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CURSO DE DOUTORADO EM ODONTOLOGIA

CAROLINA MEDEIROS DE ALMEIDA MAIA

AVALIAÇÃO *IN VITRO* DOS EFEITOS DA *Anadenanthera colubrina* (Vell.) Brenan SOBRE FATORES DE VIRULÊNCIA DE *Candida albicans*, MODULAÇÃO DA INTERAÇÃO *Candida*-HOSPEDEIRO E REGULAÇÃO DA ATIVIDADE IMUNOMODULATÓRIA

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Área de concentração: Clínica Odontológica/ Desenvolvimento e otimização de produtos relacionados ao tratamento de agravos à saúde bucal.

Orientadora: Profª. Drª. Edja Maria Melo de Brito Costa
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Coorientador: Prof. Dr. Ramiro Mendonça Murata
East Carolina University (ECU)

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“Foi o tempo que dedicastes à tua rosa que a fez tão importante.”

Antoine de Saint-Exupéry

RESUMO

OBJETIVO: Este estudo avaliou *in vitro* a atividade do extrato das cascas de *Anadenanthera colubrina* (Vell.) Brenan sobre *Candida* spp., seus efeitos sobre fatores de virulência de *Candida albicans*, modulação da resposta imune através da interação *C. albicans*-hospedeiro e regulação da atividade anti-inflamatória. **MATERIAL E MÉTODO**: A atividade antifúngica foi avaliada Método de Microdiluição em Caldo sobre cepas referências de espécies de *Candida* (*C. albicans, C. glabrata, C. tropicalis, C. dubliniensis*). O efeito anti-biofilme foi verificado em biofilme maduro de *C. albicans* e quantificado em Unidades Formadoras de Colônia/mL por peso seco de biofilme (UFC/mL/g). As atividades das enzimas proteinase e da fosfolipase foram determinadas através dos ensaios da azocaseína e da fosfatidilcolina, respectivamente. A citotoxicidade foi analisada em fibroblastos gengivais humanos (HGF ATCC® CRL-2014) e em monócitos humanos (THP-1 ATCC® TIB-202) pelo ensaio de viabilidade *Cell Titer Blue*. O modelo de cocultura de *C. albicans*-HGF foi analisado por microscopias eletrônica de varredura (MEV) e de fluorescência, pela expressão gênica das enzimas liberadas pela *C. albicans* (SAP-1, PLB-1) e das citocinas inflamatórias do hospedeiro (IL-6, IL-8, IL-1β, IL-10) avaliadas por RT-PCR, e pela liberação das citocinas, analisada por Luminex. O modelo de inflamação foi induzido através da exposição de monócitos THP-1 ao LPS (Lipopolissacarídeo), com determinação da expressão gênica e dos níveis de citocinas inflamatórias liberadas (IL-8, IL-1β e IL-10) através de RT-PCR e Luminex, respectivamente. A identificação da expressão das proteínas-chave das vias de transduções de sinais NF-κB e MAPK (NF-κB, p-38, p-NF-κB e p-p38) foi avaliada através de *Simple Western* (*WES Simple*). A caracterização do perfil fitoquímico do extrato foi realizada por HPLC-ESI-MS^n (*High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometric*) e LC-HRESIMS (*High Resolution Electrospray Ionization Mass Spectrometry*). **RESULTADOS:** O extrato apresentou efeito fungistático com CIM < 15,25 µg/mL sobre as linhagens de *Candida* testadas. O biofilme e a atividade proteolítica de *C. albicans* foram significativamente reduzidos na concentração de 312,25 µg/mL do extrato. Para o ensaio de viabilidade celular, o extrato apresentou LD_{50} = 423,3 µg/mL para a linhagem de HGF, e LD_{50} = 978,7 µg/mL para a de monócitos. As microscopias da cocultura revelaram redução substancial no crescimento de *C. albicans* com toxicidade mínima sobre as células do hospedeiro. As expressões gênicas de SAP-1/PLB-1 foram significativamente infrarreguladas e a resposta imune do hospedeiro frente à infecção por *C. albicans* foi modulada por uma diminuição significativa na secreção das citocinas pró-inflamatórias IL-6 e IL-8. Diante do estímulo por LPS, a expressão gênica e a

liberação das citocinas IL-1β e IL-10, foram infra e suprarreguladas pelo extrato, respectivamente. O extrato apresentou envolvimento nas vias NF-κB/MAPK relacionadas à ativação de TLR4 (*Toll Like Receptor*-4), através da fosforilação de NF-κB e p38, com sinal de intensidade reduzidos. O extrato de *A. colubrina* por si só não induziu resposta inflamatória. A análise fitoquímica apresentou perfil fenólico, constituído predominantemente por flavonóides, catequinas, procianidinas e taninos. **CONCLUSÃO:** O extrato de *A. colubrina* apresentou atividade antifúngica frente a cepas de *Candida*, baixa citotoxicidade, efeito antibiofilme e antiproteolítico sobre *C. albicans*, com efeitos reguladores sobre a resposta imune do hospedeiro frente à infecção e atividade anti-inflamatória.

Palavras-chave: fitoterapia; candidíase oral; *Candida albicans*; biofilmes.

ABSTRACT

OBJECTIVE: This study evaluated *in vitro* the antifungal activity of *Anadenanthera colubrina* (Vell.) Brenan barks extract on *Candida* spp., its effects on virulence factors of *Candida albicans*, modulation of immune response through *C. albicans*-host interaction and regulation from anti-inflammatory activity. **MATERIAL AND METHOD:** The antifungal activity was evaluated by Broth Microdilution Method on reference strains of *Candida* spp. (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*). Anti-biofilm effect was determined on *C. albicans* mature biofilm and quantified by Colony Forming Units/mL of biofilm dry weight (CFU/mL/g). Activities from proteinase and phospholipase enzymes were determined through azocasein and phosphatidylcholine assays, respectively. The cytotoxicity was assessed by Cell Titer Blue viability assay on human gingival fibroblasts (HGF ATCC® CRL-2014) and on human monocytes (THP-1 ATCC® TIB-202). The coculture model of *C. albicans*-HGF was analyzed by SEM (Scanning Electron Microscopy) and fluorescence microscopy, by gene expression of the enzymes released by *C. albicans* (SAP-1, PLB-1) and the host's inflammatory cytokines (IL-6, IL-8, IL-1β, IL-10) evaluated by RT-PCR; and by the release of cytokines, assessed by Luminex. The inflammation model was assessed through THP-1 cells exposed to LPS (Lipopolysaccharide), with evaluation from gene expression and levels of production of inflammatory cytokines (IL-8, IL-1β and IL-10), by RT-PCR and Luminex, respectively.
Simple Western (WES Simple) was performed to identify the expression of key proteins of NF-κB and MAPK transduction signaling pathways (NF-κB, p-38, p-NF-κB and p-p38). The phytochemical characterization of the extract was assessed by HPLC-ESI-MS^n (High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometric) and LC-HRESIMS (High Resolution Electrospray Ionization Mass Spectrometry). **RESULTS:** Extract presented fungistatic effect with MIC<15.25 µg/mL against *Candida* strains tested. Biofilm and proteolytic activity were significant reduced at 312.25 µg/mL extract concentration. For cell viability assay, the extract presented LD_{50} = 423.3 µg/mL for HGF cell line, and LD_{50} = 978.7 µg/mL for THP-1. Coculture microscopies demonstrated a substantial decreased in *C. albicans* growth and minimal toxicity against host cells. Gene expressions of SAP-1/PLB-1 were significantly down-regulated and host immune response was modulated by a significant decreased on IL-6 and IL-8 pro-inflammatory cytokines secretion. Upon LPS stimuli, gene expression and cytokines production of IL-1β and IL-10 were down and up-regulated, respectively. The extract is involved on TRL4-related NF-κB/MAPK pathways through phosphorylation of p38 and NF-κB, with decreased in the signal intensity. Extract itself

did not induce inflammatory response. Phytochemical analysis showed a phenolic profile with presence of flavonoids, cathechins, procyanidins and tannins. **CONCLUSION:** *A. colubrina* extract presented antifungal activity on *Candida* strains, low cytotoxicity, antibiofilm and anti-proteolytic enzyme effects against *C. albicans*, with modulatory effects on the host immune response in front of infection and anti-inflammatory activity.

Keywords: phytotherapy; oral candidiasis; *Candida albicans*; biofilms.

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LISTA DE ABREVIATURAS E SIGLAS

ACAM	Herbário Manuel de Arruda Câmara
ACT	Actin-like Protein
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	Análise de Variância
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid
Calceína-AM	Calceína-Acetoximetil
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CCL5	CC Motif Ligand 5
CFM	Concentração Fungicida Mínima
CIM	Concentração Inibitória Mínima
CO_2	Dióxido de Carbono
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECU	East Carolina University
ESI-MS	Electrospray Ionization Mass Spectrometry
EthD-III	Ethidium Homodimer III
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HGF	Human Gingival Fibroblast
HIV	Human Immunodeficiency Virus
HPLC-ESI-MS^n	High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometric
IL	Interleucina
LC-HRESIMS	High Resolution Electrospray Ionization Mass Spectrometry
LPS	Lipopolissacarídeo
M	Molar
MAPK	Mitogen-activated Protein Kinase
MEV	Microscopia Eletrônica de Varredura
N_2	Nitrogênio
NaOH	Hidróxido de Sódio

NF-κB	Factor Nuclear kappa B
NIH	National Institutes of Health
OD	Optical Density
PAMP	Pathogen-associated Molecular Pattern
PB	Paraíba
PBS	Phosphate-buffered Saline
PDSE	Programa de Doutorado Sanduíche no Exterior
pH	Potencial Hidrogeniônico
PLB	Phospolipase
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidene Difluoride
RIPA	Radioimmunoprecipitation Assay
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAP	Secreted Aspartyl Proteinase
SFB	Soro Fetal Bovino
SisGen	Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado
TLR	Toll Like Receptor
TOF-MS	Time of Flight Mass Spectrometry
U	Unidade de Enzima
UEPB	Universidade Estadual da Paraíba
UFC	Unidades Formadoras de Colônias
YNB	Yeast Nitrogen Base
°C	Grau Celsius
µL	Microlitro
µM	Micromolar
g	Grama
kV	Kilovolt
mL	Mililitro
mM	Milimolar
nm	Nanômetro

nM	Nanomolar
rpm	Rotação por minuto
V	Volt
v/v	volume/volume

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

A candidíase oral é uma das infecções fúngicas mais prevalentes em humanos (HERTEL et al., 2016; ROSA-GARCÍA et al., 2020), sendo causada por leveduras do gênero *Candida*, componentes naturais do microbioma de aproximadamente 70% da população saudável (WITHERDEN et al., 2017; NIKOU et al., 2019). As espécies de *Candida* são classificadas como patobiontes, ou seja, tornam-se patogênicas diante de condições que modificam o equilíbrio do microambiente e comprometem a imunidade do hospedeiro (PELLON et al., 2020). Podem causar desde infecções mucocutâneas superficiais até disseminação sistêmica para outros órgãos (LEWIS E WILLIAMS, 2017; NIKOU et al., 2019; BHATTACHARYA et al., 2020).

Estimativas globais indicam que as infecções causadas por fungos afetam mais de 1 bilhão de pessoas por ano, incluindo 2 milhões de casos de candidíase oral e 700 mil casos de candidíase sistêmica (BONGOMIN et al., 2017). Deste modo, *Candida* spp. São responsáveis por 70 a 90% das infecções fúngicas invasivas, sendo uma das causas mais frequentes de infecções sistêmicas adquiridas em ambiente hospitalar, relacionada a uma taxa de mortalidade de 58 a 81% (DELALOYE E CALANDRA, 2014; VAEZI et al., 2017). Além disso, a candidíase oral permanece como a infecção oportunista mais comum em cerca de 95% dos indivíduos portadores do vírus HIV, sendo considerada um indicador de progressão da Síndrome da Imunodeficiência Adquirida (AIDS) (MUCHI et al., 2017; PATIL et al., 2018).

A *Candida albicans* é a espécie que mais se destaca na patogênese da candidíase oral (PAPPAS et al., 2018; PELLON et al., 2020), em decorrência da expressão de fatores de virulência que favorecem sua rápida transição em patógeno, a proliferação descontrolada e a consequente invasão superficial nos tecidos (MILSSOP E FAZE, 2016; HELLSTEIN E MAREK, 2019). Dentre os fatores de patogencidade, destacam-se a capacidade de aderência às superfícies do epitélio oral ou de dispositivos médicos, através da expressão de adesinas de superfície; formação de biofilme; destruição do tecido do hospedeiro através da secreção de enzimas proteolíticas e evasão dos mecanismos de defesa do hospedeiro, devido à transformação fenotípica e à degradação de anticorpos e peptídeos antimicrobianos (HOFS et al., 2016; VILA et al., 2020).

A maioria das infecções causadas por *C. albicans* está diretamente associada à organização em forma de biofilme, em que a transição da sua morfologia de levedura para a forma filamentosa de hifa é fundamental para a formação do biofilme patogênico (TSUI et al., 2016; WALL et al., 2019). Devido à tenacidade conferida pela organização das populações

microbianas aderidas à superfície e incorporadas a uma matriz extracelular, o biofilme torna-se tolerante à difusão dos agentes antifúngicos (TSUI et al., 2016; GHANNOUM et al., 2015; HIROTA et al., 2017). Sendo assim, a habilidade da *C. albicans* em formar biofilmes é considerada o fator de virulência com implicações clínicas mais significativas (FOX et al., 2015; WALL et al., 2019).

Além do efeito mecânico causado pela proliferação de hifas e formação de biofilme, a destruição dos tecidos do hospedeiro pela *C. albicans* é intensificada pela liberação de enzimas hidrolíticas extracelulares (VILA et al.,2020). Dentre as enzimas associadas à virulência da *C. albicans*, destacam-se as Aspartil Proteases (SAPs) e Fosfolipases (PLs), envolvidas na destruição das membranas das células e invasão tecidual, aquisição de nutrientes e evasão da resposta imune, contribuindo diretamente para a progressão da infecção (SORGO et al., 2013; JABRA-RIKS et al., 2016; SWIDERGALL E FILLER, 2017).

Nesse contexto, a manutenção do estado de comensalismo da *C. albicans* está diretamente associada ao equilíbrio entre os fatores de virulência do patógeno e a resposta imune do hospedeiro (TOOYAMA et al., 2015), obtido através de um processo de "coevolução" responsável pela aquisição contínua de meios de adaptação mútua (NIKOU et al., 2019; PELLON et al., 2020). Nessas condições, a resposta imune do hospedeiro é direcionada para um estado de tolerância imunológica, com o intuito de evitar uma resposta inflamatória exacerbada e, consequentemente, prevenir a destruição e invasão tecidual (VILA et al., 2020).

Estudos de revisão a respeito da interação entre *C. albicans* e as células do hospedeiro, bem como a resposta imunomodulatória frente ao desenvolvimento da infecção, têm sido publicados com o intuito de esclarecer os mecanismos moleculares que regulam esta complexa associação (NETEA et al., 2010; HOFS et al., 2016; NAGLIK et al., 2017; NIKOU et al., 2019; SWIDERGALL, 2019; PELLON et al., 2020; VILA et al., 2020). Infecções causadas por microrganismos são consideradas um dos agentes mais comuns associados à ativação do processo inflamatório (JANG et al., 2020). Os patógenos apresentam estruturas em sua membrana ou parede celular, denominadas de Padrões Moleculares Associados a Patógenos (PAMPs), os quais interagem com Receptores de Reconhecimento de Padrões (PRRs), que são proteínas localizadas na membrana das células hospedeiras e diretamente relacionadas à ativação do sistema imune (TAKEUCHI E AKIRA, 2010; KIM et al., 2020).

Em relação à candidíase, a resposta imune do hospedeiro frente à infecção por *C. albicans* ativa uma cascata de mecanismos moleculares complexos a partir do reconhecimento do patógeno pelas células do hospedeiro (BROWN et al., 2012; PAPPAS et al., 2018). A parede celular da *C. albicans*, rica em polissacarídeos, é altamente imunogênica e facilmente

reconhecida pelos PRRs, em especial os *Toll-Like Receptors* (TLRs), como TRL2 e TRL4 (PAPPAS et al., 2018). Quando ativados, esses receptores induzem a secreção de peptídeos antifúngicos com o intuito de controlar a proliferação do patógeno (NAGLIK et al., 2017). Além disso, a ativação das vias de transdução de sinais que regulam a transcrição de genes associados à resposta inflamatória, resulta na liberação de citocinas pró-inflamatórias, principalmente IL-1α, IL-1β, IL-6, IL-8 e CCL5 (RANTES), e no recrutamento de células do sistema imune para o local de infecção (HEBECKER et al., 2014; VERMA et al., 2017; VERMA et al., 2018).

Considerando o crescimento na incidência de todas as formas de candidíase oral e consequentemente o aumento de episódios recorrentes da infecção, esta exposição intermitente tem propiciado a seleção contínua de cepas resistentes à terapia antifúngica (LUKASZUK et al., 2017). Além disso, as limitações dos agentes terapêuticos disponíveis como, seletividade sub-ótima, toxicidade considerável e, também, uma maior probabilidade de desenvolver resistência microbiana, comprometem o combate efetivo das infecções fúngicas (VILA et al., 2020). Portanto, o surgimento de cepas resistentes a drogas individuais ou grupos inteiros de antifúngicos constitui, de fato, um problema de saúde pública (BROWN et al., 2012) o que justifica o desenvolvimento de métodos alternativos de tratamento (AS-AMI et al., 2016; SANGLARD, 2016; ZAIDI et al., 2016; ALGHAMDI et al., 2018).

Assim, o potencial de novas alternativas antifúngicas tem sido investigado com o objetivo de identificar compostos bioativos que apresentem mecanismos moleculares capazes de modular os fatores de virulência associados à infecção causada por *C. albicans* e a resposta imune do hospedeiro frente ao patógeno (FRANCISCONI et al., 2020). Entre as novas estratégias, produtos naturais derivados de plantas medicinais tem sido considerados uma fonte promissora para o desenvolvimento de novas terapias antifúngicas. Entre os anos de 1940 e 2014, 40% das moléculas aprovadas pela FDA (*US Food and Drug Administration*) foram classificadas como produtos naturais (NEWMAN E CRAGG, 2016).

Dentre a biodiversidade disponível, destaca-se a utilização da *Anadenanthera colubrina* (Vell.), planta pertencente à seção Mimosoideae da família Fabaceae, ordem Fabales (WEBER et al., 2011) e conhecida popularmente como angico-branco ou angico-liso. É uma espécie arbórea que pode ser encontrada ao sul da linha do Equador, mais comumente no bioma Caatinga na região Nordeste do Brasil (WEBER et al., 2011). É bastante utilizada como planta medicinal pelas populações tradicionais locais, principalmente para o tratamento de inflamações em geral (ARAÚJO et al., 2014; ARAÚJO et al., 2015).

Estudos recentes têm demonstrado o potencial terapêutico da *A. colubrina*, em decorrência de suas propriedades antifúngicas (LIMA et al., 2014; SILVA et al., 2019), antiproliferativa (LIMA et al., 2014), anti-inflammatória (GUARNEIRE et al., 2019; CARDOSO-JUNIOR et al., 2020) e antioxidante (ARAÚJO et al., 2019; CARDOSO-JUNIOR et al., 2020). Estas propriedades terapêuticas e biológicas podem ser justificadas pela presença de compostos bioativos na composição química da *A. colubrina*, comprovadamente considerada fonte de compostos fenólicos, principalmente da classe dos flavonoides (LIMA et al., 2014; CARDOSO-JUNIOR et al., 2020). Esses compostos possuem atividade antimicrobiana contra espécies de bactérias e fungos (SILVA et al., 2012; SILVA et al., 2013; ARAÚJO et al., 2014; LIMA et al., 2014; ARAÚJO et al., 2017), e principalmente, atividade anti-inflamatória (LEE et al., 2018; CHEN et al., 2019; MALEKI et al., 2019; CARDOSO-JUNIOR et al., 2020).

O potencial antifúngico da *A. colubrina* investigado por pesquisas recentes, tem revelado um efeito inibitório sobre espécies de *Candida*, com destaque para o biofilme formado por *C. albicans* (LIMA et al., 2014; SILVA et al., 2019). Apesar da identificação desse potencial terapêutico, existem poucos dados disponíveis a respeito da atividade dessa planta sobre a expressão de fatores de virulência relacionados à patogenicidade da *C. albicans* e seu efeito modulador da interação *Candida*-hospedeiro Este é o primeiro trabalho avaliando *in vitro* os efeitos do extrato das casas da *Anadenanthera colubrina* (Vell.) Brenan sobre a interação entre *C. albicans* e hospedeiro e a regulação da atividade imunomoduladora, através de uma abordagem a nível transcricional e proteômico. Sendo assim, este trabalho foi delineado com o intuito de avançar nas análises das propriedades bioativas da *A. colubrina*, considerando seu potencial anti-*Candida*, associado à importância dos recursos naturais do nosso país como fonte sustentável de novas drogas e o largo interesse pelo uso de medicamentos de origem natural, de maneira a constituir alternativas viáveis e eficazes para a prevenção e tratamento da candidíase oral.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar *in vitro* os efeitos do extrato das cascas de *Anadenanthera colubrina* (Vell.) Brenan sobre fatores de virulência associados à infecção oral por *C. albicans*, regulação da resposta imune a partir da interação *C. albicans*-hospedeiro e modulação da atividade anti-inflamatória.

2.2 Objetivos específicos

Plano de Análise I:

- Avaliar a atividade antifúngica do extrato hidroetanólico das cascas de *A. colubrina* sobre espécies do gênero *Candida*;
- Verificar a atividade antibiofilme do extrato de *A. colubrina* em biofilme maduro de *C. albicans*;
- Identificar o potencial do extrato de *A. colubrina* em modular a liberação de enzimas proteolíticas por *C. albicans*;
- Determinar a citotoxicidade do extrato de *A. colubrina* em linhagem de fibroblastos gengivais humanos;
- Avaliar o efeito modulador do extrato de *A. colubrina* na interação *C. albicans*-hospedeiro em modelo de cocultura, através da:
    - Análise qualitativa da distribuição de *C. albicans* e das células do hospedeiro por microscopias eletrônica de varredura (MEV) e de fluorescência;
    - Análise quantitativa da regulação da expressão gênica de enzimas proteolíticas produzidas por *C. albicans*;
    - Análise quantitiativa da expressão gênica e da secreção de citocinas anti e pró-inflamatórias produzidas pelo hospedeiro.

Plano de Análise II:

- Determinar a citotoxicidade do extrato hidroetanólico das cascas de *A. colubrina* em linhagens de monócitos humanos;

- Avaliar o efeito modulador do extrato de *A. colubrina* sobre a expressão gênica e a produção de citocinas pró e anti-inflamatórias em linhagem de monócitos estimulados por LPS;
- Determinar as vias de transdução de sinais ativadas pelo extrato de *A. colubrina* após estímulo do receptor de membrana TLR4 por LPS;
- Caracterizar o perfil fitoquímico do extrato de *A. colubrina* através de HPLC-ESI-MS^n (*High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometric*) e HRESIMS (*High Resolution Electrospray Ionization Mass Spectrometry*).

3 METODOLOGIA

3.1 Material vegetal e obtenção do extrato

As cascas de *A. colubrina* foram coletadas na região do semiárido paraibano, na Serra de Bodocongó, município de Queimadas (7º 22’ 25” S, 35º 59’ 32”W), na meso região da Borborema e microrregião do Cariri Oriental, no mês de setembro. O espécime botânico testemunho da *Anadenanthera colubrina* (Vell.) Brenan encontra-se depositado no Herbário Manuel de Arruda Câmara (ACAM) da Universidade Estadual da Paraíba (UEPB), Campus I, Campina Grande, Paraíba, Brasil, sob o nº 1936/ACAM. Foi obtido o extrato hidroetanólico a 80% da casca da planta, através de maceração por 48 horas, utilizando-se a proporção de 10 mg da planta para cada 25 mL de álcool etílico 80% (CARVALHO et al., 2011). Foram realizadas três filtragens do material, seguidas de concentração a vácuo (Tecnal TE-211, Piracicaba, SP) e liofilização (Martin Christ 1-2 Ldplus, Osterode am Harz, DE). O rendimento obtido foi de 31,7%. Esta pesquisa foi conduzida sob o número de autorização SisGen A289DF4.

PLANO DE ANÁLISE I

3.2 Microrganismos

Foram utilizadas as seguintes linhagens de referência de *Candida* spp: *C. albicans* ATCC® 90028, *C. albicans* ATCC® MYA-2876, *C. glabrata* ATCC® MYA-275, *C. tropicalis* ATCC® MYA-750 e *C. dubliniensis* ATCC® MYA-646.

3.3 Teste de susceptibilidade anti-*Candida*

A atividade antifúngica do extrato de *A. colubrina* foi avaliada através do Método de Microdiluição em Caldo sobre linhagens de *Candida* spp., com determinação das concentrações inibitória mínima (CIM) e fungicida mínima (CFM) (NCCLS/M38-A2, 2002). O ensaio foi realizado em placas de 96 poços (Greiner Bio-One North America, Inc Monroe, NC, EUA) contendo 100 µL/poço de meio de cultura RPMI-1640 (Lonza Bioscience, Walkersville, MD). Cem microlitros do extrato de *A. colubrina* (8.000 µg/mL) foram adicionados ao poço inicial, e procedeu-se a microdiluição seriada, obtendo-se um intervalo de concentrações entre 2.000 e 15,62 µg/mL. O inóculo foi preparado a partir de culturas de 24 horas de cada espécie de *Candida* e padronizado em espectrofotômero (SpectraMax M3, Molecular Devices, Sunnyvale, CA), ajustando-se sua absorbância entre 0,08 e 0,10 a 625 nm, para a obtenção de uma densidade equivalente a 5,0 x 10^6 UFC/mL. A partir dessa suspensão,

foram realizadas diluições seriadas em meio de cultura RPMI-1640, obtendo-se ao final uma densidade de 5,0 x 10^3 UFC/mL. Em seguida, 100 µL do inóculo foram adicionados a cada poço, resultando em uma concentração final de 2,5 x 10^3 UFC/mL. As placas foram incubadas por 24 horas, a 37°C com 5% de CO_2 (VWR Symphony 5.3 A, Radnor, PA). Fluconazol (512 µg/mL) (Alfa Aesar, Tewksbury, MA) foi utilizado como controle positivo e Dimetil Sulfóxido 1% (DMSO, BDH Solvents, Dawsonville, GA), como veículo. A CIM foi definida como a menor concentração do extrato capaz de inibir o crescimento visível da levedura, confirmado pela mudança na cor do meio RPMI-1640. Para a determinação da CFM, uma alíquota de 10 µL de cada poço com concentrações iguais e superiores a CIM foi subcultivada em placas com meio de cultura Agar Sabouraud Dextrose (BD Difco, Franklin Lakes, NJ) e incubadas por 48 horas, a 37°C com 5% de CO_2. A CFM foi definida como a menor concentração capaz de inibir o crescimento microbiano visível nas placas de ágar. Os ensaios foram realizados em triplicata e repetidos pelo menos em três momentos diferentes.

