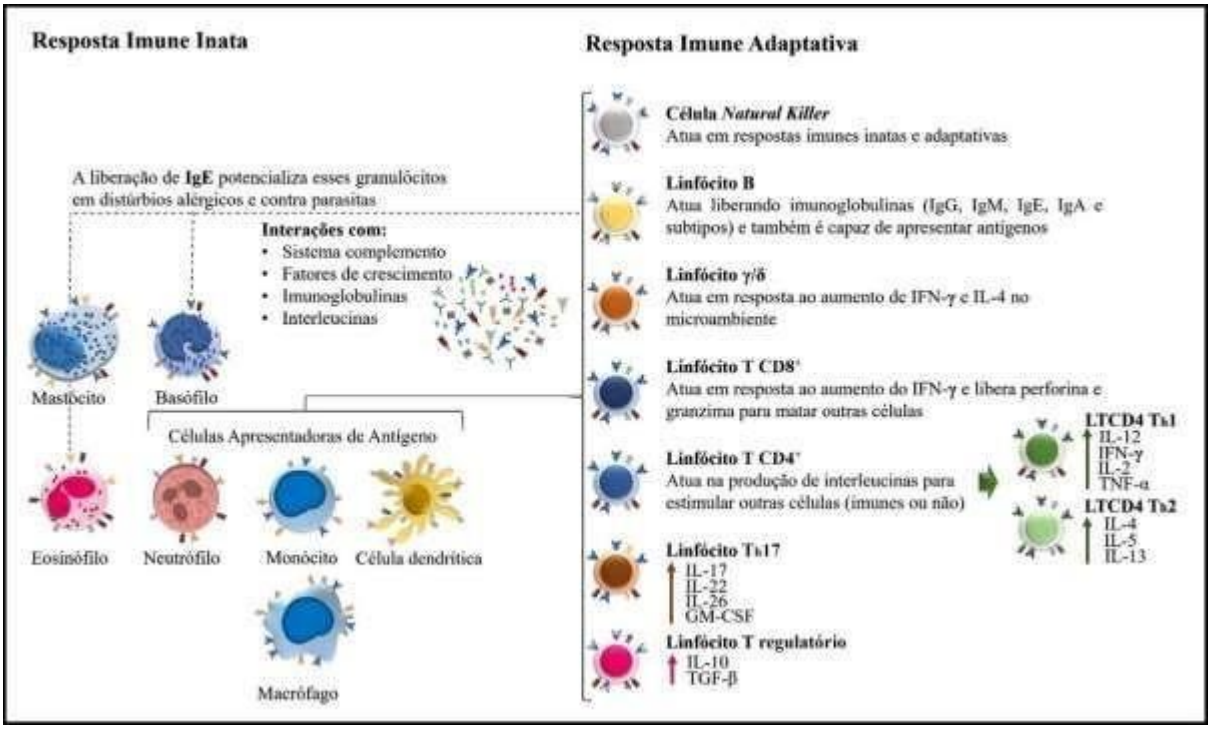
2.6 RESPOSTA IMUNOLÓGICA

A resposta imunológica é um mecanismo de defesa equilibrado voltado à proteção de um organismo contra ameaças ambientais e patógenos. Esses mecanismos são ativados por estímulos específicos, representados por estruturas moleculares estranhas ao organismo (CATANZARO et al., 2018).

Classicamente, a função imunológica é dividida em imunidade inata e adaptativa (Figura 6). A imunidade inata, própria do indivíduo desde o seu nascimento, desencadeia uma resposta rápida e inespecífica direcionada a imunógenos. É representada por barreiras físicas (pele e muco), químicas (pH e lisozima) e biológicas (microbiota), células especializadas (macrófagos, neutrófilos, células dendríticas e natural killers) e moléculas solúveis (citocinas e proteínas do sistema complemento), estando presentes no organismo independentemente de contato prévio com agentes agressores (MURPHY; WEAVER, 2016; CATANZARO et al., 2018).

Por outro lado, a imunidade adaptativa exibe especificidade e memória, e sua resposta é proporcional à exposição repetida ao estímulo. A capacidade de melhorar a resposta mediante reexposição a antígenos, gerando uma memória imunológica, confere ao organismo a capacidade de combater mais eficientemente infecções mais agressivas (PÉREZ-VÁZQUEZ; CONTRERAS-CASTILLO; LICONA-LIMÓN, 2018). Células especializadas no reconhecimento de alta especificidade no sistema adaptativo são representados pelos linfócitos T auxiliares ($CD4^+$), T citotóxicos ($CD8^+$), linfócitos B e células *Natural Killer* (NK). Essas células produzem proteínas moduladoras (imunoglobulinas e citocinas) que irão sinalizar a resposta imunológica (CRUVINEL et al., 2010; TERRA et al., 2012). Estudos demostram que são necessários pelo menos quatro tipos diferentes de leucócitos interagindo entre si, voltado ao mesmo alvo antigênico para gerar uma resposta imunológica adaptativa produtiva (CRUVINEL et al., 2010; EISENBARTH, 2018).

Figura 6. Figura esquemática da resposta imunológica e da conexão entre a imunidade inata e adaptativa.



Fonte: Adaptado de Patriota et al. (2019).

O combate inicial a antígenos envolve componentes do sistema imune inato. Após a invasão por patógenos, células residentes no tecido invadido (neutrófilos, macrófagos e células dendriticas) percebem o estímulo e iniciam a liberação de mediadores solúveis (quimiocinas, citocinas, radicais livres, aminas vasoativas e eicosanóides). As células que possuem Receptores de Reconhecimento Padrão (RRPs), como por exemplo, receptores *Toll-Like* (TLR), que reconhecem em seus alvos, Padrões Moleculares Associados a Patógenos (PAMPs), como lipopolissacarídeos, resíduos de manose e ácidos teicoicos, presentes na superfície do microrganismo (MACHADO et al., 2004; MANGINO et al., 2017). Na tentativa de eliminar o microrganismo, essas células secretam enzimas hidrolíticas, óxido nítrico (ON) e prostaglandinas, que darão início ao processo inflamatório (PÉREZ-VÁZQUEZ; CONTRERAS-CASTILLO; LICONA-LIMÓN, 2018). Uma das principais vias de sinalização pró-inflamatória está centrada na ativação do fator nuclear de transcrição *kappa* B (NF-κB), que estimula a expressão de genes pró-inflamatórios e antiapoptóticos (SUGIMOTO et al., 2019).

Os macrófagos apresentam uma variedade fenotípica e funcional, que desempenha papel fundamental em todo processo inflamatório. Essas células apresentam um status de polarização, que podem ser estimulados por sinais do microambiente, definindo dois fenótipos e funções principais: macrófagos M1 e M2. Os macrófagos M1, pró-inflamatórios, resultado de estimulação por lipopolissacarídeo (LPS) e/ou interferon γ (IFN-γ) que atuam na fagocitose e angiogênese. Os macrófagos M2 (subdivididos em M2a, M2b e M2c), anti-inflamatórios, estimulados por interleucinas 4, 10, 13 e 33 (IL-4, IL-10, IL-13 e IL- 33) que promovem a cicatrização do tecido por meio da estabilização da angiogênese e da estimulação da matriz extracelular. A regulação dessas duas respostas vai definir os resultados benéficos ou prejudiciais na resolução da inflamação e homeostase tecidual (MANGINO et al., 2017; MENDES et al., 2019; WATANABE et al., 2019).

Após a remoção do agente infeccioso, vários mecanismos reguladores são ativados para restringir o influxo de leucócitos e impedir danos teciduais do processo inflamatório em andamento (MURPHY; WEAVER, 2016). Feedback para fatores de transcrição, reguladores do gene miRNA e citocinas anti-inflamatórias atenuam a produção de mediadores pró-inflamatórios e regulam as vias de sinalização para limitar a infiltração celular (FIGUEREDO et al., 2017; ZHU et al., 2018). A remoção de granulócitos infiltrados pode ocorrer por migração reversa, drenagem linfática ou morte celular, seja ela por apoptose, ou dependendo do estado do tecido e da doença, autofagia desregulada, necrose e armadilha extracelular de neutrófilos (NET- *Neutrophil Extracellular Trap*) na resolução inflamatória (SUGIMOTO et al., 2019).

As células dendríticas são células apresentadoras de antígenos especializadas na diferenciação de células T *naive* em células T efetoras. Essas células, através da sinapse imunológica, representam a interface entre a resposta imune inata e a adaptativa (GUERROUAHEN et al., 2019). Recentemente foram classificadas ontogeneticamente em células dendríticas convencionais do tipo 1 e 2 (CDc1 e CDc2), que apesar de compartilharem um precursor de desenvolvimento comum, exigem diferentes moduladores na sua diferenciação e são fenotipicamente e funcionalmente distintos (GUILLIAMS et al., 2014; EISENBARTH, 2018). As CDc1 são as principais produtoras de interleucina 12 (IL-12), expressam o receptor 3 do tipo *Toll* (TLR3) e possuem vias celulares para processar antígeno exógeno para o complexo principal de histocompatibilidade (MHC, *Major Histocompatibility Complex*) de classe I (MHC1) apresentando antígenos majoritariamente para células T $CD8^+$. O subconjunto CDc2 tem sido amplamente associado a uma variedade de respostas de linfócitos $CD4^+$ em especial do tipo T_h1 (GUILLIAMS et al., 2016).

Os linfócitos T de memória são caracterizados pela sua capacidade de produzir e expressar citocinas específicas, que variam de fenótipos anti-inflamatórios a pró-inflamatórios. Progenitores linfoides se diferenciam em células que expressam receptores de células T (RCTs) αβ ou γδ, no qual, cada subgrupo ocupa um nicho específico e altamente preservado no sistema imunológico (EMMING et al., 2020). Os linfócitos T $CD8^+$ e $CD4^+$ direcionadas ao MHC mediam a imunidade adaptativa ao patógeno e expressam RCTs αβ. Por outro lado, as células T $\gamma\delta^+$, apesar de apresentarem características de memória imunológica, desempenham um papel principal na imunidade inata em superfícies mucosas e produzem um painel de citocinas que inclui IFN-γ, IL-4, IL-17, IL-21, IL-22, Fator Estimulador de Colônias de Granulócitos e Macrófagos (GM-CSF, *Granulocyte-macrophage colony-stimulating fator*) e fator de necrose tumoral α (TNF-α) (SUTTON; MIELKE; MILLS, 2012; MISIAK et al., 2017; EMMING et al., 2020). Além disso, dados apontam um novo subconjunto de células T consideradas hiper-inflamatórias caracterizadas pela coexpressão de RCTs αβ e γδ. Essas células são capazes reconhecer antígenos peptídicos restritos ao MHC, como células T αβ e são produtoras de citocinas características de células T γδ (IFN-γ, IL-17 e GM-CSF) em resposta ao estímulo de IL-1β e IL-23, estabelecendo uma interface entre a resposta imune adaptativa e inata (EDWARDS et al., 2020).

A ativação de células T mediada por APCs contra antígenos restritos ao MHC do tipo 1 leva à produção de linfócitos T $CD8^+$ citotóxicos, responsáveis por rastrear e destruir células que apresentem seu epítopo antigênico ativador. Estão associados ao reconhecimento de células infectadas por patógenos intracelulares como vírus, e desempenham papel importante na lise imunomediada de células cancerígenas (MURPHY; WEAVER, 2016; DELLACHERIE; SEO; MOONEY, 2019).

O impacto dos linfócitos T $CD4^+$ na resposta imunológica difere de acordo com seus subtipos (T_h0, T_h1, T_h2, T_h17 e T_{reg}). As células Th0 (células T auxiliares em repouso) ao receber o estímulo irão se diferenciar em células T_h1, T_h2, T_h17 e T_{reg} (T reguladoras) de acordo com o tipo de resposta que será desenvolvida (MURPHY; WEAVER, 2016). As células T_h1 produzem uma resposta pró-inflamatória através da produção principalmente de citocinas IL-2, IL-12, IL-1, TNF-α, IFN- γ, enquanto células do tipo T_h2 tem como principais mediadores a IL-4, a IL-5, a IL-10 e o TGF-β atuantes com um perfil de resposta anti-inflamatória. As células T_h17 produzem IL-17 que irá contribuir para uma resposta regulatória em associação com outras citocinas (IL-10, IL-35 e TGF-β) (PODGAEC et al., 2010; FIGUEREDO et al., 2017). As células T reguladoras (T_{reg}) apresentam maior especificidade para auto-antígenos, desempenhando um papel fundamental na tolerância imunológica,

regulando as respostas imunes prejudiciais e limitando o desenvolvimento de doenças autoimunes (DELLACHERIE; SEO; MOONEY, 2019).

Além disso, os linfócitos T $CD4^+$, possuem um subgrupo distinto de células T auxiliares foliculares (T_{hf}) que regulam a imunidade humoral promovendo a produção de anticorpos de alta sensibilidade e especificidade por plasmócitos e células B de memória longa. As células T_{hf} são caracterizadas pela expressão do fator de transcrição BCL-6, proteína de morte celular programada 1 (PD-1), co-estimulador indutível de células T (ICOS) e receptor de quimiocina CXC-R5 (EISENBARTH, 2018; DELLACHERIE; SEO; MOONEY, 2019). As células B, por sua vez, são indispensáveis para a imunidade humoral, produzem e secretam anticorpos quando estimuladas por citocinas inflamatórias como a IL-10, antígenos e células T $CD4^+$ ativadas que carregam seu antígeno cognato. Tais estímulos ajudam na ativação, proliferação e diferenciação das células B para gerar uma memória imunológica eficaz (CALLAN JR; MORA; HALCZAK, 2016).

2.6.1 Imunomodulação

O sistema imunológico desempenha um papel fundamental na homeostase fisiológica. Além da defesa contra patógenos, está intimamente ligado a síndromes autoimunes, alergias, patogênese e regulação do microambiente tumoral, assim como está associado a processos regenerativos e reparo de tecidos. Sendo assim, a modulação da resposta imunológica pode ser objeto de estudo para o desenvolvimento de técnicas, processos, métodos, meios e instrumentos voltadas ao tratamento das mais variadas doenças (DELLACHERIE; SEO; MOONEY, 2019).

A imunomodulação é caracterizada pela alteração da resposta imunológica fisiológica, que pode ser ativada (imunoestimulação) ou suprimida (imunossupressão), por meio de agentes externos, como compostos naturais ou sintéticos. Envolve direcionar mediadores imunológicos a controlar vias de sinalização para modificar a reatividade do hospedeiro a imunógenos específicos e minimizar efeitos colaterais potencialmente fatais (AMBROZIAK et al., 2016). A compreensão sobre os mecanismos pelos quais as respostas imunes podem ser ajustadas é crucial para impedir ou reverter o efeito prejudicial do excesso da resposta ou ativação imune crônica ou estimular uma resposta imunológica para o controle ou reparo de danos (SALIM; SERSHEN; MARCH, 2016; ALTAN-BONNET; MUKHERJEE, 2019).

A comunicação entre as células do sistema imunológico, se fosse mediada através do contato celular, seria apenas local; no entanto, para o funcionamento de um sistema de defesa

especializado, uma comunicação intercelular de longo alcance é essencial (DELLACHERIE; SEO; MOONEY, 2019). Sendo assim, as células imunológicas utilizam diversas vias de sinalização para exercer simultaneamente funções diferentes em múltiplos locais do organismo, por meio de mediadores indispensáveis para comunicação, ativação, diferenciação, proliferação e sobrevivência dessas células (SILVER et al., 2011; HUNTER; JONES, 2015).

Os mediadores imunológicos são de natureza bastante diversa, estimulam as principais etapas celulares e moleculares da resposta imunológica e podem atuar de forma parácrina e autócrina (ALTAN-BONNET; MUKHERJEE, 2019). Incluem mediadores lipídicos (mediadores pró-resolução especializados – SPMs, do inglês *Specialized pro-resolving mediators* como, lipoxinas, resolvinas, protectinas e maresinas), proteínas e peptídeos (anexina A1, hormônio adrenocorticotrópico, galectinas, citocinas), mediadores gasosos (H_2S, CO), metabolitos do ácido araquidônico (prostaglandinas, tromboxanos e leucotrienos), radicais livres (ON), aminas vasoativas (histamina, serotonina), nucleotídeos (adenosina, inosina), assim como, neuromoduladores (acetilcolina e outros neuropeptídeos) (SERHAN et al., 2015; DELLACHERIE; SEO; MOONEY, 2019; SUGIMOTO et al., 2019).

As citocinas são mediadores que desempenham um papel fundamental na homeostase imunológica, ligando-se fortemente a receptores específicos nas células-alvo, ativando uma cascata de eventos de sinalização que culmina na expressão de um conjunto de genes necessários para uma tarefa especializada (NUNES et al., 2017). Podem atuar de forma sinérgica ou antagônica, ou ainda apresentar pleiotropia funcional, sendo capazes de atuar em múltiplas resoluções e efeitos biológicos como mostra a Tabela 5 (ALTAN-BONNET; MUKHERJEE, 2019; MURAKAMI; KAMIMURA; HIRANO, 2019).

Citocinas como IFN-γ e TNF-α, por exemplo, se destacam por seu potencial antitumoral e anti-infecção pela ativação de células *natural killer* (NK) e linfócitos T citotóxicos ($CD8^+$) (PENTCHEVA-HOANG et al., 2014). IFN-γ é um mediador da ativação de macrófagos infectados por patógenos intracelulares, sendo uma molécula chave na produção de ON (SALIM; SERSHEN; MARCH, 2016). A IL-2 é um importante mediador proliferativo, que induz a expansão clonal de linfócitos T, incluindo células T $CD4^+$, de forma autócrina e atua aumentando a ação citotóxica de linfócitos T $CD8^+$. IL-6, citocina pleiotrópica, está presente na regulação da resposta de fase aguda, sendo capaz de promover a ativação de linfócitos T e a diferenciação de macrófagos associados à cicatrização de feridas (SILVER et al., 2011; HUNTER; JONES, 2015). A IL-10, por sua vez é considerada um

potente imunorregulador, inibindo a superprodução de IFN-γ e TNF-α (SAXENA et al., 2015).

Tabela 5. Principais funções imunológicas de citocinas nas respostas pró-inflamatória, anti-inflamatória e regulatória.

Citocinas	Células produtoras	Função Imunológica	Referências
IL-1β	Monócitos, células epiteliais.	Diferenciação de células T_h17.	MCGEACHY; CUA, 2008
IL-2	Células T $CD4^+$, células NK.	Melhora as respostas reguladoras e efetivas.	BUSSE et al., 2010
IL-3	Células T, mastócitos, eosinófilos.	Diferenciação de células-tronco hematopoiéticas e proliferação das células da linhagem mieloide.	REDDY et al., 2000
IL-4	Células T, células NKT, células T γδ, mastócitos.	Diferenciação de células T_h0 em células T_h2.	SCHWARTZ et al., 2016
IL-5	Células T_h2, mastócitos, eosinófilos, células NK.	Desenvolvimento de eosinófilos.	IKUTANI et al., 2011
IL-6	Células T, macrófagos, fibroblastos, células endoteliais.	Citocina pleiotrópica, amplamente relevante para respostas pró-inflamatória e anti-inflamatória.	SCHELLER et al., 2011
IL-7	Células estromais da medula óssea, células epiteliais.	Desenvolvimento de células T e homeostase.	SCHWARTZ et al., 2016
IL-8	Monócitos, células T, neutrófilos, fibroblastos, células endoteliais.	Recrutamento e ativação de neutrófilos no curso da inflamação.	MASPI; ABDOLI; GHAFFARIFAR, 2016
IL-9	Mastócitos, células T_h2, T_h17, T_h9.	Regulação da imunidade inflamatória.	GOSWAMI; KAPLAN, 2011
IL-10	Células T_h2, macrófagos, DCs, Células B.	Ações anti-inflamatórias.	COUPER; BLOUNT; RILEY, 2008
IL-11	Células estromais da medula óssea	Hematopoiese, remodelação óssea.	ALTAN-BONNET; MUKHERJEE, 2019
IL-12	Macrófagos ativados, CDs	Diferenciação de células T_h1.	SUN et al., 2015
IL-13	Células T_h2	diminui as respostas inflamatórias por meio de regulação negativa de citocinas pró-inflamatórias	WYNN, 2003
IL-15	CDs, monócitos, células epiteliais	Indução de células T e proliferação células NK.	SAEED; REVELL, 2001
IL-17	Células T_h17, células NK,	Medeia a inflamação do tecido.	MIOSSEC; KORN;

	células NKT		KUCHROO, 2009
IL-18	Macrófagos	Induz respostas T_h1 através da produção de IFN-γ.	ALTAN-BONNET; MUKHERJEE, 2019
IL-19	Monócitos	Ativação de células B.	SCHWARTZ et al., 2016
IL-20	Monócitos, granulócitos, queratinócitos, CDs, fibroblastos	Migração de macrófagos, desenvolvimento de osteoclastos.	RUTZ; WANG; OUYANG, 2014
IL-21	Células T $CD4^+$, células T_h17, células NKT	Diferenciação de células TFH.	LEONARD; ZENG; SPOLSKI, 2008
IL-22	Células T auxiliares 22 (T_h22), T_h17 e T_h1	Promove a imunidade na barreira endotelial, aumenta a função IL-17.	RUTZ; EIDENSCHENK; OUYANG, 2013
IL-23	CDs, macrófagos, células B, células endoteliais	Doença mediada por T_h17.	KASTELEIN; HUNTER; CUA, 2007
G- CSF	Monócitos, macrófagos	Estimulação da medula óssea para produção de células.	SCHWARTZ et al., 2016
GM- CSF	Células T ativadas, células NK, macrófagos	Maturação de macrófagos e granulócitos, aumento de células T e função de CD, estimulação e diferenciação de células-tronco.	SCHWARTZ et al., 2016
IFN-α	pCDs, células NK, células T, células B, macrófagos, fibroblastos, células endoteliais, osteoblastos	Aumento da atividade de macrófagos e síntese de ON.	MASPI; ABDOLI; GHAFFARIFAR, 2016
IFN-γ	Células T, células NK, células NKT	Estimula a produção de ON em macrófagos, diferenciação de células T $CD4^+$ para T_h1 e inibe o desenvolvimento de células T_h2 e T_h17.	MASPI; ABDOLI; GHAFFARIFAR, 2016
TGF-β	Células T, macrófagos	Fator de crescimento pleiotrópico com propriedades anti-inflamatórias e imunossupressoras significativas, homeostase do sistema imunológico.	WAN; FLAVELL, 2007

NK: célula *Natural Killer*; **NKT**: célula T *Natural Killer*; **CD**: célula dendrítica; **TFH**: células T auxiliares foliculares; **pCDs**: células dendríticas plasmocitóides; **ON**: óxido nítrico. **Fonte:** A autora.

2.6.2 Ativação Linfocitária

As células linfocitárias, ao serem estimuladas, iniciam uma cascata de ativação, no qual produzem uma resposta adequada ao estímulo. EROs e cálcio (Ca^{2+}) desempenham um papel fundamental de sinalização na produção da resposta efetora dos linfócitos, no entanto, a

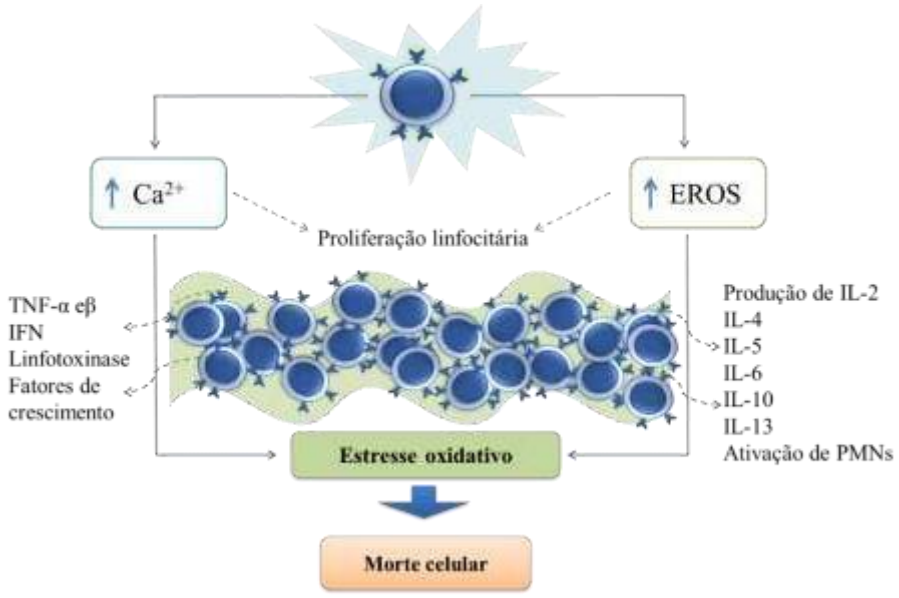
regulação desta sinalização pode indicar um sinal de vida ou de morte celular (Figura 7) (GÖRLACH et al., 2015). Em um estado em que a geração de EROs supera a capacidade antioxidante da célula, três tipos de respostas podem ser desencadeadas: a célula pode compensar o estresse oxidativo através de uma fonte de energia redutora e retornar a seu estado homeostático; a célula se diferencia e adapta-se a condições mais oxidantes; ou, quando a adaptação celular não são suficientes para reverter o estado oxidativo, o processo de morte celular é desencadeado (AGUIRRE et al., 2005).