3.4 Atividade anti-biofilme

A partir de uma cultura de 24 horas de *C. albicans* (ATCC® MYA-2876), foi preparado um inóculo em meio de cultura YNB (*Yeast Nitrogen Base*, Sigma Aldrich, Saint Luis, MO) suplementado com 50 mM de glicose (VWR Life Science, Radnor, PA), padronizado a uma concentração de 1x10^6 UFC/mL. O ensaio foi realizado em placas de cultura de células de 24 poços (Greiner Bio-One North America, Inc Monroe, NC) contendo 1 mL/poço do inóculo. Para estabelecer o crescimento inicial do biofilme, as placas foram incubadas por 24 horas, a 37°C com 5% de CO_2. Em seguida, o meio de cultura foi descartado, preservando-se o biofilme aderido ao fundo dos poços. Para remover as células não aderidas, as amostras foram lavadas com 1 mL de Solução Tampão Fosfato (PBS, Lonza Bioscience, Walkersville, MD), reabastecidos com 900 µL de meio YNB estéril e 100 µL do extrato de *A. colubrina*, em concentrações equivalentes a 10xCIM e 20xCIM. As placas foram então incubadas por 24 horas, a 37°C com 5% de CO_2. Este protocolo foi repetido uma vez ao dia, durante 72 horas. Fluconazol (10xCIM) foi utilizado como controle positivo e DMSO 1% (v/v), como veículo. Após 72 horas de tratamento, os biofilmes foram removidos do fundo dos poços, ressuspendidos em PBS e centrifugados a 10.000 rpm por 5 minutos. A biomassa (peso seco) de cada amostra de biofilme foi obtida descartando-se o sobrenadante e secando as amostras em concentrador à vácuo por 40 minutos. Para a contagem das UFCs, as suspensões do bioifilme foram diluídas seriadamente (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) e uma alíquota de 10 µL de cada diluição foi subcultivada, em triplicata, em placas de Ágar Sabouraud Dextrose. Após a

incubação das placas por 24 horas, a 37°C com 5% de CO_2, o número de colônias de *C. albicans* foi registrado e os dados foram normalizados pela relação UFC/mL/peso seco (g) de biofilme (SANTANA et al., 2013; SELEEM et al., 2016; CHEN et al., 2018).

3.5 Inibição da atividade proteolítica de Aspartil Protease e Fosfolipase

Um inóculo padronizado com densidade de $1x10^6$ UFC/mL de *C. albicans* (ATCC® MYA-2876) foi preparado em meio de cultura YNB suplementado com 50 mM de glicose e incubado em placas de cultura de células de 24 poços por 24 horas, a 37°C com 5% de CO_2. Após a formação inicial do biofilme, o meio de cultura foi descartado e o biofilme lavado com 1 mL de PBS. Em seguida, foram adicionados 900 µL de meio YNB estéril e 100 µL do extrato de *A. colubrina* (10xCIM e 20xCIM) e as placas foram então incubadas por 24 horas, a 37°C com 5% de CO_2. Após 72 horas de maturação, os biofilmes foram ressuspendidos através de sonicação por 15 segundos a 20% de amplitude com intervalos de pulsos de 5 e 10 segundos (FB120; Fischer Scientific, Pittsburgh, PA). A atividade da enzima proteinase (SAP) foi determinada pela ensaio da azocaseína. O sobrenadante da suspensão do biofilme foi incubado com 1% de azocaseína (Sigma Aldrich, Saint Luis, MO) na proporção de 9:1 (v/v), por 1 hora, a 37°C com 5% de CO_2. Em seguida, foram adicionados à suspensão 500 µL de ácido tricloroacético a 10% (VWR Life Science, Radnor, PA) para interromper a reação. A solução foi centrifugada por 5 min a 10.000 rpm. Em seguida, 500 µL do sobrenadante foram combinados com 500 µL de NaOH 0,5 M (Macron Fine Chemicals, Avantor VWR Life Science, Radnor, PA) e incubados por 15 min, a 37°C com 5% de CO_2. A absorbância da reação foi lida em espectrofotômetro a 440 nm (PANDE et al., 2006; GONÇALVES et al., 2012; SANTANA et al., 2013). A atividade da enzima fosfolipase (PL) foi analisada pelo ensaio da fosfatidilcolina. Os sobrenadantes das suspensões de biofilme (pH ajustado a 7,5) foram incubados com substrato de fosfatidilcolina (Sigma Aldrich, Saint Luis, MO) na proporção de 9:1 (v/v), por 1 hora, a 37°C com 5% de CO_2. A absorbância da reação foi lida em espectrofotômetro a 630 nm (TANIGUCHI et al., 2009). Para ambas as reações, Tripsina (Gibco, Invitrogen, Waltham, MA) foi utilizada como controle. Uma unidade de enzima foi estabelecida como a variação da absorbância (ΔOD) por minuto de reação, multiplicado por mil ($U = \Delta OD nm \times min - 1 \times 1000$). A atividade enzimática específica foi definida como a quantidade de enzima que provocou um aumento de 0,001 unidades de absorbância/minuto por peso seco do biofilme (g) (TANIGUCHI et al., 2009; SANTANA et al., 2013).

3.6 Ensaio de citotoxicidade em fibroblastos gengivais humanos

O efeito citotóxico do extrato de *A. colubrina* foi avaliado em fibroblastos gengivais humanos (HGF-1 ATCC® CRL-2014) e determinado por método fluorométrico com resazurina (*Cell Titer Blue Viability Assay*, Promega Corp, Madison, WI). As células foram mantidas em meio de cultura DMEM (Lonza Bioscience, Walkersville, MD) suplementado com 10% de soro fetal bovino (SFB), (Gibco, Invitrogen, Waltham, MA) a 37°C com 5% de CO_2. Foi preparada uma suspensão de fibroblastos padronizada em 1×10^5 células/mL, subcultivada em placa de 24 poços e incubada por 24 horas, a 37°C com 5% de CO_2, para promover a adesão das células à superfície dos poços. O extrato de *A. colubrina* foi diluído em DMSO 1%, com concentração final dentro dos poços de 0,1%, posteriormente adicionado aos poços da placa (2.500 – 0,25 µg/mL), incubadas novamente por 24 horas. *Cell Titer Blue* reagente (30 µL) foi adicionado a cada poço, e após 3 horas a fluorescência do sobrenadante foi lida em leitor de microplacas (SpectraMax M3, Molecular Devices, Sunnyvale, CA) com excitação de 555 nm, emissão de 585 nm e *cut off* de 570 nm (O'BRIEN et al., 2000).

3.7 Modelo de cocultura de fibroblastos e *C. albicans*

3.7.1 Microscopia de fluoroscência

Células de *C. albicans* (ATCC® MYA-2876) e fibroblastos gengivais foram co-cultivados, de acordo com o método proposto por Wong et al. (2014). Inicialmente, uma suspensão de fibroblastos preparada em DMEM (1×10^5 células/mL) foi subcultivada em placa de 24 poços e incubada por 24 horas, a 37°C com 5% de CO_2, para promover a adesão das células à superfície dos poços. Após o período de incubação, o meio de cultura foi substituído pelo inóculo de *C. albicans* ($5 \times 10^3 – 2{,}5 \times 10^3$ UFC/mL) preparado em DMEM sem SFB. A cocultura foi tratada com o extrato de *A. colubrina* (33,28 µg/mL) e a placa incubada a 37°C com 5% de CO_2, por 24 horas. Fluconazol (10 µg/mL) foi utilizado como controle positivo e DMSO 0,1%, como veículo. A distribuição de fibroblastos mortos e viáveis foi examinada usando o kit *Live/Dead Assay* para avaliação de citotoxicidade/viabilidade (Molecular Devices, Sunnyvale, CA), composto pelos corantes Calceína-AM e EthD-III (Etídio Homodímero). *Calcofluor White* (Sigma Aldrich, Saint Luis, MO) foi utilizado para corar as células de *C. albicans*. As imagens da coloração dupla foram capturadas através de microscopia de fluorescência (Keyence All-in-One BZ-X810 Fluoroscence Microscope, Itasca, IL).

3.7.2 Microscopia Eletrônica de Varredura (MEV)

Fibroblastos foram cocultivados com *C. albicans* em placas individuais de cultura de células (Greiner Bio-One North America, Inc Monroe, NC), seguindo o mesmo protocolo descrito acima para preparação das suspensões de células e para os tratamentos. Após o período de incubação, as amostras da cocultura foram lavadas com PBS e fixadas em glutaraldeído 3% (v/v) à temperatura ambiente por 12 horas. As células foram desidratadas em banhos sequenciais de etanol a 50%, 70%, 90% e 100%, por duas vezes em cada concentração, metalizadas em liga de ouro/paládio (Desk V Denton Vacuum, Moorestown, NJ) e observadas em microscópio eletrônico de varredura (Zeiss EVO LS10 SEM, Oberkochen, DE) (BERSAN et al., 2014).

3.7.3 Avaliação da expressão gênica através de RT-PCR

De acordo o mesmo protocolo para preparação da cocultura, e após 8 horas de tratamento com extrato de *A. colubrina*, o RNA dos fibroblastos e de *C. albicans* foram isolados e purificados utilizando o *Rneasy® Mini Kit* (Qiagen, Hilden, DE) e o RiboPure™-Yeast Kit (Invitrogen, ThermoFischer Scientific, Rockford, IL), respectivamente. O RNA total extraído foi quantificado utilizando o kit *SpectraDrop Micro-Volume Starter* (Molecular Devices, Sunnyvale, CA). RT-PCR foi realizada utilizando *QuantiFast® SYBR® Green RT-PCR Kit One Step* (Qiagen, Hilden, DE). Foram selecionados primers para os seguintes genes de *C. albicans*: Aspartil Protease-1 (*SAP-1*) e Fosfolipase B-1 (*PLB-1*). O gene *ACT-1* (*housekeeping*), a 10 µM, foi utilizado para normalizar a expressão dos genes *SAP-1* e *PLB-1*. Para avaliação da expressão gênica das citocinas inflamatórias do hospedeiro, foram selecionados primers para os genes *IL-6* (Qiagen Gene ID #3570), *IL-8* (Qiagen Gene ID #3576), *IL-10* (Qiagen Gene ID #3587) e *IL-1β* (Qiagen Gene ID #3553). Os dados da expressão desses genes foram normalizados pelo gene *housekeeping GAPDH* (Qiagen Gene ID #2597). As reações de amplificação foram conduzidas em termociclador (QuantStudio 3 Real Time PCR System, ThermoFischer Scientific, Rockford, IL) a 50°C por 10 min (etapa de transcrição reversa), 95°C por 5 min (etapa de ativação inicial da PCR), 40 ciclos de 10 segundos a 95°C (etapa de desnaturação) e 30s a 60°C (etapa de anelamento/extensão). A análise da expressão relativa de cada gene foi determinada através do método ΔΔCt (SELEEM et al., 2016; CHEN et al., 2018).

3.7.4 Análise da expressão de citocinas pró e anti-inflamatórias por Luminex

Conforme descrito anteriormente, após 8 horas de incubação com os tratamentos, os sobrenadantes da cocultura foram coletados, centrifugados por 10 minutos a 1.000 rpm e

imediatamente aplicadas para análise da secreção das citocinas pró-inflamatórias IL-6, IL-8, IL-1β, e anti-inflamatória IL-10, utilizando o *Human Magnetic Premixed Multi-Analyte Luminex Assay Kit* (R&D Systems, Minneapolis, MN), de acordo com as instruções do fabricante. Os dados foram obtidos pelo Luminex 200 Milliplex System e analisados pelo software Milliplex Analyst (ALLIN et al., 2016).

3.8 Análise estatística

Os dados foram expressos em média ± desvio padrão (Erro Padrão da Média) e analisados pelo teste de análise de variância a um critério fixo (ANOVA one way), com pós teste de Dunnet para comparação múltipla e nível de significância de 5% ($\alpha < 0.05$), utilizando o software GraphPad Prism (versão 8.02).

PLANO DE ANÁLISE II

3.9 Ensaio de citotoxicidade em linhagem de monócitos humanos

O efeito citotóxico do extrato de *A. colubrina* foi avaliado em linhagem de monócitos THP-1 (ATCC® TIB-202) e determinado por método fluorométrico com resazurina (*Cell Titer Blue Viability Assay*, Promega Corp, Madison, WI). As células foram mantidas em meio de cultura RPMI-1640, suplementado com 10% de soro fetal bovino e 2-mercaptoetanol a 50 nM (VWR Life Science, Radnor, PA), a 37°C com 5% de CO_2. Foi preparada uma suspensão de células padronizada em $2{,}5 \times 10^5$ células/mL e subcultivada em placa de 24 poços. Em seguida, o extrato de *A. colubrina* diluído em DMSO 1%, com concentração final nos poços de 0,1%, foi adicionado aos poços da placa (2.500 – 0,25 µg/mL), que foi incubada por 24 horas, a 37°C com 5% de CO_2. *Cell Titer Blue* reagente (30 µL) foi adicionado a cada poço, e após 3 horas a fluorescência do sobrenadante foi lida em leitor de microplacas com excitação de 555 nm, emissão de 585 nm e *cut off* de 570 nm (O'BRIEN et al., 2000).

3.10 Ativação de cultura de monócitos por LPS

Células THP-1 foram subcultivas ($2{,}5 \times 10^5$ células/mL), em placas de 24 poços, seguindo as mesmas condições do protocolo para ensaio da viabilidade celular. As células foram então expostas ao extrato de *A. colubrina* e LPS de *Porphyromonas gingivalis* (InvivoGen, San Diego, CA), de acordo com os seguintes grupos: estímulo com LPS (100 ng/mL), tratamento com *A. colubrina* (250 µg/mL), exposição simultânea ao LPS (100 ng/mL) e a *A. colubrina* (250 µg/mL), e grupo sem tratamento. As placas foram incubadas por 6 horas,

a 37°C com 5% de CO_2 (WEI et al., 2016). Os sobrenadantes das células foram coletados por centrifugação a 1.500 rpm por 5 min, para a avaliação quantitativa da produção de citocinas. As células mantidas no fundo dos poços foram processadas para extração de RNA e obtenção do lisado de células, para utilização nos ensaios de expressão gênica por RT-PCR e *Simple Western* (*Wes Simple*), respectivamente.

3.11 Avaliação da expressão gênica através de RT-PCR

O RNA total das células THP-1 foi isolado e purificado utilizando o *Rneasy® Mini Kit*, de acordo com as instruções do fabricante. O RNA total extraído foi quantificado utilizando o kit *SpectraDrop Micro-Volume Starter*. RT-PCR foi realizada utilizando o *QuantiFast® SYBR® Green RT-PCR Kit One Step*. Foram selecionados primers para os seguintes genes de citocinas inflamatórias do hospedeiro: *IL-8* (Qiagen Gene ID #3576), *IL-10* (Qiagen Gene ID #3587) e *IL-1β* (Qiagen Gene ID #3553). Os dados da expressão gênica foram normalizados pelo gene housekeeping GAPDH (Qiagen Gene ID #2597). As reações de amplificação foram conduzidas em termociclador a 50°C por 10 min (etapa de transcrição reversa), 95°C por 5 min (etapa de ativação aniicial da PCR), 40 ciclos de 10 segundos a 95°C (etapa de desnaturação) e 30 s a 60°C (etapa de anelamento/extensão). A expressão relativa de cada gene foi determinada através do método ΔΔCt (SELEEM et al., 2016; CHEN et al., 2018).

3.12 Análise da ativação de vias de transdução de sinais por *Simple Western* (*Wes Simple*)

As células THP-1 foram lisadas utilizando 200 µL de *Pierce RIPA Lysis Buffer* (ThermoFischer Scientific, Rockford, IL) por 5 min em temperatura ambiente. A concentração total de proteínas do lisado de células foi determinada pelo *Pierce BCA Protein Assay Kit* (Thermo Scientific, Rockford, IL). A expressão das proteínas NF-κB, p38, NF-κB fosforilada e p38 fosforilada foi detectada através do sistema *Wes Simple Western* (ProteinSimple, San Jose, CA), de acordo com as instruções do fabricante. Os anticorpos primários específicos (Cell Signaling Technology, Danvers, MA) utilizados para a detecção das proteínas analisadas, suas diluições otimizadas e seus pesos moleculares estão descritos no Quadro 1. Os dados obtidos foram analisados no software Compass™ for Simple Western versão 5.0 (ProteinSimple, San Jose, CA) (MISIEWICZ-KRZEMINSKA et al., 2018; PETOVARI et al., 2020).

Quadro 1. Lista dos anticorpos primários específicos para análise por *Wes Simple Western*.

Anticorpo Primário	Diluição	Peso Molecular (kDa)	Cat No.
NF-κB	1:1000	120	4717
p38	1:1000	40	9212
p-NF-κB	1:1000	120	4806S
p-p38	1:1000	43	4511

FONTE: Cell Singnaling Technology (www.cellsignal.com)

3.13 Análise da expressão de citocinas pró e anti-inflamatórias por Luminex

Os sobrenadantes da cultura de células THP-1 foram coletados, centrifugados por 10 minutos a 1.000 rpm e imediatamente aplicados para a análise da secreção das citocinas inflamatórias IL-8, IL-1β e IL-10, utilizando o *Human Magnetic Premixed Multi-Analyte Luminex Assay Kit* (R&D Systems, Minneapolis, MN), de acordo com as instruções do fabricante. Os dados foram obtidos pelo Luminex 200 Milliplex System e analisados pelo software Milliplex Analyst (ALLIN et al., 2016).

3.14 Caracterização fitoquímica por LC-ESI-MS^n e LC-HRESIMS

O extrato de *A. colubrina* foi analisado por HPLC (Shimadzu, Kyoto, Japão) utilizando coluna cromatográfica analítica C18 (Kromasil – 250 mm x 4,6 mm x 5 μm), acoplado a espectrômetro de massas (Ion-Trap AmazonX, Bruker ou microTOFII, Bruker, Berlim, Alemanha), com Ionização por Eletrospray (ESI). O extrato foi solubilizado em metanol (1mg/mL), com posterior filtração em filtros PVDF (fluoreto de polivinilideno), com malha de 0,45 μm. O método cromatográfico desenvolvido utilizou os solventes metanol (solvente B) de grau cromatográfico e água ultrapura tipo I (*Mili-Q*), acidificada com ácido fórmico (0,1% v/v) (solvente A), com análise em gradiente de concentração (5 a 100% de B em 60 minutos). O volume de injeção foi de 10 μL e a taxa de fluxo foi de 0,6 mL/minuto. Os parâmetros de aquisição no Ion Trap e TOF foram: capilar 4,5 kV, *offset* da placa final 500 V, gás nebulizador 35 psi, gás seco (N_2) com fluxo de 8 mL/minuto e temperatura de 300ºC. A amostra foi analisada no modo de ionização negativo e a identificação dos compostos foi baseada nos dados (MS/MS) reportados pela literatura.

3.15 Análise estatística

Os dados foram expressos em média ± desvio padrão (Erro Padrão da Média) e analisados pelo teste de análise de variância a um critério fixo (ANOVA one way), com pós teste de Dunnet para comparação múltipla e nível de significância de 5% ($\alpha < 0.05$), utilizando o software GraphPad Prism (versão 8.02).

4 RESULTADOS

4.1 Artigo 1

Yeast-Host Interactions: *Anadenanthera colubrina* modulates virulence factors of *C. albicans* and inflammatory response *in vitro*

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Keywords: phytotherapy, antifungal agents, oral candidiasis, *Candida albicans*, biofilms, virulence factors, immune response.

Abstract

Oral candidiasis is one of the most common fungal infections in humans. Its incidence has increased widely, as well as the antifungal 37yceum37fla, demanding for the search for novel antifungal therapeutic agents. *Anadenanthera colubrina* (Vell.) Brenan is a plant species that has been proven to possess pharmacological effects, including antifungal and anti-inflammatory activities. This *in vitro* study evaluated the effects of standardized *A. colubrina* extract on virulence factors of *C. albicans* and its regulation on immune response through *C. albicans*-host interaction. Antifungal activity was evaluated by Broth Microdilution Method against reference *Candida* strains (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. dubliniensis*). Antibiofilm effect was analyzed on *C. albicans* mature biofilm and quantified by CFU/mL/g of biofilm dry weight. Proleotlytic enzymatic activities of proteinase and phospholipase were assessed by Azocasein and Phosphatidylcholine assays, respectively. Cytotoxic effect was determined by Cell Titer Blue Viability Assay on human gingival fibroblasts. Cocultured model was used to analyze *C. albicans* coexisting with HGF by SEM and fluorescence microscopies. Gene expression was assessed by RT-PCR of *C. albicans* enzymes (SAP-1, PLB-1) and of host inflammatory cytokines (IL-6, IL-8, IL-1β, IL-10). Cytokines secretion was analysed by Luminex. The extract presented antifungal effect with MIC<15.62 µg/mL against *Candida* strains. Biofilm and proteolytic activity were significant reduced at 312.4 µg/mL (20 x 15.62 µg/mL) extract concentration. Cell viability was maintained higher than 70% in concentrations up to 250 µg/mL (LD_{50}=423.3 µg/mL). Coculture microscopies demonstrated a substantial decreased in *C. albicans* growth and minimal toxicity against host cells. Gene expressions of SAP-1/PLB-1 were significantly down-regulated and host immune response was modulated by a significant decrease on IL-6 and IL-8 cytokines secretion. *A. colubrina* had antifungal activity on *Candida* strains, antibiofilm and antiproteolytic enzyme effects against *C. albicans*. Presented low cytotoxicity to the host cells and modulatory effects on the host immune response.

INTRODUCTION

Oral candidiasis, one of the most common fungal infections in humans (Hertel et al., 2016; Rosa-García et al., 2020), is caused by yeasts from the genus *Candida* (Williams and Lewis, 2011), a polymorphic fungus and a commensal microorganism that colonizes the human oral cavity in healthy people (Nikou et al., 2019). However, under circumstances where host immunity is impaired, *Candida* spp. Can switch their harmless phenotype to a pathogenic form capable of breaching mucosal barriers (Dantas et al., 2016), causing from superficial mucosal infection to deep seated invasive and life-threatening disseminated disease (Lewis and Williams, 2017; Nikou et al., 2019), with high mortality rates (58 to 81%) (Vaezi et al., 2017).

Candida albicans is the species most often associated to oral candidiasis (Prieto et al., 2016; Pappas et al., 2018), accounting for up to 95% of the cases (Vila et al., 2020). Its overgrowth and invasion of superficial tissues is dependent on the host's defenses and the virulence factors of the fungus (Tooyama et al., 2015; Milssop and Faze, 2016; Hellstein and Marek, 2019), such as, adherence to oral epithelial or medical devices surfaces; biofilm formation; destruction of host tissue through secretion of proteolytic enzymes; evasion of host defenses invasion mechanisms and development of drug 38yceum38fla (Hofs et al., 2016; Vila et al., 2020).

Considering the continuing rise of resistant *Candida* spp. Strains and the limited number of antifungal agents, novel therapeutic strategies have been directed toward the identification of bioactive compounds that target virulence factors and pathogenic mechanisms to prevent *C. albicans* transition from harmless commensal to pathogen (Vila et al., 2020).

In this regard, natural products from plants are considered a potential source for the development of new antifungal therapies. Between the years of 1940 and 2014, 40% of all molecules accepted by FDA (US Food and Drug Administration) were natural products (Newman and Cragg, 2016). *Anadenanthera colubrina* (Vell.) Brenan, popularly known as Angico, is a plant species that can be found in Brazil, from the Northeastern to the Southeastern regions. It is a woody species typical of the Caatinga Brazilian biome (Weber et al., 2011) and its use by the traditional communities of Brazilian semiarid as a medicinal plant is common, which includes the treatment of inflammation in general (Araújo et al., 2014; Araújo et al., 2015). Recent studies have shown that *A. colubrina* has promising therapeutic properties, such as antifungal (Lima et al., 2014; Silva et al., 2019), antiproliferative (Lima et al., 2014), anti-inflammatory (Guarneire et al., 2019; Cardoso-Junior et al., 2020) and antioxidant (Araújo et al., 2019; Cardoso-Junior et al., 2020).

Some studies regarding the antifungal effects of *A. colubrina* have suggested an inhibitory activity among *Candida* species, mainly on *C. albicans* biofilms (Lima et al., 2014; Silva et al., 2019). Despite this therapeutic potential, there is little information available about how this plant species can regulate the expression of virulence factors of *Candida* cocultured with human cells and the host immune response during the fungal infection as well. Therefore, the present study aimed to evaluate *in vitro* the modulatory effects of *A. colubrina* extract on major virulence factors related to the pathogenicity of *C. albicans* infection through the interaction between host and pathogen.

MATERIAL AND METHODS

Plant material

The plant material was collected during the month of September in the semi-arid region of Paraíba state, Brazil (7º 22' 25" S, 35º 59' 32" W). Botanical specimens of *Anadenanthera colubrina* (Vell.) Brenan were deposited in the Manuel de Arruda Câmara Herbarium (ACAM) at the State University of Paraíba (UEPB), Campus I, Campina Grande, Paraíba, Brazil, under nº 1936/ACAM. This research was conducted under authorization number SisGen A289DF4. A hydroethanolic standardized extract was 39yceumned according to the method described by Carvalho et al. (2011). Briefly, hydroethanolic extract (80%, v/v) of the plant bark was obtained by maceration for 48 hours using the proportion of 10 mg of the plant for each 25 mL of 80% ethyl alcohol. Three filtrations of the material were performed, followed by vacuum concentration (Tecnal TE-211, Piracicaba, SP) and lyophilization (Martin Christ 1-2 Ldplus, Osterode am Harz, DE). An extraction yield of 31.7% was obtained.

Susceptibility test

The antimicrobial activity of *A. colubrina* extract was assessed by Broth Microdilution Method against the following *Candida* strains: *C. albicans* ATCC® 90028, *C. albicans* ATCC® MYA-2876, *C. glabrata* ATCC® MYA-275, *C. tropicalis* ATCC® MYA-750 and *C. dubliniensis* ATCC® MYA-646. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) were determined according to NCCLS guidelines (M38-A2). The assay was performed in 96 well-plates (Greiner Bio-One North America, Inc Monroe, NC) containing 100 µL/well of RPMI-1640 culture medium (Lonza Bioscience, Walkersville, MD). One hundred microliters of the extract were added to the initial well (8,000 µg/mL), followed by serial microdilution, obtaining concentrations between 2,000 and 15.62 µg/mL. Inoculum

concentration was standardized using a spectrophotomer (SpectraMax M3, Molecular Devices, Sunnyvale, CA), by first measuring the absorbance in the range of 0.08–0.1 at 625 nm, which yielded a yeast stock solution equivalent to $5x10^6$ CFU/mL that was then diluted in RPMI-1640 medium to a final concentration of $5x10^3$ CFU/mL. Next, 100 µL of yeast suspension was added to each well, resulting in a final concentration of 2.5×10^3 CFU/mL. The plates were incubated for 24 hours at 37°C in 5% CO_2 (VWR Symphony 5.3 A, Radnor, PA). Fluconazole (512 µg/mL) (Alfa Aesar®, Tewksbury, MA) was used as positive control. The vehicle control used was dimethyl sulfoxide 1% (DMSO, BDH Solvents, Dawsonville, GA). The MIC was defined as the lowest concentration of the sample capable of inhibiting visible microbial growth, as confirmed by the change in the color of the RPMI-1640 medium. For the determination of the MFC, an aliquot of 10 µL from each well with concentrations equal to or higher than the MIC was sub-cultivated in Sabouraud Dextrose Agar medium (BD Difco, Franklin Lakes, NJ) and incubated at 37°C, in 5% CO_2, for 48 hours. The MFC was defined as the smallest concentration that inhibited visible growth on the agar plates. All of the assays were performed in triplicates and repeated at least three different times for reproducibility (Seleem et al., 2016b).

Biofilm assay

An inoculum of $1x10^6$ CFU/mL of *C. albicans* (ATCC® MYA-2876) was grown for 24 h in a sterile 24-well plate (Greiner Bio-One North America, Inc Monroe, NC, USA) using Yeast Nitrogen Base Medium (YNB) (Sigma Aldrich, Saint Luis, MO) with 50 mM of glucose (VWR Life Science, Radnor, PA) for 24 h at 37°C in 5% CO_2 to establish initial biofilm growth. Total volume of 1 mL of inoculum was pipetted in each well. After 24 hours of incubation, the biofilms were treated once daily with 100 µL of *A. colubrina* extract at concentrations equivalents to 156.2 µg/mL (10 x 15.62 µg/mL) and 312.4 µg/mL (20 x 15.62 µg/mL), which remained incubated with the biofilms suspended in medium overnight. The vehicle control used was 1% DMSO, while positive control was Fluconazole (10xMIC). Before each treatment, biofilms were washed with Phosphate Buffer Solution (PBS) (Lonza Bioscience, Walkersville, MD) and replenished with 900 µL of fresh YNB medium in addition to 100 µL of the corresponding treatment, yielding a total volume of 1 mL in each well. After 72 hours of treatments, adhered biofilms were collected by scraping the bottom of each well plate and suspending in PBS, which was then centrifuged at 10,000 rpm for 5 minutes. Biomass (dry weight) of each biofilm sample was obtained by discarding the supernatant and placing the samples in a speed vacuum to dry for 40 minutes. Colony formation unit (CFU) was determined by submitting the biofilm suspension to serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) and plating 10 µL

of these dilutions on Sabouraud Dextrose Agar plates, which were incubated at 37°C in 5% CO_2. After 24 hours of incubation, the number of *C. albicans* colonies was counted and the data was normalized based on the CFU/ml/dry weight of biofilm sample (Santana et al., 2013; Seleem et al., 2016a; Seleem et al., 2016b; Chen et al., 2018).

Proteinase and phospholipase enzyme secretion assay

Proteinase and phospholipase enzyme secretion assays were conducted as previously performed by Santana et al. (2013) and Chen et al. (2018). Biofilms of *C. albicans* were grown for 24 h in YNB medium with 50 mM of glucose at 37°C in 5% CO_2 and treated with *A. colubrina* extract (156.2 µg/mL – 10 x 15.62 µg/mL and 312.4 µg/mL – 20 x 15.62 µg/mL). Trypsin (Gibco, Invitrogen) was used as standard. The vehicle control used was 1% DMSO. After 72 h of biofilm maturation, the enzyme secretion assays were performed on biofilms suspended in PBS, which were sonicated for 15 s at 20% amplitude with pulses at 5 and 10 seconds intervals (FB120; Fisher Scientific, Pittsburgh, PA, USA). The proteinase enzyme activity was determined by mixing the supernatant of the biofilm solution with 1% azocasein (Sigma Aldrich, Saint Luis, MO) at 9:1 (v/v) for 1 h at 37°C in 5% CO_2. Then, 500 µL of 10% trichloroacetic acid (VWR) was added to stop the reaction. The solution was centrifuged for 5 min at 10,000rpm and 500 µL of the supernatant was combined with 500 µL of 0.5 M NaOH (Macron Fine Chemicals, Avantor VWR Life Science, Radnor, PA), which was incubated for 15 min at 37°C in 5% CO_2. Absorbance was read in a spectrophotometer at 440 nm (Gonçalves et al., 2012; Pande et al., 2006; Santana et al., 2013, Chen et al., 2018). The phospholipase enzyme activity was determined by mixing the supernatant of the biofilm solution (pH corrected to 7.5) with phosphatidylcholine substrate (Sigma Aldrich, Saint Luis, MO) at 9:1 (v/v) for 1 h at 37°C in 5% CO_2 and reading the absorbance in a spectrophotometer at 630 nm (Taniguchi et al., 2009). The rates of absorbance shifts (ΔOD) for the repetitions were adjusted by the blank. One enzyme unity was arbitrarily established as the absorbance shift, by minute of reaction, by biomass, multiplied by one thousand ($U=\Delta OD nm \times min^{-1} \times 1000$). The specific enzyme activity was defined as the amount of enzyme that elicited an increase of 0.001 units of absorbance/minute of digestion by biofilm dry weight (g) (Taniguchi et al., 2009; Santana et al., 2013; Chen et al., 2018).

Cytotoxicity assay

The *in vitro* cytotoxic effect of *A. colubrina* extract was performed on human gingival fibroblasts (HGF-1 ATCC® CRL-2014) and determined by a resazurin fluorometric method

(Cell Titer Blue Viability Assay, Promega Corp, Madison, WI). Gingival fibroblasts were cultured in Dulbecco's modifed Eagle's medium (DMEM, Lonza,Walkersville, MD) with 10% fetal bovine serum (FBS Gibco, Invitrogen, Waltham, MA) at 37°C in 5% CO_2. Fibroblast cells (1×10^5 cells/mL) were first seeded in a 24-well plate in DMEM with 10% FBS, and the plates were incubated for 24 h at 37°C in 5% CO_2. The *A. colubrina* extract was diluted in DMSO 1%, with final concentration inside the wells of 0.1%, and then added to the cultured cells wells (2,500 – 0.25 µg/mL). The plates were incubated for 24 hours at 37°C in 5% CO_2. Resazurin (30 µL) was added to each well, and the cells were incubated for 3 hours. The fluorescence of the supernatant was read in a microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA), with excitation of 555 nm, emission of 585 nm and 570 nm *cut off* (O'Brien et al., 2000).

Coculture model fluorescence microscopy

A coculture model was conducted by culturing HGF-1 and *C. albicans* together in a sterile 24-well plate, as adapted by Wong et al. (2014) and Oliveira Silva et al. (2018). First, gingival fibroblasts were seeded in DMEM with 10% FBS at 37°C in 5% CO_2 for 24 hours. The medium was then replaced with an inoculum of 5 x 10^3 to 2.5 x 10^3 CFU/mL *C. albicans* (MYA 2876) grown in DMEM without FBS. HGF-1 cells and *C. albicans* were treated with 33.28 µg/mL of *A. colubrina* extract. The plate was then incubated at 37°C in 5% CO_2 for 24 hours. The vehicle control tested was 0.1% DMSO and the positive control was Fluconazole (10 µg/mL). The distribution of dead and live fibroblasts was examined using the viability/cytotoxicity Live/Dead Assay Kit for mammalian cell type (Molecular Devices, Sunnyvale, CA), which contains a mixture of Calcein AM and EthDIII. Calcofluor white (Sigma Aldrich, Saint Luis, MO) was used to stain *C. albicans*. Fluorescent images of the double staining were captured using fluorescence microscopy (Keyence All-in-One BZ-X810 Fluoroscence Microscope, Itasca, IL).

Coculture model Scanning Electron Microscopy (SEM)

Coculture model was conducted by coculturing HGF-1 cells and *C. albicans* in a sterile petri dish (Greiner Bio-One North America, Inc Monroe, NC), following the same protocol above described for coculture plating and treatments. After the period of incubation, the samples were washed twice with PBS and fixed in glutaraldehyde 3% (v/v) at room temperature for 12 h. The dehydrated cells were submitted to sequential baths of ethanol at concentrations of 50%, 70%, 90% and absolute ethanol twice, then coated with gold/palladium alloy in a Metalizer (Desk V

Denton Vacuum, Moorestown, NJ) and observed using a Scanning Electron Microscope (Zeiss EVO LS10 SEM, Oberkochen, DE) (Bersan et al., 2014).

Coculture model quantitative real-time PCR

Following the same protocol above described for coculture plating, RNA was isolated from HGF-1 cells and *C. albicans* after 8 hours of treatment with *A. colubrina* extract. The fibroblasts RNA and *C. albicans* RNA were isolated and purified using Rneasy® Mini Kit (Qiagen, Hilden, Germany) and RiboPure™-Yeast Kit (Invitrogen, ThermoFisher Scientific, Rockford, IL), respectively. SpectraDrop Micro-Volume Starter Kit (Molecular Devices, Sunnyvale, CA) was used to quantify the total RNA extracted. Real-time PCR was conducted by using QuantiFast® SYBR® Green RT-PCR One Step Kit (Qiagen, Hilden, DE). The *C. albicans* primers for the genes: *Secreted Aspartyl Proteinases-1* (*SAP-1*), *Phospholipase B-1* (*PLB-1*), and *ACT-1* (housekeeping), at 10 µM were used. *ACT-1* was the gene used to normalize *SAP-1* and *PLB-1* genes expression. The following host inflammatory cytokines genes were selected: *IL-6* (Qiagen Gene ID# 3570), *IL-8* (Qiagen Gene ID#3576), *IL-10* (Qiagen Gene ID#3587), *IL-1β* (Qiagen Gene ID#3553) and *GAPDH* (housekeeping) (Qiagen Gene ID#2597). All data from cytokines genes expression were normalized using the housekeeping gene GAPDH. PCR amplification was performed by using 25 µl reaction mix per well in 0.2 mL 8-Strip PCR tubes. The reactions were conducted in thermocycler (QuantStudio 3 Real Time PCR System, ThermoFisher Scientific, Rockford, IL) at 50°C for 10 min (Reverse Transcription Step); 95°C for 5 min (PCR Initial Activation Step); followed by 40 cycles of 10 s at 95°C (Denaturation Step) and 30 s at 60°C (Annealing/Extension Step). Analysis of relative gene expression was achieved according to the ΔΔCt method (Seleem et al., 2016a; Seleem et al., 2016b; Chen et al., 2018).

Host inflammatory cytokines analysis using Luminex

As previously described, coculture models were performed using HGF-1 cells, *C. albicans* (MYA2876), and the tested groups of *A. colubrina* extract (33.28 µg/mL), positive control (Fluconazole 10 µg/mL) and 0.1% DMSO (vehicle control). After 8 hours of incubation, the supernatants of the coculture were collected, centrifuged for 10 minutes at 1,000 rpm, and assayed immediately using Human Magnetic Premixed Multi-Analyte Luminex Assay Kit (R&D Systems, Minneapolis, MN) for secretion of pro-inflammatory (IL-6, IL-8 and IL-1β) and anti-inflammatory (IL-10) cytokines. Culture supernatants and cytokine capture bead cocktails were incubated overnight. The samples were then incubated for 1 h with biotin-labeled

antibody and for 30 min in a dilution of streptavidin-PE. Data were obtained by Luminex 200 Milliplex System and analyzed with Milliplex Analyst software (Allin et al., 2016).

Statistical analysis

Data were expressed as the mean ± SEM using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests in relation to the vehicle, using GraphPad Prism software (version 8.02). Results were considered significant if p-values were less than 0.05.

RESULTS

***In vitro* antifungal activity**

MIC and MFC values for *A. colubrina* extract and for the standard antifungal on *Candida* spp. Are presented in **Table 1**. For *A. colubrina*, the MIC were lower than 15.62 µg/mL and the MFC ranged from 250 µg/mL to higher than 2,000 µg/mL, whereas MIC and MFC for Fluconazole ranged from lower than 1 to 8 µg/mL and from 32 to higher than 128 µg/mL, respectively. The extract demonstrated strong antifungal activity against all *Candida* strains tested, based on the classification proposed by Holetz et al. (2002). According to the parameters stated by Siddiqui et al. (2013), the MFC/MIC ratio found revealed a fungistatic effect of the extract against the species tested.

TABLE 1

Biofilm inhibition

Biofilm assay showed that the treatments with *A. colubrina* extract at 312.4 µg/mL, equivalent to 20 x 15.62 µg/mL for *C. albicans* MYA 2876, and with Fluconazole at 10 µg/mL (10MIC) had significant reduction ($p < 0.05$) in fungal load, expressed as CFU/mL/g of biofilm dry weight, in comparison to the vehicle control group (0.1% DMSO) (**Figure 1**).

FIGURE 1

Proteinase and phospholipase enzymes secretion

Phospholipase enzyme activity was significantly reduced ($p < 0.05$) after treatment with *A. colubrina* at 156.2 µg/mL (10 x 15.62 µg/mL) and 312.4 µg/mL (20 x 15.62 µg/mL), when

compared to the vehicle (0.1% DMSO). On the other hand, proteinase enzyme activity was significantly decreased ($p < 0.05$) by the extract only at 312.4 µg/mL (20 x 15.62 µg/mL) in comparison to the vehicle control group (**Figures 2A** and **2B**).

FIGURE 2

Coculture model of fibroblasts and *C. albicans*

Cytotoxicity

The *A. colubrina* extract presented LD_{50} of 432.3 µg/mL and a non-toxic profile on gingival fibroblast cell culture up to a concentration of 250 µg/mL (**Figure 3**).

FIGURE 3

Proteolytic enzymes gene expression

Similar to the profile obtained from the biofilm proteinase and phospholipase assays, the expression of *SAP-1* and *PLB-1* gene by *C. albicans* MYA 2876, grown as immature biofilm in a coculture model, was significantly down-regulated ($p < 0.05$) after the exposure to *A. colubrina* extract at 33.28 µg/mL, when compared to the vehicle control group (**Figures 4A** and **4B**).

FIGURE 4

Fluorescence microscopy

In the coculture model of fibroblasts coexisting with *C. albicans*, samples treated with *A. colubrina* extract showed a considerable decrease in *Candida* growth distribution in comparison with the vehicle control, as indicated by the sparse and less dense accumulation of *C. albicans* (blue color) among viable fibroblasts (green color) in fluorescent images. In addition, fibroblasts viability was not significantly affected by the treatment with the extract, since there was no significant increase in the dead fibroblast population, indicated by the red fluorescent color, suggesting that *A. colubrina* was effective against *C. albicans* with minimal effects or toxicity against HGF-1 cells (**Figures 5A, 5B** and **5C**).

FIGURE 5

Scanning Electron Microscopy (SEM)

As seen in **Figure 6** (**A, B** and **C**), the SEM pattern was similar to the arrangement observed on fluorescence microscopy. The SEM showed density reduction of *C. albicans* biofilm, and destructuring of hyphae morphology, in comparison to the vehicle control group.

FIGURE 6

Pro and anti-inflammatory cytokines gene expression

The *A. colubrina* extract at 33.28 µg/mL presented a modulatory effect on the gene expression of host inflammatory cytokines in a coculture model. The expression of *IL-6*, *IL-1β* and *IL-10* genes were up-regulated after host cells exposure to the extract, while *IL-8* gene expression was down-regulated. However, the *A. colubrina* concentration tested showed no statistical difference ($p > 0.05$) on the gene expression of the host cytokines in comparison to the vehicle control group (**Figures 7A, 7B, 7C** and **7D**).

FIGURE 7

Pro and anti-inflammatory cytokines secretion

Coculture supernatants were assessed for expression of pro-inflammatory (IL-6, IL-8 and IL-1β) and anti-inflammatory (IL-10) cytokines, following treatments with *A. colubrina* extract (33.28 µg/mL). The extract significantly reduced ($p < 0.05$) the expression of IL-6 and IL-8 when compared to the vehicle control group. On the other hand, there was no detection of modulatory effect on the expression of IL-1β and IL-10 cytokines upon treatment with the extract (**Figure 8**).

FIGURE 8

DISCUSSION

Recently, the traditional therapeutic use of *A. colubrina* by popular communities in Brazilian culture has encouraged comprehensive studies about its biological properties to be conducted (Damascena et al., 2014; Lima et al., 2014; Barreto et al., 2016; Mota et al., 2017; Guarneire et al., 2019; Silva et al., 2019; Cardoso-Junior et al., 2020). In this study, we evaluated the *A. colubrina* extract effects on virulence factors of *C. albicans* and its modulatory effects on host immune response during the fungal infection.

In a previous study from our group (Lima et al., 2014), phytochemical analysis of *A. colubrina* extract revealed a high total polyphenol content (53.18% gallic acid equivalents); tannins (8.77% catechin equivalents) and flavonoids (0.28% quercetin equivalents). This chemical profile seems to support the biological activities of *A. colubrina* extract observed here and discussed ahead, since these classes of compounds are related to a wide range of properties, including antifungal (Ramirez et al., 2013; Kanchanapiboon et al., 2020) and immunomodulatory effects (Fu et al., 2013; Ji et al., 2019; Cardoso Junior et al., 2020)

A. *colubrina* extract has been reported to present *in vitro* antifungal effects against *Candida* strains and antibiofilm properties, mainly on *C. albicans* biofilms (Lima et al., 2014; Silva et al., 2019). The susceptibility assay from our study showed a strong anti-*Candida* effect (Holletz et al., 2011) of *A. colubrina*, exerting a fungistatic profile (Siddiqui et al. 2013) over all the strains tested in planktonic form. *Candida* spp. Are not only important components of oral microbiota, living as a common commensal in immunocompetent individuals (Qin et al., 2016), but also display an important role in shaping the oral microbiome (Xu and Dongari-Bagtzoglou, 2015; Bertolini et al., 2019). Thus, total elimination of yeasts from the body is neither desirable nor feasible (Bhattacharya et al., 2020) and their growth inhibition instead of their elimination might be positive regarding the infection control, by preventing the rise of pathogenic strains that could lead to more severe infections and antifungal resistance (Ford et al., 2015).

One of the major virulence factors related to *C. albicans* pathogenesis, with significant clinical implications, resides in its ability to form biofilms (Fox et al., 2015; Wall et al., 2019). In oral cavity, hyphae formation and adherence to oral epithelial cells and other abiotic surfaces, such as dentures, lead to the development of this structured community of surface-associated microbial populations embedded in an extracellular matrix (Ghannoum et al. 2015; Hirota et al., 2017). In our study, biofilms treated with *A. colubrina* at 312.4 µg/mL (20x15.62 µg/mL) showed significant decrease in fungal viability in comparison to the vehicle group, by reducing the CFU/mL/g parameter and altering the biofilm composition and structure integrity. For this biofilm assay model, we used *C. albicans* MYA 2876, considering the MFC result of this strain after the treatment with the extract. In addition, higher concentrations of *A. colubrina*, equivalent to 156.2 µg/mL (10x15.62 µg/mL) and 312.4 µg/mL (20x15.62 µg/mL), were used against *C. albicans* MYA 2876 strain, due to the stable envioroment of structured biofilm, which is tolerant to diffusion of antifungal agents (Tsui et al. 2016).

Once biofilm is established, the expression of *C. albicans* virulence factors increases (Jabra-Rizk et al., 2016). In this context, the release of extracellular hydrolytic enzymes by

biofilms into the local environment, contribute to candidiasis progression (Vila et al., 2020). Most notable of the secreted enzymes frequently implicated in the pathogenicity of *C. albicans* are Secreted Aspartyl Proteinases (SAPs) and Secreted Phospholipases (PLs), which are involved in host tissue invasion, nutrient acquisition, immune evasion, and organ damage (Sorgo et al, 2013; Jabra-Riks et al., 2016; Swidergall and Filler, 2017). In order to evaluate the modulatory effect of *A. colubrina* extract on these critical virulence factors, we demonstrated that phospholipase enzyme activities was significantly reduced using the extract at 156.2 µg/mL (10 x 15.62 µg/mL) and 312.4 µg/mL (20 x 15.62 µg/mL)concentrations, while proteinase activity was decreased only by the highest concentration. These results suggest that both antifungal and antibiofilm activities of *A. colubrina* could involve the inhibition of proteolytic enzymes expression as mechanism of action, considering that during *C. albicans* penetration, these enzymes affect epithelial junctions and enable degradation of cell membrane components, facilitating fungal adhesion and biofilm formation to both oral epithelium and abiotic surfaces (Naglik et al., 2011).

To confirm the results obtained on proteolytic enzyme secretion, we assessed the expression of *SAP-1* and *PLB-1* genes after treatment with *A. colubrina* at 33.28 µg/mL on coculture models of immature biofilm of *C. albicans* coexisting with gingival fibroblasts. We demonstrated a significant down-regulation on the gene expression of both enzymes in comparison to the vehicle group. These findings are consistent with the results of enzymatic secretion, considering that fungal infections usually present a higher gene expression of SAPs and PLs. Proteinases are able to degrade a variety of host factors, such as E-cadherin, present in epithelial cell junction, increasing the hyphae capacity for colonization and penetration into host tissues, and factors involved in the innate and adaptive immune responses as well, allowing *C. albicans* to overcome the host immune defenses (Kumar et al., 2017). On the other hand, phospholipases expression displays an active role in epithelial adherence and tissue invasion, since their catalytic action results in disruption of epithelial cell membrane allowing the penetration of yeasts into host cell cytoplasm (Sanitá et al. 2014). Thus, our results suggest that *A. colubrina* extract can act by interfering with yeast invasion mechanisms, which could prevent the development of oral candidiasis.

The 48yceum48flammy assay was conducted on human gingival fibroblasts (LD_{50} = 432.3 µg/mL) in order to verify the therapeutic safety level previously, regarding further *in vivo* and human clinical studies. *A. colubrina* extract maintained the cell viability at concentrations up to 250 µg/mL, representing a relatively low cytotoxic activity. This finding is consistent with results recently reported, which verified the low toxicity of the bark extract of *A. colubrina*

on human cell lines, such macrophages (Silva et al. 2019; Cardoso-Junior et al., 2020), keratinocytes and also on tumoral cell strains (Lima et al. 2014). These data corroborate the results found in this study, regarding the low toxic potential effect of this product, shown to be pharmacological safe *in vitro*, by maintaining the cell viability.

Considering the purpose to evaluate the effects of *A. colubrina* extract on *C. albicans*-host interaction, we used a coculture model to provide comprehensions about the complex system of an immature *C. albicans* biofilm coexisting with fibroblasts incubated with the tested extract. Recent *in vitro* studies had been conducted in order to evaluate the modulatory effects of natural compounds on the interaction between *C. albicans* and the host using a coculture model (Seleem et al., 2016a; Seleem et al., 2016b; Chen et al., 2018). Moreover, cocultures have demonstrated to be an effective tool to stimulate physiological conditions and induce interactions between the cells that trigger important host response pathways (Serrano et al., 2017).

Microscope images obtained from the coculture model were helpful to evaluate qualitatively the distribution of *C. albicans* and fibroblasts in response to *A. colubrina* and the controls groups. The pattern imaging observed through fluorescence microscopy demonstrated a considerable reduction in *C. albicans* growth without affecting significantly the fibroblasts viability, showing a strong antifungal effect with minimal toxicity. Similarly, the arrangement observed on coculture SEM imaging in a higher magnification and topographic view, showed significant alterations of the biofilm structure treated with *A. colubrina*. When compared to the vehicle group, it is possible to observe structural alterations, such as notable decrease of the biofilm density and destructuring of the hyphae morphology in areas where the extract was able to penetrate. In addition, hyphae decreased, indicating a potential reduction of the biofilm virulence, since the formation of hyphae is associated to tissue invasion.

Host immune defense against *C. albicans* infection requires a wide range of complex molecular mechanisms involving the recognition of fungal cell wall components, activation of host immune cell signaling cascades and release of cytokines and chemokines (Brown et al., 2012; Pappas et al., 2018). Some reviews were recently published regarding the interplay between *C. albicans*, host cells and immune response during *C. albicans* mucosal infection (Hofs et al., 2016; Naglik et al., 2017; Nikou et al., 2019; Swidergall, 2019; Vila et al., 2020), serving as guidelines to improve our understanding that relies on this complex interaction.

Therefore, to evaluate the modulatory activity of *A. colubrina* on the host inflammatory response during *Candida* infection, we employed transcriptomic and proteomic approaches in conjunction with a proteomic profiling. A panel of inflammatory markers were selected based

on the host immune response mechanisms triggered by *Candida* recognition, which involve the activation of transmembrane Toll Like Receptors (TRLs) (Salvatori et al., 2016; Pappas et al., 2018), and downstream signaling pathways that result in the release of pro-inflammatory cytokines, mainly IL-1α, IL-1β, IL-6, IL-8 and CCL5 (RANTES), by the host cells (Hebecker et al., 2014; Verma et al., 2017; Verma et al. 2018).

Our results demonstrated that *A. colubrina* affects the expression of host inflammatory cytokines by down-regulating the expression of *IL-8* gene expression and up-regulating the expression of *IL-6*, *IL-1β* and *IL-10* genes. However, there was no statistical difference when compared to vehicle control group. On the other hand, proteomic analysis disclosed significant reduction on the secretion of pro-inflammatory cytokines IL-6 and IL-8, suggesting an anti-inflammatory effect of *A. colubrina* on the host response, which could help to eradicate the fungal infection. Differences between transcriptomic and proteomic profile of IL-1β, IL-6 and IL-10 could be related to a negative feedback mechanism modulating gene expression. Cytokines gene expression may play an important role in regulating the release of them. However, as indicated by the significant reduction in expression of these molecules, there may be a negative feedback inhibition mechanism involving the transcription and translation processes, which regulates their respective gene expression. However, this hypothesis needs to be confirmed through further molecular studies.

As mentioned before, IL-6 and IL-8 play crucial roles in innate immune response. IL-8 is a known neutrophil recruiter from the circulating blood to the site of infection (Hoffs et al., 2016), whereas IL-6 is related to immune response to microorganisms and its expression is directly influenced by the secretion of other pro-inflammatory cytokines, specially IL-1 (Tanaka et al., 2014). Some reports state that *C. albicans* infection stimulates the expression of IL-1α, IL-6 and IL-8 (Feller et al., 2014; Figueira et al., 2020; Mo et al., 2020). In this regard, our findings suggest that *A. colubrina* might reduce pro-inflammatory IL-6 and IL-8 cytokines expression during the fungal infection, due to its strong antifungal activity, which modulates some putative virulence factors of *C. albicans*, such as biofilm formation and proteolytic enzyme secretion, reducing the fungus pathogenicity. In addition, the low cytotoxicity of the extract on the host cells does not induce an inflammatory response.

This is the first study reporting the effects of *Anadenanthera colubrina* (Vell.) Brenan on *C. albicans*-host interaction. *A. colubrina* extract demonstrated anti-*Candida*, antibiofilm and anti-proteolytic enzyme activities against *C. albicans*, with relatively low cytotoxicity to the host cells. It also presented modulatory effects on the host immune response, as indicated by its regulation of IL-6 and IL-8 pro-inflammatory cytokines secretion. In this regard, *A. colubrina*

could be considered as a potential source for an anti-*Candida* formulation. Further investigations about the regulation activity of *A. colubrina* on others key virulence factors of *C. albicans*, including expression of cell surface adhesins, proteolytic degradation of host immune factors and invasion and destruction of host tissue mechanisms, must to be conducted in order to reinforce and establish the extract effects on molecular and signaling pathways in *Candida* pathogenesis.