O Ca^{2+} intracelular é um segundo mensageiro envolvido na ativação celular, essencial para a ativação, proliferação e diferenciação de linfócitos T. Níveis elevados de EROs são capazes de promover o aumento imediato de cálcio citosólico através da ativação de canais catiônicos não seletivos (BOGESKI et al., 2010; PAREKH; PUTNEY, 2015). Por sua vez, níveis aumentados de Ca^{2+} ativam enzimas geradoras de EROs e formação de radicais livres. A sinalização promovida por EROs e cálcio estão interligadas, no qual as EROs regulam a sinalização celular de cálcio, enquanto a sinalização de cálcio é essencial para a produção de EROs (GRUPE et al., 2010; GÖRLACH et al., 2015).

Níveis elevados de Ca^{+2} citosólico podem levar ao aumento da captação mitocondrial deste íon durante a ativação linfocitária, desencadeando aumento da produção de EROs mitocondrial (TORRES-QUINTANILLA; GONZÁLEZ-CASTILLO; GARCÍA-RIVAS, 2017). Por outro lado, para manter a ativação celular sem elevar a produção de EROs a um nível prejudicial, o potencial de membrana ($\Delta\Psi m$) é reduzido a valores que ainda permitem a produção de ATP, mantendo os níveis de EROs potencialmente inofensivos às células (ZOROVA et al., 2018).

Ao serem ativadas, as células linfocitárias aumentam de tamanho, com o aumento dos volumes do núcleo e do citoplasma, onde serão induzidas a síntese de novos RNAs e proteínas, entrando em um estágio de diferenciação. Após diferenciação, o processo de expansão dá origem a novas células com idêntica especificidade funcional. Essas células então irão exercer seus mecanismos efetores, através de moléculas sinalizadoras, desenvolvendo uma resposta imunológica de acordo com o estímulo recebido (MELO, 2009). Em contra partida, o aumento excessivo de EROs e Ca^{2+}, irá promover um sinal de parada para as atividades efetoras desta célula e pode indicar o início de uma morte celular programada (MELO et al., 2010).

Figura 7. Mecanismo de ativação linfocitária envolvendo a produção de espécies reativas de oxigênio (EROs) e Cálcio (Ca^{2+}).



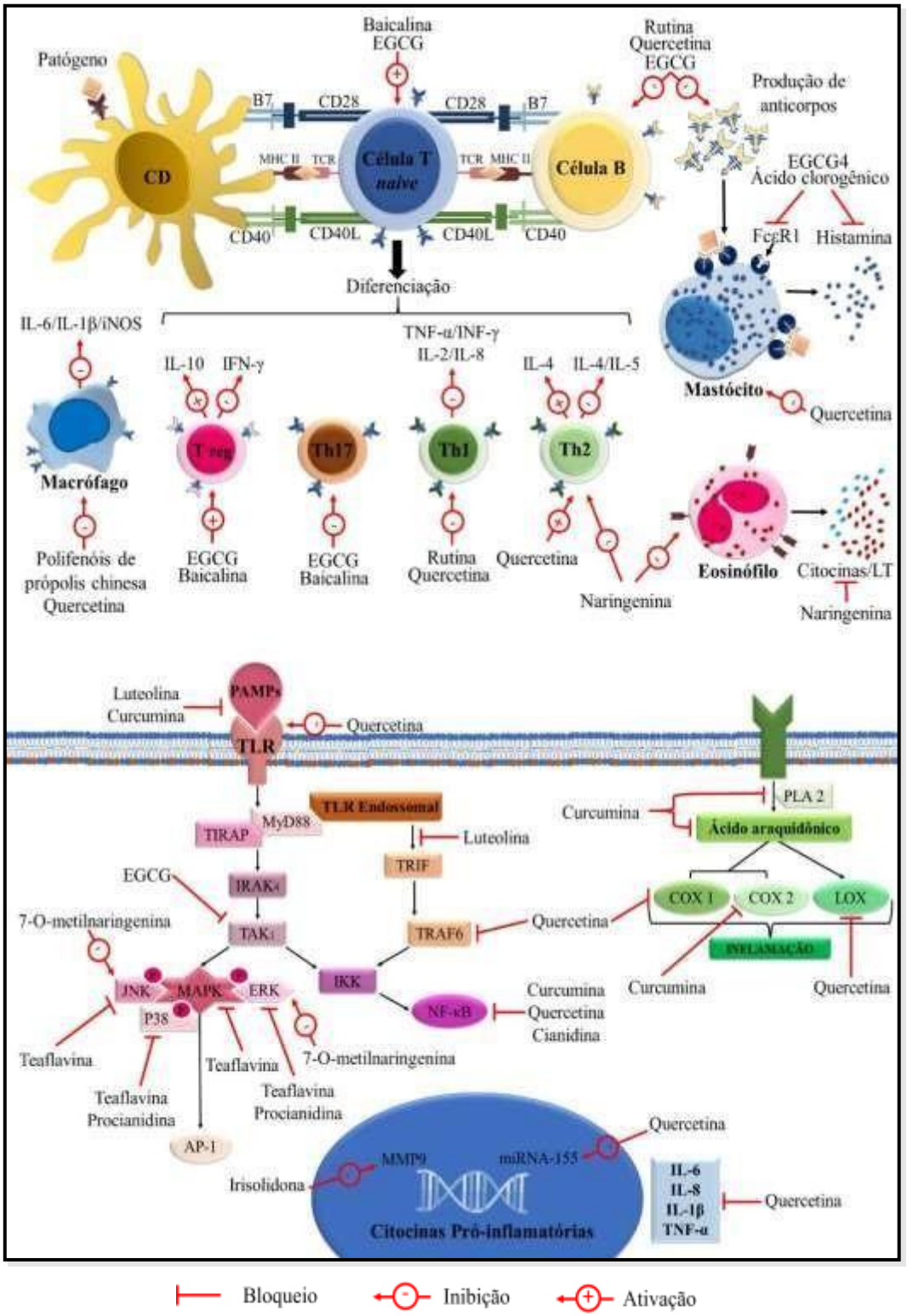
Fonte: MELO et al., 2010

2.6.3 Imunomoduladores Exógenos

Os imunomoduladores apresentam a capacidade de regular células e mediadores moleculares imunológicos. Inúmeras substâncias, como polissacarídeos, lipídios, proteínas, peptídeos, óleos voláteis e compostos fenólicos podem atuar na manutenção da saúde através da regulação da função imunológica do corpo (DELLACHERIE; SEO; MOONEY, 2019). Diferentes estratégias imunomoduladoras têm sido estudadas, tanto *in vitro*, quanto *in vivo*, na busca por terapias capazes de modular o processo inflamatório, com menos efeitos colaterais indesejados, equilibrando as respostas pró e anti-inflamatórias (SUGIMOTO et al., 2019).

Vários estudos têm demonstrado os efeitos imunomoduladores dos polifenóis por meio de diferentes mecanismos (Figura 8). Seja pela modificação da expressão e produção de imunoglobulinas, ativação e diferenciação de células imunes, potencial antioxidante ou propriedades anti-inflamatórias, cada tipo de polifenol modula a função da resposta imunológica através da ligação aos receptores da superfície celular alterando suas vias de sinalização intracelular (ZARAGOZÁ et al., 2020).

Figura 8. Mecanismos de modulação da resposta imunológica por compostos fenólicos.



AP-1: Proteína ativadora 1; **COX:** Ciclooxigenase; **EGCG:** Epigalocatequina-3-galato; **ERK**: Proteínas quinases extracelulares; **IKK:** IκB quinase; **iNOS:** óxido nítrico sintase induzível. **IRAK4:** Quinase 4 associada ao receptor de interleucina-1; **JNK:** C-Jun N-terminal quinases; **LOX:** Lipoxigenase; **LT:** Leucotrienos; **MAPK:** Proteína quinase ativada por mitogênio; **MHC:** Complexo de histocompatibilidade principal; **miR-155:**

MicroRNA-155; **MMP9:** Metaloproteinase de matriz 9; **MYD88:** Resposta primária de diferenciação mieloide 88; **NF-κB:** Fator nuclear kappa de células B ativadas; **PLA2:** Fosfolipase A2; **TAK1:** Quinase 1 ativada pelo fator de crescimento beta transformador; **TCR:** Receptor de células T; **TIRAP:** Proteína adaptadora contendo o domínio do receptor *Toll*/interleucina-1; **TLR:** Receptor *Toll*-like; **TRAF6:** Fator 6 associado ao receptor TNF; **TRIF:** domínio TIR contendo o interferon-β indutor do adaptador. **Fonte:** Adaptado de SOBHANI et al. (2020).

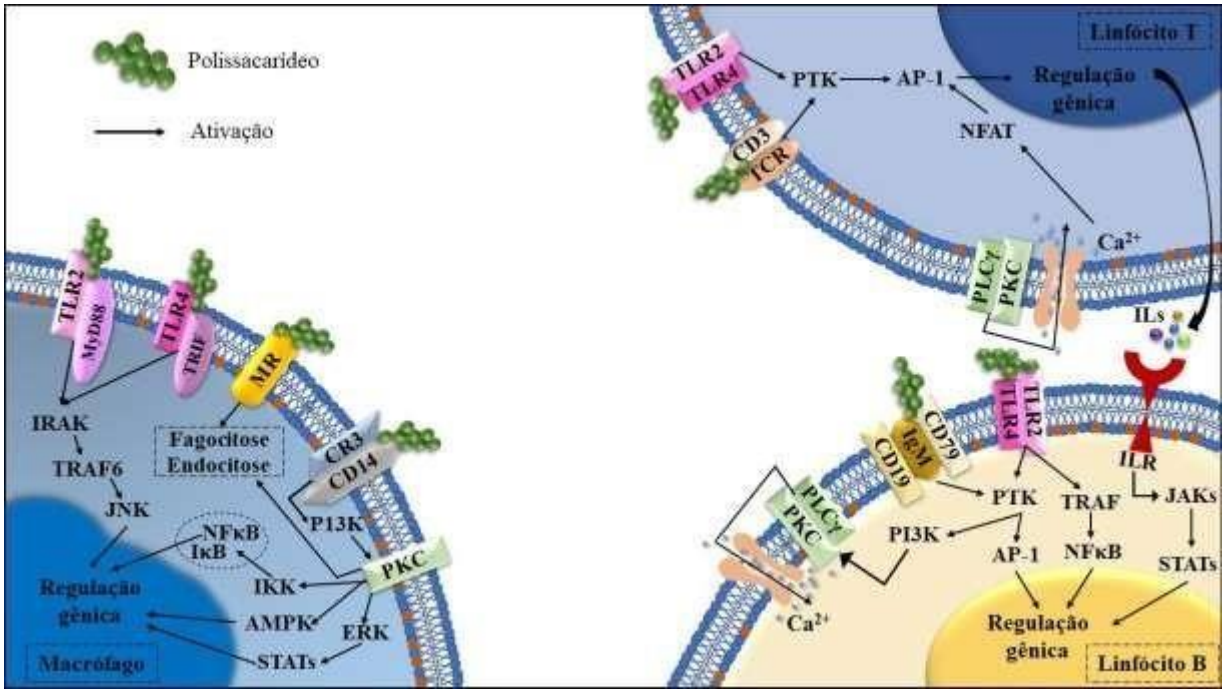
Sobhani e colaboradores (2020) relataram que a administração oral de extratos polifenólicos da fruta de *Phoenix dactylifera* estimulou a proliferação de macrófagos, células dendríticas, células T auxiliares do tipo 1 (T_h1) e células NK em camundongos e o tratamento de ratos idosos com polifenóis das flores de *Cassia auriculata* promoveu o aumento do número de células T e B esplênicas. Além disso, os polifenóis desempenham importante papel na modulação de vias de transdução de sinal nas respostas imunes, a começar pela regulação de receptores de reconhecimento padrões (PRRs), em especial o receptor do tipo *Toll* 4 (TLR4) que transduzem sinais pelas vias das Proteínas quinases ativadas por mitógenos (MAPKs) e NF-κB, envolvidas na produção de ON, TNF-α, IL-1β e IL-6. Os polifenóis também podem interferir na cascata de metabolização do ácido araquidônico e inibir as vias lipo-oxigenase (LOX) e ciclo-oxigenase (COX) e consequentemente interferir na produção de tromboxanos (TX), prostaglandinas (PG) e leucotrienos (LT) (Figura 8) (DREXLER; FOXWELL, 2010; MITJAVILA; MORENO, 2012).

De forma semelhante, os polissacarídeos podem regular a produção de citocinas, ativação de macrófagos, linfócitos T/B, células NK, produção de anticorpos e modulação de sistemas complementares (Figura 9) (HUANG et al., 2019). Polissacarídeos pécticos de cereja doce (*Prunus avium*), por exemplo, induzem a liberação de ON e expressão de mRNAs de TNFα, IL6, IL10, iNOS, COX-2 e fator estimulador de colônias de granulócitos (GCSF-*Granulocyte colony-stimulating fator*) em células RAW264.7 (CAO et al., 2018). Polissacarídeos sulfatados de *Capsosiphon fulvescens*, por sua vez, são capazes de regular uma variedade de genes relacionados à proliferação de macrófagos e liberação de citocinas além de estimular a produção de ON, prostaglandina E2 (PGE2) e expressão do óxido nítrico sintase induzida (iNOS) e ciclooxigenase 2 (COX2) (JOSE; KURUP, 2017).

Ligantes glicosil apresentam a capacidade de ligar-se a membros da família de receptores do tipo *Toll*, TLR2 e 4 principalmente, desempenhando um papel fundamental na modulação da imunidade inata e adquirida. Esses ligantes associados aos TLRs ativam o fator 6 associado ao receptor de TNF (TRAF6) sinalizando a transdução da via do NF-κB (ZHANG et al., 2020). Os receptores de manose (RM), expressos principalmente por macrófagos, são importantes para o reconhecimento de padrões e capazes de reconhecer manose, fucose e

acetil glucosamina (GAZI; MARTINEZ-POMARES, 2009). A ligação de polissacarídeos ao complexo TCR/CD3 leva a ativação da proteína tirosina quinase (PTK) e consequente resposta dos linfócitos T pela via fosfatidilinositol 3-quinase (PI3–K) ou MAPK. A proteína quinase C (PKC), ativada pela via PI3-K, afeta o fluxo de íons cálcio, que regulam o fator nuclear das células T, importantes na resposta imune das células (MIAO et al., 2005). Nas células B, a proteína tirosina quinase (PTK) é ativada quando polissacarídeos se ligam ao complexo IgM/CD79, ativando a MAPK e produzindo a proteína ativadora-1 (AP-1), que regula a expressão de genes relacionados aos linfócitos (LIU et al., 2014) (Figura 9).

Figura 9. Mecanismos de sinalização envolvidas na ativação de macrófagos, linfócitos T e linfócitos B por polissacarídeos.



AP-1: proteína ativadora-1; **CD14**: cluster de antígeno de diferenciação 14; **CD3**: cluster do antígeno de diferenciação 3; **CD79**: cluster de antígeno de diferenciação 79; **CR3**: receptor de complemento 3; **ERK**: quinase regulada por sinal extracelular; **IgM**: imunoglobulina M; **IKK**: inibidor do fator nuclear *kappa*-B quinase; **ILR**: receptor de interleucina; **IRAK**: quinase associada ao receptor de interleucina-1; **IκB**: inibidor do fator nuclear *kappa*-B; **JNK**: c-Jun Quinase N-terminal; **MR**: receptores de manose; **MyD88**: fator de diferenciação mieloide 88; **NFAT**: fator nuclear de células T ativadas; **NF-κB**: fator nuclear *kappa*-B; **PI3-K**: fosfatidilinositol 3 quinase; **PKC**: proteína quinase C; **PLC**: fosfolipases C; **PLCγ**: fosfolipase Cγ; **PTK**: proteína tirosina quinase; **STAT**: transdutores de sinal e ativadores de transcrição; **TCR**: receptor de células T; **TLR2**: receptor 2 semelhante a *Toll*; **TLR4**: receptor 4 semelhante a *Toll*; **TRAF6**: fator 6 associado ao receptor do fator de necrose tumoral; **TRIF**: domínio *Toll*/IL-1 contendo adaptador de indução de interferon β. **Fonte:** Adaptado de HUANG et al. (2019).

2.7 CONOCARPUS ERECTUS

Os manguezais são ecossistemas altamente produtivos de grande importância ecológica. Espécies vegetais de mangues apresentam características físicas, biológicas e fisiológicas que permitem se desenvolverem em condições extremas de alta salinidade, temperatura, radiação e baixa concentração de nutrientes (GHOSH et al., 2015; REDDY; GRACE, 2016).

Conocarpus erectus L. var. *erectus* é uma planta característica desse ecossistema, considerada uma espécie de transição do mangue por estar geralmente localizada nas partes mais altas do estuário. É uma árvore perene que varia de 2 a 6 m de altura, popularmente conhecido como ―mangue-de-botão‖, ―mangue-negro‖ ou ―amora-do-mar‖ devido às características do seu fruto (NETTEL et al., 2008; RAZA et al., 2016) (Figura 10). É encontrada nas regiões costeiras das áreas tropicais e subtropicais ao redor do mundo (NASEER et al., 2017), distribuída geograficamente ao longo da costa do sul da Flórida, Bahamas, Antilhas, México, América Central e Ilhas Galápagos. Também é nativa das áreas costeiras da África Ocidental; na América do Sul, desenvolvendo-se desde o Equador até o Brasil (MARQUETE; VALENTE, 1997; NETTEL et al., 2008; VON LINSINGEN; CERVI; GUIMARÃES, 2009; NASEER et al., 2017).

Figura 10. Diferentes órgãos de *Conocarpus erectus* L. Var. *erectus*.

Fonte: A autora.

É utilizada no sul da Flórida como planta ornamental (ALLEN, 1998) e suas hastes são usadas como combustível (carvão vegetal ou lenha) para uso doméstico (CARNEIRO; BARBOZA; MENEZES, 2010) e como madeira para móveis, cercas (ATHEULL et al., 2009)

e construção de barcos (AL-HUMAID; MOFTAH, 2007). A casca desta árvore tem sido usada para curtimento de couro e como piscicida (ADONIZIO et al., 2006).

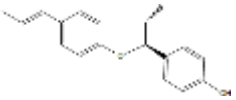
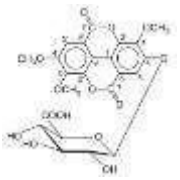
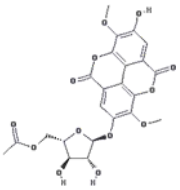
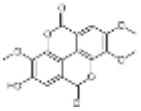
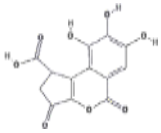
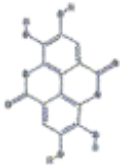
Diferentes partes de *C. erectus* têm sido usadas pela população para tratar diversas condições patológicas. Em regiões da África e Porto Rico, as folhas são comidas ou formulações aquosas usadas para febre (ADONIZIO et al., 2006). Suas folhas também são um medicamento tradicional para doenças respiratórias, dor de cabeça, inchaço, orquite e úlceras de pele (ABDEL-HAMEED; BAZAID; SABRA, 2013; RAZA et al., 2016). O chá da casca e das frutas é usado para diarreia, feridas, hemorroidas e diabetes (CARNEIRO; BARBOZA; MENEZES, 2010). Sua madeira também é utilizada para o tratamento de conjuntivite, diarreia (AYOUB, 2010; ABDEL-HAMEED et al., 2012), sífilis e gonorreia (NASCIMENTO et al., 2016).

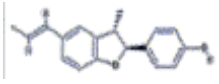
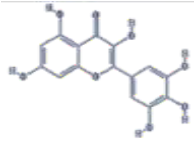
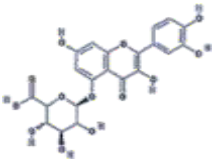
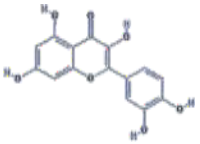
Numerosos compostos, principalmente compostos fenólicos, foram identificados e isolados de diferentes partes de *C. erectus* (Tabela 6). Essas moléculas exibem múltiplas atividades farmacológicas e são consideradas as principais substâncias bioativas dessa espécie (HAYASHI; THOMSON, 1975; AYOUB, 2010; ABDEL-HAMEED et al., 2012; SANTOS et al., 2018a).

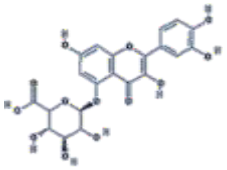
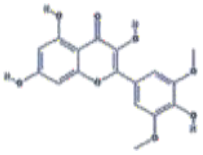
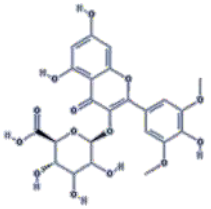
Potenciais antioxidante (ABDEL-HAMEED et al., 2014) hepatoprotetor, (ABDEL-HAMEED; BAZAID; SABRA, 2013) anticâncer (ABDEL-HAMEED et al., 2012), imunomodulador (SANTOS et al., 2018a) antimicrobianos (SHOHAYEB et al., 2013, SILVA, 2004) e de inibição de fatores de virulência de bactérias são descritos (ADONIZIO; KONG; MATHEE, 2008). Resultados promissores revelam também a eficácia do extrato de folhas desta planta para controlar elevados níveis glicêmicos (RAZA et al., 2018). Além disso, extratos etanólico e hexânico demonstraram ser uma rica fonte de agentes terapêuticos para prevenção e reparo do processo de envelhecimento e tensões oxidativas (AYOUB, 2010).

Estudos anteriores realizados por nosso grupo demonstraram que o extrato aquoso de folhas de *C. erectus* foi capaz de modular a resposta imunológica de PBMCs para uma resposta pró-inflamatória (SANTOS et al., 2018a), assim como os extratos em acetato de etila e etanólico apresentaram boa atividade antioxidante (SANTOS et al., 2018b), indicando que as folhas dessa espécie são ricas em moléculas com potencial antioxidante e imunomodulador. Sendo assim, neste trabalho buscamos identificar e isolar metabólitos das folhas desta planta, além de elucidar suas características químicas e avaliar o seu potencial quanto a moléculas antioxidantes e imunoestimulantes.

Tabela 6. Compostos identificados e/ou isolados de extratos a partir de diferentes tecidos de *C. erectus*.

Composto	Fórmula/Estrutur molecular	Tecido	Extração	Referência
2,3-Dimetil-1,4-pentadieno [id]	C_7H_{12}	Folhas	Hexânica	SANTOS et al., 2018b
2,4-Hexadieno, 2-metil [id]	C_7H_{12}	Folhas	Hexânica	SANTOS et al., 2018b
2‘-metoxi-conocarpol [is]		Madeira	Metanólica	HAYASHI; THOMSON, 1975
3-*O*-metilviolanona [id]	$C_{18}H_{18}O_6$	Folhas	Metanólica	SANTOS et al., 2018b
Ácido 3,3′,4′-tri-*O*-metilelágico 4-*O*-β [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Ácido 3,3′-dimetoxielágico [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Ácido 3,4,3′-trimetoxielágico [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Ácido cafeico [id]	$C_9H_8O_4$	Folhas	Aquosa	SANTOS et al., 2018a
Ácido carboxílico de brevifolina [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Ácido clorogênico [id]	$C_{16}H_{18}O_9$	Folhas	Aquosa	SANTOS et al., 2018a
Ácido elágico [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Ácido fertárico [id]	$C_{14}H_{14}O_9$	Folhas	Aquosa	SANTOS et al., 2018a
Ácido gálico [id, is]	$C_7H_6O_5$	Folhas	Metanólica Acetato de Etila	SANTOS et al., 2018b
Ácido protocatecuico [id]	$C_7H_6O_4$	Folhas	Acetato de Etila	SANTOS et al., 2018b

Ácido quilaico [id]	$C_{30}H_{46}O_5$	Folhas	Metanólica	SANTOS et al., 2018b
Apigenina-7-O-glucosídeo [id]	$C_{21}H_{20}O_{10}$	Folhas	Metanólica	SANTOS et al., 2018b
Apigenina [id]	$C_{15}H_{10}O_5$	Frutas	Metanólica Aquosa	ABDEL-HAMEED et al., 2014 SANTOS et al., 2018a
Artabsina [id]	$C_{15}H_{20}O_3$	Folhas	Acetato de Etila	SANTOS et al., 2018b
Cafestol [id]	$C_{20}H_{28}O_3$	Folhas	Metanólica	SANTOS et al., 2018b
Catequina [id]	$C_{15}H_{14}O_6$	Frutas	Metanólica	ABDEL-HAMEED et al., 2014
Conocarpan [is]		Madeira	Metanólica	HAYASHI; THOMSON, 1975
Eicosano, 2-metil [id]	$C_{21}H_{44}$	Folhas	Hexânica	SANTOS et al., 2018b
Eicosano, 7-hexil [id]	$C_{26}H_{54}$	Folhas	Hexânica	SANTOS et al., 2018b
Elagitanino [id]	$C_{44}H_{32}O_{27}$	Frutas	Metanólica	ABDEL-HAMEED et al., 2014
Galocatequina [id]	$C_{15}H_{14}O_7$	Folhas	Acetato de Etila Metanólica Aquoso	SANTOS et al., 2018b SANTOS et al., 2018a
Kaempferol-3-O-rutinosídeo [id]	$C_{27}H_{30}O_{15}$	Frutas	Metanólica	ABDEL-HAMEED et al., 2014
Kaempferol-3-O-β-D-glucosídeo [id]	$C_{27}H_{30}O_{16}$	Frutas	Metanólica	ABDEL-HAMEED et al., 2014
Luteolina [id]	$C_{15}H_{10}O_6$	Folhas	Metanólica	SANTOS et al., 2018b
Miricetina [is]		Folhas	Hidroalcoólica Aquosa	AYOUB, 2010 SANTOS et al., 2018a
Miricetina 3-O-glucuronídeo [is]		Folhas	Hidroalcoólica Aquosa	AYOUB, 2010 SANTOS et al., 2018a
Octano, 3,4,5,6-tetrametil [id]	$C_{12}H_{26}$	Folhas	Hexânica	SANTOS et al., 2018b
Olean-12-en-3-one [id]	$C_{30}H_{48}O$	Folhas	Hexânica	SANTOS et al., 2018b
Olean-12-eno [id]	$C_{30}H_{50}$	Folhas	Hexânica	SANTOS et al., 2018b
Pauciflorol	$C_{20}H_{34}O_2$	Folhas	Aquosa	SANTOS et al., 2018a
Quercetina [is]		Folhas	Hidroalcoólica Aquosa	AYOUB, 2010 SANTOS et al., 2018a

Quercetina 3-O-glucuronídeo [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Quercetina-3-O-β-D-glucosídeo [id]	$C_{21}H_{20}O_{12}$	Frutas	Metanólica	ABDEL-HAMEED et al., 2014
Robinina [id]	$C_{33}H_{40}O_{1}$	Folhas	Metanólica Acetato de Etila	SANTOS et al., 2018b
Rutina [id]	$C_{27}H_{30}O_{16}$	Frutas	Metanólica	ABDEL-HAMEED et al., 2014
Seringetina [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Seringetina 3-O-glucuronídeo [is]		Folhas	Hidroalcoólica	AYOUB, 2010
Taxifolina [id]	$C_{15}H_{12}O_{7}$	Frutas	Metanólica	ABDEL-HAMEED et al., 2014
α-Bisabolol [id]	$C_{15}H_{26}O$	Folhas	Metanólica	SANTOS et al., 2018b

[id] Identificado; [is] Isolado;

Fonte: A autora.

3 OBJETIVOS

3.1 GERAL

Investigar atividades antioxidante e imunomoduladora *in vitro* de frações semipurificadas contendo compostos fenólicos e de pectina e lignina isoladas das folhas de *C. erectus*.

3.2 ESPECÍFICOS

- Obter e caracterizar a composição química de frações contendo compostos fenólicos a partir de extrato aquoso de folhas de *C. erectus*.
- Purificar e caracterizar lignina e pectina de folhas de *C. erectus*.
- Avaliar o potencial antioxidante das frações e dos compostos isolados.
- Avaliar a citoxicidade das frações para esplenócitos de camundongos Balb/c e da lignina e da pectina para células mononucleares de sangue periférico humano (PBMCs).
- Avaliar a lignina e a pectina quanto à capacidade de promover proliferação de PBMCs.
- Avaliar a resposta imunológica de esplenócitos de camundongos e PBMCs humanas tratados com as frações ou compostos isolados, respectivamente, por meio da quantificação de citocinas e óxido nítrico.
- Avaliar a ocorrência de diferenciação, ativação e/ou inibição de linfócitos e monócitos tratados com a lignina ou pectina.
- Investigar possíveis mecanismos de ativação de PBMCs tratados com lignina ou pectina.
- Avaliar o potencial pré-biotico da pectina de folhas de *C. erectus*.

4 ARTIGO 1 – IMMUNOSTIMULATORY AND ANTIOXIDANT ACTIVITIES OF A LIGNIN ISOLATED FROM *CONOCARPUS ERECTUS* LEAVES

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Immunostimulatory and antioxidant activities of a lignin isolated from *Conocarpus erectus* leaves

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ABSTRACT

In this work, we investigated the antioxidant and immunomodulatory activities of a lignin isolated from *Conocarpus erectus* leaves. The lignin was characterized by FTIR, ^{1}H NMR, ^{13}C NMR and gel permeation chromatography analysis as well as ultraviolet/visible absorption spectra. The lignin was evaluated for total antioxidant activity (TAA), DPPH and $ABTS^{+}$ scavenging abilities, and by a lipid peroxidation inhibition assay. Immunomodulatory activity of the lignin (10 μg/mL) on human peripheral blood mononuclear cells (PBMCs) was determined. The *C. erectus* lignin was found to be of the guaiacyl-syringyl-*p*-hydroxyphenyl (G-S-H) type, with an average molecular weight of 2709 Da (polydispersity index: 2.1). It showed low TAA (17.92%) and moderate antioxidant activity against DPPH and $ABTS^{+}$ (IC_{50}: 231.16 and 356.03 μg/mL, respectively). It also inhibited lipid peroxidation by 42.14%. The lignin promoted an increase in mitochondrial ROS levels as well as cytosolic Ca^{2+} in PBMCs. In addition, it promoted the differentiation and activation of $CD8^{+}$ T lymphocytes, differentiation of $CD14^{+}$ monocytes, and stimulated the release of nitric oxide and cytokines, mainly those linked to a Th1 response. The results showed that the *C. erectus* lignin may be used in future studies in which the modulation of the immune response is a key factor.

1. Introduction

Lignins are among the main structural components of the plant cell wall, consisting of three-dimensional amorphous phenolic polymers composed of three basic units: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) [1,2]. They are involved in the mechanical resistance of plants; defense against microorganisms and transport of water, nutrients and metabolites [2,3]. Lignins have been studied as promising biomaterials for manufacturing of pharmaceutical formulations with higher bioavailability and lower side effects. For example, hardwood lignin was used as excipient in aspirin tablets in order to increase the release rate [4]. Similarly, lignin and carboxylated lignin were evaluated as excipients in tablets containing paracetamol and the results showed that the carboxylated lignin formulation had the highest drug release rate, which was attributed by the authors to a faster disintegration and lower hardness of the tablet [5]. Studies have also described lignins with various pharmacological properties including antioxidant [6], immunomodulatory [3,7], antitumor [8], antiviral [9] and antibacterial [10] activities. Studies on their cytotoxicity have shown that lignins usually do not promote deleterious effects on human and murine cells [11,12].

Conocarpus erectus L. belongs to the Combretaceae family and is widely distributed among the tropics [13]. It is a plant found at the more external regions of mangroves, resistant to adverse conditions such as high temperature and salinity as well as infertile soil [14,15]. In folk medicine, it is used to treat infections and inflammatory conditions such as syphilis, gonorrhea, orchitis, fever and edema [16,17]. Several extracts obtained from different tissues of this plant have shown antioxidant [18], antimicrobial [15,19], hepatoprotective [16], anticancer [20] and immunomodulatory [21] activities. Ayoub [22] isolated phenolic compounds of *C. erectus* leaves, such as gallic acid, myricetin 3-O-β-glucupyranuronide, quercetin 3-O-glucupyranuronide, syringetin 3-O-β-glucupyranuronide, and 3,3′,4′-trimethoxyellagic acid 4-O-β-glucupyranuronide, that showed high antioxidant potential.

Finding ways of purifying active compounds responsible for the bioactivities of medicinal plants and employing them as chemotherapeutic or adjuvants in the treatment of diseases have been the goals of many scientific studies. In the present work, we investigated the chemical characteristics, antioxidant effect and immunomodulatory potential of

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a lignin isolated from *C. erectus* to determine if it was the active component responsible for the described biological properties of this plant.

2. Materials and methods

2.1. Plant material

Leaves from *C. erectus* var. *erectus* were collected on 24 March 2017 in a mangrove at Ilha de Itamaracá, Pernambuco, Brazil (7°48'43.249" S, 34°51'15.896" W). Green leaves were selected that were visually intact, free of pests, diseases and did not show altered color. A voucher specimen was deposited in the Herbarium Geraldo Mariz of the *Universidade Federal de Pernambuco* under the number 75457. The access was recorded (AC84C40) at the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen).

2.2. Extraction of lignin from C. erectus leaves

The dried leaves of *C. erectus* (100 g) were initially milled in a knife mill (Pulverisette 14; FRITSCH GmbH - Milling and Sizing, Idar-Oberstein, Germany) and sieved with 100 mm granulation. Then, the powder was submitted to extraction in a Soxhlet apparatus for 8 h, using a toluene: ethanol (38:62) system to remove the extractives. The mass, free of extractives, was then subjected to acid hydrolysis with 1% H_3PO_4 at 121 °C for 1 h, at a 1:10 solid: liquid ratio. The resulting material (acid hydrolysate) was then submitted to alkaline delignification with 1% NaOH under the same conditions of the acid hydrolysis. The resulting supernatant (black liquor) was acidified with H_2SO_4 to pH 2.0 at 30 °C, and let stand for 12 h. The mixture was filtered on filter paper and thoroughly washed with water (2 L) until neutralized. The solid lignin was dried at 70 °C for approximately 72 h. For use in biological assays, the lignin was dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) to a concentration of 1 mg/mL (stock solution) and then diluted in distilled water.

2.3. Lignin acetylation

Acetylation was performed according to the procedure described by Lenz [23]. About 30 mg of lignin was dissolved in 5 mL pyridine (100%) and 5 mL acetic anhydride (100%). The mixture was bubbled for 15 min with nitrogen and the bottle was sealed. The system was left at 28 °C in the dark for 50 h. At the end of the reaction, the excess of acetic anhydride was removed by the addition of 4 mL of methanol. The solvents were evaporated under reduced pressure using azeotrope formation with toluene and ethanol. Finally, the samples were completely dried for 3 days under vacuum.

2.4. Chemical characterization of lignin from C. erectus leaves

Fourier transform infrared spectroscopy (FTIR) analysis of *C. erectus* lignin (20 mg) was performed on a Bruker Tensor 27 spectrometer (Bruker AXS, Inc., Madison, WI, USA) using the attenuated total reflectance accessory (Platinum ATR). The spectra were recorded in the spectral range from 4000 to 500 cm^{-1}, with a resolution of 2 cm^{-1} and 20 scans. The sample was placed on the diamond crystal of the ATR accessory for measurement.

Nuclear magnetic resonance spectroscopy (^1H and ^{13}C NMR) of acetylated lignin was also performed. Acetylated lignin (20 mg) was dissolved in $CDCl_3$ and subjected to ^1H nuclear magnetic resonance on a Bruker Avance 300 spectrometer (Bruker AXS, Inc., Madison, WI, USA) operating at 400 MHz frequency with a 5 mm probe.

The average molecular weight, the numeric molecular weight, and polydispersity index of the lignin was evaluated using a chromatographic system of gel permeation. The lignin (0.4 mL; 2.0 mg/mL) was injected in a Sephadex G-50 (GE Healthcare Life Sciences, Sweden) column (57.0 × 1.8 cm) using 0.5 M NaOH as mobile phase. Fractions of 4 mL were collected in a flow rate of 0.4 mL/min and the absorbance of each fraction was monitored at 280 nm. The number average molecular weight (Mn) and average molecular weight (Mw) were calculated according to Rocha et al. [24]. The polydispersity index corresponded to the ratio Mw/Mn. The chromatographic column was calibrated with proteins of known molecular mass.

For spectrophotometric analysis of the lignin in the ultraviolet-visible region, samples were solubilized in 0.01 M sodium hydroxide solution (pH 12.0) to a concentration of 0.5 g/mL and spectra were obtained in a range from 190 to 400 nm in a spectrophotometer.

2.5. Determination of the total phenolic content in lignin from C. erectus leaves

Total phenolic content was measured in accordance with Li et al. [25]. The Folin-Ciocalteau solution (2 mL; 1:10, v/v) was added to 0.2 mL of lignin (1.0 mg/mL). After 4 min, 1.6 mL of sodium carbonate (7.5%, w/v) was added followed by incubation for 120 min in the dark at 28 °C. The absorbance at 765 nm was measured against a blank (10% DMSO). A calibration curve was prepared using gallic acid (3.9–500 μg/mL) and the following linear equation ($y = 0.0023x + 0.014$; $R^2 = 0.9858$). The assay was performed in triplicate and the phenol content was expressed as gallic acid equivalents (mg/g GAE).

2.6. Antioxidant activity assays

2.6.1. Total antioxidant activity

The total antioxidant activity of the lignin was determined according to Li et al. [26]. Lignin, at 500 μg/mL (0.3 mL), was mixed with 3 mL of phosphomolybdenum solution (600 mM sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated in water at 95 °C for 90 min. After returning to room temperature (28 °C), the absorbance at 695 nm was measured in a spectrophotometer against a blank (3 mL of solution and 0.3 mL of 5% DMSO). Ascorbic acid and butylated hydroxytoluene (BHT) were used as standard molecules in the same concentrations as lignin. Total antioxidant activity (TAA) was calculated by the formula $TAA\ (\%) = [(A_t - A_{tb}) / (A_{aa} - A_b)] \times 100$, where: A_b was the absorbance of the blank, A_t was the absorbance of the test and A_{aa} was the absorbance of the control using ascorbic acid. The assay was performed in triplicate.

2.6.2. DPPH radical scavenging activity

The ability of scavenging the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was measured as described by Blois [27], with modifications. Two milliliters of a 1 mM DPPH solution in methanol (absorbance at 570 nm = 0.650 ± 0.050) was added to 0.32 mL of lignin solution at different concentrations (3.9, 7.8, 15.6, 31.25, 62.5, 125, 200 and 500 μg/mL). After a 30 min incubation time protected from light, the absorbance at 517 nm was measured in a spectrophotometer. In the control, the DPPH solution was added to 5% DMSO. Methanol was used as the blank. The sequestration of DPPH radicals was calculated by the formula: $\text{Scavenging effect}\ (\%) = [(A_c - A_t) / (A_c)] \times 100$, where A_c was the absorbance in the control assay and A_t was the test. The concentration required to inhibit the DPPH by 50% (IC_{50}) was determined by linear regression analysis. Ascorbic acid and BHT were used as standards in the same concentrations as lignin. The assay was performed in triplicate.

2.6.3. $ABTS^+$ scavenging activity

The method reported by Cano et al. [28] was performed to analyze the antioxidant activity against the $ABTS^+$ radical. The $ABTS^+$ radical solution was prepared from 5 mL of 7 mM $ABTS^+$ [2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] in methanol and 88 μL of 140 mM potassium persulfate. The mixture was kept in the dark at room temperature (28 °C) for 16 h. Thereafter, 1 mL of $ABTS^+$ solution was diluted in ethyl alcohol to an absorbance of 0.70 ± 0.05 at 734 nm. Then, 30 μL of

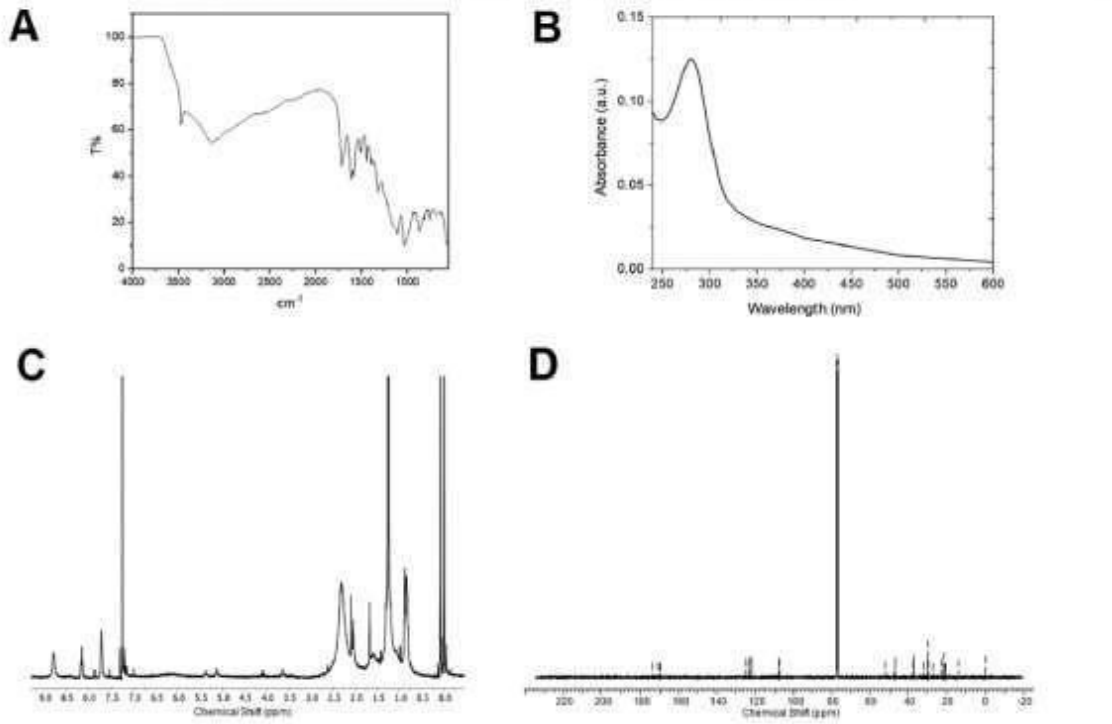

Fig. 1. Chemical analysis of *Conocarpus erectus* lignin by Fourier transform infrared spectroscopy (A), ultraviolet–visible (UV–Vis) spectrophotometry (B), proton nuclear magnetic resonance, ^{1}H NMR (C) and carbon-13 nuclear magnetic resonance, ^{13}C NMR (D).

different concentrations of lignin (3.9, 7.8, 15.6, 31.25, 62.5, 125, 200 and 500 μg/mL) were added to 3.0 mL of $ABTS^{+}$ diluted solution. After a 30 min incubation time protected from light, the absorbance at 730 nm was measured. The lignin was replaced by 5% DMSO in the control and ethanol was used as the blank. The percent inhibition was calculated according to the formula: Scavenging effect (%) = $[(A_c - A_t) / (A_c)] \times 100$, where A_c was the absorbance in the control and A_t was the absorbance in the test. The IC_{50} was calculated as described in the previous section. Ascorbic acid and BHT were used as standards in the same concentrations as lignin. The assay was performed in triplicate.

2.6.4. Lipid oxidation inhibition assay

The ferric thiocyanate (FTC) method, used to measure the amount of peroxide in initial stages of lipid oxidation, was performed as described by Kikuzaki and Nakatani [29]. An emulsion of linoleic acid was prepared by homogenizing 1 mg of linoleic acid in 50 mL of ethanol. An aliquot of the lignin (0.2 mL at 0.5 mg/mL) was individually mixed with 0.204 mL of the linoleic acid emulsion, 0.4 mL of 2.5 M phosphate buffer pH 7.0 and 0.196 mL of distilled water, packed in glass vials and incubated in an air circulation oven at 40 °C. Every 24 h, 50 μL of the assay was withdrawn and added to 50 μL of 75% ethanol, 50 μL of 30% ammonium thiocyanate and 50 μL of a chloride solution of 0.02 M iron in 3.5% HCl. After 3 min at room temperature (28 °C), the absorbance at 500 nm was measured. The degree of inhibition of linoleic acid peroxidation was calculated using the equation: Inhibition (%) = $[(A_c - A_t) / A_c] \times 100$, where A_c corresponded to the final mean absorbance in the control and A_t was the final mean absorbance in the test. Gallic acid was used as the 100% activity control and water was used as the blank. Ascorbic acid and BHT were used as standard molecules in the same concentrations as lignin. The assay was performed in triplicate.

2.7. Evaluation of cytotoxicity and immunomodulatory activity

2.7.1. Culture of human PBMCs

The isolation of lymphocytes and monocytes from human peripheral blood mononuclear cells (PBMCs) was performed according to Santos et al. [21]. Experimental protocols were approved by the Ethics Committee from the *Universidade Federal de Pernambuco* (no. 1.870.360/2016; CAAE 62119716.5.0000.5208). Blood samples were collected from healthy volunteers (non-smokers who were not using medication) that signed a 'Term of Free and Informed Consent'. PBMCs were obtained after centrifugation (400 ×g, 30 min) under a Ficoll-Hypaque (1.077 g/mL; GE Healthcare Life Sciences, Sweden) separation gradient. The cells were washed twice with PBS (400 ×g, 10 min) and then cultivated in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (w/v) fetal bovine serum (Sigma-Aldrich) in

Table 1
Relative distribution (%) of the signal areas in the chemical shift regions (ppm) of *Conocarpus erectus* lignin ^{1}H NMR spectra.

Regions	Range (ppm)	Area	%
Unoxygenated aliphatic	1.7–0.6	1.0	45.9
Aliphatic hydroxyl	2.2–1.7	0.24	11.0
Phenolic hydroxyl	2.5–2.2	0.4	18.3
Aliphatic	3.3–2.5	0.27	12.4
Methoxyl	4.0–3.3	0.21	9.6
Aliphatic	5.2–4.1	0.09	4.1
Benzene cyclic	5.7–5.2	0.09	4.1
Non-cyclic benzene	6.2–5.7	0.11	5.0
Aromatic	8.1–6.2	0.16	7.3
Carboxylic acids and aldehydes	10.0–8.1	0	0.0
Solvent (deuterated chloroform)	7.3–7.2	0.39	17.9
Total	10.0–0.6	2.18	100.0

Table 2
Antioxidant activities promoted by *C. erectus* lignin and its total phenol content.

Sample	DPPH	ABTS	TAA	FTC	Total phenols
	IC_{50} (μg/mL)	IC_{50} (μg/mL)	% (0.5 mg/mL)	% (0.5 mg/mL)	(mgGAE/g)
Lignin	231.16	356.03	17.92 ± 0.41	42.14 ± 0.64	465.90 ± 1.07
Ascorbic acid	240.30	90.93	100	71.77 ± 0.33	–
BHT	140.56	7.67	98.80 ± 1.24	50.64 ± 0.62	–

DPPH – 2,2-diphenyl-1-picrylhydrazyl radical.
ABTS – 2,2′-azino-bis(3-ethylbenzothiazoline)- 6-sulfonic acid radical.
TAA – Total antioxidant activity.
FTC – Ferric thiocyanate method.
BHT – butylated hydroxytoluene.

48-well plates at 5% CO_2 and 37 °C. Cell cultures (10^6 cells/well) were used for subsequent assays.

2.7.2. Analysis of cell viability

PBMCs were treated with the lignin (2.5, 5.0, 10.0, 20.0, 40.0 or 80.0 μg/mL) or concanavalin A (Con A, 5 μg/mL; Sigma-Aldrich) for 24 h and then cell death was analyzed using the FITC Annexin V Apoptosis Detection Kit I from BD Biosciences (Franklin Lakes, NJ, USA), according to the manufacturer's instructions. Cells cultured in the presence of 1% DMSO were used as the negative control. Flow cytometry was performed in a FACS Calibur platform (BD Biosciences) with a minimum of 10,000 events acquired. The results were analyzed using Flowing Software version 2.5.1 (Cell Imaging Core, Turku Center for Biotechnology, Finland). The experiment was performed six times.

2.7.3. Cell proliferation assay

Cell proliferation analysis was performed using the CFSE (carboxyfluorescein succinimidyl ester) method as described by Cruz-Filho et al. [12]. A suspension of PBMCs was adjusted to 10^6 cells/mL in complete RPMI 1640 medium, 2 μL of 5 mM CFSE was added, and the cells were incubated for 10 min at 25 °C in the dark. Then, the cells were washed twice with sterile PBS and treated for 24 h with the lignin (2.5, 5 and 10 μg/mL) or Con A (5 μg/mL). Cells cultured in the presence of 1% DMSO were used as the negative control. After this period, the cells were obtained by centrifugation (400 ×g, 5 min), resuspended in PBS and 10,000 events were acquired on the FACS Calibur platform. Lymphocyte and monocyte subsets were identified based on the forward scatter (FSC) *vs.* side scatter (SSC) plots. The results were analyzed using Flowing Software version 2.5.1. The experiment was performed six times.