CONFLICT OF INTEREST

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be regarded as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: RMM, EMMBC and CMAM. Investigation and Data curation: CMAM. Formal analysis: RMM, EMMBC and CMAM. Writing – original draft: CMAM. Writing – review and editing: CMAM, EMMBC and RMM.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

REFERENCES

Allin, N., Cruz-Almeida, Y., Velsko, I., Vovk, A., Hovemcamp, N., Harrison, P., et al. (2016). Inflammatory Response Influences Treatment of Localized Aggressive Periodontitis. *J Dent Res*. 95 (6), 635-641. Doi: 10.1177/0022034516631973

Araújo, D. R. C., Silva, L. C. N., Silva, A. G., Araújo, J. M., Macêdo, A. J., Correia, M. T. S., et al. (2014). Comparative analysis of anti-S*taphylococcus aureus* action of leaves and fruits of *Anadenanthera colubrina* var. *cebil* (Griseb.) Altschul. *Afr J Microbiol Res*. 8, 2690-2696. Doi: 10.5897/AJMR2014.6901

Araújo, D. R. C., Silva, T. D., Harand, W., Lima, C. S. A., Ferreira Neto, J. P., Ramos, B. A., et al. (2019). Bioguided Purification of Active Compounds from Leaves of *Anadenanthera colubrina* var. *cebil* (Griseb.) *Altschul. Biomolecules*. 9 (10), 590. Doi: 10.3390/biom9100590

Araújo, T. A. S., Castro, V. T. N. A., Solon, L. G. S., Silva, G. A., Almeida, M. G., Costa, J. G. M., et al. (2015). Does rainfall affect the antioxidant capacity and production of phenolic compounds of an important medicinal species? *Ind Crop Prod*. 76, 550-556. Doi: 10.1016/j.indcrop.2015.07.008

Barreto, H.M., Coelho, K. M., Ferreira, J. H., Santos, B. H., Abreu, A. P., Coutinho, H. D., et al. (2016). Enhancement of the antibiotic activity of aminoglycosides by extracts from *Anadenanthera colubrina* (Vell.) Brenan var. cebil against multi-drug resistant bacteria. *Nat Prod Res*. 30 (11), 1289–1292. Doi: 10.1080/14786419.2015.1049177

Bersan, S. M.F., Galvão, L. C. C., Góes, V. F. F., Sartoratto, A., Figueira, G. M., Rehder, V. L. G., et al. (2014). Action of essential oils from Brazilian native and exotic medicinal species on oral biofilms. *BMC Complement Altern Med*. 14, 151. Doi: 10.1186/1472-6882-14-451

Bertolini, M., Ranjan, A., Thompson, A., Diaz, P. I., Sobue, T., Maas, K., et al. (2019) *Candida albicans* induces mucosal bacterial dysbiosis that promotes invasive infection. *PloS Pathog*. 15 (4), e1007717. Doi: 10.1371/journal.ppat.1007717

Bhattacharya, S., Sae-Tia, S., Fries, B. C. (2020). Candidiasis and Mechanisms of Antifungal Resistance. *Antibiotics (Basel)*. 9 (6), 312. Doi: 10.3390/antibiotics9060312

Brown, G. D., Denning, D. W., Levitz, S. M. (2012). Tackling human fungal infections. *Science*. 336 (6082), 647. Doi: 10.1126/52yceum52.1222236

Cardoso-Junior, O., Lima, N. M., Silva, M. G. A., Aguiar, V. B., Carli, G. P., Scherrer, E., et al. (2020). *In vitro* and *in vivo* evaluation of anti-inflammatory activity and free radical scavenging potential of leaves extract from *Anadenanthera colubrina*. *Nat Prod Res*. 1-5. Doi: 10.1080/14786419.2020.1727472

Carvalho, D. D. C., Alves, E., Camargos, R. B., Oliveira, D. F., Scolforo, J. R. S., Carvalho, D. A., et al. (2011). Plant extracts to control *Alternaria alternata* in Murcott tangor fruits. *As Iberoame Micol*. 28, 173-178. Doi: 10.1016/j.riam.2011.05.001

Chen, E., Benso, B., Seleem. D., Nunes, L. E., Pasetto, S., Pardi, V., et al. (2018). Fungal-Host Interaction: Curcumin Modulates Proteolytic Enzyme Activity of *Candida albicans* and Inflammatory Host Response *In Vitro*. *Int J Dent*. 2018, 2393146. Doi: 10.1155/2018/2393146

Damascena, N. P., Souza, M. T., Almeida, A. F., Cunha, R. S., Damascena, N. P., Curvello, R. L., et al. (2014). Antioxidant and orofacial anti-nociceptive activities of the stem bark aqueous

extract of *Anadenanthera colubrina* (Velloso) Brenan (Fabaceae). *Nat Prod Res*. 28 (10), 753–756. Doi: 10.1080/14786419.2013.877902

Dantas, A., Lee, K. K., Raziunaite, I., Schaefer, K., Wagener, J., Yadav, B., et al. (2016). Cell biology of *Candida albicans*-host interactions. *53yceum53fl Microbiol*. 34, 111–8. Doi: 10.1016/j.mib.2016.08.006.

Feller, L., Khammissa, R. A. G., Chandran, R., Altini, M., Lemmer, J. (2014). Oral candidosis in relation to oral immunity. *J Oral Pathol Med*. 43 (8), 563–569. Doi: 10.1111/jop.12120

Figueira, L. M. D., Ricomini Filho, A. P., Silva, W. J., Cury, A. A. D. B., Ruiz, K. G. S. (2020). Glucose effect on *Candida albicans* biofilm during tissue invasion. *Arch Oral Biol*. 117, 104728. Doi: 10.1016/j.archoralbio.2020.104728

Ford, C. B., Funt. J. M., Abbey, D., Issi, L., Guiducci, C., Martinez, D. A., et al. (2015). The evolution of drug resistance in clinical isolates of *Candida albicans*. *Elife*. 4, e00662. Doi: 10.7554/eLife.00662.001

Fox, E., Bui, C., Nett, J., Hartooni, N., Mui, M., Andes, D., et al. (2015). An expanded regulatory network temporally controls *Candida albicans* biofilm formation. *Mol Microbiol*. 96 (6), 1226–1239. Doi: 10.1111/mmi.13002

Francisconi, R. S., Huacho, P. M. M., Tonon, C. C., Bordini, E. A. F., Correia, M. F., Sardi, J. C. O., et al. (2020). Antibioflm efficacy of tea tree oil and of its main component 53yceum53fl-4-ol against *Candida albicans*. *Braz Oral Res*. 34, e050. Doi:10.1590/1807-3107bor-2020.vol34.0050

Freires, I. A., Murata, R. M., Furletti, V. F., Sartoratto, A., Alencar, S. M., Figueira, G. M., et al. (2014). *Coriandrum sativum* L. (Coriander) Essential Oil: Antifungal Activity and Mode of Action on Candida spp. And Molecular Targets Affected in Human Whole-Genome Expression. *Plos One*. 9 (6), e99086. Doi:10.1371/journal.pone.0099086

Fu, R. H., Liu, S. P., Chu, C. L., Lin, Y. H., Ho, Y. C., Chiu, S. C., et al. (2013). Myricetin attenuates lipopolysaccharidestimulated activation of mouse bone marrow-derived dendritic cells through suppression of IKK/NF-kB and MAPK signalling pathways. *J Sci Food Agric*. 93 (1), 76–84. Doi: 10.1002/jsfa.5733

Ghannoum, M., Roilides, E., Katragkou, A., Petraitis, V., Walsh, T. (2015). The role of echinocandins in *Candida* biofilm–related vascular catheter infections: In vitro and in vivo model systems. *Clin Infect Dis*. 6, S618–S621. Doi: 10.1093/cid/civ815

Gonçalves, L. M., Del Bel Cury, A. A., Sartoratto, A., Rehder, V. J. G., Silva, W. J. (2012). Effects of Undecylenic Acid Released from Denture Liner on *Candida* Biofilms. *J Dent Res*. 91 (10), 985-989. Doi: 10.1177/0022034512458689

Guarneire, G. J., Cardoso Junior, O., Lima, N. M., Santos, E. A., Schulz, C. A., Silva, W. P., et al. (2019). Effect of *Anadenanthera colubrina* protease inhibitors a Paulinos an inflammatory mediator. *Nat Prod Res*. 1-6. Doi: 10.1080/14786419.2019.1624962

Hebecker, B., Naglik, J. R., Hube. B., Jacobsen, I. D. (2014). Pathogenicity mechanisms and host response during oral *Candida albicans* infections. *Expert Rev Anti-Infect Ther*. 12 (7), 867–879. Doi: 10.1586/14787210.2014.916210

Hellstein, J. W., Marek, C. L. (2019). Candidiasis: red and white manifestations in the oral cavity. *Head Neck Pathol*. 13 (1), 25–32. Doi: 10.1007/s12105-019-01004-6

Hertel, M., Schmidt-Westhausen, A. M., Strietzel, F. P. (2016). Local, systemic, demographic, health-related factors influencing pathogenic yeast spectrum and antifungal drug administration frequency in oral candidiasis: a retrospective study. *Clin Oral Investig*. 20, 1477-1486. Doi: 10.1007/s00784-015-1631-0

Hirota, K., Yumoto, H., Sapaar, B., Matsuo, T., Ichikawa, T., Miyake, Y. (2017). Pathogenic factors in *Candida* biofilm-related infectious diseases. *J Appl Microbiol*. 122 (2), 321–330. Doi: 10.1111/jam.13330

Höfs, S., Mogavero, S., Hube, B. (2016). Interaction of *Candida albicans* with host cells: Virulence factors, host defense, escape strategies and the microbiota. *J. Microbiol*. 54 (3), 149–169. Doi:10.1007/s12275-016-5514-0

Holetz, F. B., Pessini, G. L., Sanches, N. R., Cortez, D. A., Nakamura, C. V., Filho, B. P. (2002). Screening of Some Plants Used in the Brazilian Folk Medicine for the Treatment of Infectious Diseases. *Mem Inst Oswaldo Cruz*. 97 (7), 1027-1031. Doi: 10.1590/S0074-02762002000700017

Jabra-Rizk, M. (2011). Pathogenesis of polymicrobial biofilms. *Open Mycol J*. 5, 39–43. Doi: 10.2174/1874437001105010039

Ji. R., Sun, H., Peng, J., Ma, X., Bao, L., Fu, Y., et al. (2019). Rosmarinic acid exerts an antagonistic effect on vascular calcification by regulating the Nrf2 signalling pathway. *Free Radic Res*. 53 (2), 187–197. Doi: 10.1080/10715762.2018.1558447

Kanchanapiboon, J., Kongsa, U., Pattamadilok, D., Kamponchaidet, S., Wachisunthon, D., Poonsatha, S., et al. (2020). Boesenbergia rotunda extract inhibits *Candida albicans* biofilm formation by pinostrobin and pinocembrin. *J Ethnopharmacol*. 261, 113193. Doi: 10.1016/j.jep.2020.113193

Lewis, M. A. O., Williams, D. W. (2017). Diagnosis and management of oral candidosis. *Br Dent J*. 223 (9), 675–681. Doi:10.1038/sj.bdj.2017.886

Lima, R. F., Alves, E. P., Rosalen, P. L., Ruiz, A. L. T., Duarte, M. C. T., Góes, V. F. F., et al. (2014). Antimicrobial and Antiproliferative Potential of *Anadenanthera colubrina* (Vell.) Brenan. *Evid Based Complement Alternat Med*. 2014, 802696. Doi: 10.1155/2014/802696

Lionakis, M. S., Iliev, I. D., Hohl, T. M. (2017). Immunity against fungi. J*CI Insight*. 2 (11), e93156. Doi: 10.1172/jci.insight.93156

Millsop, J. W., Faze, L. N. (2016). Oral candidiasis. *Clin Dermatol*. 34 (4), 487–494. Doi: 10.1016/j.clindermatol.2016.02.022

Mo, F., Ma, J., Yang, X., Zhang, P., Li, Q., Zhang, J. (2020). *In vitro* and *in vivo* effects of the combination of myricetin and miconazole nitrate incorporated to thermosensitive hydrogels, on *C. albicans* biofilms. *Phytomedicine*. 71, 153223. Doi: 10.1016/j.phymed.2020.153223

Mota, G. S., Sartori, C. J., Miranda, I., Quilho, T., Mori, F. A., Pereira, H. (2017). Bark anatomy, chemical composition and ethanol-water extract composition of *Anadenanthera peregrina* and *Anadenanthera colubrina*. *Plos One*. 12 (12), e0189263. Doi: 10.1371/journal.pone.0189263

Naglik, J. R., König, A., Hube, B., Gaffen, S. L. (2017). *Candida albicans*-epithelial interactions and induction of mucosal innate immunity. *55yceum55fl Microbiol*. 40, 104–112. Doi: 10.1016/j.mib.2017.10.030

Naglik, J. R., Moyes, D. L., Wachtler, B., Hube, B. (2011). *Candida albicans* interactions with epithelial cells and mucosal immunity. *Microbes Infect*. 13 (12-13), 963-976. Doi: 10.1016/j.micinf.2011.06.009

National Committee for Clinical Laboratory Standards (NCCLS). (2002). Reference method for broth dilution antifungal susceptibility testing of yeast fungi. Approved standard M38-A2. Wayne: National Committee for Clinical Laboratory Standards.

Newman, D. J., Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 191 to 2014. *J Nat Prod*. 79, 629-661. Doi: 10.1021/acs.jnatprod.5b01055

Nikou, S., Kichik, N., Brown, R., Ponde, N. O., Ho, J., Naglik, J. R., et al. (2019). *Candida albicans* Interactions with Mucosal Surfaces during Health and Disease. *Pathogens*. 8 (2), 53. Doi:10.3390/pathogens8020053

O'Brien, J., Wilson, I., Orton, T., Pognan, F. (2000). Investigation of the Alamar Blue (resazurin) fluorescentdye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem*. 267, 5421–5426. Doi: 10.1046/j.1432-1327.2000.01606.x

Oliveira Silva, V., Pereira, L. J., Pasetto, S., Silva, M. P., Meyers, J. C., Murata, R. M. (2018). Effects of monolaurin on oral microbe-host transcriptome and metabolome. *Front. Microbiol*. 9:2638. Doi: 10.3389/fmicb.2018.02638

Pande, M., Dubey, V. K., Yadav, S. C., Jagannadham, V. (2006). A Novel Serine Protease Cryptolepain from *Cryptolepis buchanani*: Purification and Biochemical Characterization. *J Agric Food Chem*. 54 (26), 10141-50. Doi: 10.1021/jf062206a

Pappas, P. G., Lionakis, M. S., Arendrup, M. C., Ostrosky-Zeichner, L., Kullberg, B. J. (2018). Invasive candidiasis. *Nat As Dis Primers*. 4, 18026. Doi: 10.1038/nrdp.2018.26

Prieto, D., Correia, I., Pla, J., Roman, E. (2016). Adaptation of *Candida albicans* to commensalism in the gut. *Future Microbiol*. 11 (4),567-583. Doi: 10.2217/fmb.16.1

Qin, Y., Zhang, L., Xu, Z., Zhang, J., Jiang, Y., Cao, Y., et al. (2016). Innate immune cell response upon *Candida albicans* infection. *Virulence*. 7 (5), 512-526. Doi: 10.1080/21505594.2016.1138201

Rocha, E. A. L. S. S., Medeiros, A. C. D., Castro, R. D., Saraiva, K. L. A., Godoy, G. P., Silva, L. R. A. S. et al. (2017). Antifungal Activity, Phytochemical Characterization and Thermal Profile of *Anadenanthera colubrina* (Vell.) Brenan. *Pesqui Bras Odontopediatria Clin Integr*. 17(1):e3389. Doi: 10.4034/PBOCI.2017.171.12

Rosa-García, E., Olade-Hernández, M. J., Irigoyen-Camacho, M. E., Mondragón-Padilla, A., Mendoza-Juache, A., Sánchez-Vargas, L. O. (2020). Antifungal susceptibility of oral isolates of *Candida* species from chronic kidney disease patients on chronic dialysis. *J Mycol Med*. 101009, 1-6. Doi: 10.1016/j.mycmed.2020.101009

Salvatori, O., Puri, S., Tati, S., Edgerton, M. (2016). Innate immunity and saliva in *Candida albicans*-mediated oral diseases. *J Dent Res*. 95 (4), 365–371. Doi: 10.1177/0022034515625222

Sanitá, P. V., Zago, C. E., Pavarina, A. C., Jorge, J. H., Machado, A. L., Vergani, C. E. (2014). Enzymatic activity profile of a Brazilian culture collection of *Candida albicans* isolated from diabetics and non-diabetics with oral candidiasis. *Mycoses*. 57 (6), 351–357. Doi:10.1111/myc.12162

Santana, I. L., Gonçalves, L. M., Vasconcellos, A. A., Silva, W. J., Cury, J. A., Del Bel Cury, A. A. (2013). Dietary Carbohydrates Modulate *Candida albicans* Biofilm Development on the Denture Surface. *Plos One*. 8 (5), e64645. Doi: 10.1371/56yceum.pone.0064645

Seleem, D., Benso, B., Noguti, J., Pardi, V., Murata. R. M. (2016a). *In Vitro* and *In Vivo* Antifungal Activity of Lichochalcone-A against *Candida albicans* Biofilms. *Plos One*. 11 (6), e0157188. Doi: 10.1371/journal.pone.0157188

Seleem, D., Chen, E., Benso, B., Pardi, V., Murata, R. M. (2016b). *In vitro* evaluation of antifungal activity of monolaurin against *Candida albicans* biofilms. *Peer J*. 4, e2148. Doi: 10.7717/peerj.2148

Serrano, R., González-Menéndez, V., Rodriguez, L., Martin, J., Tormo, J. R., Genilloud, O. (2017). Co-culturing of Fungal Strains Against *Botrytis cinerea* as a Model for the Induction of Chemical Diversity and Therapeutic Agents. *Front Microbiol*. 8, 649. Doi: 10.3389/fmicb.2017.00649

Siddiqui, Z. N., Farooq, F., Musthafa, T. N. M., Ahmad, A., Khan, A. U. (2013). Synthesis, characterization and antimicrobial evaluation of novel halopyrazole derivatives. *J Saudi Chem Soc*. 17 (2), 237–243. Doi: 10.1016/j.jscs.2011.03.016

Silva, D. R., Rosalen, P. L., Freires, I. A., Sardi, J. C. O., Lima, R. F., Lazarini, J. G., et al. (2019). *Anadenanthera Colubrina* vell Brenan: anti-*Candida* and antibiofilm activities, toxicity and therapeutical action. *Braz Oral Res*. 33, e023. Doi: 10.1590/1807-3107bor-2019.vol33.0023.

Sorgo, A. G., Heilmann, C. J., Brul, S., Koster, C. G., Klis, F. M. (2013). Beyond the wall: *Candida albicans* secretIs to survive. *FEMS Microbiol Lett*. 338 (1), 10–17. Doi: 10.1111/1574-6968.12049

Swidergall, M., Filler, S. G. (2017). Oropharyngeal candidiasis: fungal invasion and epithelial cell responses. *PloS Pathog*. 13 (1), e1006056. Doi: 10.1371/journal.ppat.1006056

Swidergall. M. (2019). *Candida albicans* at Host Barrier Sites: Pattern Recognition Receptors and Beyond. *Pathogens*. 8 (1), 40. Doi: 10.3390/pathogens8010040

Tanaka, T., Narazaki, M., Kishimoto, T. (2014). IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harb Perspect Biol*. 6 (10), a016295. Doi: 10.1101/cshperspect.a016295

Taniguchi. L., Faria, B. F., Rosa, R. T., Carvalho, A. P., Gursky, L. C., Elifio-Esposito, S. L., et al. (2009). Proposal of a low-cost protocol for colorimetric semi-quantification of secretory phospholipase by *Candida albicans* grown in planktonic and biofilm phases. *J Microbiol Methods*. 78 (2), 171-174. Doi: 10.1016/j.mimet.2009.05.012

Kumar, R., Breindel, C., Saraswat, D., Cullen, P. J., Edgerton, M. (2017). *Candida albicans* Sap6 amyloid regions function in cellular aggregation and zinc binding, and contribute to zinc acquisition. *Sci Rep*. 7 (1), 2908. Doi: 10.1038/s41598-017-03082-4

Tooyama, H., Matsumoto, T., Hayashi, K., Kurashina, K., Kurita, H., Uchida, M., et al. (2015). *Candida* concentrations determined following concentrated oral rinse culture reflect clinical oral signs. *BMC Oral Health*. 15, 150. Doi:10.1186/s12903-015-0138-z

Tsui, C., Kong, E. F., Jabra-Rizk, M. A. (2016). Pathogenesis of *Candida albicans* Biofilm. *Pathog Dis*. 74 (4), ftw018. Doi: 10.1093/femspd/ftw018

Vaezi, A., Fakhim, H., Khodavaisy, S., Alizadeh, A., Nazeri, M., Soleimani, A., et al. (2017). Epidemiological and mycological characteristics of candidemia in Iran: A systematic review and meta-analysis. *J Mycol Med*. 27 (2), 146–152. Doi: 10.1016/j.mycmed.2017.02.007

Verma, A. H., Richardson, J. P., Zhou, C., Coleman, B. M., Moyes, D. L., Ho, J., et al. (2017). Oral epithelial cells orchestrate innate type 17 responses to *Candida albicans* through the virulence factor candidalysin. *Sci Immunol*. 2 (17), eaam8834. Doi: 10.1126/sciimmunol.aam8834

Verma, A. H., Zafar, H., Ponde, N. O., Hepworth, O. W., Sihra, D., Aggor, F. E. Y., et al. (2018). IL-36 and IL-1/IL-17 drive immunity to oral candidiasis via parallel mechanisms. *J Immunol*. 201 (2), 627–634. Doi: 10.4049/jimmunol.1800515

Vila, T., Sultam, A. S., Montelongo-Jauregui, D., Jabra-Rizk, M. A. (2020). Oral Candidiasis: a Disease of Opportunity. *J Fungi (Basel)*. 6 (1), 15. Doi:10.3390/jof6010015

Wall, G., Montelongo-Jauregui, D., Bonifacio, B. V., Lopez-Ribot, J. L., Uppuluri, P. (2019). *Candida albicans* biofilm growth and dispersal: contributions to pathogenesis. *58yceum58fl Microbiol*. 52, 1–6. Doi:10.1016/j.mib.2019.04.001

Weber, C. R., Soares. C. M., Lopes, A. B., Silva, T. S., Nascimento, M. S., Ximenes, E. C. (2011). *Anadenanthera colubrina*: a therapeutic potential study. *Rev Bras Farm*. 92, 235-244.

Williams, D., Lewis, M. (2011). Pathogenesis and treatment of oral candidosis. *J Oral Microbiol*. 3, 1-11. Doi:10.3402/jom.v3i0.5771

Wong, S. S., Kao, R. Y., Yuen, K. Y., Wang, Y., Yang, D., Samaranayake, L. P, et al (2014). *In vitro* and *in vivo* activity of a novel antifungal small molecule against *Candida* infections. *PloS One*. 9 (1), e85836. Doi: 10. 1371/58yceum.pone.0085836

Xu, H., Dongari-Bagtzoglou, A. (2015). Shaping the oral mycobiota: Interactions of opportunistic fungi with oral bacteria and the host. *58yceum58fl Microbiol*. 26, 65–70. Doi: 10.1016/j.mib.2015.06.002

FIGURE CAPTIONS

FIGURE 1. Fungal viability of *C. albicans* MYA 2876 72-hours-biofilm expressed in CFU/ml/grams of dry weight after treatment with *A. colubrina* extract (312.5 µg/mL – 20 x 15.62 µg/mL; 156.2 µg/mL – 10 x 15.62 µg/mL). DMSO 0.1%: vehicle control; Fluconazole (10 µg/mL): positive control. Values shown with asterisk (*) are statiscally significant when compared to the vehicle control ($p < 0.05$).

FIGURE 2. *C. albicans* MYA 2876 72-hours-biofilm proteolytic enzymes secretion expressed in U/grams of dry weight after treatment with *A. colubrina* extract (312.5 µg/mL – 20 x 15.62 µg/mL; 156.2 µg/mL – 10 x 15.62 µg/mL). **(A)** Proteinase activity; **(B)** Phospholipase activity. DMSO 0.1%: vehicle control; Fluconazole (10 µg/mL): positive control. Values shown with asterisk (*) are statistically significant when compared to the vehicle control ($p < 0.05$).

FIGURE 3. (A) Cytotoxic effect of *A. colubrina* extract (2,500 µg/mL – 0.25 µg/mL) on human gingival fibroblasts after 24 hours of treatment, **(B)** LD_{50} = 432.3 µg/mL. HGF: only cells; DMSO 0.1%: vehicle control. (Values shown with asterisk (*) are statistically significant when compared to the vehicle control ($p < 0.05$).

FIGURE 4. Real-time quantitative information about relative gene expression of **(A)** *SAP-1* and **(B)** *PLB-1* after 8 hours of treatment with *A. colubrina* extract treatment (33.28 µg/mL) in human gingival fibroblasts infected by *C. albicans*. DMSO 0.1%: vehicle control. Values shown with asterisk (*) are statistically significant when compared to the vehicle control ($p < 0.05$).

FIGURE 5. Coculture fluorescence microscopy after 24 hours of treatment with *A. colubrina* extract. **(A)** *A. colubrina* extract (33.28 µg/mL); **(B)** Vehicle control (DMSO 0.1%) and **(C)** Positive control (Fluconazole – 10 µg/mL). Scale bar set at 100 µm at 100x magnification power.

FIGURE 6. Coculture SEM after 24 hours of treatment with *A. colubrina* extract. **A)** *A. colubrina* extract (33.28 µg/mL); **(B)** Vehicle control (DMSO 0.1%) and **(C)** positive control (Fluconazole – 10 µg/mL). Scale bar set at 30 µm at 1000x magnification power.

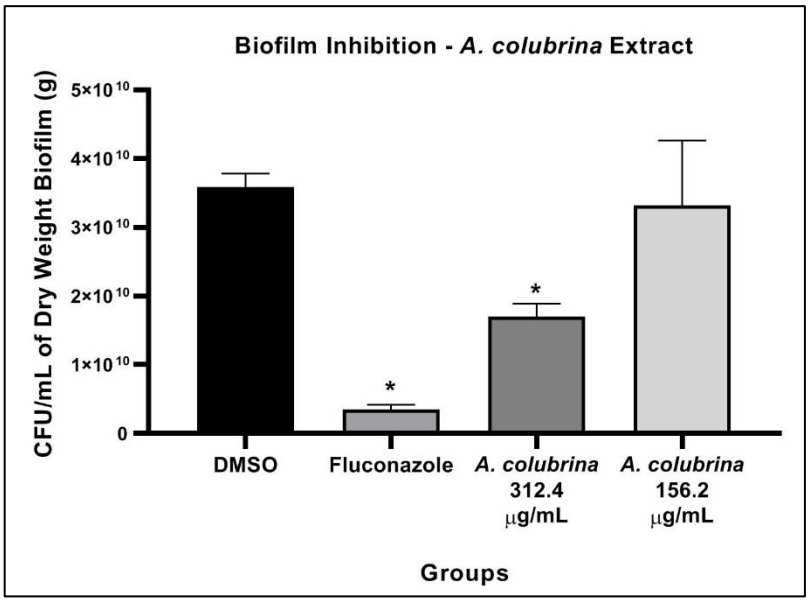
FIGURE 7. Real-time quantitative information about relative gene expression of **(A)** *IL-6*; **(B)** *IL-8*; **(C)** *IL-1β* and **(D)** *IL-10* of human gingival fibroblasts after 8 hours of infection by *C. albicans* and treatment with *A. colubrina* extract (33.28 µg/mL). DMSO 0.1%: vehicle control; Fluconazole (10 µg/mL): positive control. Values are shown as the fold-change relative to the vehicle control group. Values shown with asterisk (*) are statistically significant when compared to the vehicle control ($p < 0.05$).