2.7.4. Measurement of cytosolic calcium concentration

Cytosolic Ca^{2+} levels ($[Ca^{2+}]_{cyt}$) were measured using the Fluo-3AM probe (Thermo Fisher Scientific, Waltham, MA, USA). PBMCs were treated with the lignin (10 μg/mL) for 24 h or 1% DMSO as the negative control. After the incubation, the cells were washed with PBS and centrifuged (400 ×g, 5 min). The supernatant was discarded and cell pellets were incubated (5% CO_2, 37 °C, 40 min) with Fluo-3 AM (5 μM), pluronic acid F-127 (1 μM; Sigma-Aldrich) and bovine serum albumin

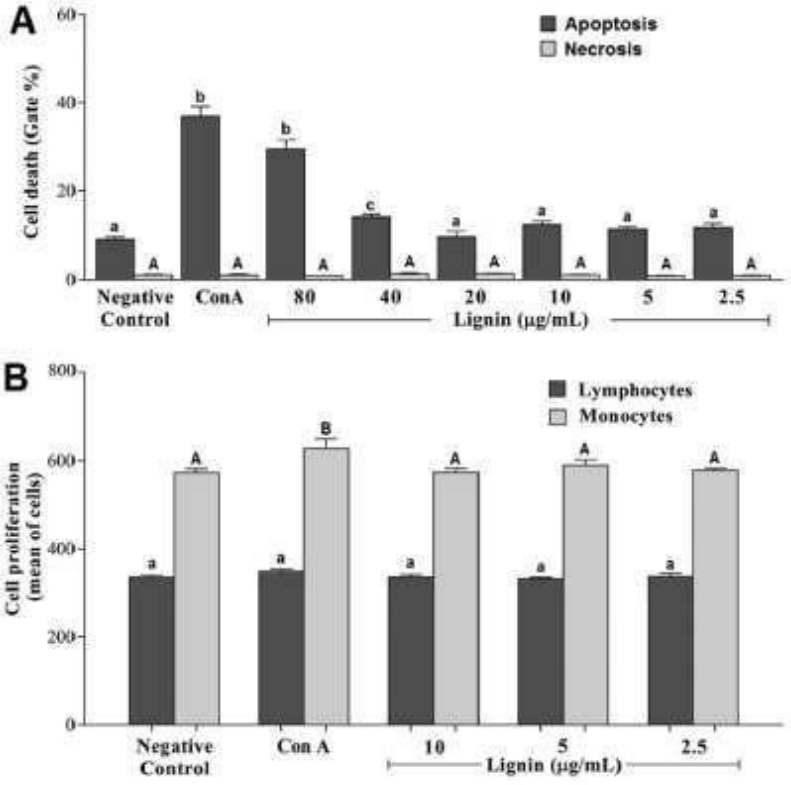

Fig. 2. Effects of *Conocarpus erectus* leaf lignin on the viability and proliferation of human peripheral blood mononuclear cells (PBMCs). (A) Evaluation of the cytotoxic effect of lignin (2.5–80 μg/mL) by flow cytometry using Annexin V (AnnV) and propidium iodide (PI). AnnV−/PI+ cells were considered to be necrotic and AnnV+/PI− cells were considered to be apoptotic. (B) Evaluation of the proliferation of lymphocytes and monocytes from human PBMCs, using the 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) probe, incubated with or without the lignin (2.5, 5 or 10 μg/mL). For the negative control, cells were incubated with 1% dimethyl sulfoxide (DMSO) while concanavalin A (Con A, 5 μg/mL) was used as the positive control. Both assays were performed after 24 h of treatment. The bars represent the average of six independent experiments. Different uppercase or lowercase letters indicate significant differences ($p < 0.05$) between treatments.

(30 μg/mL; Sigma-Aldrich). The cells were then washed with PBS, centrifuged (400 ×g, 5 min) and transferred to cytometer tubes. A minimum of 10,000 events was collected and the fluorescence intensity was analyzed on the FACS Calibur cytometer with excitation set at 395 nm and emission at 525 nm. Maximum fluorescence was measured using cells incubated with 1 μM ionomycin for 2 min, and minimal fluorescence was measured using cells incubated with 8 mM EDTA for 2 min. The results were analyzed using Flowing Software version 2.5.1 and $[Ca^{2+}]_{cyt}$ was calculated using the equation: $[Ca^{2+}]_{cyt} = K_d$ (test fluorescence − minimal fluorescence) / maximum fluorescence − test fluorescence, where K_d was 390 nM [30,31]. The experiment was performed five times.

2.7.5. Evaluation of reactive oxygen species (ROS) levels

Cytosolic and mitochondrial levels of reactive oxygen species (ROS) were measured by flow cytometry, using the dihydroethidium (DHE) (Sigma-Aldrich) and MitoSox Red (Thermo Fisher Scientific) probes, respectively [31]. PBMCs (10^6 cells/mL) were incubated for 24 h with lignin (10 μg/mL) or 1% DMSO as the negative control. After incubation, cells were washed with PBS (400 ×g, 5 min), transferred to cytometer tubes, and incubated in a CO_2 incubator with 5 μM DHE (40 min) or with 5 μM MitoSox Red (10 min). After incubation, 1 mL of PBS was added, cells were centrifuged (400 ×g, 5 min), the supernatant was discarded and the pellet was resuspended in 0.3 mL of PBS. Fluorescence intensity was analyzed using a FACS Calibur flow cytometer, with excitation at 488 nm and emission at 620 nm. A minimum of 10,000 events was collected and the results were analyzed using Flowing Software version 2.5.1. The experiment was performed six times.

2.7.6. Determination of the mitochondrial transmembrane potential (ΔΨm)

Changes in mitochondrial transmembrane potential (ΔΨm) were measured using the MitoStatus probe (BD Biosciences). PBMCs were incubated with the lignin (10 μg/mL) for 24 h or 1% DMSO as the negative control. After incubation, the cells were washed with PBS, centrifuged (400 ×g, 5 min) and the pellet was transferred to cytometer tubes. The cells were then incubated in a CO_2 incubator with 100 nM of MitoStatus for 30 min. After incubation, cells were washed with PBS, centrifuged (400 ×g/5 min) and transferred to cytometer tubes. A minimum of 10,000 events was collected using a FACS Calibur flow cytometer and the results were analyzed using Flowing Software version 2.5.1. The experiment was performed six times.

2.7.7. Immunophenotyping assay

Immunophenotyping assays of lymphocytes and monocytes were performed according to Santos et al. [19]. After 24 h of treatment with the lignin (10 μg/mL), the cells were removed from the plates using ice-cold PBS and transferred to polypropylene tubes (BD Biosciences) with 6 mL of PBS for centrifugation (400 ×g, 10 min). After discarding the supernatant, cells were washed with 2 mL of PBS and centrifuged (400 ×g, 10 min). The supernatant was discarded and surface monoclonal antibodies were added to the tubes and incubated for 30 min. Then, two washing steps were performed with 1 mL of PBS followed by centrifugation (400 ×g, 5 min). Supernatants were discarded and cells were fixed for 15 min with 150 mL of Cytofix solution (BD Biosciences) and then washed with 2 mL of PBS followed by centrifugation (400 ×g, 5 min). After discarding the supernatant, 300 μL of PBS was added to each tube and 10,000 events were acquired on the FACS Calibur platform and the results were analyzed using Flowing Software version 2.5.1. The positive control corresponded to cells treated with Con A (5 μg/mL) and the negative control to cells treated with 1% DMSO. The monoclonal antibodies used were anti-CD4-PerCP, anti-CD8-FITC, anti-CD-28-PE, anti-CTLA-4-APC, anti-CD14-FITC, anti-CD80-PE, anti-CD-86-APC and anti-HLA-DR-PerCP (BD Biosciences). The experiment was performed six times.

2.7.8. Measurement of cytokines and nitrite release

Cytokines and nitrite levels were quantified in supernatants of PBMC cultures treated with the lignin (10 μg/mL) and Con A (5 μg/mL) for 24 h or cells incubated with 1% DMSO as the negative control. Cytokine assessment was carried out using the Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine kit (BD Biosciences) for detection of interleukin (IL)-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ according to the manufacturer's instructions. The data were acquired on the FACS Calibur platform and the results were analyzed using Flowing Software 2.5.1.

Nitrite concentration was estimated by the Griess method [32] using a standard curve of sodium nitrite ($y = 0.007x + 0.0256$; $R^2 = 0.9978$). The absorbance at 595 nm was measured in a microplate Multiskan spectrophotometer (Thermo Fisher Scientific). The experiment was performed five times.

2.8. Statistical analysis

The GraphPad Prim 7 software (GraphPad Software, San Diego, CA) was used for statistical analysis. The Shapiro–Wilk test was applied to test the normality of the hypothesis and the means of replicates were analyzed by non-parametric tests. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA). The differences were considered significant when $p < 0.05$.

3. Results and discussion

The bands obtained in the FTIR spectrum for *C. erectus* lignin can be seen in Fig. 1A. The analysis showed bands at 3453 cm^{-1} (corresponding to the OH groups), 2842 cm^{-1} (C—H stretch in methyl groups), 1715 cm^{-1} (stretch C = O in unconjugated ketones, carbonyls and ester groups), 1682 cm^{-1} (stretch C = O unconjugated), 1593 cm^{-1} (aromatic skeletal vibrations influenced by a C = O stretch), 1515 cm^{-1} (aromatic skeletal vibrations), 1457 cm^{-1} (asymmetric C—H deformations), 1430 cm^{-1} (aromatic skeletal vibrations combined with C—H deformation in the plane), 1327 cm^{-1} (syringyl ring C—O), 1221–1230 cm^{-1} (C—C, C—O, C = O stretch), 1166 cm^{-1}

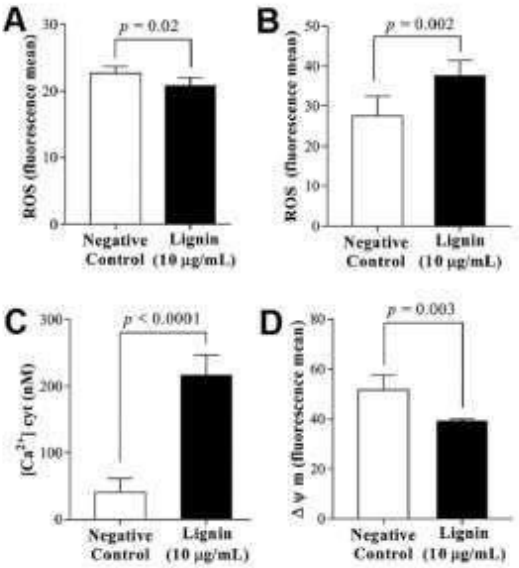

Fig. 3. Evaluation of the effects of *Canocarpus erectus* leaf lignin (10 μg/mL) treatment on cytosolic (A) and mitochondrial (B) reactive oxygen species (ROS) production, cytosolic calcium concentration (C), and membrane mitochondrial potential (ΔΨm) (D) on human lymphocytes after incubation for 24 h. Bars represent the mean ± standard deviation of six independent experiments.

(C = O stretch in conjugated ester groups), 1030–1035 cm^{-1} (aromatic C—H plane strain, primary alcohol C—O strain, influenced by an unconjugated C = O stretch) and 832 cm^{-1} (off-plane C—H deformation at positions 2, 5 and 6 of unit G) [33–37]. Therefore, it was possible to identify that this lignin was of the guaiacyl-syringyl-*p*-hydroxyphenyl (GSH) type.

The UV–Vis spectrum (Fig. 1B) revealed that *C. erectus* lignin showed a maximum absorption around 280 nm indicating a greater number of G-type monomers, corresponding to the electronic transition π → π* in the aromatic ring, typical of lignin units [38,39]. This bathochromic shift shows high G-unit content. S-type lignins have absorption maximums in the region of 268–276 nm, while absorption in the region between 310 and 320 nm is indicative of the presence of type H units and at 320 nm there is absorption of the - CH = CHCOOH units [39].

The ^{1}H NMR spectrum of *C. erectus* lignin (Fig. 1C) was integrated into defined regions and signals were identified according with the works of Gonçalves et al. [39], García et al. [40] and Rocha et al. [24]. Signals with a chemical shift in the region of aromatic hydrogens, around 7.00 ppm, were attributed to the protons of the aromatic ring of alcohols (*p*-coumaryl, coniferyl and synaphyl) as well as vinyl protons. Signals at 6.7–6.8 ppm and 7.0 ppm were assigned to aromatic protons in syringyl propanol and guaiacyl propanol units, respectively. The side-chain protons from syringyl units appeared in the 6.60 ppm region. This was observed with respect to signals in the region 6.0–6.2 ppm that were vinyl protons. Hydrogens attached to the α and β carbons showed signals at 5.1 and 4.7 ppm, respectively. Signals at 4.2 and 4.4 ppm corresponded to the H-γ of various lignin units. A characteristic signal of methoxyl protons was observed at 3.65 ppm. Signals between 3.1 and 3.20 ppm were characteristic of H-β in β-β structures. The 1.60 ppm and 2.60 ppm signals originated from aliphatic and phenolic hydroxyls, respectively. Signals <1.7 ppm were related to CH_3 and CH_2 groups in saturated aliphatic chains. Table 1 shows the relative distribution (%) and signal area in the chemical shift regions (ppm).

^{13}C NMR signals for *C. erectus* leaves lignin (Fig. 1D) were similar to those observed in other lignins [12,41–43]. Signals for the γ-methyl as well as α and β-methylene groups of the lignin n-propyl side chains appear in the spectrum between 13 and 40 ppm. From 20.3 to 31.8 ppm, methyl terminal groups, aliphatic ring methylene groups and

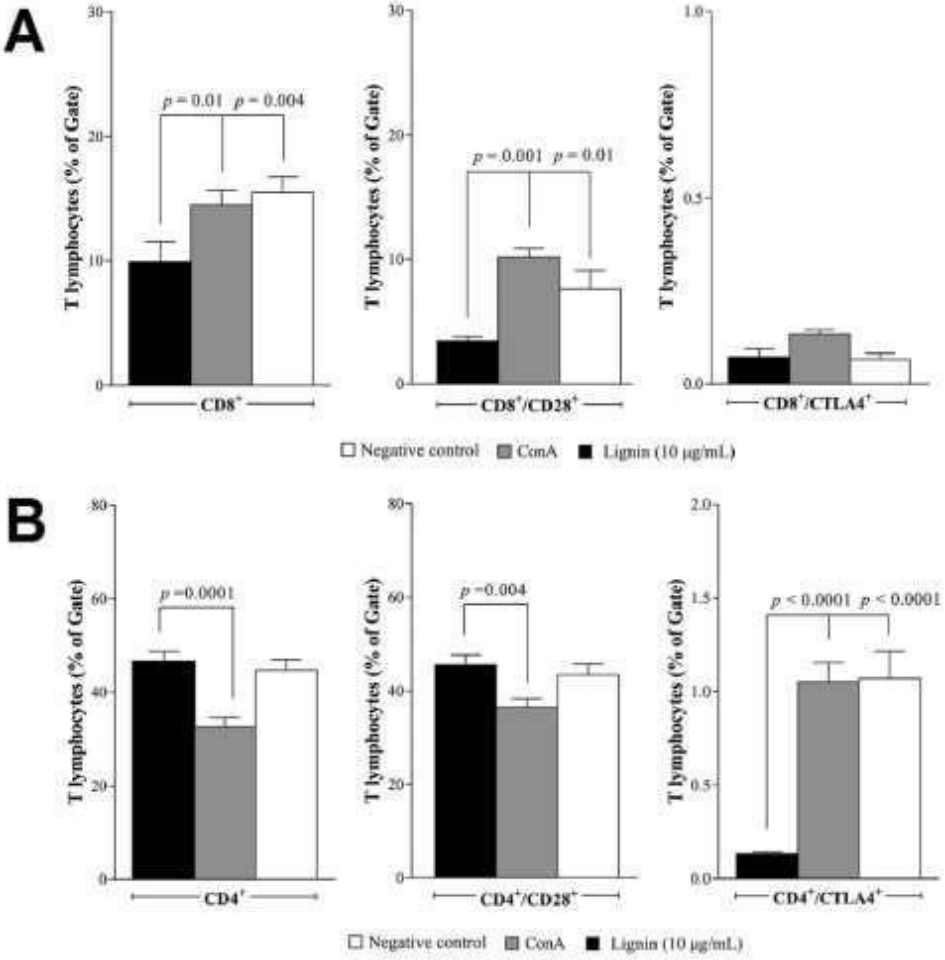

Fig. 4. Immunophenotyping of T lymphocytes (A, B) and monocytes (C) in cultures treated with the *Conocarpus erectus* lignin (10 μg/mL). Numbers of differentiated $CD8^+$ T (A) or $CD4^+$ T (B) lymphocytes as well as cells expressing the co-stimulatory receptor CD28 or the co-inhibitory receptor CTLA4. (C) Numbers of differentiated $CD14^+$ monocytes expressing the co-stimulatory receptors CD80 and CD86 or the human leukocyte antigen of the DR isotype (HLA-DR). For the negative control, cells were incubated with 1% dimethyl sulfoxide (DMSO) while concanavalin A (Con A, 5 μg/mL) was used as the positive control. The bars represent the average of six independent experiments.

methylene groups can be observed in long alkyl chains. The region between 46 and 53 ppm is assigned to C-β in β-β and β-5 units. The methoxyl groups in units of syringyl (S) and guaiacyl (G) types are assigned around 56.1 ppm. Signals between 76 and 78 ppm refer to the solvent $CDCl_3$. The region between 106.2 and 107.7 ppm is assigned to C-2/C-6 in syringyl units with α-CO, 123.0 to 123.3 ppm assigned to C5 (Cα-OAc and –OR) and Cβ in acetylated guaiacyl units. At 121.8 ppm the signal refers to C-1 and C-6 in Ar ~ C (= O) C ~ C. Signs of p-coumaric acid are observed at 124.6–124.7 ppm. From 169,1 at 169.7 ppm and from 170 at 173 ppm signals attributed to CO in aromatic acetyl and CO in aliphatic acetyl are observed, respectively.

The lignin showed an average molecular weight of 2709 Da, a number average molecular weight of 1279 Da and a polydispersity index value of 2.1. A low polydispersity gives the compound higher solubility [44] and indicates that most lignin fragments have similar molecular weight [24].

Table 2 shows the results of total antioxidant activity assays, the IC_{50} values found for the DPPH and $ABTS^+$ scavenging activities, and the results of the FTC method. The lignin showed a low total antioxidant activity in comparison with ascorbic acid and BHT. In the DPPH assay, the IC_{50} for lignin was near to that of ascorbic acid but higher than that of BHT. In the $ABTS^+$ assay, the lignin showed weak activity, with an IC_{50} 3.9- and 46.4-fold higher than the values for ascorbic acid and BHT, respectively. The lignin exhibited a moderate ability to inhibit lipid peroxidation, similar to BHT and lower than ascorbic acid.

The lignin contained a total phenol content of 465.90 mg/g GAE and the phenols present in its structure could be responsible for the detected antioxidant power. Polyphenolic compounds have been widely studied for their ability to prevent the propagation of reactive oxygen species (ROS) and the damage caused by them [45]. There are reports that free phenolic hydroxyl groups present in lignins are crucial for their antioxidant activity and the radical scavenging property depends on the exchange potential of a free radical for a hydrogen atom of a phenol molecule [46,47]. In contrast to the *C. erectus* lignin, other studies have shown lignins as promising molecules with high antioxidant potential, such as rice straw lignin (*Oryza sativa*), which promoted high scavenging of DPPH (88%) and $ABTS^+$ (99%) radicals at a concentration of 80 μg/mL [48]. In addition, the corn cob lignin showed an IC_{50} of 260 μg/mL and 28 μg/mL in the DPPH and $ABTS^+$ assays, respectively [49].

The *C. erectus* lignin did not induce death (apoptosis or necrosis) of human PBMCs when tested until the concentration of 20 μg/mL (Fig. 2A). Similar results were found for the lignins from *Opuntia ficus-indica* and *Opuntia cochenillifera*, which did not induce the death of BALB/c mouse splenocytes [12]. The Con A positive control induced apoptosis of about 36% of the PBMC population. The *C. erectus* lignin did not stimulate the proliferation of lymphocytes and monocytes (Fig. 2B) when compared to the negative control, but also did not cause downregulation of cell growth. Con A stimulated monocyte proliferation as expected [50] but did not stimulate lymphocyte proliferation.

For immunomodulatory assays, the lignin was tested at a concentration of 10 μg/mL because this is a low, non-toxic concentration usually used in other studies and would be enough to induce activity; in addition, lower doses are indicated to avoid immunosuppression due to intolerance by immune cells [51–53].

The oxidative stress response, without inducing cell death, is crucial for differentiation and activation of immune cells. Lymphocytes treated with the *C. erectus* lignin showed a small reduction in cytosolic ROS levels (Fig. 3A), but a significant increase in mitochondrial ROS (Fig. 3B) and cytosolic Ca^{2+} levels (Fig. 3C). Moreover, lignin promoted a decrease in the mitochondrial membrane potential (Fig. 3D).

Reactive oxygen species have been reported as molecules that are involved in signaling and regulation of immune responses, acting as mediators of cell activation, differentiation and proliferation [54,55]. Ca^{2+} also regulates fundamental cellular processes, including immune cell metabolism, differentiation, gene expression, and plays a regulatory role in cell survival and death processes [31,56]. Elevated cytosolic Ca^{2+} levels may lead to increased uptake of Ca^{2+} by mitochondria during lymphocyte activation, triggering the increase of mitochondrial ROS production [57]. However, to maintain cellular activation without exacerbated enhancement of ROS production (which can be detrimental to the cell), the mitochondrial transmembrane potential is reduced to values that still allow ATP production while keeping ROS levels potentially harmless to cells [58]. The *C. erectus* lignin promoted increased cytosolic Ca^{2+} levels, followed by increased mitochondrial ROS, with a decrease in the mitochondrial transmembrane potential.

The *C. erectus* lignin induced differentiation of the $CD8^+$ T lymphocyte subset as well as its activation ($CD8^+/CD28^+$) (Fig. 4A). While the lignin did not induce differentiation of the co-inhibitory CTLA-4 receptor by T $CD4^+$ lymphocytes, it increased its expression (Fig. 4B). In addition, the lignin induced $CD14^+$ monocyte differentiation but the expression of CD80, CD86 and HLA-DR molecules in lignin-treated monocytes were not significantly different when compared to the negative control (Fig. 4C).

Studies describe the important role of the adaptive immune system, especially the action of $CD8^+$ T cells in the immune response against

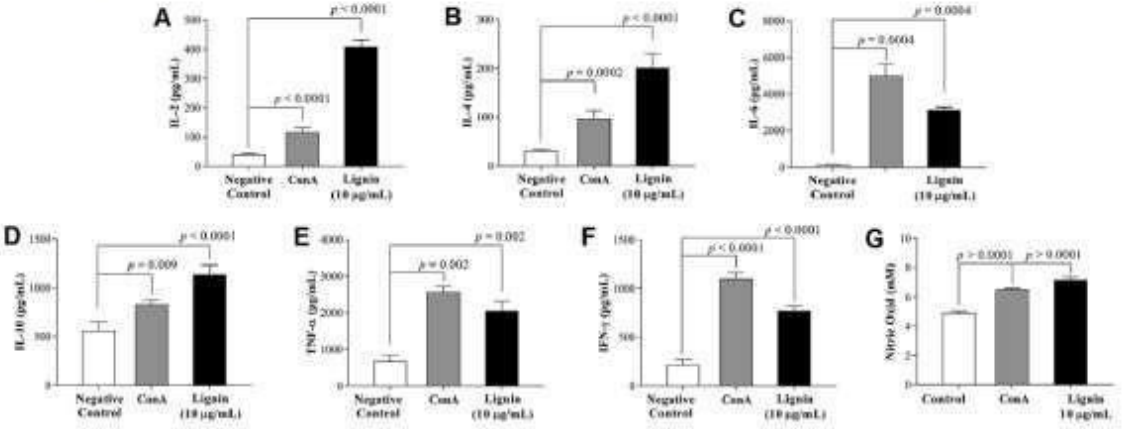

Fig. 5. Cytokines [IL-2 (A), IL-4 (B), IL-6 (C), IL-10 (D), TNF-α (E), IFN-γ (F)] and nitric oxide (G) release by PBMCs treated with the *Consolea erectus* lignin (10 μg/mL). For the negative control, cells were incubated with 1% dimethyl sulfoxide (DMSO) while concanavalin A (Con A, 5 μg/mL) was used as the positive control. The bars represent the average of six independent experiments.

tumors [59]. Antitumor strategies have been developed based on the use of molecules that lead to increased immunity based on increased $CD8^+$ T cell expression [60]. The ability of polyphenols to enhance differentiation and activation of cytotoxic $CD8^+$ T cells and cytokine production has been reported [61–63]. $CD8^+$ T lymphocytes are also recruited to infected tissues to help eliminate intracellular pathogens by cytolysis [64] while high $CD14^+$ expression is responsible for the efficacy of the human peripheral blood monocyte response to antigens [65].

C. erectus lignin stimulated the release of all evaluated cytokines in PBMCs (Fig. 5A–F). A large increase in the levels of IL-2, IL-4, IL-6, TNF-α and IFN-γ was observed, while a moderate increase of IL-10 was observed in response to lignin stimulation. There was a larger increase in the levels of cytokines secreted by cells involved in a Th1 response. Considering two Th1/Th2 response indexes, the ratios of IFN-γ/IL-4 and TNF-α/IL-10 were approximately 2 and 4, respectively, indicating a predominant pro-inflammatory response. In addition, there was a significant increase in the release of nitric oxide (NO) when compared to the control (Fig. 5G).

Similar results are found for lignins isolated from other plants. A previous study showed that cacao husk lignin induces the release of the pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ as well as NO by RAW264.7 macrophage cells [7]. Cellulase-treated lignin from barley husk stimulates bone marrow derived dendritic cells to produce TNF-α and IL-12p40 [66]. A lignin-carbohydrate complex from the mushroom *Inonotus obliquus* also stimulates NO release and phagocytic activity in RAW 264.7 cells [67].

The pro-inflammatory cytokines IFN-γ and TNF-α are important in the immune response due to their antitumor potential and role in infection control by the activation of natural killer (NK) cells and cytotoxic T lymphocytes; in addition, IFN-γ is a key mediator in NO production by activated macrophages [68]. Similar to our results, Cruz-Filho et al. [12] also showed that lignins isolated from *Opuntia ficus-indica* and *Opuntia cochenillifera* induce the release of TNF-α, IL-6 and IL-10 and suggested that these lignins can be evaluated in future investigations involving wound repair, antiviral therapies and immunological tools.

4. Conclusion

A G-S-H type lignin was isolated from *C. erectus* leaves with antioxidant potential and immunomodulatory ability. The lignin promoted differentiation of lymphocytes and monocytes, activated $CD8^+$ T cells, and promoted the release of nitrite and cytokines linked to both Th1 and Th2 responses, but mainly those involved in a proinflammatory response. These results prove that the lignin is an active component involved in the biological properties of *C. erectus*. The data stimulate future studies on the *in vivo* immunomodulatory potential of *C. erectus* lignin in infections, healing, and tumor models.

CRediT authorship contribution statement

Dayane Kelly Dias do Nascimento Santos: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Bárbara Rafaela da Silva Barros:** Data curation, Investigation. **Lethícia Maria de Souza Aguiar:** Data curation, Investigation. **Iranildo José da Cruz Filho:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. **Virgínia Maria Barros de Lorena:** Methodology, Resources, Validation. **Cristiane Moutinho Lagos de Melo:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Thiago Henrique Napoleão:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing - review & editing.

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5 ARTIGO 2 – PECTIN-LIKE POLYSACCHARIDE EXTRACTED FROM THE LEAVES OF *CONOCARPUS ERECTUS* LINNAEUS PROMOTES ANTIOXIDANT, IMMUNOMODULATORY AND PREBIOTIC EFFECTS

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Pectin-like polysaccharide extracted from the leaves of *Conocarpus erectus* Linnaeus promotes antioxidant, immunomodulatory and prebiotic effects

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ABSTRACT

Pectins are complex heteropolysaccharides made up of a linear main chain of repeated units of galacturonic acid that can promote different biological activities. This work aimed to isolate and elucidate the chemical structure of a polysaccharide similar to pectin, obtained from *C. erectus* leaves. Furthermore, we evaluate the antioxidant and immunomodulatory potential promoted by this macromolecule. The physical-chemical analyzes showed that the pectin obtained showed low degree of esterification and low molecular weight. In addition, it presented in its structure galacturonic acid, glucose, xylose, rhamnose and arabinose. In antioxidant assays *in vitro* the pectin expressed high activity for the DPPH and ABTS methods. In addition, the pectin obtained was able to promote the growth of probiotic microorganisms *in vitro*. Immunological tests have shown that pectin was able to stimulate immune cells through the oxidative stress mechanism, promoting lymphocyte proliferation and activation TCD4+ and inhibit lymphocyte activation TCD8+ and stimulate its effector mechanisms through the production of cytokines and nitric oxide, mainly producing a pro-inflammatory immune response, without causing damage to cells. These results highlight the potential use of pectin extracted from *C. erectus* leaves as an antioxidant, prebiotic and immunomodulatory agent.

1. Introduction

Conocarpus erectus Linnaeus is a mangrove plant, popularly known as mangrove bud, found in Africa, Central and South America. This plant belongs to the family Combretaceae and has high resistance to the adverse conditions typically present in this environment (Linnigen et al., 2009). *C. erectus* is used in folk medicine to treat intestinal disorders and infectious diseases, as well as being used as an anti-inflammatory agent (Sabi-Ur-Rehman et al., 2019). Studies with leaves and fruits of *C. erectus* demonstrated high content of phenolic compounds, mainly flavonoids and tannins (Sabi-Ur-Rehman et al., 2019; Santos et al., 2018). Recently Santos et al. (2020), characterized the leaf lignin and verified its immunomodulatory potential. In another study Santos et al. (2018) featuring the *C. erectus* leaves verified the presence of different polyoses, among them a polysaccharide in which the authors named pectin.

Pectins are water-soluble dietary fibers and represent one of the main components of plant cell walls. The structure and degree of methylation of pectins are different in each plant, and their biological properties are related to the conditions of extraction, sources and stage of maturation of the plant (Mercheb et al., 2019; Nisar et al., 2018). Some studies have shown that pectins can be applied as a nutritional supplement (Ciriminna et al., 2016). Pectin has nutraceutical benefits, being used as a prebiotic potential that has shown favorable effects on human health (Amorim et al., 2016). As a source of dietary fiber, it is associated with gastrointestinal health, glucose tolerance, lipid digestion and consequent weight control (Ciriminna et al., 2016; Lefnih et al., 2018).

Pectins are described in the literature as immunomodulatory agents (Mercheb et al., 2019), antioxidants (Nisar et al., 2018) and anti-inflammatory compounds (Ramachandran et al., 2017). We also point out that the absence of toxicity, the ability to gel and the presence of functional groups in its structure, which can be easily modified

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(-COOH, -OH), allow its application as an excipient in drug delivery systems (Zhang et al., 2015).

Immunomodulatory molecules are able to alter the activities of the immune system through the regulation of effector molecules such as cytokines, hormones, neurotransmitters and other peptides. In addition to activation, differentiation of immune cells (Nair et al., 2019). The search for better treatments for related diseases or immunological changes has increased, and the elucidation of the immunomodulatory effects of natural compounds as a way to discover new immunotherapeutic strategies has increased (Ortuño-Sahagún et al., 2017).

In this work, we report the isolation and partial elucidation of the chemical structure of the pectin obtained from *C. erectus* leaves. In addition, we sought to study *in vitro* the antioxidant and immunomodulatory potential in order to propose it in the future as a low-cost therapeutic agent.

2. Results and discussion

2.1. Physico-chemical characterization of pectin from leaves of C. erectus

2.1.1. Yield of extraction and analysis of compositional pectin

The yield of obtaining the pectin under study was 3.3 ± 0.2% (w/w dry matter). This value was close to those obtained by Yuliarti, Goh, Matia-Merino, Mawson, and Brennan (2015) for golden kiwi pectin (*Actinidia chinensis*) that was 3.62% (w/w), and by Chan and Choo (2013) who obtained extraction yields that varied from 3.33 ± 0.23 to 3.92 ± 0.24 (w/w). When compared to that obtained by Liu et al. (2006) with orange peel pectins (2.2% w/w), the pectin yield of *C. erectus* leaves was considered greater. However, when compared to Santos et al. (2013) who obtained income that varied from 4.61 to 19.2% for pectins obtained from sisal residues, it was considered minor. The difference in the yield value is directly related to the extraction method and the source of obtaining the pectins (Chan & Choo, 2013; Santos et al., 2013).

The pectin obtained from *C. erectus* leaves presented moisture content in its composition 01.4 ± 0.3% and total ash from 14.7 ± 0.5%. In addition, it was possible to determine the content of different monosaccharides present in the chemical structure, these being galacturonic acid (35.90 ± 0.03%), glucose (3.93 ± 0.01%), xylose (14.45 ± 0.06%), rhamnose (9.86 ± 0.1%) and arabinose (16.17 ± 0.5%). Furfural were also quantified (6.5 ± 0.6%) and 5-hydroxymethylfurfural (13.0 ± 0.07%). Degradation products of monosaccharides with five and six carbons, formed during acid hydrolysis with trifluoroacetic acid. The same behavior has been described in obtaining pectins in eggplant food processing (Kazemi et al., 2019), grapefruit peels (Wang et al., 2016), citrus peels (Jiang et al., 2012), pectin obtained from *Tamarindus indica* L. pulp (Sharma et al., 2015), artichoke by-products pectin (Sabater et al., 2020) and broccoli (Petkowicz & Williams, 2020).

The results obtained in the composition of the monosaccharides allowed to determine the contents of two different polysaccharides present in the structure of the pectin, HG (Homogalacturonan) and RG-I (Rhamnogalacturonan-I). The values obtained for these polysaccharides were 71.8 ± 0.1% to (RG-I) and 26.1 ± 0.5% to (HG). Other authors have also determined the values of these polysaccharides. However, these levels may vary according to the method of extraction and quantification of the monosaccharides, and the source of obtaining the pectin. (Jiang et al., 2012; Petkowicz & Williams, 2020; Sabater et al., 2020; Sharma et al., 2015; Wang et al., 2016).

2.1.2. Structural characterization by fourier transform infrared spectroscopy (FT-IR)

Through FTIR analysis (Fig. 1) was possible to identify the main functional groups, the pectin extracted from *C. erectus* leaves. The bands found were previously assigned by Oberemko et al. (2019), Macial et al. (2015), Cerná et al. (2003) and Gnanasambandam and Proctor (2000) for the characterization of different pectins.

The pectin spectrum under study showed a wide absorption band

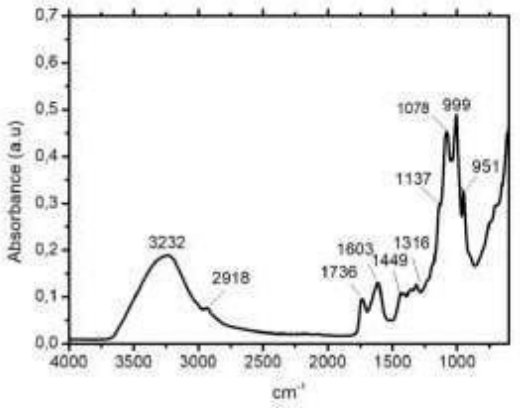

Fig. 1. FTIR spectrum of pectin leaves from *C. erectus*.

between 3100 and 3500 cm^{-1} attributed to axial deformation of hydroxyl groups in alcohol intermolecular hydrogen bonds, commonly found in polysaccharides (Cerná et al., 2003). The band in 2929 cm^{-1} attributed to the axial strain of the connection C-H, found in the region between 3000 and 2840 cm^{-1} (Oberemko et al., 2019). The band found in the region between 1790 and 1720 cm^{-1} is associated with esterified carboxylic groups (-$COOCH_3$) and bands between 1660 and 1590 cm^{-1}, associated with free carboxylic groups (-COOH) (Gnanasambandam & Proctor, 2000; Oberemko et al., 2019). The band in 1347 cm^{-1} is attributed to the asymmetric deformation of C-O (Macial et al., 2015). The absorption bands between 1200 and 1000 cm^{-1} are associated with connections COC and CC in the pectin molecules (Cerná et al., 2003). Finally, bands in 1000 - 600 cm^{-1} are assigned to connections β-glycosides and α-glycosides (Gnanasambandam & Proctor, 2000; Oberemko et al., 2019; Cerná et al., 2003).

The pectin in this study showed a low degree of esterification (37.5 ± 0.3%), that is, the value obtained is less than 50% (Thakur et al., 1997). This result of a low degree of esterification is similar to that found in other studies, such as sisal residue pectin (*Agave sisalana*) (44.35%) (Yang et al., 2018), dragon fruit peel pectin (34.78%) (Nguyen & Pirak, 2019), common fig bark (*Ficus carica*) (39.42%) (Gharibzahedi et al., 2019a), Common fig bark (33.65%) (Gharibzahedi et al., 2019b), *Opuntia ficus indica* cladodes (41.42%) (Bayar et al., 2017) and *Suaeda fruticosa* (33%) (Mzoughi et al., 2018). Low esterification pectins are characterized by forming gels in the presence of divalent cations such as calcium, in a pH range of 2.5–7, not requiring large amounts of sugars, which is ideal for the preparation of gels dietary (Thakur et al., 1997).

2.1.3. Proton nuclear magnetic resonance spectroscopy analysis (^1H NMR)

Spectroscopy of ^1H RMN was used to elucidate the chemical structure of pectin. The signals obtained in the spectrum of ^1H NMR (Fig. 2) for the pectin in this study were previously assigned by Marcon et al. (2005), Cheng and Neiss (2012) and Shakhmatov et al. (2019) evaluating different pectins.

Through the spectrum presented in Fig. 2, it is possible to observe the signals related to the anomeric hydrogens H-1 and H-5 of methyl esters (-$COOCH_3$), galacturonic acids are located in the region between 4.9 and 5.25 ppm, respectively. The signal with chemical displacement in 4.60 ppm is related to the pectin dissolving solvent D_2O (deuterated water). O protons related to carbons two and three (H-2 and H-3) are related to the signals that appear around 3.7 ppm and 4.0 ppm (Marcon et al., 2005). Thus, the signals that appear in 3.72 ppm and 3.97 ppm, are assigned to protons H-2 and H-3, respectively (Shakhmatov et al., 2019). In 4.4 ppm there are protons of carbon four (H-4). The signal in 3.6 ppm was

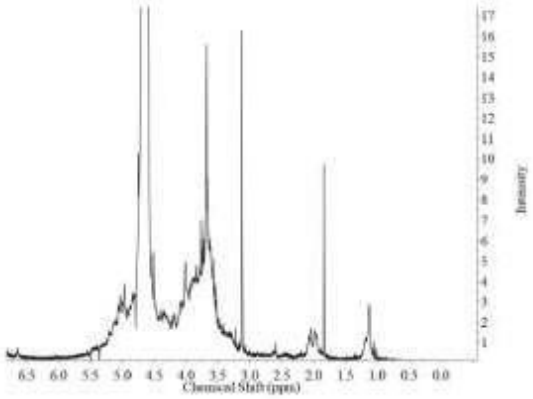

Fig. 2. ^{1}H NMR spectrum of pectin leaves from *C. erectus*.

assigned to methoxyl groups linked to carboxylic groups of galacturonic acid. Around 2.0 ppm there are two signs refer to acetyl groups attached to the 2-O- e 3-O- Galacturonic acid and finally a signal in 1.14 ppm assigned to methyl groups (Cheng & Neim, 2012; Marcon et al., 2005; Shakhmatov et al., 2019).

2.1.4. Thermogravimetric analysis

The thermogravimetric analysis (TGA) provided information on the loss of mass of the material (%) proportional to the increase in temperature. Fig. 3 shows the mass loss curves (TGA) and the rate of mass loss represented by the first derivative (DTG) obtained for the pectin under this study.

The TGA curve shows that degradation of pectin from *C. erectus* occurs in three stages. This degradation profile has also been reported by other authors evaluating different pectins (Combo et al., 2013; Mangiacapra et al., 2006; Wang et al., 2016; Zhou et al., 2011). The results show a first loss of mass between 30 °C and 100 °C, being attributed to the evaporation of the residual and structural water, a fact directly associated with the hydrophilic nature of the pectin functional groups. (Jiang et al., 2020). In this first phase of decomposition, a loss of mass of around 5.3% occurred. The second mass loss event is in the range of 100 °C–370 °C and is associated with primary and secondary decarboxylation, breakdown of bonds or functional groups and breakdown of the main pectin chain, resulting in a 76.5% loss of mass (Wang et al., 2016). And finally, a third event above 400 °C related to the degradation of decarboxylation by-products (Zhou et al., 2011). The residual mass value was 18.3%, a value close to that obtained for the total ash determined by the composition analysis.

The DTG curve showed that the *C. erectus* pectin showed a maximum degradation temperature around 249 °C. In the literature we find results similar to ours. Qiu et al. (2019) evaluated the effect of pre-drying methods on the structure and properties of the pectins extracted from the Chinese quince, showed a maximum degradation temperature ranging from 233 to 240 °C. Jiang et al. (2020) pectin extracted from persimmon obtained maximum temperature ranging from 236.91 to 242.13 °C. Güzel and Akpınar (2019) evaluated pectins from fruit peels obtained maximum temperature of degradation in the range of 210–250 °C.

2.1.5. X-ray diffraction analysis and viscosimetric molecular weight

Fig. 4 shows the diffractogram for the pectin of *C. erectus* leaves. This analysis was carried out with the objective of obtaining information about the structure of the pectin (amorphous or crystalline). The signals obtained in the 10 to 50° region are characteristic of polysaccharides and show that the pectin under study has a predominance of crystalline regions. Other pectins reported in the literature showed a predominance of crystalline regions. Among these we can mention: pectin obtained from eggplant food processing (Kazemi et al., 2019), those found in grapefruit peels (Wang et al., 2016), in citrus fruit peels (Jiang et al., 2012) and that obtained from the *Tamarindus indica* pulp (Sharma et al., 2015). However, not all pectins have a predominance of crystalline regions. Chomto and Nunthanid (2017) featuring different citrus pectins, found they were an amorphous solid. The amorphous or crystalline behavior of pectins is directly related to the source of extraction and the types of extraction method (Jiang et al., 2012; Kazemi et al., 2019).

The molecular mass of pectins also varies according to the parameters cited. The pectin obtained from the *C. erectus* leaves presented molecular weight of 24.0 ± 0.2 kDa value close to those obtained by Morris et al. (2000) and Morris et al. (2008) for commercial pectins.

2.2. Determination of antioxidant potential and total phenol content in vitro

The phenolic content found in pectins is directly related to the extraction method. These compounds during the process of obtaining can adhere to the pectin structure and promote an increase in antioxidant activity (Hosseini et al., 2019; Khan et al., 2010). Furthermore, low phenolic values are indicative of purity (Hosseini et al., 2019). The *C. erectus* pectin presented a content of total phenolic compounds around 23 ± 0.7 mg GAE/g of pectin. In the works reported by Kazemi

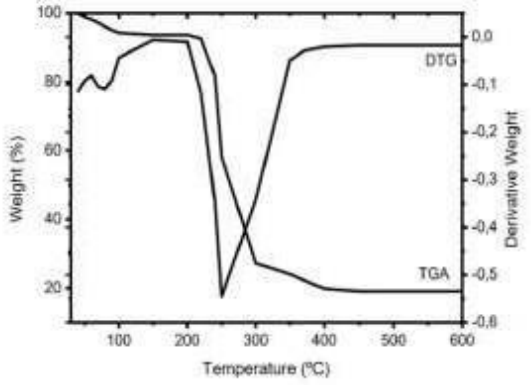

Fig. 3. TGA and DTG curves for leave pectin from *C. erectus*.

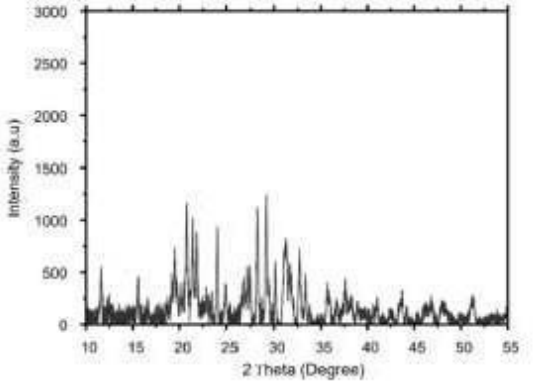

Fig. 4. X-ray diffraction pattern of leaves pectin from *C. erectus*.

et al. (2019) for pistachio green shell pectin (18.18 mg GAE/g of pectin) and Hosseini et al. (2019) for *Citrus aurantium* bark pectin (39.95 ± 3.13 mg GAE/g pectin) were approximately that obtained for the pectin under study.

The phenolic content in the chemical structure of *C. erectus* pectin can promote greater antioxidant effect. However, this effect was enhanced due to the presence of free hydroxyl groups. Studies have shown that free hydroxyl groups can promote good antioxidant activity when the viscosity is not too high (Huang et al., 2017; Mirzanova et al., 2018; Ro et al., 2013). This fact was confirmed by the low viscosimetric mass found for the pectin in this study. In addition, the composition and arrangement of monosaccharides can also contribute to increased antioxidant activity (Encalada et al., 2019; Eça et al., 2015; Nisar et al., 2018; Oguro & Um, 2016; Ramachandran et al., 2017). The monosaccharides present in pectin can act as reducing agents, as they can provide protons, which can react with radicals and form a stable compound to eliminate the radical reaction (Eça et al., 2015; Oguro & Um, 2016).

The *C. erectus* pectin showed high antioxidant potential (dose-dependent) in the concentration of 500 μg/mL in the ABTS test with percent inhibition of 96.02 ± 0.41% similar to the natural pattern, ascorbic acid (92.16 ± 0.25%) and with activity close to the standard synthetic BHT (95.55 ± 0.07%) with a difference of less than 10% (9.53%) according to Fig. 5A. Pectin also showed moderate activity (dose-dependent) in the concentration of 500 μg/mL (76.54 ± 0.14%) in the DPPH assay, when compared to the BHT and ascorbic acid standards (Fig. 5B). The values of IC_{50} calculated for *C. erectus* pectin in DPPH and ABTS assays can be seen in Table 1.

The results presented in Table 1 show that pectin can act as a proton donor and can eliminate free radicals DPPH and ABTS. Similar to our results, modified citrus pectin (MCP), the association of pectin films with fruit extracts, sweet potato pectin and the association of citrus pectin films with clove essential oil showed potent antioxidant activities (Encalada et al., 2019; Eça et al., 2015; Nisar et al., 2018; Oguro & Um, 2016; Ramachandran et al., 2017).

2.3. Immunomodulatory activity promoted by pectin from *C. erectus* leaves

2.3.1. Cytotoxicity and proliferation assays

Cytotoxicity assays showed that *C. erectus* pectin at the concentrations under study (2.5–80.0 μg/mL) did not promote cell death of human PBMC (Fig. 6A). Hizn et al. (2018) also found similar behavior investigating human lymphocytes treated with pectin-guar-zinc oxide gum (PEC-GG-ZnO) in concentrations from 25 to 200 μg/mL. In addition, normal NIH-3T3 fibroblasts treated with water-soluble pectin of *Opuntia ficus-indica* at higher concentrations (0.1 a 2 mg/mL) also showed no significant increase in cell death (Lefsih et al., 2018). Pectin promoted the proliferation of immune cells in a non-significant way when compared to the negative control (Fig. 6B). Since no concentration had a cytotoxic effect, the lowest concentrations of pectin were chosen (2.5; 5.0 e 10 μg/mL) to assess the proliferation index of immune cells.

Common criteria for screening immunomodulatory molecules indicate that lower doses should be sufficient to induce activity without producing toxicity (Liu et al., 2010; Northrup et al., 2016; Zheng et al., 2016). In addition, these compounds in high concentration can cause immunosuppression, suggesting that lower doses are needed to induce immunological tolerance (Northrup et al., 2017; Wong et al., 2015). So, we chose the concentration of 10 μg/mL commonly used to assess the effects of cell activation and immunomodulation of natural products.

2.3.2. Changes in the levels of reactive oxygen species, cytosolic calcium and in the mitochondrial membrane potential promoted by pectin in PBMCs

Studies investigating the cellular immune response show that calcium ions and reactive oxygen species contribute to lymphocyte activation, differentiation and effector functions (Peske, 2007; Lewis, 2001; Melo et al., 2010). In addition, mitochondrial signaling dependent on ROS levels controls numerous biological responses associated with the cell cycle and adaptation to stress (Martinez-Reyes et al., 2016).

The *C. erectus* pectin did not cause changes in the levels of cytosolic ROS Figure 7A when compared to the negative control, however it was able to increase levels of mitochondrial ROS (Fig. 7B) and cytosolic $[Ca^{2+}]$ (Fig. 7C) In addition, it was able to promote a decrease in the mitochondrial membrane potential ($\Delta\Psi m$) of immune cells treated with pectin (Fig. 7D). On the other hand, Con A lectin promoted increases in the membrane potential $\Delta\Psi m$ and in the concentration of cytosolic Ca^{2+}, but did not interfere in the levels of mitochondrial and cytosolic

Table 1
Antioxidant activity was assessed by DPPH and ABTS assay. Significance levels are represented as treated with *C. erectus* pectin vs treated groups with BHT and Ascorbic acid, ***$p < 0.0001$.

Sample	DPPH	ABTS	Total phenolic content
	IC_{50} (μg/mL)	IC_{50} (μg/mL)	mg GAE/g
Pectin	109.21	240.25	23 ± 0.7
BHT	140.56	7.67	–
Ascorbic acid	69.51	90.93	–

DPPH – 2,2-diphenyl-1-picrylhydrazyl radical.
ABTS – 2,2'-azino-bis(3-ethylbenzothiazoline)- 6-sulfonic acid radical.
BHT – butylated hydroxytoluene.

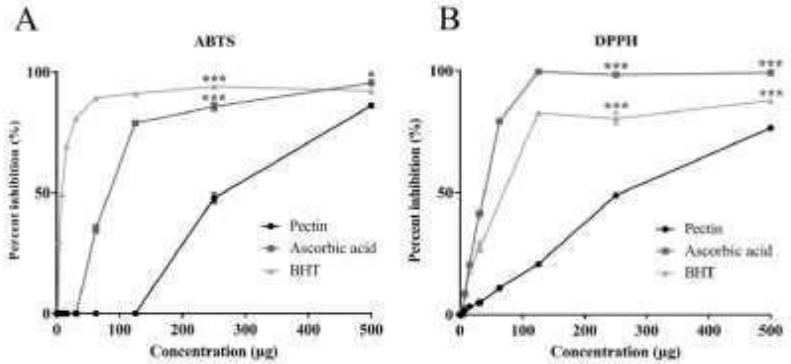

Fig. 5. Percentage inhibition of free radicals assessed by ABTS (A) and DPPH (B) assay. Significance levels are represented as treated with *C. erectus* pectin vs treated groups with BHT and Ascorbic acid in the concentrations of 250 and 500 μg; *$p < 0.02$, ***$p < 0.0001$. The differences were considered significant when $p < 0.05$.