FIGURE 8. Pro and anti-inflammatory cytokines expression of IL-6, IL-8, IL-1β and IL-10 by human gingival fibroblasts after 8 hours of infection with *C. albicans* and treatment with *A. colubrina* extract (33.28 µg/mL). HGF: only cells; DMSO 0.1%: vehicle control; Fluconazole (10 µg/mL): positive control. Values shown with asterisk (*) are statistically significant when compared to the vehicle control ($p < 0.05$).

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of *A. colubrina* extract against *Candida* strains.

	A. colubrina			**Fluconazole**		
***Candida* Strain**	**MIC (µg/mL)**	**MFC (µg/mL)**	**MFC/MIC *ratio***	**MIC (µg/mL)**	**MFC (µg/mL)**	**MFC/MIC *ratio***
***Candida albicans* ATCC® 90028**	< 15.62	> 2000	> 4	< 1	64-128	> 4
***Candida albicans* ATCC® MYA-2876**	< 15.62	2000	> 4	< 1	64-128	> 4
***Candida glabrata* ATCC® MYA-275**	< 15.62	> 2000	> 4	8	> 128	> 4
***Candida tropicalis* ATCC® MYA-750**	< 15.62	250	> 4	4	> 128	> 4
***Candida dubliniensis* ATCC® MYA-646**	< 15.62	> 2000	> 4	< 1	32-64	> 4

Note: MFC/MIC < 4: fungicidal profile / MFC/MIC > 4: fungistatic profile



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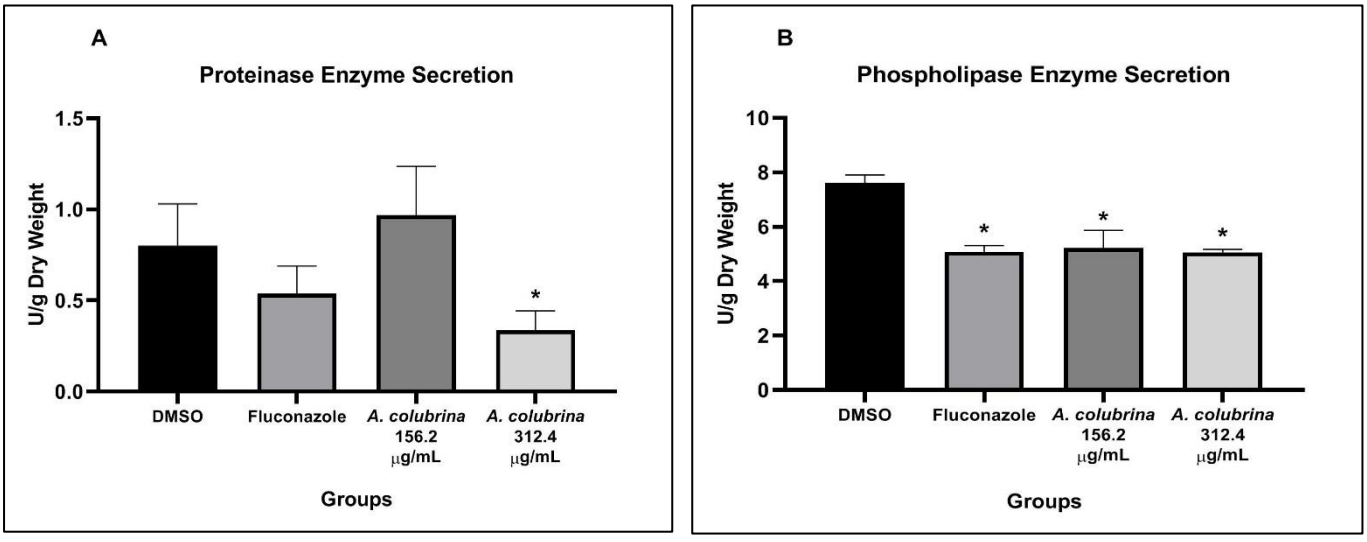

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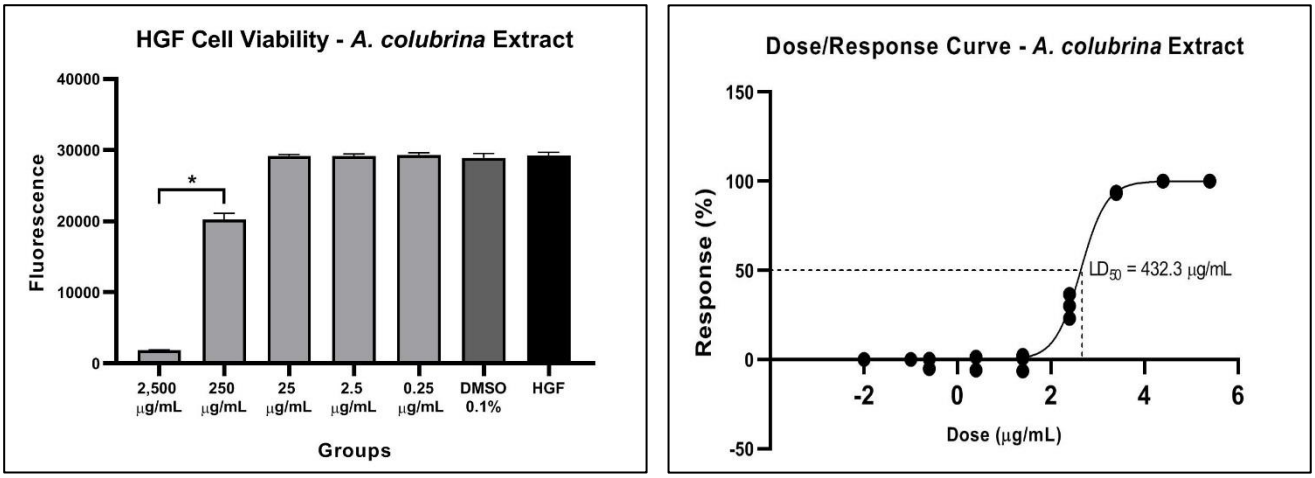

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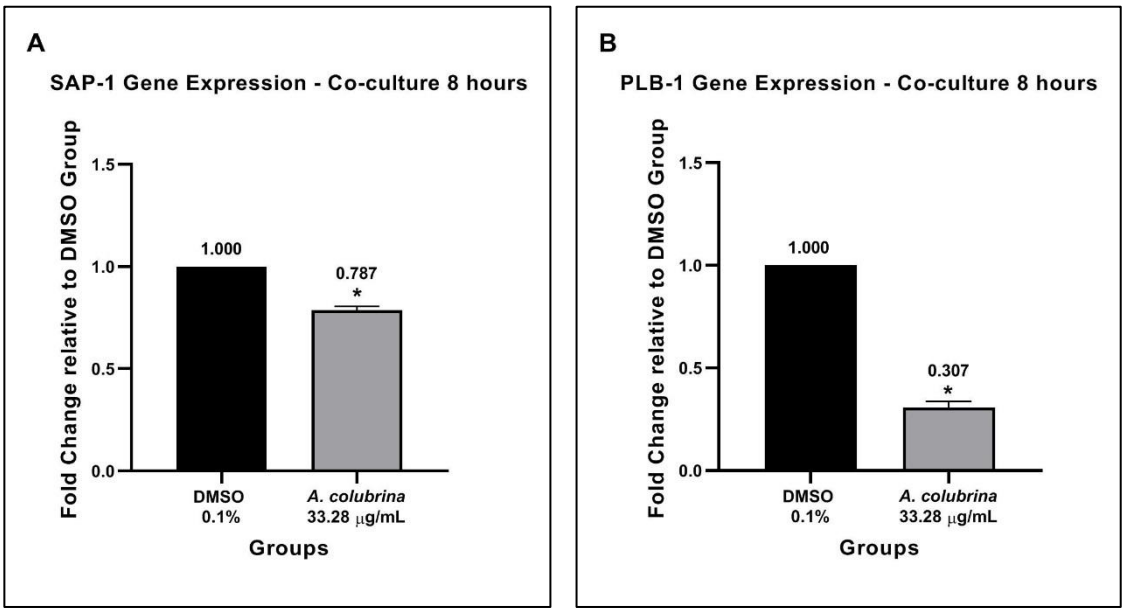

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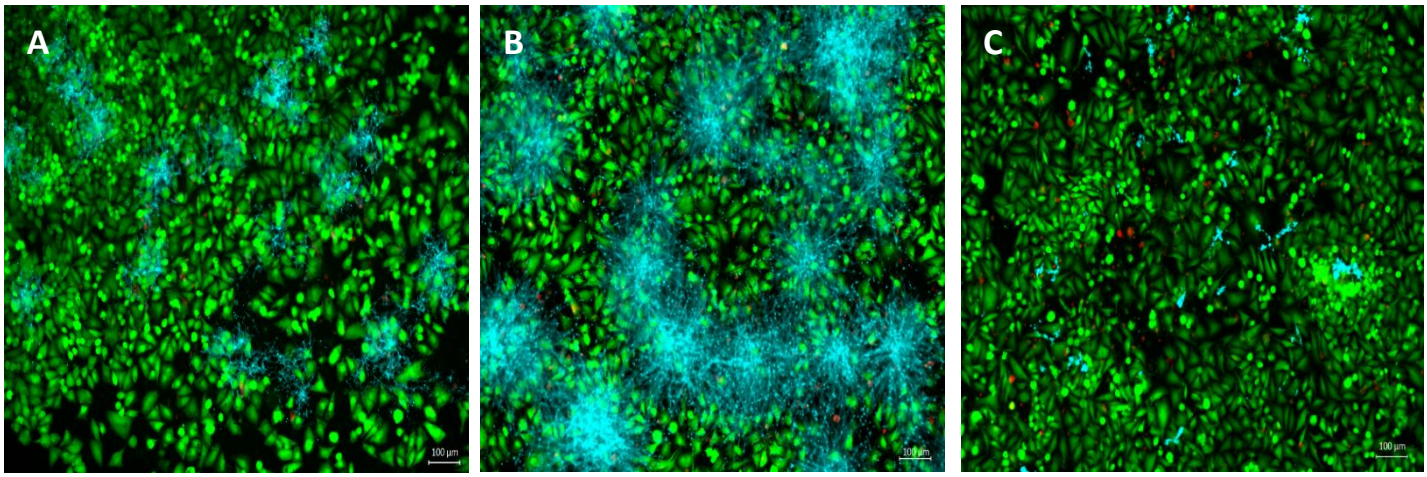

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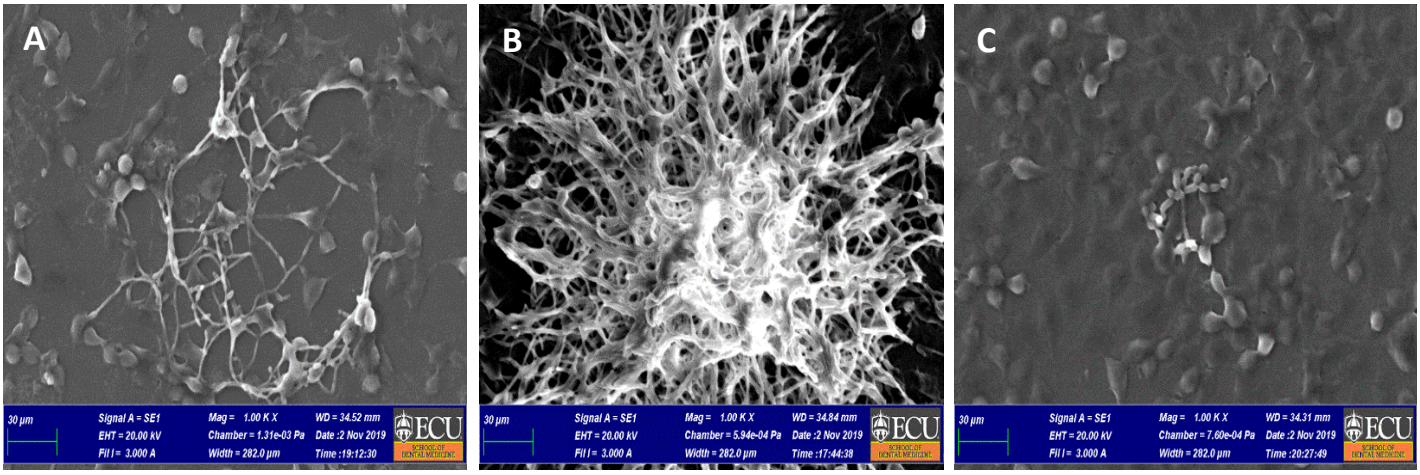

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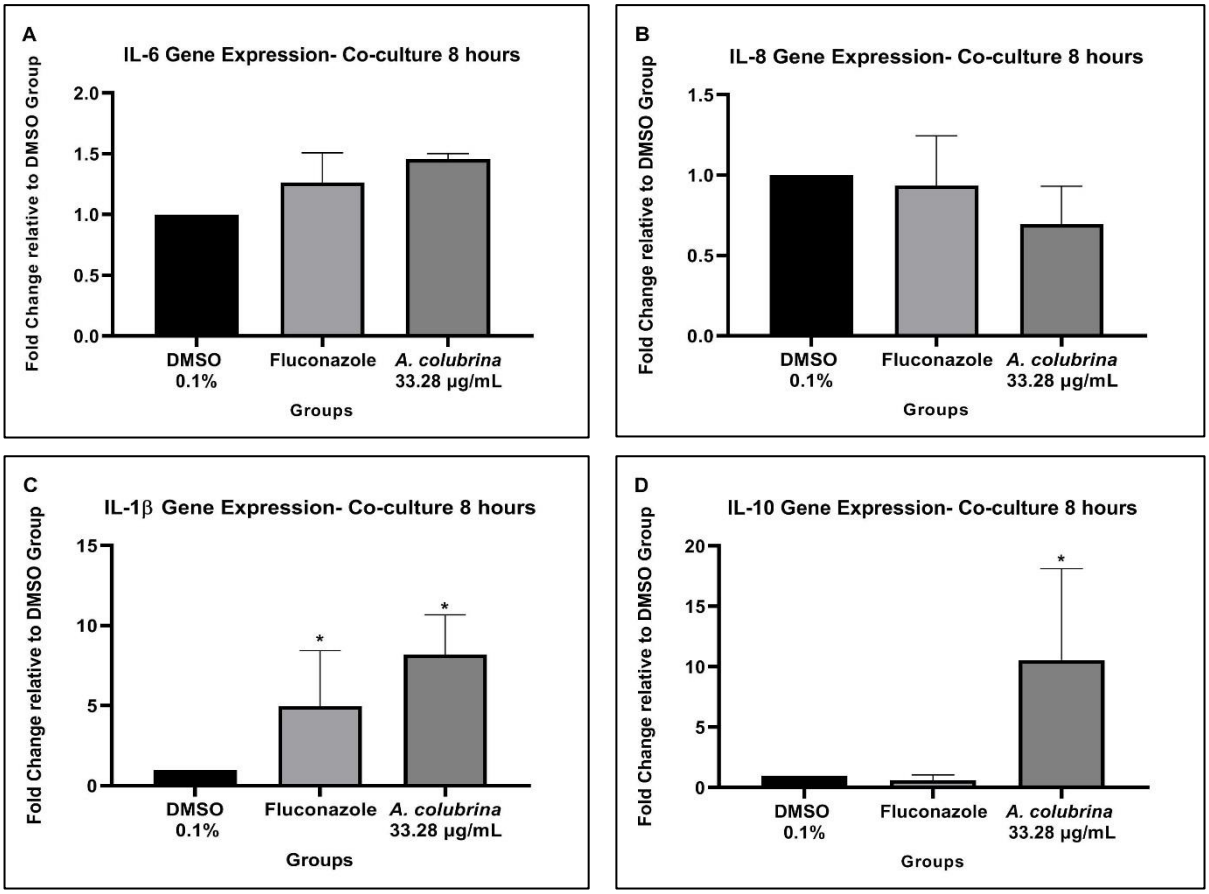

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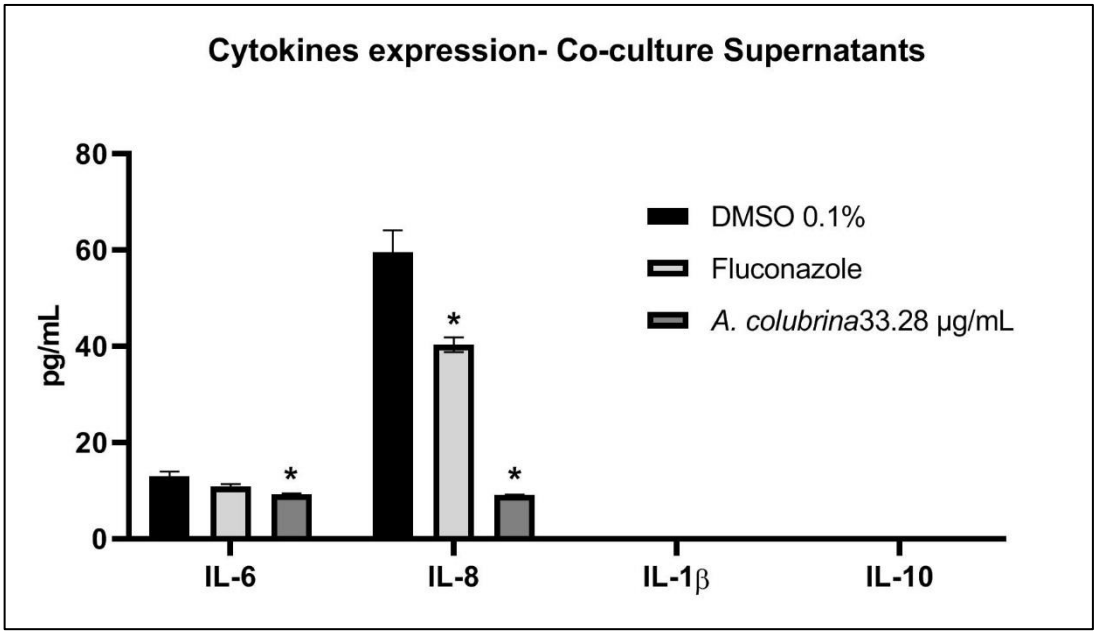

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4.2 Artigo 2

Polyphenols from *Anadenanthera colubrina* exerts anti-inflammatory activity by suppressing NF-κB and p38-MAPK signaling pathways

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Conceptualization: RMM, EMMBC, CMAM. Investigation and Data curation: CMAM, WCG, JPRS. Formal analysis: RMM, EMMBC, CMAM, WCG, JPRS, JTF. Writing – original draft: CMAM. Writing – review and editing: CMAM, RMM, EMMBC.

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Abstract

Anadenanthera colubrina (Vell.) Brenan, popularly known as “angico”, is a plant species typical of the Brazilian Caatinga biome, and widely applied in folk medicine for the treatment of general inflammation. *A. colubrina* has been proven to have anti-inflammatory effects. Phytochemical analysis have identified the presence of polyphenolic compounds which are known for potent anti-inflammatory properties. The aim of this *in vitro* study was to determine the chemical profile and evaluate the effects of *A. colubrina* extract on the expression of inflammatory cytokines and key proteins from immunoregulory signaling pathways on LPS-induced THP-1 monocytes cells. The cytotoxic effect of *A. colubrina* extract was determined by Cell Titer Blue Viability Assay on THP-1 monocytes (THP-1 ATCC® TIB-202). To establish an inflammatory model, LPS-induced THP-1 cells were treated with the extract for 6 hours. RT-PCR and Luminex Assays were performed to detect the gene expression and the levels of IL-8, IL-1β and IL-10 inflammatory cytokines, respectively. Key proteins of NF-κβ and MAPK transductions signaling pathways (NF-κβ, p-38, p-NF-κBand p-p38) were detected by Simple Western (WES Simple). Phytochemical profile was assessed by HPLC-ESI-MS^n (High-Performance Liquid Chromatography with Electrospray Ionization Mass Spectrometry) and LC-HRESIMS (High Resolution Electrospray Ionization Mass Spectrometry). Cell viability was not significantly affected in concentrations up to 250 µg/mL (LD_{50} = 978.7 µg/mL). Upon LPS stimuli, gene expression and cytokine production of IL-1β and IL-10 were down and up-regulated, respectively. The extract is involved in TLR4-related NF-κβ/MAPK pathways through phosphorylation of p38 and NF-κβ, with decreases in the signal intensity. The extract itself did not induce inflammatory response. Phytochemical analysis showed a phenolic profile, composed predominantly of flavonoids, cathechins, procyanidins and tannins. *A. colubrina* extract demonstrated low cytotoxicity and anti-inflammatory effects by modulating IL-1β and IL-10 inflammatory cytokines gene expression and secretion, through regulation of intracellular NF-κBand p38-MAPK signaling pathways.

Keywords: Phytotherapy; Immune Response; Anti-inflammatory Agents; LPS; Inflammatory Cytokines; TLR4 Receptor; THP-1 Cells.

Introduction

Inflammation is a type of immune response characterized by protective or healing process to tissue damage on the body [1, 2]. It triggers a complex cascade of molecular mechanisms in response to several agents such as pathogens and trauma, in order to eliminating them and

repairing injured cells or tissues [3, 4]. The activation of immune response leads to the accumulation of immune cells at the site of injury [5], stimulating the production of inflammatory mediators such as, prostaglandins, nitric oxide, interleukins (IL-1β, IL-6, IL-8, TNF-α) and reactive oxygen species (ROS) [6].

Microbial infection is one of the most common causes activating the inflammatory process [4]. Pathogen species present structures on their cell membrane or cell wall, called Pathogen-Associated Molecular Patterns (PAMPs), which interact with inflammation-related Pattern Recognition Receptors (PRRs) on the host cells membrane, giving rise to the initiation of intracellular signaling process for the onset of inflammation [7, 8].

Toll-Like Receptors (TLRs) are transmembrane proteins and a type of PRR, which bind to a specific PAMP, depending on the type of pathogen [4]. Lipopolysaccharide (LPS), for example, is the major glycolipid within the outer membrane from Gram-negative bacteria and the best characterized type of PAMPs, which is recognized by the specific PRR TLR4 [9]. This interaction between TLR4 and LPS initiates successive intracellular signaling responses critical for inflammation, stimulating the release of cytokines and inflammatory mediators that exacerbate the immune response and inflammatory damage to local tissues [10, 11]. In addition, TLR4 plays a crucial role in innate immunity and inflammation, triggering the activation of NF-κB and MAPK (ERK, p38 and JNK kinases) signaling transduction pathways, which regulate the genes transcription and synthesis of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α [12–14].

Despite the importance of immune response, when these inflammatory pathways are overactive, they may increase the risk of several diseases, including cancer and autoimmune diseases [15]. Thus, the suppression of these pathways and the appropriate modulation of inflammation is critical for homeostasis maintenance and the development of effective anti-inflammatory drugs is strongly required [8, 16]. Considering the pharmacological significance of plant-derived substances, much attention has been drawn to them, especially for the discovery of potential therapeutic agents for treating a wide range of disorders, as alternative choices [2, 17].

In this regard, *Anadenanthera colubrina* (Vell.) Brenan is a plant species that occurs to the South of the line of the Equator, and in Brazil, from the Northeastern to the Southeastern regions, more typically on Caatinga biome [18]. It´s popularly known as “angico” and widely applied in folk medicine for the treatment of inflammation in general [19, 20]. *A. colubrina* has been proven to have pharmacological and biological effects, including antifungal [21, 22], antiproliferative [21], anti-inflammatory [23, 24] and antioxidant [24, 25]. Moreover,

phytochemical analyses of *A. colubrina* have identified the presence of polyphenolic compounds, mainly flavonoids [21, 24], which are known for their potent anti-inflammatory and antioxidant properties [26, 27, 28].

Despite anti-inflammatory properties of *A. colubrina*, only limited data exist about its immunomodulatory effects at transcriptome and proteome levels, as well as, the transductions signaling molecular mechanisms. Therefore, this study was carried out to investigate the phytochemical profile and the *in vitro* immunomodulatory effect of *A. colubrina* barks extract and underlying cell signaling transduction pathways in LPS-stimulated monocytes.

Material and methods

Preparation of the extract

Barks of *Anadenanthera colubrina* (Vell.) Brenan were collected during the month of September in the semi-arid region of Paraíba state, Brazil (7º 22’ 25” S, 35º 59’ 32” W). Botanical specimens were deposited in the Manuel de Arruda Câmara Herbarium (ACAM) at the State University of Paraíba (UEPB), Campus I, Campina Grande, Paraíba, Brazil, under nº 1936/ACAM. This research was conducted under authorization number SisGen A289DF4. The plant material was dried, ground and immersed in 80% ethanol for 48 hours (10 mg : 25 mL) in order to obtain a hydroethanolic extract [29]. The material was filtered three times, vacuum concentrated (Tecnal TE-211, Piracicaba, SP) and lyophilized (Martin Christ 1-2 Ldplus, Osterode am Harz, DE), with extraction yield of 31.7%.

Cell viability assay

The effect of *A. colubrina* extract on cell viability was evaluated on THP-1 monocytes (THP-1 ATCC® TIB-202) and assessed by a resazurin fluorometric method (Cell Titer Blue Viability Assay, Promega Corp, Madison, WI). THP-1 cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640, VWR Life Science, Radnor, PA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, Invitrogen, Waltham, MA), Penicillin (10,000 U/mL) and Streptomycin (10,000 µg/mL), and 2-mercaptoethanol 50 nM (VWR Life Science, Radnor, PA), at 37°C in 5% CO_2. THP-1 cells (2.5 x 10^5 cells/mL) were seeded in each well of a 24-well plate (Greiner Bio-One North America, Inc Monroe, NC, USA) in RPMI with 10% FBS. The *A. Colubrina* extract was diluted in Dimethyl Sulfoxide 1% (DMSO) (BDH Solvents, Dawsonville GA), with final concentration inside the wells of 0.1%, and then added to the cultured cells wells (2,500 – 0.25 µg/mL). The plates were incubated for 24 hours at 37°C in

5% CO_2. Resazurin (30 µL) was added to each well, and the plates were incubated for 3 hours. The fluorescence of supernatant was read in a microplate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA), with excitation of 555 nm, emission of 585 nm and 570 nm *cut off* [30].