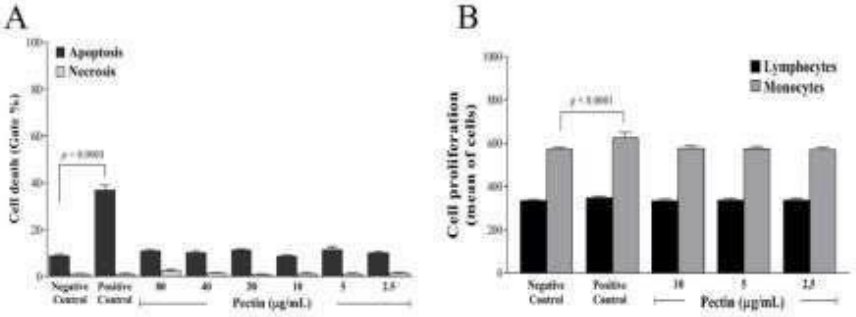

Fig. 6. Effects of *C. erectus* leave pectin on the viability and proliferation of human peripheral blood mononuclear cells (PBMCs) in 24 h of incubation. A – Cytotoxic effect of pectin in human PBMC. B – Proliferation induction by pectin in PBMC. In negative control, cells were incubated only in culture medium while in positive control cells were treated with concanavalin A (5 µg/mL). The bars represent the average of the experiment performed in quintuplicate. The differences were considered significant when $p < 0.05$.

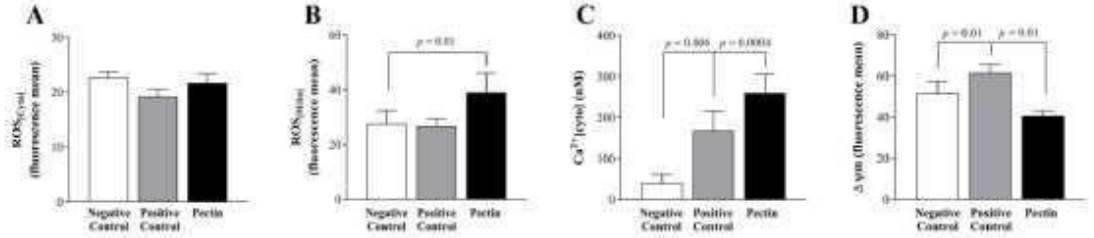

Fig. 7. Evaluation of the effects of *C. erectus* leave pectin (10 µg/mL) treatment on (A) cytosolic and (B) mitochondrial reactive oxygen species (ROS) levels, (C) cytosolic calcium concentration $[Ca^{2+}]_{cyto}$ and (D) membrane mitochondrial potential ($\Delta\Psi m$) (D) on human peripheral blood mononuclear cells (PBMCs) after incubation for 24 h. In negative control, cells were incubated only in culture medium while in positive control cells were treated with concanavalin A (5 µg/mL). The bars represent the average of the experiment performed in quintuplicate. The differences were considered significant when $p < 0.05$.

ROS when compared to the negative control. Increased levels of mitochondrial ROS and cytosolic calcium associated with decreased mitochondrial membrane potential promoted by *C. erectus* pectin indicate a mechanism of lymphocyte activation.

2.3.3. Immunophenotyping assays and production of cytokines and nitric oxide

The immunomodulatory effects of pectins are directly related to their chemical structure. Studies have shown that pectins that have galacturonic acid levels below 75% and molecular weight below 100 kDa have high antioxidant and immunostimulatory potential. In addition, these pectins are characterized by a large branched portion, represented by rhamnogalacturonan -I (RG-I) (Ho et al., 2016; Klosterhoff et al., 2016).

Immunophenotyping assays for lymphocytes and monocytes showed that *C. erectus* pectin promoted the proliferation and activation of $TCD4^+$ lymphocytes (Fig. 8A) This result was confirmed by the inhibition assay since pectin is not able to stimulate inhibition of $TCD4^+$ (Fig. 8B). The pectin did not stimulate the activation of $TCD8^+$ lymphocytes (Fig. 8C) result confirmed by inhibition tests (Fig. 8D). Regarding monocytes, there was no stimulus for the proliferation of $CD14^+$, $CD14^+/CD80^+$, $CD14^+/CD86^+$ e $CD14^+/HLADR^+$. The Con A, in turn, promoted only the proliferation of monocytes ($CD14^+$) (Fig. 8E).

The evaluation of cytokine production showed that cells treated with pectin produced higher levels of all investigated cytokines compared to the negative control. As shown Fig. 9, the pectin stimulates the production de IL-2 with concentration of 124.14 ± 16.55 pg/mL (Fig. 9A), IL-4 with 525.79 ± 23.90 pg/mL (Fig. 9B), IL-6 with 4202.89 ± 330.72 pg/mL (Fig. 9C), IL-10 with 970.96 ± 263.45 pg/mL (Fig. 9D), TNF- α with 2734.17 ± 965,69 pg/mL (Fig. 9E) and IFN-γ with 1478,30 ± 67,19 pg/mL (Fig. 9F).

When analyzing the relationships IFN-γ: IL-4 (ca. 3: 1) and TNF- α: IL-10 (ca. 3: 1), it can be seen that the Th1 response was more prevalent compared to Th2. High nitrite production (Fig. 9G) is also an indicator of a prevalent Th1 response. Different cytokines were produced by cells treated with *C. erectus* pectin.

The results showed the prevalence of a Th1 response associated with high stimulation of $TCD4^+$ lymphocytes. Similar to our results, Mezhebi et al. (2019) investigating the insertion of natural (CP) and modified (MCP) citrus pectins in the diet of Balb/c mice, they found increased levels of IL-17, IFN-γ and TNF-α, also suggesting a Th1 response stimulus. Pectin-guar-zinc oxide gum (PEC-GG-ZnO) induced a significant increase in cytokines IFN-γ, IL-2 and TNF-α in lymphocytes CD3, CD8 and CD56 (Hira et al., 2015). The water-soluble heteropolysaccharide of *Plantago asiatica* L. leaves stimulated the production of pro-inflammatory cytokines, including TNF-α and IL-1β in macrophage RAW264.7 cells (Yin et al., 2019). And a pectic edible jambo arabinogalactan (Fruits of Syzygium jambos) showed immunomodulatory properties with intrinsic activation capacity to induce cytokine secretion (TNF-α, IL-1β and IL-10) by THP-1 macrophages (Tamiello et al., 2019).

Galactose-rich pectins have modulating effects on the cellular and humoral immune response (Hira et al., 2015). Studies have shown that these molecules influence macrophage activity by stimulating phagocytic activity and increasing the secretion of cytokines that mediate innate immunity (Mezhebi et al., 2019; Yamasaki et al., 2015). The cellular modulation mechanisms of these molecules are based on the possibility that this polymer binds to the same cell receptors for bacterial polymers due to its structural similarity (Riede et al., 2013). Therefore,

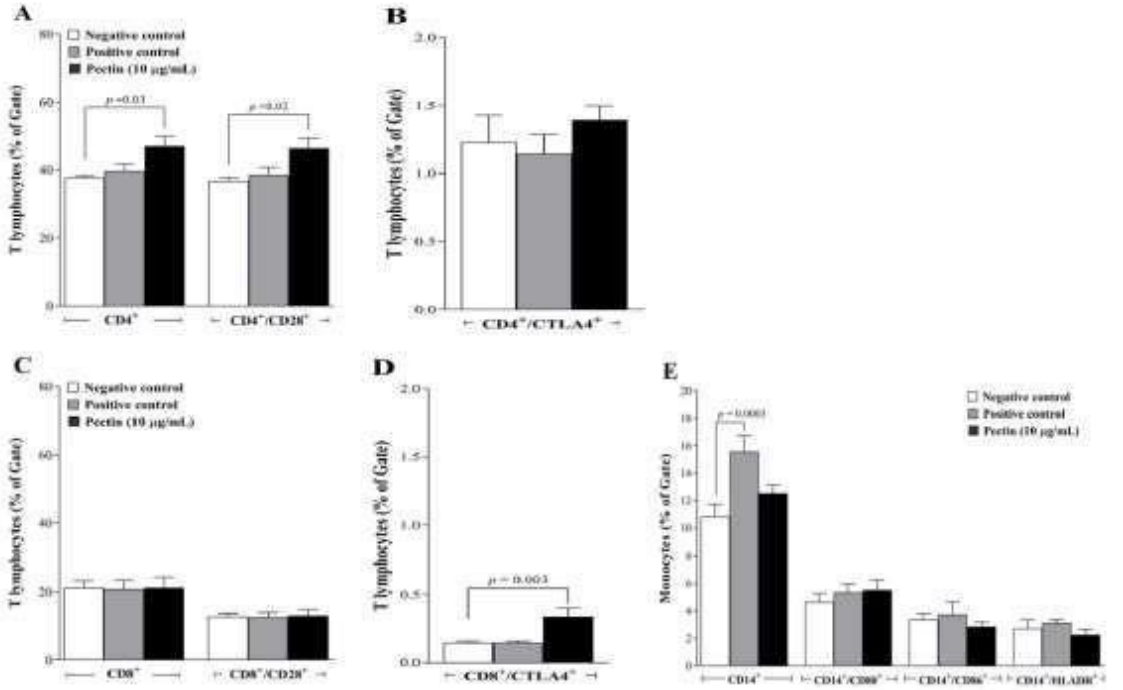

Fig. 8. Immunophenotyping of T lymphocytes and monocytes in cultures treated with *C. erectus* pectin (10 µg/mL). In negative control, cells were incubated only in culture medium while in positive control cells were treated with concanavalin A (5 µg/mL). Vertical bars represent the average of one experiment performed in quintuplicate. The differences were considered significant when $p < 0.05$.

considering that plant polysaccharides have low toxicity and do not cause significant side effects (Seyfried et al., 2016), pectins are potential candidates for the development of new therapeutic agents with immunomodulating properties.

2.3.4. *In vitro tests of prebiotic activity*

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or some microorganisms in the intestinal colon (Caleffi et al., 2015; Huebner et al., 2008; Kalidas et al., 2017). During growth, prebiotics are hydrolyzed to monomers by bacterial glycosidases to monomers which during microbial metabolism are degraded to short-chain fatty acids (acetic, lactic butyric and propionic acids) and different gases (Ho et al., 2016). Different pectic polysaccharides have been isolated and their effects as prebiotics evaluated (Ho et al., 2017; Zhang et al., 2018). Fig. 10 shows the results of growth in individual cultures of *Lactobacillus* (*L. paracasei* and *L. rhamnosus*) in culture media with the carbon source of inulin (commercial probiotic) and the *C. erectus* pectin at 0, 24 and 48 h.

Inulin-based culture media promoted the growth of *L. paracasei* (Fig. 10 A, B, C) and *L. rhamnosus* (Fig. 10 D, E, F) as expected. The *C. erectus* pectin (low degree of esterification, < 50%) was also able to promote the growth of the cited lactobacilli. Similar results were obtained by Li et al. (2016) evaluating the prebiotic potential of pectin oligosaccharides found that low degrees of esterification favor microbial growth.

Different studies evaluating pectin polysaccharides as prebiotics have shown that in addition to promoting the growth of beneficial bacteria, they act directly on the immune system. Van Doan et al. (2018) and Van Doan et al. (2019) using orange peel pectins as prebiotics they found that they positively stimulated the immune system favoring the growth of tilapia fish. Similar results have recently been obtained by Hoseinifar et al. (2021) adding dietary pectin derived from apple peel to carp fish feed. Homogalacturonan oligomers also demonstrated a probiotic effect and were able to stimulate human peripheral blood mononuclear cells producing IL-10 anti-inflammatory cytokine in *in vitro* assays (Chung et al., 2017). Singh et al. (2020) found that linear and branched oligosaccharides of different types of pectin, had immuno- and bacterio-modulatory effects. These results indicate that the pectin obtained can promote beneficial effects on human and animal health.

3. Conclusion

In the present work, a water-soluble polysaccharide was isolated and purified from the *Conocarpus erectus* leaves. Structural elucidation studies, as well as the antioxidant and immunostimulant potentials were investigated for the first time. The results of the chemical characterization showed that the polysaccharide obtained had a heterogeneous and quite complex structure with a predominance of galacturonic acid, which allowed it to be compared to the pectin. In vitro assays significantly promoted DPPH and ABTS radical sequestration in a dose dependent manner within the tested concentration range. The pectin obtained favored the growth of probiotic microorganisms and can be considered as prebiotic agents. In addition to promoting microbial proliferation, he was able to exhibit immunomodulatory effects promoting cell activation, lymphocyte proliferation and increased production of pro-inflammatory cytokines. These results provide a scientific basis for further studies on the use of this natural molecule which can be used as an adjuvant in immunological therapies and functional food. Thus promoting benefits to human and animal health.

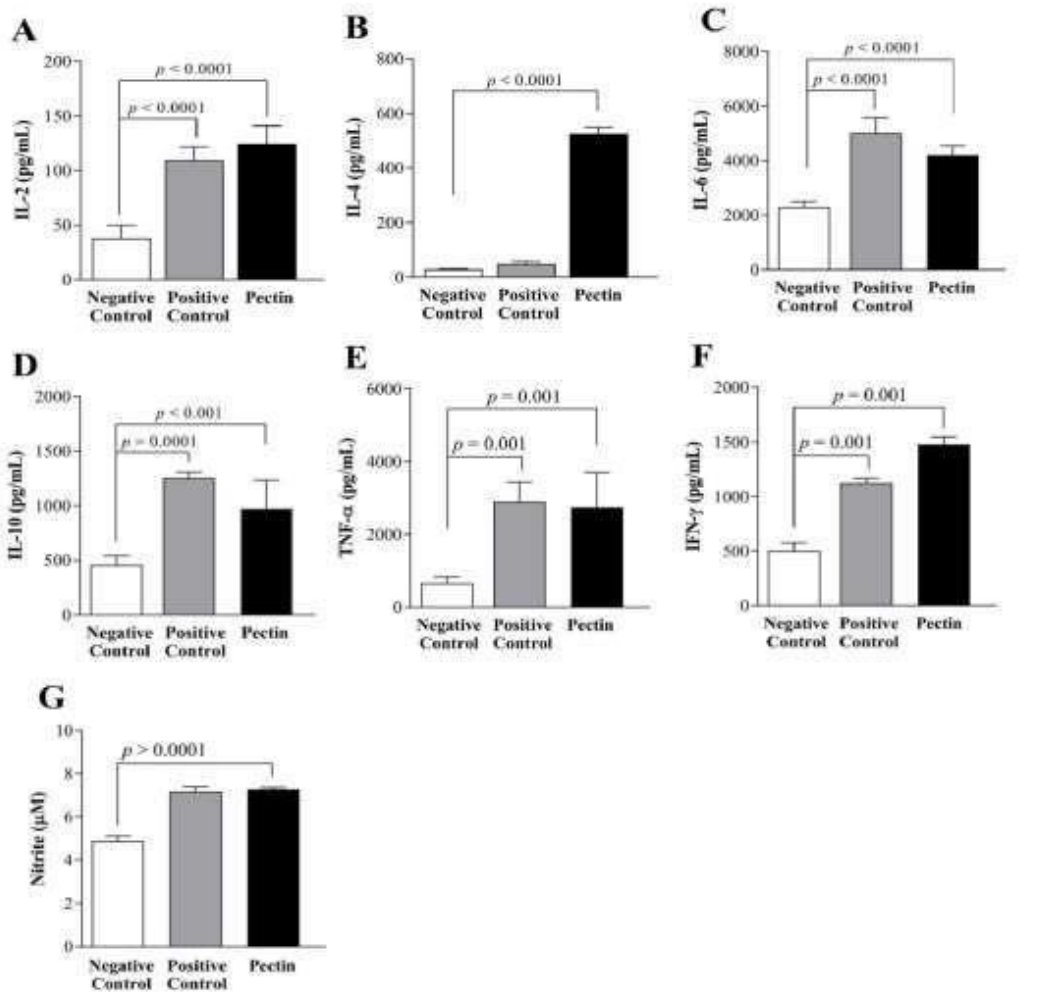

Fig. 9. Cytokine (A–F) and nitric oxide (G) levels in supernatants of PBMCs cultures treated with *C. erectus* pectin for 24 h. In negative control, cells were incubated only in culture medium while in positive control cells were treated with concanavalin A (5 μg/mL). Vertical bars represent the average of the experiment performed in quintuplicate. The differences were considered significant when $p < 0.05$.

4. Experimental

4.1. Plant material

The *Conocarpus erectus* Linnaeus leaves were obtained from mangroves located on Itamaracá Island, Pernambuco, Brazil (S 7° 40'43.249 ", W 34° 51'15.096"). A voucher sample is deposited at the Herbário Geraldo Mariz of Universidade Federal de Pernambuco under the number 75457. Access was registered (AC84C40) in the National System of Management of Genetic Heritage and Associated Traditional Knowledge (SisGen).

4.2. Extraction of pectin from leaves of *C. erectus*

Only visually intact green leaves were selected, free from pests and diseases. These were dried at 60 °C in an oven, ground in a knife mill (Fritsch-pulverisette 14) and sieved in 100 mesh granulations. The material obtained was subjected to extraction in a Soxhlet apparatus, using toluene/absolute ethanol 38:62 v/v (Merck Millipore, CAS108-88-3, Merck Millipore, CAS 64-17-5) as an extractor system for 8 h. The purpose of this step was to reduce the number of phenolic molecules and other constituents present in the leaves. Then, the resulting solid was subjected to an aqueous extraction in 2 L Erlenmeyer flasks under the following conditions: ratio 1:10 solid: liquid (g/L) at 60 °C, 1200 rpm for 4 h. After aqueous extraction, the system was filtered and the liquid fraction was subjected to a 1: 2 precipitation in ethanol (soluble fraction/ethanol). The precipitate obtained was dried at 70 °C for approximately 24 h. The pectin yield was calculated using Equation (1) proposed by Yulianti et al. (2015).

$$\text{Yield (\%)} = (\text{pectin mass / dry leaf mass}) * 100\%. \quad (1)$$

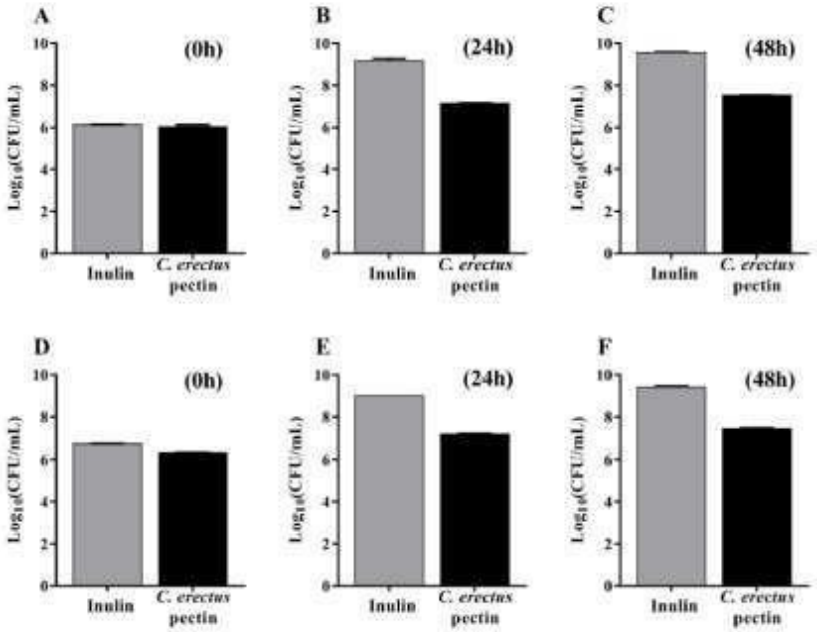

Fig. 10. Evaluation of prebiotic activity *in vitro* over a 48 h period. Growth of L. *paracasei* (A, B and C) and *L. rhamnosus* (D, E and F) in inulin and pectin in *C. erectus* respectively. The differences were considered significant when $p < 0.05$.

4.3. Physicochemical characterization of pectin

4.3.1. Determination of humidity and ash content of pectin

The moisture content of the pectin obtained after precipitation in absolute ethanol was determined by gravimetry after drying the pectin in an oven at 105 °C for 8 h. The ash content present in the pectin was also determined in a gravimetric way by measuring the residue obtained after incineration in a muffle at 650 °C for 4 h. All tests were performed in triplicate.

4.3.2. Determination of monosaccharides and galacturonic acid content present in pectin

Pectin (25 mg) was subjected to acid hydrolysis carried out at 100 ± 5 °C in a thermostatic bath (Nova Instruments - NI 1246) using 2.5 mL of trifluoroacetic acid (Merck Millipore, CAS 76-05) (4 M) for 8 h. After hydrolysis, the samples were filtered through a microporous membrane (0,22 μm).

The neutralized hydrolyzate was analyzed in a high-performance liquid chromatograph (Agilent, series 1100). The identification and quantification of monosaccharides (glucose, arabinose, galacturonic acid, xylose and rhamnose) were performed using the following operating system: mobile phase (H_2SO_4 5 mM), injection volume (5 μL), flow of 0.6 mL/min, at 60 °C, column Aminex HPX87H (Bio-Rad) and refractive index (IR) detector. The identification and quantification of furfural and 5-hydroxymethylfurfural (HMF) degradation products were performed using a reverse phase column (C-18) (Agilent Technologies), with a mobile phase composed of a 1:8 solution of acetonitrile-water containing 1% acetic acid, injection volume (5 μL), flow of 0.5 mL/min and detector UV/Vis (274 nm) at 25 °C.

Through the results obtained by the quantification of monosaccharides present in the hydrolyzate it was possible to determine the percentage of polysaccharides such as Homogalacturonan (HG) and Rhamnogalacturonan-I (RG-I). This percentage was previously determined by Alba et al. (2015) using Equations (2) and (3).

$$\text{HG (\% molar)} = \text{Galacturonic acid (\%)} - \text{Rhamnose (\%)} \quad (2)$$

$$\text{RG - I (\%)} = 2\,\text{Rhamnose (\%)} + \text{Arabinose (\%)} + \text{Galactose (\%)} \quad (3)$$

4.3.3. Fourier transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR)

The FT-IR analysis of *C. erectus* pectin was performed on a spectrometer Bruker Tensor 27 (Bruker AXS, Inc., Madison, WI, EUA), using attenuated full reflectance accessory (Platinum ATR).

4.3.4. Determination of the degree of esterification by FTIR

The determination of the degree of pectin esterification was carried out by integrating the bands of the infrared spectrum using the methodology proposed by Monsoor et al. (2001). His studies compared the determination of the degree of esterification by gas chromatography with the results obtained by integrating the areas of the bands of esterified carboxylic groups (between 1844 and 1682 cm^{-1}), attributed to the axial deformation of the carbonyl group, C = O, and non-esterified carboxylic groups, attributed to the axial deformation of the carboxylate ions, COO^-, (between 1682 and 1532 cm^{-1}) in the infrared spectrum through Equation (4):

$$DE = \left(\frac{A_{est}}{Anest + A_{est}}\right) \times 100\% \quad (4)$$

where: DE the degree of esterification (%); A_{est}, the peak area of the esterified carboxylic groups; A_{nest}, the peak area of non-esterified carboxylic groups.

4.3.5. Nuclear magnetic resonance spectroscopy (^{1}H NMR)

The pectin dissolved in D_2O heated to 60 °C was subjected to Nuclear magnetic resonance spectroscopy (^{1}H NMR) on a Bruker Avance 300 spectrometer (Bruker AXS, Inc., Madison, WI, USA) according to Cheng and Neiss (2012), Marcon et al. (2005) and Shakhmatov et al. (2019).

4.3.6. Thermogravimetric analysis

The thermogravimetric analysis of the pectin was performed in a nitrogen atmosphere using a thermogravimetric analyzer (TGA-50, Shimadzu, Kyoto, Japão). For the test, 20 mg of pectin were placed in a crucible, then the sample was heated from 25 °C to 600 °C at a linear rate of 10 °C/min. The test was performed in triplicate.

4.3.7. X-ray diffraction (XRD)

To obtain information about the structure of the pectin (amorphous or crystalline), the X-ray diffraction technique was performed. Tests were performed on an automatic diffractometer (XRD-6000/Shimadzu) (operating at 40 kV and 40 mA) in the angular range of 5–45° (2θ) (step size = 0.04 and time per step = 353 s) at a temperature of 30 °C.

4.3.8. Average viscosimetric molecular mass

The average molecular weight of pectin at different concentrations (1.0–9.0 g/L) was estimated through viscosity in water. For this, an Ostwald viscometer was used, applying the methodology proposed by Arslan (1995). The pectin was dissolved in water heated to 60 °C.

4.4. Tests of antioxidant activity and determination of total phenols

The antioxidant activity of pectin was determined based on the DPPH and ABTS radical scavenging activity. The tests were carried out according to the methodology proposed by Santos et al. (2020) with modifications. Pectin was tested in different concentrations (3.9; 7.8; 15.6; 31.25; 62.5; 125; 200 and 500 μg/mL), the ascorbic acid and the butylated hydroxytoluene (BHT) were used as standard molecules in the same concentrations of pectin. Absorbances were measured on a Hewlett-Packard spectrophotometer, model 8453. And all tests were performed in triplicate. The total phenolic content was measured according to the protocol described by Santos et al. (2020) through the Folin-Ciocalteu method. A calibration curve resulted in the linear equation ($y = 0.0023x + 0.014$; $R^2 = 0.9858$). The test was carried out in triplicate and the phenol contents were expressed in gallic acid equivalent (mg/g GAE).

4.5. Evaluation of cytotoxicity and immunomodulatory activity

4.5.1. Culture of human peripheral blood mononuclear cells (PBMC)

Experimental protocols for isolation of human PBMC lymphocytes and monocytes were approved by the Ethics Committee of the Federal University of Pernambuco (number 1.870.360/2016). The procedure for obtaining the cells was described by Santos et al. (2020) and with these, the cytotoxicity, proliferation, oxidative stress, and evaluation of immunomodulatory activity tests were carried out.

4.5.2. Cell viability analysis

PBMCs were treated with pectin (2.5; 5.0; 10.0; 20.0; 40.0 and 80.0 μg/mL) or concanavalin A (5 μg/mL; Sigma-Aldrich®) for cell viability analysis according to Santos et al. (2020), using BD Biosciences FITC Annexin V Apoptosis Detection Kit I (Franklin Lakes, NJ, EUA).

4.5.3. Immunomodulatory activity

Assays to assess the immunomodulatory potential promoted by *C. erectus* pectin were performed according to Santos et al. (2020) with few modifications. The analysis of cell proliferation was performed by the CFSE method (succinimidyl ester carboxyfluorescein). The immunophenotyping assays of lymphocytes and monocytes were performed by means of analyzes with monoclonal antibodies (anti-CD4-PerCP, anti-CD8-FITC, anti-CD-28-PE, anti-CTLA-4-APC, anti-CD14-FITC, anti-CD80-PE, anti-CD-86-APC and anti-HLA-DR-PerCP (BD Biosciences®). Cytosolic Ca^{2+} levels ([Ca^{2+}] cit) were determined using the fluo-3AM probe (Thermo Fisher Scientific®), the levels of reactive oxygen species (ROS) cytosolic and mitochondrial were determined by flow cytometry, using dihydroethide probes (DHE) (Sigma Aldrich®) and red MitoSox (Thermo Fisher Scientific®), respectively. Changes in transmembrane mitochondrial potential (ΔΨm) were determined using the MitoStatus probe (BD Biosciences®). To evaluate cytokine production, the Human Th1/Th2 Cytokine Cytokine (BD Biosciences) Bead Array (CBA) kit was used. The cytokines determined were: interleukins IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ. Finally, the production of nitric oxide (NO) produced by the cells was also determined using the Griess method (Ding et al., 1988). The levels of NO produced were determined using a standard sodium nitrite curve ($y = 0.007x + 0.0256$; $R^2 = 0.9978$). All tests were carried out in five independent experiments.

4.6. Evaluation of prebiotic activity in vitro

A polysaccharide is considered prebiotic when it is able to stimulate the growth and activity of beneficial bacteria in the intestinal microbiota, resulting in improved health (Huebner et al., 2008). To evaluate the prebiotic activity of the pectin obtained in this study, a methodology proposed by Kalailas et al. (2017) with few modifications. Two probiotic strains of Lactobacillus (*L. paracasei* and *L. rhamnosus*) were grown in MRS broth (Man, Rogosa and Sharpe) and incubated at 37 °C for a period of 24 h. After the incubation period, the broth containing the bacteria was centrifuged at 11000 rpm for 20 min at 4 °C. The cell precipitate was suspended in 0.85% saline and standardized to a cell concentration of 10^7 CFU/mL. The pectin and inulin samples (positive control) were filtered through a membrane (0,22 μm, Millipore) and added to 100 mL of MRS broth to obtain a 0.25% (w/v) solution. The initial cell concentration in the assay was 10^6 CFU/mL. The assays were incubated at 37 °C for 24 in 48 h. After cultivation, aliquots were removed and diluted from 10^{-1} to 10^{-6}, in tubes containing sterile distilled water. Then 10 μL rates were plated in Petri dishes containing MRS agar medium and were again incubated at 35 °C for 24 h the growth results were expressed in CFU (colony forming unit)/mL.

4.7. Statistical analysis

GraphPad Prism 7® software was used to perform the statistical analysis. To test the normality of the hypothesis, the Shapiro-Wilk test and non-parametric tests were used to analyze the mean of the repetitions. The unilateral analysis of variance (ANOVA) was performed to analyze the statistical differences between the groups. The differences were considered significant when $p < 0.05$.

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CRediT authorship contribution statement

Dayane Kelly Dias do Nascimento Santos: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Bárbara Rafaela da Silva Barros:** Investigation, Validation. **Iranildo José da Cruz Filho:** Investigation, Validation, Methodology, Writing – original draft. **Maria do Carmo Alves de Lima:** Methodology, Resources, Writing – review & editing, Supervision. **Thiago Henrique Napoleão:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Cristiane Moutinho Lagos de Melo:** Conceptualization, Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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6 ARTIGO 3 – PHENOLIC SUBFRACTIONS OF THE AQUEOUS EXTRACT FROM CONOCARPUS ERECTUS L. VAR. ERECTUS LEAVES SHOWS ANTIOXIDANT AND IMMUNOMODULATORY ACTIVITIES

Phenolic subfractions of the aqueous extract from *Conocarpus erectus* L. *var. erectus* leaves shows antioxidant and immunomodulatory activities

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Abstract

The discovery of new biomolecules based on their ethnopharmacological use has brought unprecedented benefits to the population. This study evaluated phenolic subfractions from aqueous extract from *Conocarpus erectus* leaves for chemical composition, antioxidant properties and immunostimulatory effects on mice splenocytes. The chemical analysis was performed by preparative HPLC, and colorimetric methods were used for the quantification of phenolic compounds. The free radical scavenging power was evaluated by the DPPH, $ABTS^{+}$ and phosphomolybdenum complex assays. Cultures of Balb/c mice splenocytes were

evaluated for cell viability and cytokine and nitric oxide levels when incubated or not the subfractions. The subfractions presented mainly flavonoids in their composition. The fractions presented percentages of free radical sequestration similar to synthetic (BHT) and natural (ascorbic acid) antioxidant standards in DPPH method and better to ABTS. F01 and F03 induced an immunosuppressive profile in splenocytes, while the F02 fraction stimulated a proinflammatory profile. In conclusion, the subfractions of phenolic compounds from the aqueous extract from *C. erectus* leaves are promising sources of antioxidant and immunostimulatory compounds.

Keywords: cytokines; flavonoids; buttonwood; free radical scavenger.

1 Introduction

The development of drugs based on natural products results from the popular knowledge in traditional medicine, in which plants are the main therapeutic agents used to treat diseases (Jain & Das, 2016). Researchers around the world have shown a growing interest in how compounds synthesized by plants can modulate the functioning of the human body and improve health (Perez-Vizcaino & Fraga, 2018).

The redox balance of cells provides a stable microenvironment for the normal functions of various macromolecules in the body (Zhang et al., 2021). However, the excessive production of free radicals can cause oxidative damage to these biomolecules and eventually lead to many chronic diseases, such as atherosclerosis, cancer, diabetes and other degenerative diseases (Xu et al., 2016). Currently, antioxidant supplementation has been a strategy in nutrition, aging or disease treatment (Meng et al., 2020). Synthetic antioxidants have been widely used because they are effective and cheaper, however, the safety and toxicity of these compounds has been a concern in the healthcare sector (Wang et al., 2021). In this regard, emerging research has suggested that natural antioxidants can reduce oxidative damage and subsequently stimulate healthy longevity (Meng et al., 2020; Zhang et al., 2021).

The immune system is fundamental in homeostatic processes as well as disease. Manipulating innate and adaptive immune responses holds great promise for correcting the pathological processes of a wide range of human diseases, including alternative and tissue regeneration, cancer, autoimmune syndromes, and chronic modifications (Dellacherie, Seo and Mooney, 2019; Song, Scholtemeijer & Shah, 2020; Petroni et al., 2020). The potential of natural molecules for the management of pathological conditions in immune-mediated

diseases has been the subject of large studies due to their capacity for modulating immune functions (Gandhi et al., 2018).

Mangrove plants have physiological adaptations that allow them to survive in peculiar conditions of their environment (Kathiresan & Bingham, 2001). Communities traditionally use these plants to cure diseases such as gastrointestinal disorders, diarrhea, ulcers, skin diseases, bacterial and viral infections (Bashir et al., 2015; Chandrakala & Rajeswari, 2017; Bibi et al., 2019). Furthermore, it has been shown that these species are rich in bioactive compounds with biotechnological potential for the development of new drugs (Nascimento et al., 2016). *Conocarpus erectus* L., belonging to the Combretaceae family, is a mangrove plant popularly known as a buttonwood, used in folk medicine against diarrhea, fever, rash, swelling, gonorrhea, conjunctivitis, and syphilis (Abdel-Hameed et al., 2013; Ramadan et al., 2017). The leaves are eaten fresh or like teas by local communities (Santos et al., 2018a), being phenolic compounds, especially flavonoids and tannins, the main active compounds reported for this species (Abdel-Hameed et al., 2014, Azam et al., 2019).

Few studies on bioactive properties of isolated molecules from *C. erectus* are found in the literature. Recent studies have demonstrated the bioactive potential of two metabolites isolated from the leaves of *C. erectus*, lignin (Santos et al., 2020) and pectin (Santos et al., 2021) showed antioxidant potential and stimulating capacity for immunomodulatory response. Here, we carried out a study with secondary metabolites of the phenolic class obtained from the leave aqueous extract of this species, to determine their chemical characteristics, antioxidant potential and immunostimulating properties.

2 Material and methods

2.1 Plant material

Conocarpus erectus var. *erectus* leaves were collected in May 2018 in the mangrove of Itamaracá island, Pernambuco, Brazil (7°48‘43.249‖ S, 34°51‘15.896‖ W). A voucher specimen was identified by Marlene Barbosa and is deposited in the Herbarium Geraldo Mariz from the *Universidade Federal de Pernambuco* (register no. 75457). Plant collection was authorized by *Agência Estadual de Meio Ambiente*, Recife, Pernambuco (register no. CA DFRB N. 120/2014) and the study was recorded (AC84C40) at the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen).

2.2 Preparation of aqueous extract

Fresh leaves of *C. erectus* (250 g) were ground, added to distilled water (500 mL) and maintained at 40 °C for 30 min. After passing the suspension through filter paper (7 µm pore), the extract obtained was lyophilized and stored at 4 °C.

2.3 High performance liquid chromatography (HPLC)

An aliquot of 500 µL (1 mg/mL) of the extract was transferred to a 2 mL volumetric flask and the volume was completed with ultrapure water (Purelab, ELGA LabWater, High Wycombe, UK). The solution was filtered through a 0.45 µm PVDF filter for vials. The analysis was performed on Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA), coupled to a photodiode array detector (DAD) and equipped with binary pump (HPG-3x00RS), degasser and sampler equipped with a 20 µL loop (ACC-3000). The wavelength was set at 254 nm for the detection of tannins and 350 nm for the detection of flavonoids. Chromatographic separations were obtained with a Dionex C18 column (250 mm x 4.6 mm d.i, 5 µm) equipped with Phenomenex pre-column (C18 of 4 mm x 3.9 cm). The separations were carried out at a temperature of 25 °C. The mobile phase consisted of ultrapure water (A) and methanol (B), both acidified with 0.05% trifluoroacetic acid, and flow adjusted to 0.7 mL/min. A gradient program was applied as follows: 0-10 min, 5-20% B; 10-13.5 min, 20-25% B; 13.5-18 min, 25-40% B; 18-25 min, 40-80% B; 25-30 min, 80% B; 30-34 min, 80-5% B; 34-36 min, 5% B. For the analysis and processing of the data, Chromeleon software 6.8 (Thermo Fisher Scientific) was used.

2.4 Analysis by flash chromatography system

About 50 mg of the sample was weighed and solubilized in methanol. To obtain subfractions, it was used a semi-preparative Isolera LS chromatographic system (Biotage, Uppsala, Sweden) equipped with degasser, binary gradient pump, UV/VIS detector, automatic injector and control software. The chromatographic conditions used are described in Table 1. The purification method used was the same employed for the HPLC. The fractions (F01, F02 and F03) were collected in assay tubes (15 mm x 1.5 mm) and those of interest were

concentrated in rotary evaporator (RV10 Basic; IKA, Staufen, Germany) under reduced pressure until complete elimination of the organic solvent.

Table 1 - Chromatographic conditions used for purification of the fractions from the aqueous extract of *C. erectus*.

Parameters	**Conditions employed**
Stationary phase	SNAP Ultra C_{18} 12 g
Flow	10 mL/min
Solvent A	H_2O + TFA 0,05%
Solvent B	MeOH + TFA 0,05%
Detection mode	UV1 + UV2
Wavelength 1	270 nm
Wavelength 2	350 nm
Threshold	10 mAU
Slope Mode	Medium
Collection volume	18 mL

TFA: trifluoroacetic acid

2.5 Investigation of total phenolic content

The method used to measure total phenols content was in accordance with Santos et al. (2018b). A calibration curve was prepared by plotting the absorbance as a function of the gallic acid concentration (3.9-500 μg/mL) and then finding the linear equation ($y = 0.0023x + 0.014$; $R^2 = 0.9858$). The assay was performed in five replicates and the phenols contents are expressed in gallic acid equivalent (mg/g GAE).

2.6 Investigation of flavonoid content

The determination of total flavonoid content was performed according to a method described by Santos et al. (2018b). A standard quercetin curve (3.9-500 μg/mL) was performed to obtain the equation ($y = 0.0016x + 0.0517$; $R^2 = 0.9858$). The assay was performed in five replicates and the flavonoid content is expressed as quercetin equivalent (mg/g QE).

2.7 Investigation of tannin content

For the dosage of tannins, the method described by Patay et al. (2016) was adopted. A standard tannic acid curve (3.9-500 µg/mL) was performed to obtain the equation ($y = 0.0287x + 0.1652$; $R^2 = 0.9776$). The assay was performed in five replicates and the flavonoid content is expressed as quercetin equivalent (mg/g TAE).

2.8 DPPH scavenging assay

The antioxidant activity of the fractions was evaluated on the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) according to Blois (1958). Acid ascorbic and butylated hydroxytoluene (BHT) were used as standards. The concentration of the sample required to inhibit 50% (IC_{50}) of DPPH was calculated. The assay was performed in three replicates.

2.9 Cation ($ABTS^+$) radical elimination

ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6- sulfonic acid) radical cation elimination activity was performed according to Jiang et al. (2018). The concentration of the sample required to inhibit 50% (IC_{50}) of ABTS was calculated. The assay was performed in three replicates.

2.10 Total antioxidant activity

The total antioxidant activity according to Pietro et al. (1999) and determined as a function of ascorbic acid, compound considered with 100% activity. The assay was performed in three replicates.

2.11 Animals

All experimental procedures were approved by Ethics Committee of Animal Use (CEUA) of the *Universidade Federal de Pernambuco*, UFPE (protocol no. 0048/2016). Female BALB/c mice (6–8 weeks old) were raised and maintained at the animal facilities of the *Laboratório de Imunopatologia Keizo Asami* of UFPE. Mice were kept under standard laboratory conditions (20– 22 °C and 12 h dark/light cycle) with free access to a standard diet (Labina/Purina, Campinas, Brazil) and water.

2.12 Evaluation of cytotoxicity to mice splenocytes

Splenocytes were obtained according to the methodology described by Aguiar et al. (2019). The cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was >98%. The cytotoxicity was evaluated with Propidium iodide (PI, 20 μg/mL) which was added to each cytometer tube containing mice splenocytes (10^6 cells) treated and untreated with the fractions in 50, 25, 12.5, 6 and 3 μg/mL. Flow cytometry was performed in a FACS Calibur flow cytometer (Becton Dickinson Biosciences) and analyzed using CELL QUEST PRO software (Becton Dickinson). Result analysis was performed in graphs by dot plot. PI positive cells were considered dead cells and negatives were considered viable cells.

2.13 Nitrite analysis

The supernatants from cultures of splenocytes incubated with the fractions (12.5 μg/mL) for 24 h were used for nitrite analysis by the colorimetric Griess method (Ding, et al., 1988). NO concentration was estimated using a standard curve ($y = 0.005 + 0.016$; $R^2 = 0.9912$) (3.12–100.0 μmol/mL). The reading was performed in a microplate spectrophotometer at 595 nm.

2.14 Cytokine production measurement in splenocytes supernatants

Supernatants of splenocyte cultures treated or not treated with fractions at 12.5 μg/mL for 24 h, were collected for quantification of cytokines using the Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (Becton Dickinson Biosciences, USA) for simultaneous detection of interleukins (IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17. Assays were performed according to the manufacturer's instructions, data were acquired on the FACSCalibur platform and results were analyzed using FCAP 3.1 software (Becton Dickinson Biosciences, USA).

2.15 Statistical analysis

GraphPad Prim 7 software was used for statistical analysis. The Shapiro–Wilke test was applied to test the normality of the hypothesis on the variable of this study, the means of samples were analyzed by non-parametric tests and the statistical difference between groups

was analyzed by one-way analysis of variance (ANOVA). All the results were considered with a significance level of 5%.

3. Results and discussion

3.1 Phytochemical analysis of chromatography fractions from aqueous extract of Conocarpus erectus leaves

Subfractions from aqueous extract of *C. erectus* leaves were obtained concentrating majority chemical substances present in the extract. After phytochemical screening by HPLC, the samples were collected according to the similarities, obtaining 3 subfractions (F01, F02, F03) with total yield of 4.2%, 33% and 1.2% respectively (Table 2). The three fractions were analyzed by HPLC, in which it was possible to identify the presence of polyphenolic compounds. Compounds were identified according to retention time and UV spectra corresponding to the molecules analyzed (Table 3). In fraction F01, as shown in the chromatogram (Figure 1), the presence of several peaks was verified, being the peaks 1, 2, 3, 5, 6 and 8 indicatives of flavonoid compounds and peaks 4, 7 and 9 indicatives of ellagitannins, confirmed by the scanning spectra obtained for each peak (Figure 2A and Table 3). Eight peaks were identified in the F02 fraction. The peaks 1, 2, 3, 4 and 7 indicative of flavonoid compounds and the peaks 5, 6 and 8 indicatives of ellagitannins as shown in the chromatogram (Figure 1), evidenced by their respective sweep spectra (Figure 2B and Table 3). The F03 fraction presented four peaks (Figure 1) indicative of flavonoid compounds, evidenced by the sweep spectra obtained for the peaks (Figure 2C and Table 3).

Table 2 - Yield of the fractionation of the aqueous extract from leaves of *C. erectus* and dosage of total phenolic compounds, flavonoids and tannins.

Chromatographic Fraction	Extraction yield (mg of dry weight)	Extraction yield (w/w % of dry weight)	Phenolic compounds (mg/g GAE)	Total flavonoid (mg/g QE)	Total tannins (mg/g TA)
CE F01	2.1	4.2	412.99 ± 0.24	986.76 ± 0.03	11.53 ± 0.34
CE F02	16.5	33	146.77 ± 1.09	275.22 ± 0.01	6.33 ± 0.02
CE F03	0.6	1.2	229.20 ± 0.37	781.47 ± 0.07	11.52 ± 0.05

Figure 1 - Chromatogram of F01, F02 and F03 subfractions of the aqueous extract from *Conocarpus erectus* at 350 nm.

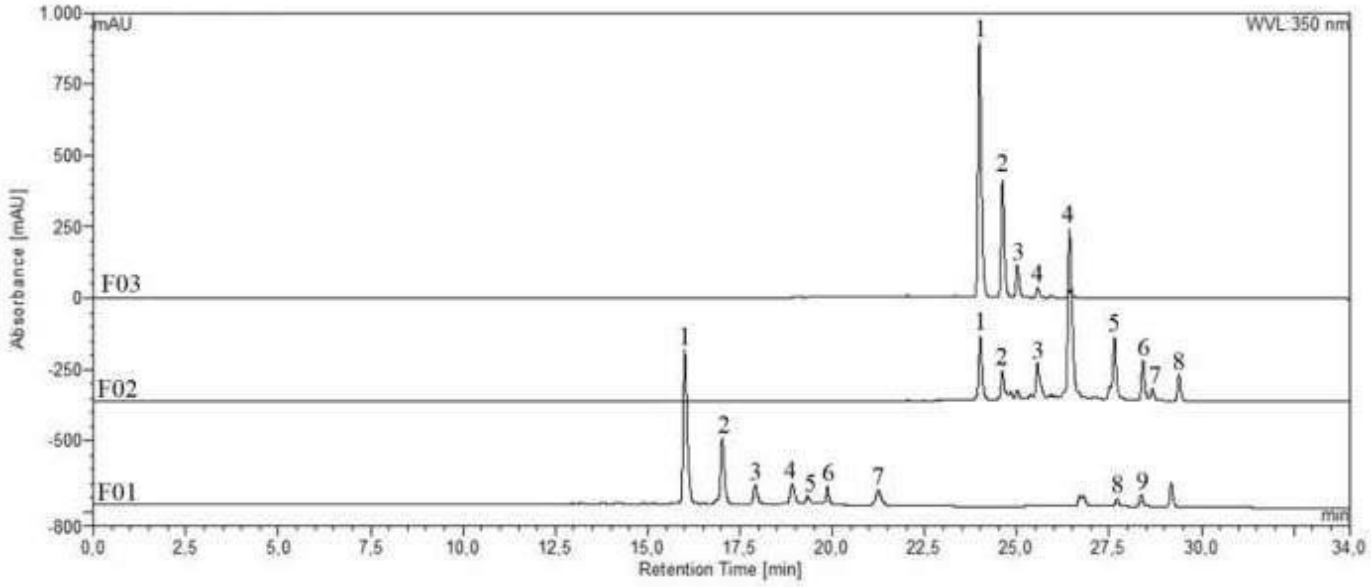
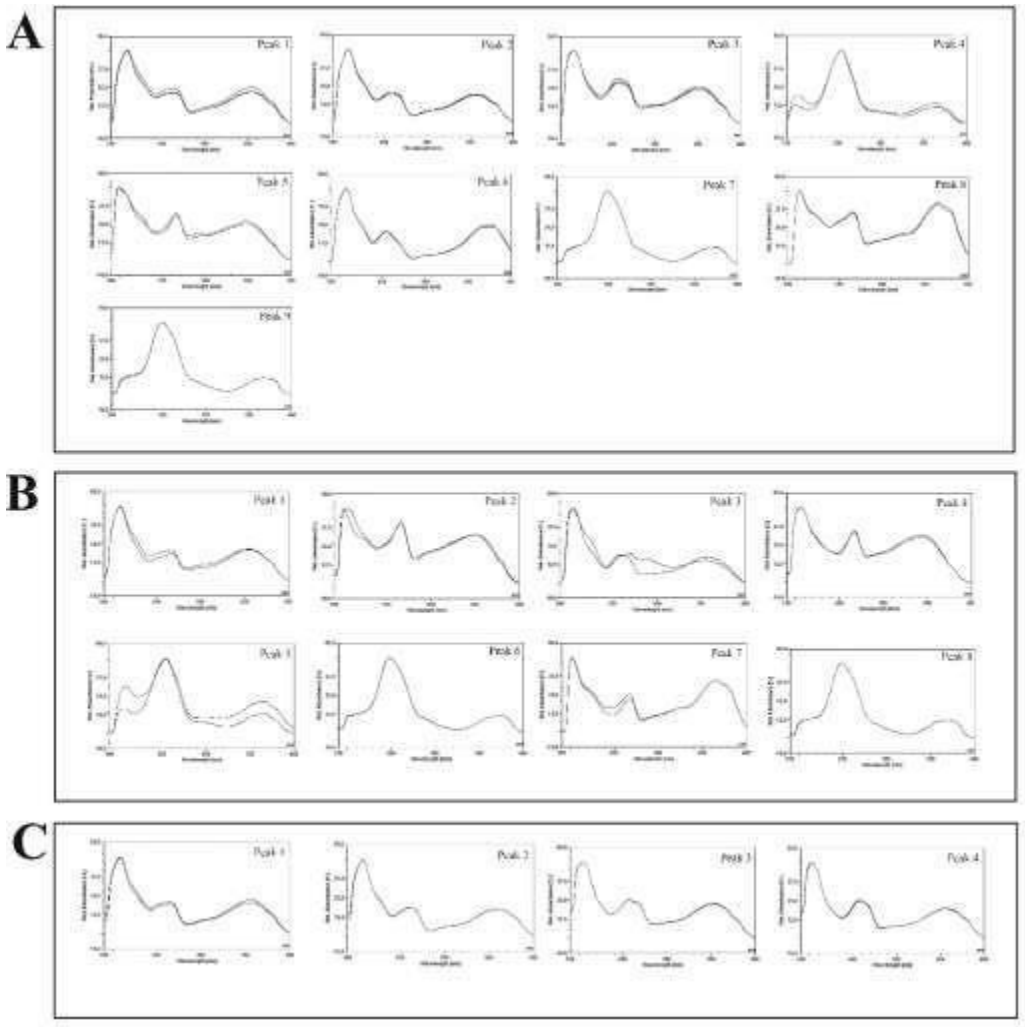


Table 3 - Chemical characteristics of the compounds identified in the phenolic fractions from the aqueous extract of *C. erectus*.