Cell treatment and LPS-induced inflammation assay

THP-1 cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well, following the same culture conditions from cell viability assay protocol, as mentioned above. Then, the cells were exposed to *A. colubrina* extract and LPS from *Porphyromonas gingivalis* (InvivoGen, San Diego, CA), according to the following groups: stimulation with LPS (100 ng/mL), treatment with *A. colubrina* (250 µg/mL), simultaneous exposure to LPS (100 ng/mL) and *A. colubrina* (250 µg/mL), and a control group with no treatment. The plates were incubated for 6 hours, at 37°C in 5% CO_2 (VWR Symphony 5.3 A, Radnor, PA) [31]. Cell supernatants were collected by centrifugation at 1,500 rpm for 5 min, for quantitative analysis of cytokines. The cells kept on the bottom of the wells were processed for RNA extraction and whole-cells lysate obtaining, for gene expression by RT-PCR and Wes Simple Western assays, respectively.

Real-Time Quantitative PCR

Total RNA was isolated from LPS-induced THP-1 cells and treated with *A. colubrina* using Rneasy® Mini Kit (Qiagen, Hilden, DE), according to manufacturer's instructions. SpectraDrop Micro-Volume Starter Kit (Molecular Devices, Sunnyvale, CA) was used to quantify the total RNA extracted. The following host inflammatory cytokines genes were selected: *IL-8* (Qiagen Gene ID #3576), *IL-10* (Qiagen Gene ID #3587), *IL-1β* (Qiagen Gene ID #3553) and *GAPDH* (housekeeping) (Qiagen Gene ID #2597). All data from genes expression were normalized using the housekeeping gene GAPDH. PCR amplification was performed by using 25 µL reaction mix per well in 0.2 mL 8-Strip PCR tubes. The reactions were conducted in thermocycler (QuantStudio 3 Real Time PCR System, ThermoFischer Scientific, Rockford, IL) at 50°C for 10 min (Reverse Transcription Step); 95°C for 5 min (PCR Initial Activation Step); followed by 40 cycles of 10 s at 95°C (Denaturation Step) and 30 s at 60°C (Annealing/Extension Step). Analysis of relative gene expression was achieved according to the ΔΔCt method [32, 33].

Luminex for quantitative analysis of inflammatory cytokines

Supernatants from TPH-1 cells treated with *A. colubrina* and exposed to LPS were collected and assayed immediately using Human Magnetic Premixed Multi-Analyte Luminex Assay Kit (R&D Systems, Minneapolis, MN) for expression of pro-inflammatory (IL-8 and IL-1β) and anti-inflammatory (IL-10) cytokines. Culture supernatants and cytokine capture bead cocktails were incubated overnight. Samples were then incubated for 1 h with biotin-labeled antibody and for 30 min in a dilution of streptavidin-PE. Data were obtained by Luminex 200 Milliplex System and analyzed with Milliplex Analyst software [34].

WES Simple capillary immunoassay of NF-κB and p38-MAPK

THP-1 cells were washed with PBS and lysed with 200 µL of ice-cold Pierce RIPA Lysis Buffer (ThermoFischer Scientific, Rockford, IL), for 5 min at room temperature, in order to obtain the whole-cell lysate. Total protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions, in order to normalize the lysates protein content. The protein expression of NF-κB, p38, phosphorylated NF-κB and phosphorylated p38 was detected by Wes Simple Western system (ProteinSimple, San Jose, CA), a capillary-based semiquantitative protein analysis Toll. Separation Module of 12–230 kDa, Anti-Rabbit Detection Module and capillary cartridges (ProteinSimple, SM-W002-1, San Jose, CA) were applied according to the manufacturer's protocol. Lysate samples were reduced in 0.4 M dithiothreitol (DTT), mixed with Fluorescent Master Mix (1:4) and heated for denaturation at 95°C for 5 min. A biotinylated ladder (12-230 kDa) was used for molecular weight determination. Specific primary antibodies for protein detection of NF-κB and p-NF-κB (MW: 120 kDa), p38 (MW: 40kDa) and p-p38 (MW:43 kDa) (Cell Signaling Technology, Danvers, MA), were used according to the optimized dilutions (1:1000). The samples, the blocking reagent, the primary antibodies, the HRP-conjugated secondary antibodies and the chemiluminescent substrate were added to the plate, loaded into a WES machine (ProteinSimple, San Jose, CA) coupled to capillary cartridge and run together with fluorescent standards, which permit molecular weight normalization to the ladder. The data were analyzed with Compass™ for Simple Western Software version 5.0 (ProteinSimple, San Jose, CA) [35, 36].

Phytochemical profile analysis by LC-ESI-MS^n and LC-HRESIMS

A. colubrina extract was analysed using a HPLC (Shimadzu, Kyoto, JP) equipped with C18 column (Kromasil – 250 mm x 4.6 mm x 5 µm) coupled to Ion-Trap (Amazon X,

Bruker, Berlin, DE) or microTOF mass spectrometers (Bruker, Berlin, DE) with on Electrospray Ionization (ESI). Extract was diluted in methanol (1 mg/mL) and then filtered in 0.45 µM PVDF (Polyvinylidene Difluoride) membrane. The chromatographic method used methanol (solvent B) and ultrapure Mili-Q water (solvent A), acidified with formic acid (0.1% v/v), following gradient elution of concentration (5 to 100% of solvent B in 60 minutes). Injection volume was 10 µL and flow set to 0.6 mL/min. Ion Trap and TOF acquisition parameters were as follows: negative ionization mode; spray voltage of 4.4 kV; offset of 500V; sheath gas at 35 pi; drying gas N_2; flow of 8mL/min and heater temperature of 300°C. Identification of the compounds was based on MS/MS data available on literature.

Statistical analysis

Data were expressed as the mean ± SEM using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison tests in relation to the vehicle, using GraphPad Prism software (version 8.02). Results were considered significant if p-values were less than 0.05.

RESULTS

Cytotoxicity

The *A. colubrina* extract presented LD_{50} of 978.7 µg/mL and a non-toxic profile on THP-1 cells culture, with no significant effect on cell viability in concentrations up to 250 µg/mL, when compared to the vehicle and the cell control (Fig. 1).

Fig. 1. Cytotoxic effect of *A. colubrina* extract (2,500 µg/mL – 0.25 µg/mL) on THP-1 monocytes after 24 hours of treatment (LD_{50} = 978.7 µg/mL). THP-1: only cells; DMSO 0.1%: vehicle control. Values shown with asterisk (*) are statistically significant when compared to the vehicle control ($p < 0.05$).

Modulatory effect on inflammatory cytokines gene expression

A. colubrina extract at 250 µg/mL presented modulatory effects on the gene expression of inflammatory cytokines. The extract itself up-regulated the gene expression of *IL-1β* cytokine. Upon LPS stimuli, gene expression of *IL-1β* and *IL-10* were respectively,

down and up-regulated by *A. colubrina*, while, it was not effective in modulating *IL-8* gene expression (Fig. 2 – A, B, C).

Fig. 2. Real-time quantitative information about relative gene expression of **A**) *IL-8*, **B**) *IL-1β* and **C**) *IL-10* of THP-1 cells after 6 hours of treatment with *A. colubrina* extract (250 µg/mL) and stimulation by LPS (100 ng/mL). Values are shown as the fold-change relative to the negative control group (6 hours). Values shown with asterisk (*) are statistically significant when compared to the negative control ($p < 0.05$).

Pro and anti-inflammatory cytokines secretion

THP-1 culture supernatants were assessed for the expression of pro-inflammatory (IL-8 and IL-1β) and for anti-inflammatory (IL-10) cytokines, after treatments with *A. colubrina* extract (250 µg/mL). The extract itself did not induce any inflammatory response. On the other hand, the extract was not effective in reducing IL-8 cytokine secretion from LPS-induced THP-1 cells group ($p < 0.05$). On the LPS-stimulated group treated with *A. colubrina*, IL-1β and IL-10 cytokines levels expression were significantly reduced and increased, respectively ($p < 0.05$), when compared to the LPS-induced only cells group (Fig. 3 – A, B, C).

Fig. 3. Pro and anti-inflammatory cytokines expression of **A**) IL-8, **B**) IL-1β and **C**) IL-10 by THP-1 cells after 6 hours of treatment with *A. colubrina* extract (250 µg/mL). Values shown with asterisk (*) are statistically significant when compared to the LPS-induced cells group ($p < 0.05$).

Regulatory mechanism on signaling pathways

Simple Western assay data demonstrated that the phosphorylated forms of NF-κB and p38 were detected at all groups tested, with visual differences on bands signal intensity. LPS-induced cells group triggered NF-κB and p-38 phosphorylated forms, in which bands signal intensity are visually stronger than the other groups. *A. colubrina* also activated the MAPK pathway by itself and in conjunction with LPS, through phosphorylation of p38. NF-κB pathway was also activated by the same groups, however the bands signal intensity are visually lower when compared to LPS-induced group (Fig. 4 – A, B, C, D).

Fig. 4. Western blot analysis of **A**) NF-κB, **B**) p-NF-κB, **C**) p-38 and **D**) p-p38 for THP-1 cells treated for 6 hours with *A. colubrina* extract (250 µg/mL) and stimulated by LPS (100 ng/mL).

Phytochemical profile

The phytochemical analysis from hydroethanolic extract of barks of *A. colubrina* assessed by HPLC-ESI-MS^n and HRESIMS disclosed a phenolic profile, composed predominantly of flavonoids, cathechins, procyanidins and tannins (Table 2). The base peak chromatogram is shown in Fig 5.

Fig. 5. Base peak chromatogram of *A. colubrina* extract.

Table 2. Phytochemical compounds identified in *A. colubrina* extract by HPLC-ESI-MS^n and HRESIMS.

Peak	R.T.	$[M-H]^-$	Formula	Error (ppm)	MS^n m/z	Assignment	References
1	5,3	341,1088	$C_{12}H_{22}O_{11}$	0,4	MS^2 [341,0]: 178,9; 160,8; 148,8; 142,8; 130,8 MS^3 [341,0 → 178.7]: 160,8; 130,9; 142,8; 118,9; 100.8; 88,9	Sucrose	Taylor et al, 2005; Verardo; Duse; Callea, 2009; Jin et al. 2018
2	17,4	451,1244	$C_{21}H_{24}O_{11}$	0,3	MS^2 [451,0]: 288,9, 244,9, MS^3 [451,0 → 288,9]: 244,9; 204,9; 178,9	Catechin-O-hexoside	Zhao et al, 2014; Kang et al. 2016
3	21,4	577.1360	$C_{30}H_{26}O_{12}$	-1,6	MS^2 [577,0]: 425,0; 406,9; 288,9	ICatechin-ICatechin	Bubba et al, 2012; Lin et al, 2014; Maia et al, 2019
4	22,0	561, 1401	$C_{30}H_{26}O_{11}$	0,2	MS^2 [561,0]: 451,0; 409,0; 391,0; 288,9; 271,0; 244,9	(E)Fisetinidol–(E)Catechin I	Venter et al, 2012; Lin et al, 2014; Mateos-Martín et al, 2014;
5	23,8	561, 1402	$C_{30}H_{26}O_{11}$	0,1	MS^2 [561,0]: 450,9; 409,0; 391,0; 289,0; 271,0; 245,0	(E)Fisetinidol–(E)Catechin II	Venter et al, 2012; Lin et al, 2014; Mateos-Martín et al, 2014
6	25,2	833.2087	$C_{45}H_{38}O_{16}$	-0,0	MS^2 [833,2]: 681,0; 663,0; 561,0; 543,0; 408,9; 390,9	B-type Proanthocyanidin Trimer I	De Souza et al, 2008; Omar; Mullen; Crozier, 2011; Mateos-Martín et al, 2014; Xiong et al, 2017
7	25,4	561, 1405	$C_{30}H_{26}O_{11}$	-0,4	MS^2 [561,0]: 451,0; 408,9; 391,0; 288,9; 270,9; 245,0	(E)Fisetinidol–(E)Catechin III	Venter et al, 2012; Lin et al, 2014; Mateos-Martín et al, 2014
8	25,7	545.1448	$C_{30}H_{26}O_{10}$	1,0	MS^2 [545,0]: 527,0; 435,0; 409,0; 288,9	(E)Guibourtinidol-(E)Catechin I	Sobeh et al, 2017; Maia et al, 2019
9	26,1	833.2085	$C_{45}H_{38}O_{16}$	0,2	MS^2 [833,2]: 681,0; 663,0; 561,0; 543,1; 409,0; 391,0	B-type Proanthocyanidin Trimer II	De Souza et al, 2008; Omar; Mullen; Crozier, 2011; Mateos-Martín et al, 2014; Xiong et al, 2017
10	26,9	545.1450	$C_{30}H_{26}O_{10}$	0,6	MS^2 [545,1]: 527,0; 434,9; 408,9; 288,9	(E)Guibourtinidol-(E)Catechin II	Sobeh et al, 2017 ;Maia et al, 2019

11	27,6	713.1506	$C_{37}H_{30}O_{15}$	0,8	MS^2 [713,0]: 561,0; 408,9; 391,0; 288,9; 271,0; 245,0	Galloyl-I-afzelechin/fisetinidol –I-Catechin	Zhang; Sun; Chen 2016; Schmeda-Hirschmann et al, 2019
12	28,2	833.2082	$C_{45}H_{38}O_{16}$	0,6	MS^2 [833,2]: 681,0; 663,0; 561,0; 543,1; 409,0; 391,0	B-type Proanthocyanidin Trimer III	De Souza et al, 2008; Omar; Mullen; Crozier, 2011; Mateos-Martín et al, 2014; Xiong et al, 2017
13	29,3	833.2089	$C_{45}H_{38}O_{16}$	-0,2	MS^2 [833,2]: 681,0; 663,0; 561,0; 543,1; 409,0; 390,9	B-type Proanthocyanidin Trimer IV	De Souza et al, 2008; Omar; Mullen; Crozier, 2011; Mateos-Martín et al, 2014; Xiong et al, 2017
14	30,9	817.2141	$C_{45}H_{38}O_{15}$	-0,4	MS^2 [817,1]: 665,0; 561,0; 545,0; 409,0; 288,9	(E) -Guibourtinidol – (E) -Afzelechin-(E)-Catechin	Schmeda-Hirschmann et al, 2019; Sobeh et al, 2018
15	32,7	801.2179	$C_{45}H_{38}O_{14}$	1,3	MS^2 [801,2]: 765,0; 545,0; 408,9; 288,9	(E)-Guibourtinidol-(E)-Guibourtinidol-(E)-Catechin	Schmeda-Hirschmann et al, 2019; Sobeh et al, 2018
16	33,1	447, 0935	$C_{21}H_{20}O_{11}$	-0,5	MS^2 [447,1]: 284,9; 255,0; 162,9 MS^3 [447,1→284,9]: 254,8; 162,8;	Kaempferol-O-hexoside	Engels et al, 2012
17	36,1	463,0891	$C_{21}H_{20}O_{12}$	-1,9	MS^2 [463,0]: 300,9 MS^3[463,0 →300,9]: 270,8; 254,8; 178,8; 150,8;	Quercetin-O-hexoside	Goveia; Castilho, 2011; Jaiswal; Jayasingheb; Kuhnert, 2011
18	44,1	285,0402	$C_{15}H_{10}O_{6}$	0,9	MS^2 [285,0]: 242,9; 240,9; 216,9; 198,9; 174,9; 150,9; 132,9	Luteolin	Kang et al, 2016

Discussion

A. colubrina is commonly used as medicinal plant by traditional communities, which includes the treatment of inflammatory disorders [19, 20]. Thus, several *in vitro* and *in vivo* studies have been conducted in order to evaluate and confirm the anti-inflammatory potential of this plant species [23, 24, 37]. In this research, we employed for the first time a transcriptomic-proteomic approach to investigate the immunomodulatory effect of *A. colubrina* extract and its regulation on underlying cell signaling transduction pathways in LPS-induced THP-1 monocytes.

Cytotoxicity screening of plant extracts intended for therapeutic applications is an important step when investigating potential alternatives or developing new compounds for the treatment of disorders [2]. Therefore, we initially investigated the cytotoxicity of *A. colubrina* on THP-1 monocytes, with the purpose to verify its therapeutic safety level. According to our study, the extract did not affect cell viability at concentrations up to 250 µg/mL, representing a relatively low cytotoxic activity. Several reports also confirmed the low toxicity *A. colubrina* on macrophages [22-24], keratinocytes and tumoral cell strains [21].

In this study, human monocytic leukemia cell line THP-1 was set as the *in vitro* cell model to evaluate the immunomodulatory effects of *A. colubrina*, since these cells possess regulatory proteins which initiate inflammation upon stimulation by LPS [38]. Monocytes, along with macrophages, play a major role in inducing several inflammatory responses, being involved in phagocytosis, release of inflammatory chemokines, reactive oxygen species (ROS) and triggering the adaptive immunity [39]. This cell line has been widely used to study immune responses since cells are not only in the monocyte state but also in the macrophage-like state and has become a suitable model to estimate modulation of monocyte and macrophage activities [1, 40-43]. Several studies have investigated the immunomodulatory effects of medicinal plant extracts using THP-1 cells [38, 39, 44-46], thus supporting its application as an *in vitro* model to study human inflammatory diseases.

To evaluate the immunomodulatory activity of *A. colubrina*, we assessed its effects on the regulation of pro-inflammatory cytokines expression during LPS stimulation, through gene expression (transcriptional level) and secretion (proteomic level) of these inflammation mediators. Considering that TLRs exert pro-inflammatory effects when activated, and anti-inflammatory when down-regulated or suppressed, they are thought to play a central role in both mediating and modulating inflammatory response [47]. In this regard, we selected a panel of pro-inflammatory markers, since the interaction between TLR4 receptor and LPS induces the activation of NF-κB/MAPK signaling pathways and the release of TNF-α, IL-1β, IL-6 and

IL-8 [14, 48, 49]. Additionally, we evaluated the secretion levels of IL-10, as an anti-inflammatory marker.

The assessment of gene 79yceum79flan demonstrated that *A. Colubrina* was not effective in reducing the gene expression of pro-inflammatory cytokine IL-8 in LPS-induced group. However, upon LPS stimuli, *A. colubrina* affected the expression of inflammatory cytokines by down-regulating *IL-1β* and up-regulating *IL-10* genes expression, which suggests an anti-inflammatory effect of the extract on the transcriptional level of regulation. In addition, our results showed that LPS stimulus was effective in up-regulating the gene expression of pro-inflammatory cytokines *IL-8* and *IL-1β*, which proves its effect on inducing transcription of genes related to inflammatory responses, upon interaction with TLR4 [50].

The analysis for secretion of pro-inflammatory and anti-inflammatory cytokines showed that *A. colubrina* itself did not induce any type of inflammatory response, which is positive considering that the extract did not significantly affect THP-1 cells viability and functions. Upon *A. colubrina* treatment, secretion levels of IL-8 were affected in LPS-induced groups, suggesting that the extract was not effective in decreasing this pro-inflammatory cytokine during the inflammatory stimuli produced by LPS. On the other hand, the extract significantly reduced the secretion of IL-1β on LPS-induced cells, as well as significantly enhanced the synthesis production of anti-inflammatory cytokine IL-10 on LPS-induced cells.

IL-1β is vital for the host inflammatory response against pathogens, since this cytokine is involved in the recruitment of immune cells to the site of infection [51] and aggravates injury during acute tissue insult and chronic diseases [48]. Therefore, a decrease in IL-1β expression by *A. colubrina* may prevent additional inflammatory response induced by the recruitment of immune cells. In addition, IL-10 is a representative anti-inflammatory cytokine that plays a critical role in the control of immune responses and is reported to be involved in inhibition of IL-1β production [52, 53], which is consistent with our results. Therefore, these findings suggest that *A. colubrina* may regulate the immune response by modulating the secretion levels of cytokines IL-1β and IL-10.

To examine *A. colubrina*-mediated-signal transduction and determine which pathways were affected on the regulation of pro-inflammatory cytokines gene expression, Simple Western assay was used for analyzing the expression of key proteins involved on NF-κB/MAPK signaling pathways through detection of phosphorylated forms of NF-κB and p38. MAPK (JNK, ERK and p38) and NF-κB are crucial intracellular signaling pathways leading to the inflammatory response [54]. These biological responses are mediated by their transcription factors, such as activator protein-(AP-)1 and NF-κB subunit IκBα, which are phosphorylated

and translocated from the cytoplasm to the nucleus, resulting in an inflammatory action through the expression of target genes Iing proinflammatory cytokines IL-1β, IL-6, and TNF-α, as well as iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) proteins [55, 56].

In this study, we evidenced the boosted activity of phosphorylated forms of NF-κB and p38 upon 100 ng/mL of LPS stimulation alone in THP-1 cells, which proves the activation from NF-κB/MAPK pathways by LPS, resulting in over-production of inflammatory mediators. In addition, we observed phosphorylation of NF-κB and p38, with an attenuated signal intensity bands regarding *A. colubrina* itself and in conjunction with LPS stimuli, especially on NF-κB transduction factor. These findings suggest that the extract may exerts molecular mechanisms involving the modulation of these signaling pathways, particularly on NF-κB protein activation.

Indeed, IL-1β is commonly induced by the activation of the inflammatory transcription factor NF-κB, which regulates responses such as cell proliferation, migration, adhesion, and lymphocyte development [57]. Thus, the decreased expression of IL-1β cytokine in Luminex analysis in our study is consistent with the down-regulation of NF-κB signaling pathway. Furthermore, considering that the extract combined with LPS was not effective in decreasing IL-8 levels, but significantly decreased IL-1β secretion, we hypothesized that phosphorylation of p38 in conjunction with an attenuated activation of NF-κB, were responsible for the interaction between these pathways, determining this final biological response after LPS stimulation [58].

This approach may also help to identify potential therapeutic targets in pathophysiological contexts of exacerbated or chronic inflammation [8, 59]. Thus, we reinforce that further analysis to measure the phosphorylation levels of NF-κB and p38 proteins are necessary, in order to verify on quantitative terms how *A. colubrina* extract can regulate the expression of these key proteins related to signaling pathways, which triggers the expression of pro-inflammatory cytokines. Besides, other transmembrane receptors, signaling pathways and key regulators could be implicated in these effects and must be evaluated.

Besides the regulation of IL-1β, IL-6 and IL-8, NF-κB/MAPK signaling cascades also monitor the expression of pro-inflammatory cytokine TNF-α [39]. Our study did not evaluate the expression TNF-α. However, recent studies evaluating the anti-inflammatory effects of *A. colubrina* leaves extract [24] and of protease inhibitors also extracted from the leaves [23] showed significant reductions on the expression of TNF-α and nitric oxide (NO) in LPS-induced macrophages, assessed by ELISA assays. Indeed, high levels of TNF-α and IL-1β strongly stimulate iNOS enzyme, which results in elevation of intracellular NO levels [60].

Along with these findings, our results can contribute to a better understanding of the molecular mechanisms underlying the anti-inflammatory effects of *A. colubrina*.

Previously, we evaluated the phytochemical composition of *A. colubrina* barks extract [21], in which was found a high total polyphenol content (53.18% gallic acid equivalents); tannins (8.77% catechin equivalents) and flavonoids (0.28% quercetin equivalents). In this current study, we also assessed the chemical profile of the extract by HPLC-ESI-MS^n and HRESIMS, and the results were characterized by the presence of flavonoids, mainly cathechins and proanthocyanidins, and also Quercetin and Kaempferol. Flavonoids are polyphenolic compounds, commonly present in most plants, and have been proven to have anti-inflammatory properties [26-28], which could support the immunomodulatory properties of *A. colubrina* observed in the present study. Flavonoids can modulate the immune response through inhibition of molecules that play important roles in the modulation of mediators related to inflammatory responses, including regulatory enzymes and transcription factors, such as NF-κB [28, 61-63].

Several studies have reported that extracts containing flavonoids, such as catechin and glycosylated derivatives of quercetin, can modulate several inflammatory and oxidative stress mediators through the negative regulation of pro-inflammatory cytokines and chemokines (TNF-α, IL-6, IL-1β, IL-8), NO and COX-2 in LPS-activated macrophages [26, 27, 64, 65]. Thus, consistent with these studies, the immunomodulatory effects of *A. colubrina* extract observed in the present study could be related to the presence of flavonoids.

Conclusion

A. colubrina extract demonstrated *in vitro* low cytotoxicity and anti-inflammatory properties in LPS-induced THP-1 cells. These effects can be related to modulations in the secretion of IL-1β and IL-10 cyotkines, through the regulation of intracellular NF-κB and p38-MAPK signaling pathways. Thereby, further understandings on additional signaling pathways and activation effects implicated on extract properties, might provide novel insights into the mechanisms of immunomodulation and new opportunities for *A. colubrina* rational application.

Competing Interests

The authors have declared that no competing interests exist.

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Author Contributions

Conceptualization: RMM, EMMBC, CMAM. Investigation and Data curation: CMAM, WCG, JPRS. Formal analysis: RMM, EMMBC, CMAM, WCG, JPRS, JTF. Writing – original draft: CMAM. Writing – review and editing: CMAM, RMM, EMMBC.