Subfraction	Peak	Retention time (min)	λ_{max} (nm)	Compound
F01	1	16.003	210.1; 264.2; 353.6	Flavonoid
	2	17.020	208.3; 259.2; 357.0	Flavonoid
	3	17.913	204.8; 256.2; 351.4	Flavonoid
	4	18.920	201.8; 253.9; 364.4	Ellagitannin
	5	19.327	199.5; 265.7; 347.5	Flavonoid
	6	19.863	207.6; 366.9; 377.0	Flavonoid
	7	21.247	248.0; 366.9; 375.9	Ellagitannin
	8	27.703	203.1; 265.6; 365.5	Flavonoid
	9	28.363	248.4; 367.0	Ellagitannin
F02	1	24.070	209.0; 265.9; 354.4	Flavonoid
	2	24.853	205.7; 265.5; 349.5	Flavonoid
	3	25.407	204.8; 257.7; 357.5	Flavonoid
	4	25.600	205.7; 266.8; 343.6	Flavonoid
	5	25.960	208.8; 254.5; 365.0	Ellagitannin
	6	27.600	248.2; 367.3; 374.6	Ellagitannin
	7	28.453	202.1; 266.8; 365.7	Flavonoid
	8	28.703	248.5; 367.3	Ellagitannin
F03	1	23.963	209.1; 263.5; 355.2	Flavonoid
	2	24.610	207.6; 259.6; 358.9	Flavonoid
	3	25.013	204.3; 256.5; 352.9	Flavonoid
	4	25.563	203.6; 257.2; 356.7	Flavonoid

Figure 2 - Peak scanning spectra evidenced in the F01 (A), F02 (B) and F03 (C) fraction chromatogram at 350 nm.



The quantitative analysis of total phenolic compounds, flavonoids and tannins (Table 2) of the fractions were performed by spectrophotometric methods and corroborated with the data obtained in the chromatography. The fractions F01 and F03 have the highest concentrations of flavonoids and tannins and consequently of phenolic compounds. Studies have shown that *C. erectus* leaves are rich in phenolic compounds, especially flavonoids and tannins (Santos et al., 2018b, Safwat et al., 2018). New evidence shows that these compounds, individually or in combination, may act on multiple signaling cascades exerting their modulatory properties on different types of cells (Ballard & Maróstica, 2019).

3.2 Antioxidant potential

The percentage of free radical sequestration referring to 0.5 mg of the substance and IC_{50} were determined according to Table 4. The fractions F03 and F01 presented the highest percentage of sequestration of the DPPH radical, with values similar to the synthetic antioxidant BHT. Previous studies have shown that ethanolic and ethyl acetate fractions of leaves of *C. erectus* also presented high percentage of sequestration of DPPH with values of 71.27 ± 6.85 and 71.82 ± 6.87, respectively (Santos et al., 2018b). The fractions F01, F02 and F03 were promising for the ABTS cation sequestration, the three fractions showed higher sequestration percentages than ascorbic acid, natural antioxidant tested. F02 and F03 also showed a higher antioxidant potential than BHT, and F01 similar to this, for ABTS. In addition, fractions F01 and F03 also presented total antioxidant activity close to BHT. The free radicals are products of essential processes for energy supply and signaling, however, can act as toxic products involved in cellular and organic dysfunction (Barapatre et al., 2016). An antioxidant molecule is a substance that significantly delays or prevents oxidation of an oxidizable substrate (Carocho et al., 2018). In this sense, plant phenols and polyphenols have often been evaluated for their potential antioxidants, in terms of their ability to eliminate free radicals and prevent damage (Barapatre et al., 2016). The catecholic B-ring is characteristic of most flavonoids and tannins and is the main determinant of the antioxidant capacity of these compounds (Gourlay & Constabel, 2019). In addition, they act as antioxidants via transfer of hydrogen atoms or single electron transfer mechanisms (Velderrain-Rodríguez et al., 2018).

Table 4 – Antioxidant activity promoted by fractions semi-purified of aqueous extract from leaves of *C. erectus*.

Sample	DPPH	ABTS	TAA
	IC_{50} (µg/mL)	**IC_{50} (µg/mL)**	**% (0.5 mg/mL)**
CE F01	293.56	47.52	46.71 ± 0.11
CE F02	888.20	157.44	33.52 ± 0.07
CE F03	139.83	21.57	49.16 ± 0.14
Ascorbic acid	240.30	90.93	100
BHT	140.56	7.67	67.59 ± 0.17

3.4 Cytotoxicity and immunostimulatory activity of the chromatographic fractions of C. erectus under mice splenocytes

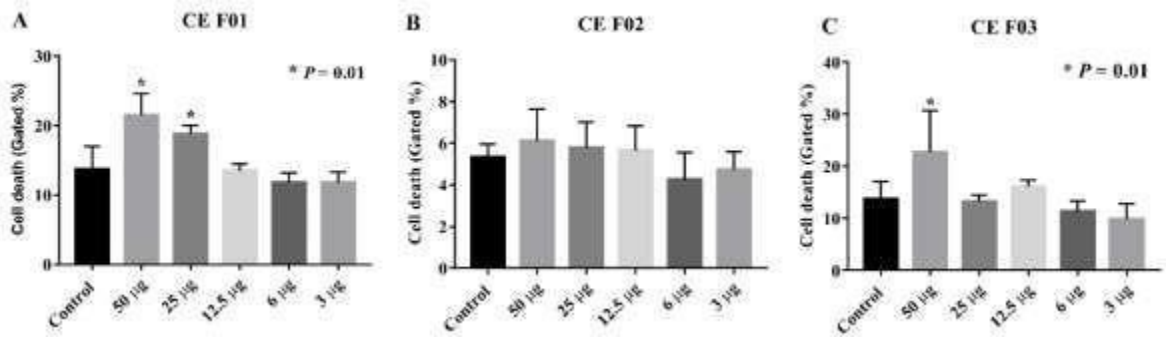
Mice splenocytes were cultured with the fractions in different concentrations (50 to 3 µg/mL) to measure the cytotoxicity promoted by these substances. Cytokines production and NO concentration also were investigated in supernatants of mice splenocytes cultures treated with 12.5 µg/mL of the samples, considering that recent studies on biological property promoted by natural compounds have indicate that smaller doses should be enough to stimulate cells (Zheng et al., 2016).

The F01 fraction did not promote significant cell death relative to the control in the concentrations of 12.5 to 3µg/mL (figure 3 - A), while F03 was not cytotoxic in the concentrations of 25 µg/mL down (figure 3 - B). However, the F02 fraction can be used safely at all concentrations tested in this study (Figure 3 - C). Human lymphocytes treated with aqueous extract of leaves of *C. erectus* at different concentrations also remained alive with cell viability upper to 95% (Santos et al. 2018a).

F01 and F03 fractions were similar in relation to the suppression of IL-6, TNF-α and IL-17 production, presenting an immunosuppressive characteristic, while levels of the IL-4, IL-10 and IFN-γ cytokines remained similar to the control (figure 4). F03 also stimulated the high release of nitric oxide, an important microbicidal agent released by M1 macrophages (Das et al., 2015) and an important mediator of signaling in the immune system (Cruz Filho et al., 2019). Cytokines profile obtained for phenolic fraction F02 was a proinflammatory status associated with high IL-6, TNF-α and IFN-γ production, the IL-4 and IL-17 cytokines and the nitric oxide were produced in basal values, similar to control (Figure 4). Although high IL-10 production was observed in our results, the proinflammatory status was prevalent. IL-10 has been reported as an important regulatory cytokine in the inflammatory process exacerbated by pathogens (Belkaid and Hand, 2014). Studies have shown that extracts of polyphenolic compounds and flavonoids are able to reduce proinflammatory effects by inhibiting cytokines expression, especially IL-1b, TNF-α, IL-6, IL-17, and IFN-γ, in chemically induced colitis (Mileo, Nisticò and Miccadei, 2019). Santis et al. (2016) have shown that quercetin induces a significant reduction in TNF-α secretion by dendritic cells stimulated with Lipopolysaccharide (LPS) and promotes tissue repair by interfering in the onset of inflammatory bowel disease. In addition, baicalein reduces the production of IL-1β, IL-6, IL-8 and TNF-α cytokines in macrophages stimulated by *P. aeruginosa* (Luo et al., 2016).

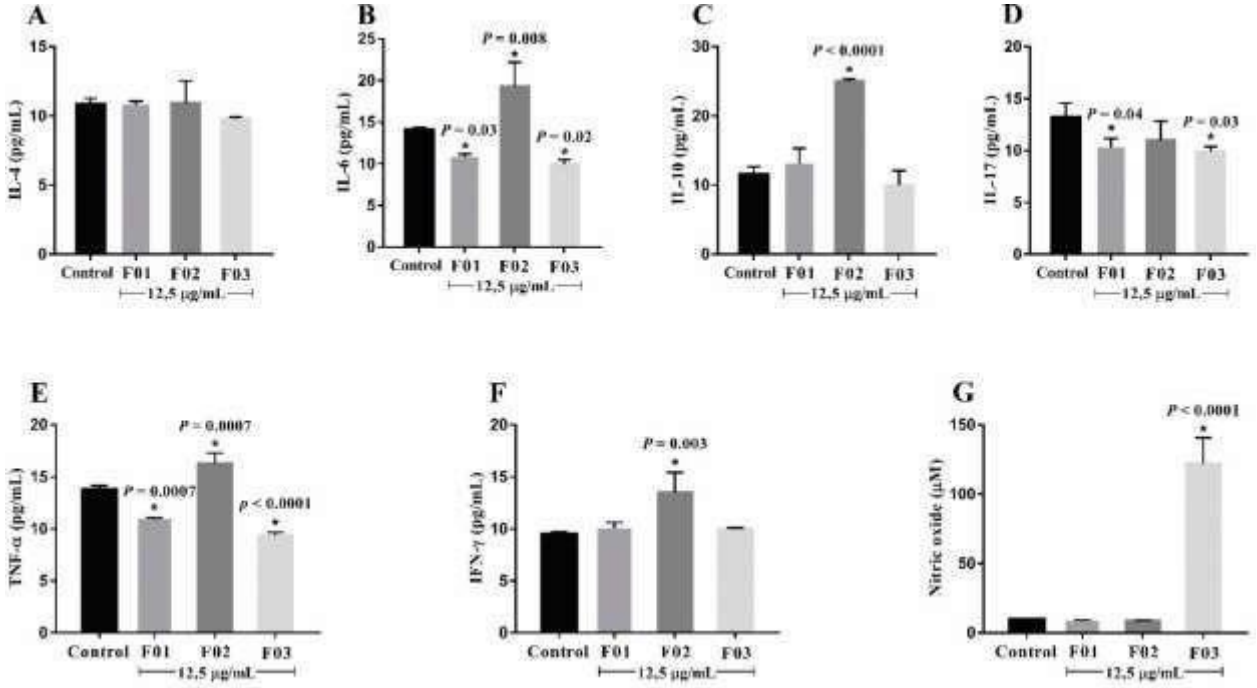
Figure 3 - Cytotoxicity investigation against mice splenocytes promoted by phenolic fractions from aqueous extract of *C. erectus* leaves using propidium iodide-PE staining in flow

cytometry. Vertical bars represent the average of two independent experiments performed in triplicate.



Our results to F02 fraction resemble the results obtained previously for the aqueous extract of *C. erectus* leaves, which presented a proinflammatory profile with the production of IL-2, TNF-α, IFN-γ and IL-10 for PBMCs (Santos et al., 2018b). Phenolic compounds extracted from *Opuntia ficus-indica* and *Opuntia cochenillifera*, plants adapted to environments of extreme adverse conditions, also stimulate a pro-inflammatory response in splenocytes through the production of TNF-α, IL-6 and IL-10 (Cruz Filho et al., 2019). Curgali et al. (2018) analyzing the effect of quercetin in rats, detected elevated levels of TNF-α and IL10 in blood serum, in addition to increased expression of TNF-α and IL10 in jejunal tissue of these animals. In order, the increased production of proinflammatory cytokines, such as IL-6, IFN-γ, TNF-α and IL-4, is an important indicator of the healing process of lesions, according to studies by Ligi et al. (2016). Moreover, Rodrigues-Alves et al. (2018), classified IL-10 as an efficient biomarker in the monitoring of cutaneous wound healing time. In this sense, polyphenol compounds have been shown to be promising molecules with immunostimulatory activities, for your modulation of the production of cytokines, chemokines and activation of immunological cells (Mileo, Nisticò and Miccadei, 2019).

Figure 4 - Cytokines produced in splenocytes cultures treated in vitro with 12.5 µg/mL of the fraction F01, F02 and F03. F01 and F03 Fraction induced suppression of IL-6 (B), IL-17 (D) and TNF-α (E) cytokines. F02 Fraction induced IL-6 (B), IL-10 (C), TNF-α (E) and IFN-γ (F) cytokines production. Nitric oxide (G) production was stimulated by F03 in relation to control. Vertical bars represent two independent experiments performed in triplicate.



4. Conclusion

Conocarpus erectus is a plant rich in phenolic compounds used by the population for the treatment of various diseases and, mainly, infections. The phenolic fractions obtained from aqueous extract of *C. erectus* leaves are essentially composed of flavonoids, present high antioxidant. Furthermore, the fractions stimulated immune cells, F2 to release proinflammatory cytokines and F1 and F3 stimulated a suppression of cytokine production, and only the F3 fraction produced nitric oxide significantly. Our findings indicate that these fractions are promising sources of substances that may have a pharmacological potential for the treatment of infections and can actuate as supporting agents in wound repair.

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Declarations of interest: none

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CONCLUSÕES

- As frações obtidas do extrato aquoso de folhas de *C. erectus* apresentaram em sua composição compostos fenólicos identificados por suas características espectroscópicas.
- As características estruturas da lignina e pectina isoladas de folhas de *C. erectus* foram confirmadas.
- As frações e os compostos isolados apresentaram um bom potencial antioxidante comparados aos antioxidantes naturais e sintéticos.
- As frações não foram tóxicas para esplenócitos de camundongos Balb/c, assim como a lignina e a pectina não foram tóxicas para células mononucleares de sangue periférico humano (PBMCs) em concentrações $\leq$ 12,5 μg/mL.
- A lignina e a pectina não promoveram proliferação de PBMCs.
- As frações fenólicas F01 e F03 suprimiram a produção de citocinas por esplenócitos, enquanto que a fração F02 estimulou a produção de citocinas do tipo T_h1. F03 foi a única fração que estimulou a produção de óxido nítrico. A lignina e a pectina estimularam a produção de todas as citocinas e óxido nítrico. No entanto, a produção de citocinas do tipo T_h1 sobrepõe a produção de citocinas T_h2 sugerindo uma resposta majoritariamente pró-inflamatória dessas moléculas.
- A lignina estimulou a proliferação de linfócitos T $CD8^+$, assim como sua ativação ($CD28^+$), não estimulou a proliferação, mas inibiu células T $CD4^+$ e proliferou células $CD16^+$. A pectina estimulou a proliferação de linfócitos T $CD4^+$, assim como sua ativação ($CD28^+$), não estimulou a proliferação, mas inibiu células T $CD8^+$ e não proliferou células $CD16^+$.
- PBMCs tratados com lignina ou pectina possivelmente foram ativados pelo aumento dos níveis cálcio citosólico, o qual pode ter contribuído para o aumento de EROs mitocondrial, enquanto a diminuição do potencial de membrana mitocondrial pode ter mantido os níveis de EROs citosólico para não promover danos celulares.
- A Pectina de folhas de *C. erectus* apresentou potencial pré-biotico semelhante a inulina.

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ANEXO 1

AUTORIZAÇÃO DE COLETA DO MATERIAL BOTÂNICO

CPRH Agência Estadual de Meio Ambiente

CA DRFB Nº 120/2014 24 de outubro de 2014.

Ao Senhor
JEYMESSON RAPHAEL CARDOSO VIEIRA

Prezado Senhor,

Cumprimentando Vossa Senhoria, nos reportamos ao Processo CPRH nº 013520/2014, referente ao requerimento para realização de atividades de pesquisa na APA de Santa Cruz e na APA Estuarina de Santa Cruz, sendo o local de coleta às margens do Canal de Santa Cruz e Rio Paripe, próximo a Vila Velha, Ilha de Itamaracá - PE.

Após análise da documentação apresentada vimos informar que o projeto **"Tecnologia Morfológica Aplicada à Inovação Terapêutica: Uma Perspectiva de Investigação de Produtos Naturais da Região de Mangue"** foi aprovado, estando autorizada a realização da citada pesquisa. No entanto, em caso de realização de atividades em áreas particulares, estas somente poderão ocorrer mediante a anuência do proprietário das terras. Salientamos ainda, que esta autorização permite as atividades de campo e coleta de material para estudo, entretanto, o acesso ao patrimônio genético deverá ser autorizado pelos órgãos competentes.

Informamos que a Unidade de Conservação APA de Santa Cruz possui sede e administração local, desta forma, caso haja necessidade de apoio para a realização das atividades de campo, faz-se necessário contato prévio com a equipe de gestão da Unidade.

Atenciosamente,

CINTHIA RENATA VIEIRA DE LIMA
Diretoria de Recursos Florestais e Biodiversidade

ANEXO 2

PARECER DO COMITÊ DE ÉTICA

Comitê de Ética em Pesquisa Envolvendo Seres Humanos

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

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

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

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

DADOS DO PARECER

Número do Parecer: 1.870.360

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

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

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

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

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

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

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

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

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

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

Avaliação dos Riscos e Benefícios:

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

Comentários e Considerações sobre a Pesquisa:

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

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

Considerações sobre os Termos de apresentação obrigatória:

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

Recomendações:

Sem recomendações

Conclusões ou Pendências e Lista de Inadequações:

Sem pendências

Considerações Finais a critério do CEP:

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

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

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

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

Continuação do Parecer: 1.870.360

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

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

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

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

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

RECIFE, 16 de Dezembro de 2016

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador)

ANEXO 3

UNIVERSIDADE FEDERAL DE PERNAMBUCO
TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO
(PARA MAIORES DE 18 ANOS OU EMANCIPADOS - Resolução 466/12)

Convidamos o (a) Sr. (a) para participar como voluntário (a) da pesquisa **Investigação imunológica e antitumoral de compostos naturais extraídos de plantas**, que está sob a responsabilidade do (a) pesquisador (a) Profa. Dra. Cristiane Moutinho Lagos de Melo, Av. Prof. Moraes Rego, s/n.º - Cidade Universitária – Recife – PE – CEP 50.670.420, fone: 2126.8866, e-mail: cristianemout@gmail.com. Também participam desta pesquisa os alunos:

Bruno Rafael Barboza, telefone: 997738245; Jéssica de Santana Brito, telefone: 998746208; Leydianne Leite de Siqueira Patriota Queiroz, telefone: 997990623; Glêzia Renata da Silva Lacerda, telefone: 997132402; Dayane Kelly Dias do Nascimento Santos, telefone: 993947520; Vanessa Silva de Almeida, telefone: 986991635; Camila Joyce Alves da Silva, telefone: 996855413 e Bárbara Rafaela da Silva Barros, telefone: 985554155 e está sob a orientação da Profa. Dra. Cristiane Moutinho Lagos de Melo, fone: 2126.8866.

Caso este Termo de Consentimento contenha informações que não lhe sejam compreensíveis, as dúvidas podem ser tiradas com a pessoa que está lhe entrevistando e apenas ao final, quando todos os esclarecimentos forem dados, caso concorde com a realização do estudo pedimos que rubrique as folhas e assine ao final deste documento, que está em duas vias, uma via lhe será entregue e a outra ficará com o pesquisador responsável.

Caso não concorde, não haverá penalização, bem como será possível retirar o consentimento a qualquer momento, também sem nenhuma penalidade.

INFORMAÇÕES SOBRE A PESQUISA:

Convidamos o (a) Sr (a) para participar da Pesquisa Investigação imunológica e antitumoral de compostos naturais extraídos de plantas, a qual pretende analisar o efeito de compostos naturais extraídos de plantas sobre o sistema imunológico e em células humanas de câncer. Sua participação é necessária, pois, como não portador de doenças infecciosas e tumorais, o seu sangue será utilizado como controle negativo. Sua participação é voluntária e se dará através de dois itens: (1) coleta de até 9 (nove) colheres de chá de sangue (4 ml) através de um tubo adaptado a uma agulha, estéril e descartável. Esse procedimento é praticamente isento de risco, pois todo material utilizado é descartável, porém, poderá causar dor ou mancha vermelha (hematoma). Caso ocorra qualquer desconforto ou promoção de hematoma local você receberá ajuda da equipe de coleta através de cuidados paliativos. O sangue será utilizado para a realização do cultivo das células do sangue quando em contato diversos compostos naturais com o objetivo de: (i) verificar produção de citocinas e quimiocinas (substâncias envolvidas no sistema de defesa contra doenças), (ii) analisar a presença de marcadores de superfície celular (que identifica quais células são e se houve aumento ou diminuição da população de células), (iii) dosar a quantidade de óxido nítrico (que também es tá envolvido na defesa contra as doenças).

Todas as informações desta pesquisa serão confidenciais e serão divulgadas apenas em eventos ou publicações científicas, não havendo identificação dos voluntários, a não ser entre os responsáveis pelo estudo, sendo assegurado o sigilo sobre a sua participação. As amostras sanguíneas coletadas nesta pesquisa serão processadas e ficarão armazenadas em freezer a -20°C, sob a responsabilidade do Profa. Dra. Cristiane Moutinho Lagos de Melo, no endereço acima informado pelo período de mínimo 5 anos.

Nada lhe será pago e nem será cobrado para participar desta pesquisa, pois a aceitação é voluntária, mas fica também garantida a indenização em casos de danos, comprovadamente decorrentes da participação na pesquisa, conforme decisão judicial ou extra-judicial. Se houver necessidade, as despesas para a sua participação serão assumidas pelos pesquisadores (ressarcimento de transporte e alimentação).

Em caso de dúvidas relacionadas aos aspectos éticos deste estudo, você poderá consultar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da UFPE no endereço: **(Avenida da Engenharia s/n – 1º Andar, sala 4 - Cidade Universitária, Recife-PE, CEP: 50740-600, Tel.: (81) 2126.8588 – e-mail: cepccs@ufpe.br).**

_____
(assinatura do pesquisador)

CONSENTIMENTO DA PARTICIPAÇÃO DA PESSOA COMO VOLUNTÁRIO (A)

Eu,_____, CPF_____, abaixo assinado, após a leitura (ou a escuta da leitura) deste documento e de ter tido a oportunidade de conversar e ter esclarecido as minhas dúvidas com o pesquisador responsável, concordo em participar do estudo **Investigação imunológica e antitumoral de compostos naturais extraídos de plantas**, como participante da pesquisa. Fui devidamente informado (a) e esclarecido (a) pelo(a) pesquisador (a) sobre a pesquisa, os procedimentos nela envolvidos, assim como os possíveis riscos e benefícios decorrentes de minha participação. Foi-me garantido que posso retirar o meu consentimento a qualquer momento, sem que isto leve a qualquer penalidade.

Local e data _____

Assinatura do participante: _____

Impressão digital (opcional)

Presenciamos a solicitação de consentimento, esclarecimentos sobre a pesquisa e o aceite do voluntário em participar. (02 testemunhas não ligadas à equipe de pesquisadores):

Nome:	Nome:
Assinatura:	Assinatura:

ANEXO 4

PARECER DO COMITÊ DE ÉTICA

Universidade Federal de Pernambuco
Centro de Biociências
Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
Fone: (55 81) 2126 8340 | 2126 8351
Fax: (55 81) 2126 8350
www.ccb.ufpe.br

Recife, 27 de dezembro de 2016.

Ofício nº 134/16

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.ª Cristiane Moutinho Lagos de Melo**
Departamento de Antibióticos
Centro de Biociências
Universidade Federal de Pernambuco
Processo nº **0048/2016**

Certificamos que a proposta intitulada **"Investigação imunológica e antitumoral de compostos naturais extraídos de plantas"**, registrada com o nº **0048/2016**, sob a responsabilidade de Prof.ª **Cristiane Moutinho Lagos de Melo** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE PERNAMBUCO (UFPE), em reunião de 07/12/2016.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	Até 01/2019
Espécie/linhagem/raça	Camundongos Swiss e BALB/c
Nº de animais	32
Peso/Idade	25-40g/ 60 dias
Sexo	Fêmeas
Origem	Biotério do Departamento de Antibióticos – CB/UFPE

Atenciosamente,

Prof. Dr. Pedro V. Carelli
Presidente da CEUA / CCB - UFPE
SIAPE 1501584