References

1. Salaverry LS, Parrado AC, Mangone FM, Dobrecky CB, Flor AS, Lombardo T, et al. In vitro anti-inflammatory properties of Smilax campestris aqueous extract in human macrophages, and characterization of its flavonoid profile. J Ethnopharmacol. 2019; 247:112282. https://doi.org/10.1016/j.jep.2019.112282
2. Adegbaju OD, Otunola GA, Afolayan AJ. Anti-inflammatory and cytotoxic evaluation of extracts from the flowering stage of Celosia argentea. BMC Complement Med Ther. 2020; 20:152. DOI: https://doi.org/10.1186/s12906-020-02941-4
3. Kim JH, Yi YS, Kim MY, Cho JY. Role of ginsenosides, the main active components of Panax ginseng, in inflammatory responses and diseases. J. Ginseng Res. 2017; 41(4):435–443. https://doi.org/10.1016/j.jgr.2016.08.004.
4. Jang J, Lee JS. Jang Y, Choung ES, Li WL, Lee SW, et al.. Sorbaria kirilowii Ethanol Extract Exerts Anti-Inflammatory Effects *In Vitro* and *In Vivo* by Targeting Src/Nuclear Factor (NF)-κB. Biomolecules. 2020; 10(5):741. https://doi.org/10.3390/biom10050741
5. Oguntibeju OO. Type 2 diabetes mellitus, oxidative stress and 82yceum82flamm: examining the links. Int J Physiol Pathophysiol Pharmacol. 2019; 11(3):45-63.
6. Hamidzadeh K, Christensen SM, Dalby E, Chandrasekaran P, Mosser DM. Macrophages and the recovery from acute and chronic 82yceum82flamm. Annu Rev Physiol. 2017; 79:567–592. https://doi.org/10.1146/annurev-physiol-022516-034348
7. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010; 140(6):805–820. https://doi.org/10.1016/j.cell.2010.01.022

8. Kim H, Shin KK, Kim HG, Jo M, Kim JK, Lee JS, et al. Src/NF-κB-Targeted Anti-Inflammatory Effects of Potentilla glabra var. Mandshurica (Maxim.) Hand.-Mazz. Ethanol Extract. Biomolecules. 2020; 10(4):648. Ttps://doi.org/10.3390/biom10040648
9. Li S, Guo Q, Li S, Zheng H, Chi S, Xu Z, et al. Glutamine protects against LPSinduced inflammation via adjusted NODs signaling and enhanced immunoglobulins secretion in rainbow trout leukocytes. Dev Comp Immunol. 2019; 98:148–156. https://doi.org/10.1016/j.dci.2019.05.006
10. Davino-Chiovatto JE, Oliveira-Junior MC, MacKenzie B, Santos-Dias A, Almeida-Oliveira AR, Aquino-Junior JCJ, et al. Montelukast, leukotriene inhibitor, reduces LPS-induced acute lung inflammation and human neutrophil activation. Arch Bronconeumol. 2019; 55(11):573–580. https://doi.org/10.1016/j.arbres.2019.05.003
11. Yin P, Zhang Z, Li J, Shi Y, Jun N, Zou W, et al. Ferulic acid inhibits bovine endometrial epithelial cells against LPS-induced inflammation via suppressing NK-jB and MAPK pathway. ResVet Sci. 2019; 126:164–169. https://doi.org/10.1016/j.rvsc.2019.08.018
12. Yim M, Lee JM, Choi G, Lee D, Park WS, Jung W, et al. Anti-Inflammatory Potential of Carpomitra costata Ethanolic Extracts via Inhibition of NF-kB and AP-1 Activation in LPS-Stimulated RAW264.7 Macrophages. Evid Based Complement Alternat Med. 2018; 26,2018:6914514. https://doi.org/10.1155/2018/6914514
13. Elmarakby AA, Ibrahim AS, Katary MA, Elsherbiny NM, El-Shafey M, Abd-Elrazik AM, et al. A dual role of 12/15-lipoxygenase in LPS-induced acute renal inflammation and injury. Biochim Biophys Acta Mol Cell Biol Lipids. 2019; 1864(11):1669–1680. https://doi.org/10.1016/j.bbalip.2019.07.009
14. Zhu Z, Xueying L, Chunlin L, Wen X, Rongrong Z, Jing H, et al. Effect of berberine on LPS-induced expression of NF-kB/MAPK signalling pathway and related inflammatory cytokines in porcine intestinal epithelial cells.. Innate Immun. 2020; 1753425920930074. https://doi.org/10.1177/1753425920930074
15. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. Oncotarget. 2018; 9(6):7204–18. https://doi.org/10.18632/oncotarget.23208
16. Sugimoto MA, Sousa LP, Pinho V, Perretti M, Teixeira MM. Resolution of Inflammation: What Controls Its Onset? Front. Immunol. 2016;7:160. https://doi.org/10.3389/fimmu.2016.00160
17. Sadhegi A, Bastin AR, Ghahremani H, Doustimotlagh AH. The 83yceum83 of rosmarinic acid on oxidative stress parameters and 83yceum83flamm cytokines in ipopolysaccharide-induced

peripheral blood mononuclear cells. Mol Biol Rep. 2020; 47:3557-3566. https://doi.org/10.1007/s11033-020-05447-x

18. Weber CR, Soares CM, Lopes AB, Silva TS, Nascimento MS, Ximenes EC. Anadenanthera colubrina: a therapeutic potential study. As Bras Farm. 2011; 92, 235-244.
19. Araújo DRC, Silva LCN, Silva AG, Araújo JM, Macêdo AJ, Correia MTS, et al. Comparative analysis of anti-Staphylococcus aureus action of leaves and fruits of Anadenanthera colubrina var. cebil (Griseb.) Altschul. Afr J Microbiol Res. 2014; 8:2690-2696. https://doi.org/10.5897/AJMR2014.6901
20. Araújo TAS, Castro VTNA, Solon LGS, Silva GA, Almeida MG, Costa JGM, et al. Does rainfall affect the antioxidant capacity and production of phenolic compounds of an important medicinal species? Ind Crop Prod. 2015; 76:550-556. https://doi.org/10.1016/j.indcrop.2015.07.008
21. Lima RF, Alves EP, Rosalen PL, Ruiz ALT, Duarte MCT, Góes VFF, et al. Antimicrobial and Antiproliferative Potential of Anadenanthera colubrina (Vell.) Brenan. Evid Based Complement Alternat Med. 2014; 2014:802696. https://doi.org/10.1155/2014/802696
22. Silva DR, Rosalen PL, Freires IA, Sardi JCO, Lima RF, Lazarini JG, et al. Anadenanthera Colubrina vell Brenan: anti-Candida and antibiofilm activities, toxicity and therapeutical action. Braz Oral Res. 2019; 33:e023. https://doi.org/10.1590/1807-3107bor-2019.vol33.0023.
23. Guarneire GJ, Cardoso Junior O, Lima ,NM, Santos EA, Schulz CA, Silva WP, et al. Effect of Anadenanthera colubrina protease inhibitors a Paulinos an inflammatory mediator. Nat Prod Res. 2019; 1-6. https://doi.org/10.1080/14786419.2019.1624962
24. Cardoso Junior O, Lima NM, Silva MGA, Aguiar VB, Carli GP, Scherrer E, et al. In vitro and in vivo evaluation of anti-inflammatory activity and free radical scavenging potential of leaves extract from Anadenanthera colubrina. Nat Prod Res. 2020; 1-5. https://doi.org/10.1080/14786419.2020.1727472
25. Araújo DRC, Silva TD, Harand W, Lima CSA, Ferreira Neto JP, Ramos BA, et al. Bioguided Purification of Active Compounds from Leaves of Anadenanthera colubrina var. cebil (Griseb.) Altschul. Biomolecules. 2019; 9(10):590. https://doi.org/10.3390/biom9100590
26. Lee HN, Shin SA, Choo GS, Kim HJ, Park YS, Kim BS, et al. Anti-inflammatory effect of quercetin and galangin in LPS-stimulated RAW264.7 macrophages and DNCB-induced atopic dermatitis animal models. Int J Mol Med. 2018; 41(2):888–898. https://doi.org/10.3892/ijmm.2017.3296

27. Chen GL, Fan MX, Wu JL, Li N, Guo MQ. Antioxidant and anti-inflammatory properties of flavonoids from lotus plumule. Food Chem. 2019; 277:706-712. https://doi.org/10.1016/j.foodchem.2018.11.040
28. Maleki SJ, Crespo JF, Cabanillas B. Anti-inflammatory effects of flavonoids. Food Chem. 2019; 299:125124. https://doi.org/10.1016/j.foodchem.2019.125124
29. Carvalho DDC, Alves E, Camargos RB, Oliveira DF, Scolforo JRS, Carvalho DA, et al. Plant extracts to control Alternaria alternata in Murcott tangor fruits. Rev Iberoame Micol. 2011. 28:173-178. https://doi.org/10.1016/j.riam.2011.05.001
30. O'Brien. J, Wilson I, Orton T, Pognan F. Investigation of the Alamar Blue (resazurin) fluorescentdye for the assessment of mammalian cell cytotoxicity. Eur J Biochem. 2000; 267:5421–5426. https://doi.org/10.1046/j.1432-1327.2000.01606.x
31. Wei W, Xiao H, Bao W, Ma D, Leung C, Han X, et al. TLR-4 may mediate signaling pathways of Astragalus polysaccharide RAP induced cytokine expression of RAW264.7 cells. J Ethnopharmacol. 2016; 179:243-252. https://doi.org/10.1016/j.jep.2015.12.060
32. Seleem D, Chen E, Benso B, Pardi V, Murata RM. In vitro evaluation of antifungal activity of monolaurin against Candida albicans biofilms. Peer J. 2016; 4:e2148. https://doi.org/10.7717/peerj.2148
33. Chen E, Benso B, Seleem D, Nunes LE, Pasetto S, Pardi V, et al. Fungal-Host Interaction: Curcumin Modulates Proteolytic Enzyme Activity of Candida albicans and Inflammatory Host Response In Vitro. Int J Dent. 2017; 2018:2393146. https://doi.org/10.1155/2018/2393146
34. Allin N, Cruz-Almeida Y, Velsko I, Vovk A, Hovemcamp N, Harrison P, et al. Inflammatory Response Influences Treatment of Localized Aggressive Periodontitis. J Dent Res. 2016; 95(6):635-641. https://doi.org/10.1177/0022034516631973
35. Misiewicz-Krzeminska I, Corchete LA, Rojas EA, Martínez-López J, García-Sanz R, Oriol A, et al. A novel nano-immunoassay method for quantification of proteins from CD138-purified myeloma cells: biological and clinical utility. Haematologica. 2018; 103(5):880-889. https://doi.org/10.3324/haematol.2017.181628.
36. Petovari G, Danko T, Krencz I, Hujber Z, Rajani H, Vetlenyi E, et al. Inhibition of Metabolic Shift can Decrease Therapy Resistance in Human High-Grade Glioma Cells. Pathol Oncol Res. 2020; 26(1):23-33. https://doi.org/10.1007/s12253-019-00677-2
37. Santos JS, Marinho RR, Ekundi-Valentim E, Rodrigues L, Yamamoto MH, Teixeira AS, et al. Beneficial effects of Anadenanthera colubrina (Vell.) Brenan extract on the inflammatory and nociceptive responses in rodent models. J Ethnopharmacol. 2013; 148(1):218–222. https://doi.org/10.1016/j.jep.2013.04.012

38. Sullivan AC, Pangloli P, Dia VP. Kafirin from Sorghum bicolor inhibition of inflammation in THP-1 human macrophages is associated with reduction of intracellular reactive oxygen species. Food Chem Toxicol. 2018; 111:503–510. https://doi.org/10.1016/j.fct.2017.12.002
39. Albrahim T, Alnasser MM, Al-Anazi MR, ALKahtani MD, Alkahtani S, Al-Qahtani AA. Potential anti-inflammatory and anti-apoptotic effect of Coccinia grandis plant extract in LPS stimulated-THP-1 cells. Environ Sci Pollut Res Int. 2020; 27(17):21892-21904. https://doi.org/10.1007/s11356-020-08445-5
40. Huang X, Huang Q, He Y, Chen S, Li T. Mycophenolic acid enhanced lipopolysaccharide-induced interleukin-18 release in THP-1 cells via activation of the NLRP3 inflammasome. Immunopharmacol Immunotoxicol. 2019; 41(5):521-526. https://doi.org/10.1080/08923973.2019.1652913
41. Liu Y, He H, Fan L, Yuan J, Huang H, Yang W, et al. Compound C attenuates NLRP3 inflammasome despite AMPK knockdown in LPS plus palmitate-induced THP-1 cells. Naunyn Schmiedebergs Arch Pharmacol. 2020; 393(10:67-76. https://doi.org/10.1007/s00210-019-01712-4
42. Sun L, Li X, Xu M, Yang M, Yang F, Wang W, et al. In vitro immunomodulation of magnesium on monocytic cell toward anti-inflammatory macrophages. Regen Biomater. 2020; 7(4):391-401. https://doi.org/10.1093/rb/rbaa010
43. Cheeran V, Munuswamy-Ramanujam G. Sesquiterpene lactone Zaluzanin D alters MMP-9 promoter methylation in differentiated THP-1 monocytic cells and down regulates inflammatory cytokines IL-1β and TNF-α. Int Immunopharmacol. 2020; 87:106803. https://doi.org/10.1016/j.intimp.2020.106803
44. Gusman GS, Campana PRV, Castro L, Castilho RO, Teixeira M, Braga FC. Evaluation of the Effects of Some Brazilian Medicinal Plants on the Production of TNF- α and CCL2 by THP-1 Cells. Evid Based Complement Alternat Med. 2015; 2015(1):497123. https://doi.org/10.1155/2015/497123
45. Schink A, Neumann J, Leifke AL, Ziegler K, Froehlich-Nowoisky J, Cremer C, et al. Screening of herbal extracts for TLR2-and TLR4-dependent anti-inflammatory effects. Plos One. 2018; 13(10):e0203907. https://doi.org/10.1371/journal.pone.0203907
46. Zhang G, Gao X, Zeng H, Li Y, Guo X. Virosecurinine induces apoptosis in human leukemia THP-1 cells and other underlying molecular mechanisms. Oncol Lett 2018; 15(1):849–854. https://doi.org/10.3892/ol.2017.7437

47. Calabrese EJ, Agathokleous E, Kapoor R, Kozumbo WJ, Rattan SIS. Re-analysis of herbal extract data reveals that inflammatory processes are mediated by 87yceum87f mechanisms. Chem Biol Interact. 2019; 314:108844. https://doi.org/10.1016/j.cbi.2019.108844.
48. Park HS, Vick EJ, Gao Y, He C, Almosnid NM, Farone M, et al. Cis- and trans-gnetin H from Paeonia suffruticosa suppress inhibitor kappa B kinase phosphorylation in LPS-stimulated human THP-1 cells. J Ethnopharmacol 2016; 189:202–209. https://doi.org/10.1016/j.jep.2016.05.042
49. Lee JO, Choi E, Shin KK, Hong YH, Kim HG, Jeong D, et al. Compound K, a ginsenoside metabolite, plays an 87yceum87flammatory role in macrophages by targeting the AKT1-mediated signaling pathway. J Ginseng Res. 2019; 43(1):154–160. https://doi.org/10.1016/j.jgr.2018.10.003
50. Elramakby AA, Ibrahim AS, Katary MA, Elsherbiny NM, El-Shafey M, Abd-Elrazik AM, et al. A dual role of 12/15-lipoxygenase in LPS-induced acute renal inflammation and injury. Biochim Biophys Acta Mol Cell Biol Lipids. 2019; 1864(11):1669–1680. https://doi.org/10.1016/j.bbalip.2019.07.009
51. Mantovani A, Dinarello CA, Molgora M, Garlanda C. Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity. Immunity. 2019; 50(4):778–795. https://doi.org/10.1016/j.immuni.2019.03.012
52. Hara K, Shirasuna K, Usui F, Karasawa T, Mizushina Y, Kimura H, et al. Interferon-tau attenuates uptake of nanoparticles and secretion of interleukin-1β in macrophages. PloS One. 2014; 9(12):e113974. https://doi.org/10.1371/journal.pone.0113974
53. Yamazaki T, Ohshio K, Sugamata M, Morita Y. Lactic acid bacterium, Lactobacillus paracasei KW3110, suppresses inflammatory stressinduced caspase-1 activation by promoting interleukin-10 production in mouse and human immune cells. Plos One. 2020; 15(8):e0237754. https://doi.org/10.1371/journal.pone.0237754
54. Jang H, Kim K, Park E, Kang JA, Yun B, Lee S, et al. Anti-Inflammatory Activity of Diterpenoids from Celastrus orbiculatus in Lipopolysaccharide-Stimulated RAW264.7 Cells. J Immunol Res. 2020; 2020:7207354. https://doi.org/10.1155/2020/7207354
55. Cui L, Wang H, Lin J, Wang Y, Dong J, Li J, et al. Progesterone inhibits inflammatory response in E. coli- or LPS-stimulated bovine endometrial epithelial cells by NF-kB and MAPK pathways. Dev Comp Immunol. 2020; 105:103568. https://doi.org/10.1016/j.dci.2019.103568
56. Li C, Yu J, Ai K, Li H, Zhang Y, Zhao T, et al. Iκβα phosphorylation and associated NF-κB activation are essential events in lymphocyte activation, proliferation, and anti-bacterial

adaptive immune response of Nile tilapia. Dev Comp Immunol. 2020; 103:103526. https://doi.org/10.1016/j.dci.2019.103526

57. Liu T, Zhang L, Joo D, Sun S. NF-κB signaling in inflammation. Signal Transduct Target Ther. 2017; 2:17023. https://doi.org/10.1038/sigtrans.2017.23
58. Hsieh YH, Deng JS, Pan HP, Liao JC, Huang SS, Huang GJ. Sclareol ameliorate lipopolysaccharide-induced acute lung injury through inhibition of MAPK and induction of HO-1 signaling. Int Immunopharmacol. 2017; 44:16–25. https://doi.org/10.1016/j.intimp.2016.12.026
59. Afonina IS, Zhong Z, Karin M, Beyaert R. Limiting inflammation – The negative regulation of NF-κB and the NLRP3 inflammasome. Nat. Immunol. 2017; 18(8):861-869. https://doi.org/10.1038/ni.3772
60. Govindaraj J, Pillai SS. Rosmarinic acid modulates the antioxidant status and protects pancreatic tissues from glucolipotoxicity mediated oxidative stress in high-fat diet: streptozotocininduced diabetic rats. Mol Cell Biochem. 2015; 404(1–2):143–159. https://doi.org/10.1007/s11010-015-2374-6
61. Chen, L., Teng, H., Jia, Z., Battino, M., Miron, A., Yu, Z, et al. Intracellular signaling pathways of inflammation modulated by dietary flavonoids: The most recent evidence. Critical Reviews in Food Science and Nutrition. 2017; 1–17. https://doi.org/10.1080/10408398.2017.1345853
62. Sharma A, Sharma R, Kumar D, Padwad Y. Berberis 88yceum Royle fruit extract mitigates oxi-inflammatory stress by suppressing NF-κB/MAPK signalling cascade in activated macrophages and Treg proliferation in splenic lymphocytes. Inflammopharmacology. 2020; 28(4):1053-1072. https://doi.org/10.1007/s10787-018-0548-z.
63. Ji R, Sun H, Peng J, Ma X, Bao L, Fu Y, et al. Rosmarinic acid exerts an antagonistic effect on vascular calcification by regulating the Nrf2 signalling pathway. Free Radic Res. 2019; 53(2):187–197. https://doi.org/10.1080/10715762.2018.1558447
64. Lin W, Wang W, Wang D, Ling W. Quercetin protects against atherosclerosis by inhibiting dendritic cell activation. Mol Nutr Food Res. 2017; 61(9). https://doi.org/10.1002/mnfr.201700031
65. Balkrishna A, Ranjan R, Sakat SS, Sharma VK, Shukla R, Joshi K, et al. Evaluation of polyherbal ayurvedic formulation 'Peedantak Vati' for anti-inflammatory and analgesic properties. J. Ethnopharmacol. 2019; 235:361-374. https://doi.org/10.1016/j.jep.2019.01.028

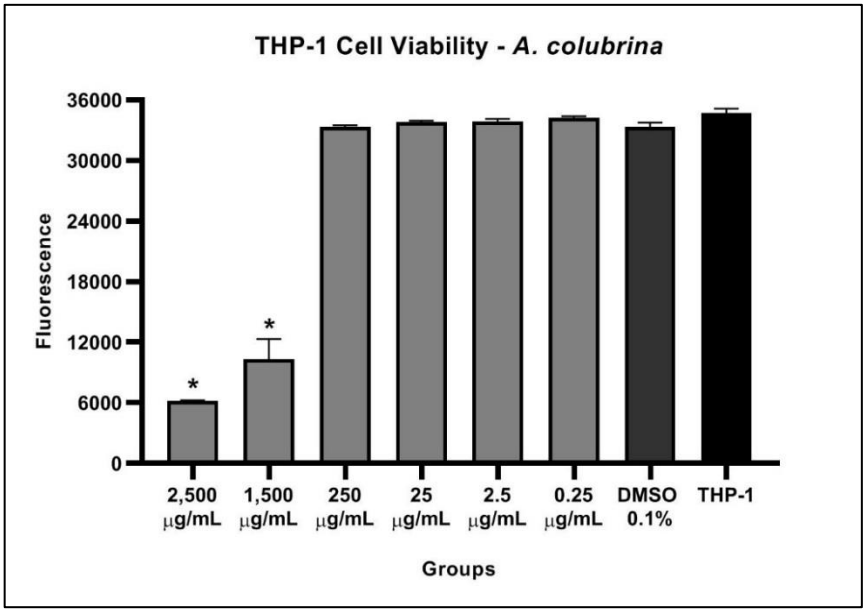

Fig. 1. Cytotoxicity effect of *A. colubrina* extract (2,500 µg/mL – 0.25 µg/mL) on THP-1 monocytes after 24 hours of treatment (LD_{50} = 978.7 µg/mL). THP-1: only cells; DMSO 0.1%: vehicle control. Values shown with asterisk (*) are statistically significant when compared to the vehicle control ($p < 0.05$).

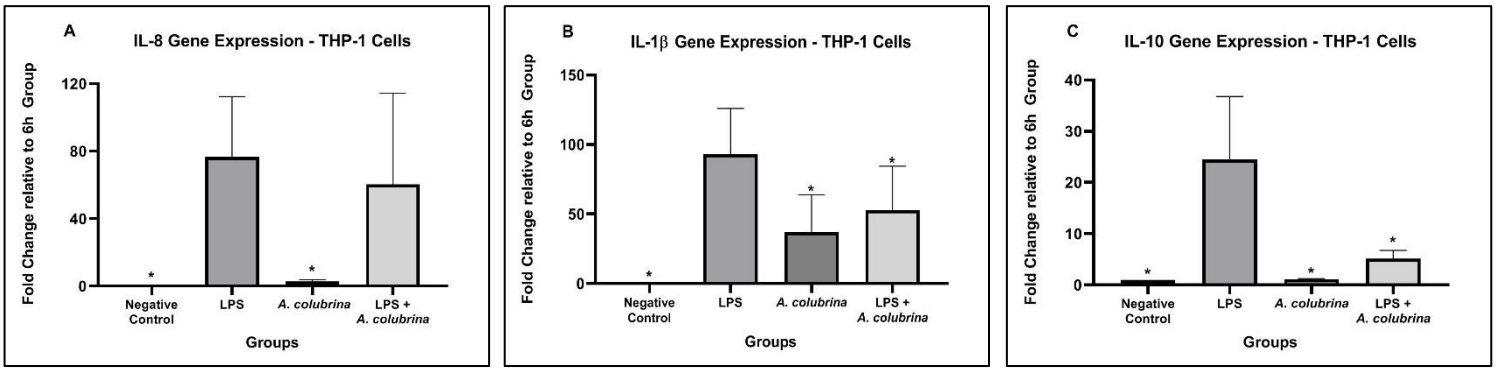

Fig. 2. Real-time quantitative information about relative gene expression of **A**) IL-8, **B**) IL-1β and **C**) IL-10 of THP-1 cells after 6 hours of treatment with *A. colubrina* extract (250 µg/mL) and stimulation by LPS (100 ng/mL). Values are shown as the fold-change relative to the negative control group (6 hours). Values shown with asterisk (*) are statistically significant when compared to the negative control ($p < 0.05$).

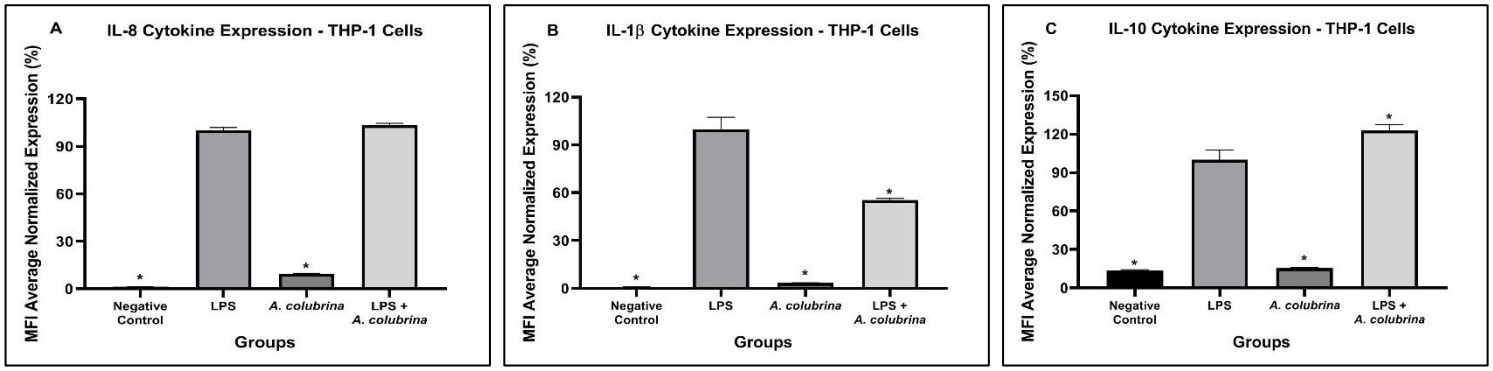

Fig. 3. Pro and anti-inflammatory cytokines expression of **A**) IL-8, **B**) IL-1β and **C**) IL-10 by THP-1 cells after 6 hours of treatment with *A. colubrina* extract (250 µg/mL). Values shown with asterisk (*) are statistically significant when compared to the LPS-induced cells group ($p < 0.05$).

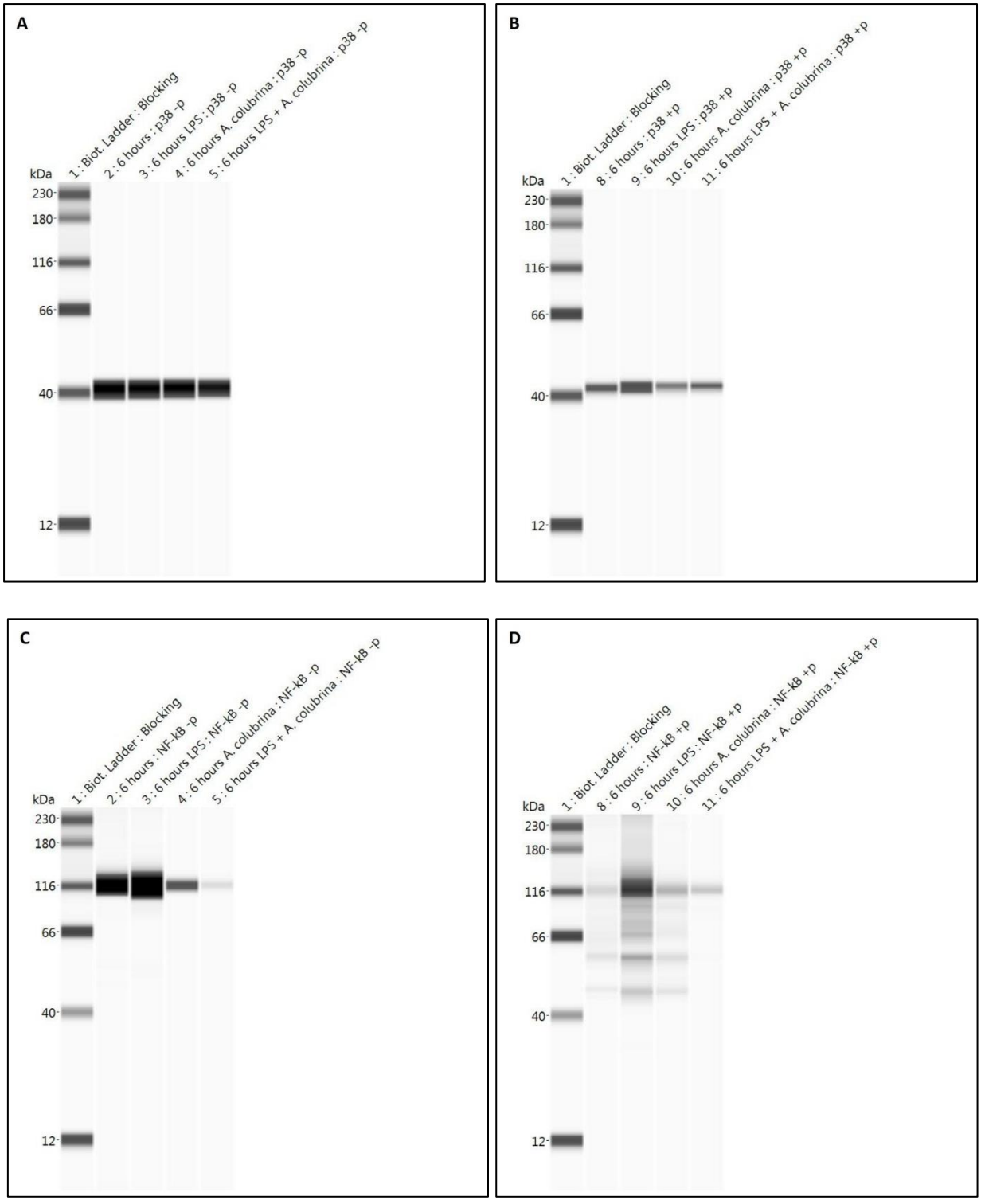

Fig. 4. Western blot analysis of **A**) NF-κB, **B**) p-NF-κB, **C**) p-38 and **D**) p-p38 for THP-1 cells treated for 6 hours with *A. colubrina* extract (250 µg/mL) and stimulated by LPS (100 ng/mL).

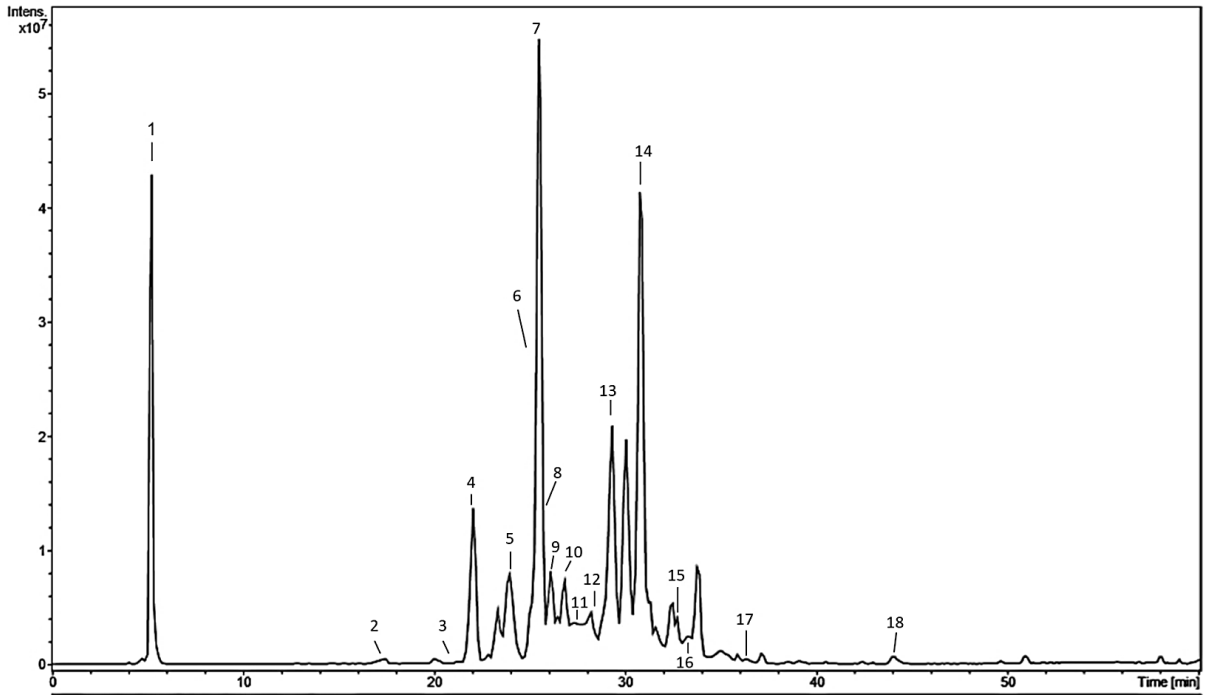

Fig. 5. Base peak chromatogram of *A. colubrina* extract.

5 CONSIDERAÇÕES FINAIS

Este é o primeiro trabalho avaliando *in vitro* os efeitos do extrato das cascas da *Anadenanthera colubrina* (Vell.) Brenan sobre a interação entre *C. albicans*-hospedeiro e sobre a regulação da atividade imunomoduladora, através de uma abordagem a nível transcricional e proteômico.

O extrato da *A. colubrina* demonstrou atividade anti-*Candida* e efeitos inibitórios sobre biofilme formado por *C. albicans*, bem como sobre a liberação extracelular de enzimas proteolíticas associadas à destruição e invasão tecidual pela levedura. Em modelo de cocultura, reduziu significativamente a distribuição no crescimento de *C. albicans*, com relativa baixa citotoxicidade às células do hospedeiro, apresentando efeito modulador sobre a resposta imune do hospedeiro através da regulação da liberação das citocinas pró-inflamatórias IL-6 e IL-8.

Além destes resultados, o extrato de *A. colubrina* apresentou atividade anti-inflamatória, indicada pela modulação da liberação das citocinas pró-inflamatória IL-1β e anti-inflamatória IL-10, por meio da regulação intracelular das vias de transdução sinais NF-κB e p38-MAPK. A caracterização fitoquímica do extrato das cascas de *A. colubrina* revelou a presença de um perfil fenólico, composto predominantemente por flavonoides, procianidinas e taninos, justificando as propriedades biológicas do extrato observadas neste estudo.

Diante dos resultados encontrados, sugere-se a realização de estudos adicionais a respeito da regulação da *A. colubrina* sobre outros fatores de virulência associados à patogenicidade da *C. albicans*, como a expressão de adesinas de superfície, a degradação de moléculas do sistema imune do hospedeiro e os mecanismos de destruição e invasão tecidual, de forma a estabelecer e reforçar os efeitos do extrato a nível molecular na progressão da patogênese da *C. albicans*. Além disso, avaliações futuras sobre a participação do extrato nos mecanismos de ativação de vias adicionais de sinalização associadas à reposta imune, bem como em outros modelos de inflamação, podem fornecer novas informações sobre o efeito imunomodulador *in vitro* da *A. colubrina*.

Em termos ecológicos representa a possibilidade de um manejo conservacionista para a espécie. Economicamente representa uma nova fonte de renda para as populações tradicionais que sobrevivem na região semiárida do Brasil e socialmente garante melhoria da qualidade de vida viabilizando a sobrevivência a partir dos recursos naturais do seu próprio ambiente.

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REFERÊNCIAS

ALGHAMDI, S.; ATEF SHEBL, N.; ASLANPOUR, Z. SHIBL, A.; BERROU, I. Hospital Adoption of Antimicrobial Stewardship Programmes in Gulf Cooperation Council Countries: A Review of Existing Evidence. *J Glob Antimicrob Resist*, V. 15, P. 196-209, 2018.

ALLIN, N.; CRUZ-ALMEIDA, Y.; VELSKO, I.; VOVK, A.; HOVEMCAMP, N.; HARRISON, P.; et al. Inflammatory Response Influences Treatment of Localized Aggressive Periodontitis. *J Dent Res*, v. 95, n. 6, p. 635-641, 2016.

ARAÚJO, D. R. C.; SILVA, L. C. N.; SILVA, A. G.; ARAÚJO, J. M.; MACÊDO, A. J.; CORREIA, M. T. S, et al. Comparative analysis of anti-*Staphylococcus aureus* action of leaves and fruits of *Anadenanthera colubrina* var. cebil (Griseb.) Altschul. *Afr J Microbiol Res*, v. 8, p. 690-2696, 2014.

ARAÚJO, D. R. C.; SILVA, T. D.; HARAND, W.; LIMA, C. S. A.; FERREIRA NETO, J. P.; RAMOS, B. A.; et al. Bioguided Purification of Active Compounds from Leaves of *Anadenanthera colubrina* var. cebil (Griseb.) Altschul. *Biomolecules*, v. 9, n. 10, p. 590, 2019.

ARAÚJO, T. A. S.; CASTRO, V. T. N. A.; SOLON, L. G. S.; SILVA, G. A.; ALMEIDA, M. G.; COSTA, J. G. M.; et al. Does rainfall affect the antioxidant capacity and production of phenolic compounds of an important medicinal species? *Ind Crop Prod*, v. 76, p. 550-556, 2015.

BEN-AMI, R.; ZIMMERMAN, O.; FINN, T.; AMIT, S.; NOVIKOV, A.; WERTHERIMER, N.; et al. Heteroresistance to fluconazole is a continuously distributed phenotype among candida glabrata clinical strains associated with *in vivo* persistence. *MBio*, v. 7, n. 4, 2016.

BERSAN, S. M. F.; GALVÃO, L. C. C.; GÓES, V. F. F.; SARTORATTO, A.; FIGUEIRA, G. M.; REHDER, V. L. G.; et al. Action of essential oils from Brazilian native and exotic medicinal species on oral biofilms. *BMC Complement Altern Med*, v. 14, p. 151, 2014.

BHATTACHARYA, S.; SAE-TIA, S.; FRIES, B. C. Candidiasis and Mechanisms of Antifungal Resistance. *Antibiotics (Basel)*, v. 9, n. 6, p. 312, 2020.

BONGOMIN, F.; GAGO, S.; OLADELE, R.O; DENNING, D.W. Global and Multi-National Prevalence of Fungal Diseases - Estimate Precision. *J. Fungi*, v. 3, p. 57, 2017.

BROWN, G. D.; DENNING, D. W.; LEVITZ, S. M. Tackling human fungal infections. *Science*, v. 336, n. 6082, p. 647, 2012.

CARDOSO JUNIOR, O.; LIMA, N. M.; SILVA, M. G. A.; AGUIAR, V. B.; CARLI, G. P.; SCHERRER, E.; et al. *In vitro* and *in vivo* evaluation of anti-inflammatory activity and free radical scavenging potential of leaves extract from *Anadenanthera colubrina*. *Nat Prod Res*, v. 1-5, 2020.

CARVALHO, D. D. C.; ALVES, E.; CAMARGOS, R. B.; OLIVEIRA, D. F.; SCOLFORO, J. R. S.; CARVALHO, D. A.; et al. Plant extracts to control *Alternaria alternata* in Murcott tangor fruits. *Rev Iberoame Micol*. v. 28, p. 173-178, 2011.

CHEN, E.; BENSO, B.; SELEEM, D.; NUNES, L. E.; PASETTO, S.; PARDI, V.; et al. Fungal-Host Interaction: Curcumin Modulates Proteolytic Enzyme Activity of *Candida albicans* and Inflammatory Host Response *In Vitro. Int J Dent*, v. 2018, n. 2393146, 2018.

CHEN, G. L.; FAN, M. X.; WU, J. L.; LI, N.; GUO, M. Q. Antioxidant and anti-inflammatory properties of flavonoids from lotus plumule. *Food Chem*, v. 277, p. 706-712, 2019.

DELALOYE, J.; CALANDRA, T. Invasive candidiasis as a cause of sepsis in the critically ill patient. *Virulence*, v. 5, n. 1, p. 161-169, 2014.

FOX, E.; BUI, C.; NETT, J.; HARTOONI, N.; MUI, M.; ANDES, D.; et al. An expanded regulatory network temporally controls *Candida albicans* biofilm formation. *Mol Microbiol*, v. 96, n, 6, 2015.

FRANCISCONI, R. S.; HUACHO, P. M. M.; TONON, C. C.; BORDINI, E. A. F.; CORREIA, M. F.; SARDI, J. C. O.; et al. Antibioflm effcacy of tea tree oil and of its main component terpinen-4-ol against *Candida albicans*. *Braz Oral Res*, v. 34, n. e050, 2020.

GHANNOUM, M.; ROILIDES, E.; KATRAGKOU, A.; PETRAITIS, V.; WALSH, T. The role of echinocandins in *Candida* biofilm–related vascular catheter infections: In vitro and in vivo model systems. *Clin Infect Dis*, n. 6, p. S618–S621, 2015.

GONÇALVES, L. M.; DEL BEL CURY, A. A.; SARTORATTO, A.; REHDER, V. J. G; SILVA, W. J. Effects of Undecylenic Acid Released from Denture Liner on *Candida* Biofilms. *J Dent Res*, v. 91, n, 10, p. 985-989, 2012.

GUARNEIRE, G. J.; CARDOSO JUNIOR, O.; LIMA, N. M.; SANTOS, E. A.; SCHULZ, C. A.; SILVA, W. P.; et al. Effect of *Anadenanthera colubrina* protease inhibitors a Paulinos an inflammatory mediator. *Nat Prod Res*, v. 2019, p. 1-6, 2019.

HEBECKER, B.; NAGLIK, J. R.; HUBE. B.; JACOBSEN, I. D. Pathogenicity mechanisms and host response during oral *Candida albicans* infections. *Expert Rev Anti-Infect Ther*, v. 12, n. 7, p. 867–879, 2014.

HELLSTEIN, J. W.; MAREK, C. L. Candidiasis: Red and white manifestations in the oral cavity. *Head Neck Pathol*, v. 13, n. 1, p. 25–32, 2019.

HERTEL, M.; SCHMIDT-WESTHAUSEN, A. M.; STRIETZEL, F. P. Local, systemic, demographic, health-related factors influencing pathogenic yeast spectrum and antifungal drug administration frequency in oral candidiasis: a retrospective study. *Clin Oral Investig*, v. 20, p. 1477-1486, 2016.

HIROTA, K.; YUMOTO, H.; SAPAAR, B.; MATSUO, T.; ICHIKAWA, T.; MIYAKE, Y. Pathogenic factors in *Candida* biofilm-related infectious diseases. *J Appl Microbiol*, v. 122, n. 2, p. 321–330, 2017.

HÖFS, S.; MOGAVERO, S.; HUBE, B. Interaction of *Candida albicans* with host cells: Virulence factors, host defense, escape strategies and the microbiota. *J. Microbiol*, v. 54, n. 3, p. 149–169, 2016.

JABRA-RIZK, M. Pathogenesis of polymicrobial biofilms. *Open Mycol J*, v. 5, p. 39–43, 2011.

JANG, J.; LEE, J. S.; JANG, Y.; CHOUNG, E. S.; LI, W. L.; LEE, S. W.; et al. *Sorbaria kirilowii* Ethanol Extract Exerts Anti-Inflammatory Effects In Vitro and In Vivo by Targeting Src/Nuclear Factor (NF)-κB. *Biomolecules*, v. 10, n. 5, p. 741, 2020.

KIM, H.; SHIN, K. K.; KIM, H. G.; JO, M.; KIM, J. K.; LEE, J. S.; et al. Src/NF-κB-Targeted Anti-Inflammatory Effects of *Potentilla glabra* var. Mandshurica (Maxim.) Hand.-Mazz. Ethanol Extract. *Biomolecules*, v. 10, n. 4, p. 648, 2020.

LEE, H. N.; SHIN, S. A.; CHOO, G. S.; KIM, H. J.; PARK, Y. S.; KIM, B. S.; et al. Anti-inflammatory effect of quercetin and galangin in LPS-stimulated RAW264.7 macrophages and DNCB-induced atopic dermatitis animal models. *Int J Mol Med*, v. 41, n. 2, p. 888–898, 2018.

LEWIS, M. A. O.; WILLIAMS, D. W. Diagnosis and management of oral candidosis. *Br Dent J*, v. 223, n. 9, p. 675–681, 2017.

LIMA, R. F.; ALVES, E. P.; ROSALEN, P. L.; RUIZ, A. L. T.; DUARTE, M. C. T.; GÓES, V. F. F.; et al. Antimicrobial and Antiproliferative Potential of *Anadenanthera colubrina* (Vell.) Brenan. *Evid Based Complement Alternat Med*, v. 2014, n. 802696, 2014.

LUKASZUK, C.; KRAJEWSKA-KULAKA, E.; KULAK, W. Retrospective observation of drug susceptibility of *Candida* strains in the years 1999, 2004, and 2015. *Peer J*, v. 5, n. e3038, 2017.

MALEKI, S. J.; CRESPO, J. F.; CABANILLAS, B. Anti-inflammatory effects of flavonoids. *Food Chem*, v. 299, p. 125124, 2019.

MUSHI, M, F.; BADER, O.; TAVERNE-GHADWAL, L.; BII, C.; GROB, U.; MSHANA, S. E. Oral candidiasis among African human immunodeficiency virus-infected individuals: 10 years of systematic review and meta-analysis from sub-Saharan Africa. *J Oral Microbiol*, v. 9, n. 1, p. 1317579, 2017.

MILLSOP, J. W.; FAZE, L. N. Oral candidiasis. *Clin Dermatol*, v. 34, n. 4, p. 487–494, 2016.

MISIEWICZ-KRZEMINSKA, I.; CORCHETE, L. A.; ROJAS, E. A.; MARTÍNEZ-LÓPEZ, J.; GARCÍA-SANZ, R. ORIOL, A.; et al. A novel nano-immunoassay method for quantification of proteins from CD138-purified myeloma cells: biological and clinical utility. *Haematologica*, v. 103, n. 5, p. 880-889, 2018.

NAGLIK, J. R.; KÖNIG, A.; HUBE, B.; GAFFEN, S. L. *Candida albicans*-epithelial interactions and induction of mucosal innate immunity. *Curr Opin Microbiol*, v. 40, p. 104–112, 2017.

National Committee for Clinical Laboratory Standards (NCCLS). Reference method for broth dilution antifungal susceptibility testing of yeast fungi. Approved standard M38-A2. Wayne: National Committee for Clinical Laboratory Standards, 2002.

NETEA, M. G.; MARÓDI, L. Innate immune mechanisms for recognition and uptake of *andida* species. *Trends Immunol*, v. 31, n. 9, p. 346-353, 2010.

NEWMAN, D. J.; CRAGG, G. M. Natural Products as Sources of New Drugs from 191 to 2014. *J Nat Prod*, v. 79, p. 629-661, 2016.

NIKOU, S.; KICHIK, N.; BROWN, R.; PONDE, N. O.; HO, J.; NAGLIK, J. R.; et al. *Candida albicans* Interactions with Mucosal Surfaces during Health and Disease. *Pathogens*, v. 8, n. 2, p. 53, 2019.

O'BRIEN, J; WILSON, I.; ORTON, T.; POGNAN, F. Investigation of the Alamar Blue (resazurin) fluorescentdye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem*, v. 267, p.5421–5426, 2000.

PANDE, M.; DUBEY, V. K.; YADAV, S. C.; JAGANNADHAM, V. A Novel Serine Protease Cryptolepain from *Cryptolepis buchanani*: Purification and Biochemical Characterization. *J Agric Food Chem*, v. 54, n. 26, p. 10141-10150, 2006.

PAPPAS, P. G.; LIONAKIS, M. S.; ARENDRUP, M. C.; OSTROSKY-ZEICHNER, L.; KULLBERG, B. J. Invasive candidiasis. *Nat Rev Dis Primers*, v. 4, p. 18026, 2018.

PATIL, S.; MAJUMDAR, B.; SARODE, S.C.; SARODE, G.S.; AWAN, K.H. Oropharyngeal candidosis in HIV-infected patients-An update. *Front. Microbiol*, v. 9, p. 980, 2019.

PELLON, A.; NASAB, S. D. S.; MOYES, D. L. New Insights in *Candida albicans* Innate Immunity at the Mucosa: Toxins, Epithelium, Metabolism, and Beyond. *Front Cell Infect Microbiol*, v. 10, p. 81, 2020.

PETOVARI, G.; DANKO, T.; KRENCZ, I.; HUJBER, Z.; RAJANI, H.; VETLENYI, E.; et al. Inhibition of Metabolic Shift can Decrease Therapy Resistance in Human High-Grade Glioma Cells. *Pathol Oncol Res*, v. 26, n. 1, p. 23-33, 2020.

ROSA-GARCÍA, E.; OLADE-HERNÁNDEZ, M. J.; IRIGOYEN-CAMACHO, M. E.; MONDRAGÓN-PADILLA, A.; MENDOZA-JUACHE, A.; SÁNCHEZ-VARGAS, L. O. Antifungal susceptibility of oral isolates of *Candida* species from chronic kidney disease patients on chronic dialysis. *J Mycol Med*, v. 101009, 1-6, 2020.

SANGLARD, D. Emerging threats in antifungal-resistant fungal pathogens. *Front Med*, v. 3, n. 11, 2016.

SANTANA, I. L.; GONÇALVES, L. M.; VASCONCELLOS, A. A.; SILVA, W. J.; CURY, J. A.; DEL BEL CURY, A. A. Dietary Carbhydrates Modulate *Candida albicans* Biofilm Development on the Denture Surface. *Plos One*, v. 8, n. 5, p. e64645, 2013.

SELEEM, D.; CHEN, E.; BENSO, B.; PARDI, V.; MURATA, R. M. *In vitro* evaluation of antifungal activity of monolaurin against *Candida albicans* biofilms. *Peer J*, v.4, p. e2148, 2016.

SILVA, D. R.; ROSALEN, P. L.; FREIRES, I. A.; SARDI, J. C. O.; LIMA, R. F.; LAZARINI, J. G.; et al. *Anadenanthera Colubrina* vell Brenan: anti-*Candida* and antibiofilm activities, toxicity and therapeutical action. *Braz Oral Res*, v. 33, p, e023, 2019.

SORGO, A. G.; HEILMANN, C. J.; BRUL, S.; KOSTER, C. G.; KLIS, F. M. Beyond the wall: *Candida albicans* secret(e)s to survive. *FEMS Microbiol Lett*, v. 338, n. 1, p. 10–17, 2013.

SWIDERGALL, M.; FILLER, S. G. Oropharyngeal candidiasis: Fungal invasion and epithelial cell responses. *PLoS Pathog*, v. 13, n. 1, p. e1006056, 2017.

SWIDERGALL, M. *Candida albicans* at Host Barrier Sites: Pattern Recognition Receptors and Beyond. *Pathogens*, v. 8, n. 1, p. 40, 2019.

TAKEUCHI, O.; AKIRA, S. Pattern recognition receptors and inflammation. *Cell*, v. 140, n. 6, p. 805–820, 2010.

TANIGUCHI, L.; FARIA, B. F.; ROSA, R. T.; CARVALHO, A. P.; GURSKY, L. C.; ELIFIO-ESPOSITO, S. L.; et al. Proposal of a low-cost protocol for colorimetric semi-quantification of secretory phospholipase by *Candida albicans* grown in planktonic and biofilm phases. *J Microbiol Methods*, v. 78, n. 2, p. 171-174, 2009.

TOOYAMA, H.; MATSUMOTO, T.; HAYASHI, K.; KURASHINA, K.; KURITA, H.; UCHIDA, M.; et al. *Candida* concentrations determined following concentrated oral rinse culture reflect clinical oral signs. *BMC Oral Health*, v. 15, p. 150, 2015.

TSUI, C.; KONG, E. F.; JABRA-RIZK, M. A. Pathogenesis of *Candida albicans* Biofilm. *Pathog Dis*, v. 74, n. 4, 2016.

VAEZI, A.; FAKHIM, H.; KHODAVAISY, S.; ALIZADEH, A.; NAZERI, M.; SOLEIMANI, A.; et al. Epidemiological and mycological characteristics of candidemia in Iran: A systematic review and meta-analysis. *J Mycol Med*, v. 27, n. 2, p. 146–152, 2017.

VERMA, A. H.; RICHARDSON, J. P.; ZHOU, C.; COLEMAN, B. M.; MOYES, D. L.; HO, J.; et al. Oral epithelial cells orchestrate innate type 17 responses to *Candida albicans* through the virulence factor candidalysin. *Sci Immunol*, v. 2, n. 17, eaam8834, 2017.

VERMA, A. H.; ZAFAR, H.; PONDE, N. O.; HEPWORTH, O. W.; SIHRA, D.; AGGOR, F. E. Y.; et al. IL-36 and IL-1/IL-17 drive immunity to oral candidiasis via parallel mechanisms. *J Immunol*, v. 201, n. 2, p. 627–634, 2018.

VILA, T.; SULTAM, A. S.; MONTELONGO-JAUREGUI, D.; JABRA-RIZK, M. A. Oral Candidiasis: A Disease of Opportunity. *J Fungi (Basel)*, v. 6, n. 1, p. 15, 2020.

WALL, G.; MONTELONGO-JAUREGUI, D.; BONIFACIO, B. V.; LOPEZ-RIBOT, J. L.; UPPULURI, P. *Candida albicans* biofilm growth and dispersal: Contributions to pathogenesis. *Curr Opin Microbiol*, v. 52, p. 1–6, 2019.

WEBER, C. R.; SOARES. C. M.; LOPES, A. B.; SILVA, T. S.; NASCIMENTO, M. S.; XIMENES, E. C. *Anadenanthera colubrina*: a therapeutic potential study. *Rev Bras Farm*, v. 92, p. 235-244, 2011.

WEI, W.; XIAO, H.; BAO, W.; MA, D.; LEUNG, C.; HAN, X.; et al. TLR-4 may mediate signaling pathways of Astragalus polysaccharide RAP induced cytokine expression of RAW264.7 cells. *J Ethnopharmacol*, v. 179, p. 243-252, 2016.

WITHERDEN, E. A.; SHOAIE, S.; HALL, T. A.; MOYES, D. L. The Human Mucosal Mycobiome and Fungal Community Interactions. *J Fungi (Basel)*, v. 3, n. 4, p. 56, 2017.

WONG, S. S.; KAO, R. Y.; YUEN, K. Y.; WANG, Y.; YANG, D.; SAMARANAYAKE, L. P.; et al. *In vitro* and *in vivo* activity of a novel antifungal small molecule against *Candida* infections. *PLoS One*, v. 9, n. 1, e85836, 2014.

ZAIDI, K.U.; MANI, A.; THAWANI, V.; MEHRA, A. Total protein profile and drug resistance in *Candida albicans* isolated from clinical samples. *Mol Biol Int*, v. 2016, 4982131, 2016.

ANEXO A – Normas de publicação do periódico *Frontiers in Pharmacology*

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Autism spectrum disorders are a group of neurodevelopmental disorders that affect up to 1 in 100 individuals. People with autism display an array of symptoms encompassing emotional processing, sociability, perception and memory, and present as uniquely as the individual. No theory has suggested a single underlying neuropathology to account for these diverse symptoms. The Intense World Theory, proposed here, describes a unifying pathology producing the wide spectrum of manifestations observed in autists. This theory focuses on the neocortex, fundamental for higher cognitive functions, and the limbic system, key for processing emotions and social signals. Drawing

on discoveries in animal models and neuroimaging studies in individuals with autism, we propose how a combination of genetics, toxin exposure and/or environmental stress could produce hyper-reactivity and hyper-plasticity in the microcircuits involved with perception, attention, memory and emotionality. These hyper-functioning circuits will eventually come to dominate their neighbors, leading to hyper-sensitivity to incoming stimuli, over-specialization in tasks and a hyper-preference syndrome. We make the case that this theory of enhanced brain function in autism explains many of the varied past results and resolves conflicting findings and views and makes some testable experimental predictions.

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The text should be legible and of high quality. The smallest visible text should be no less than 8 points in height when viewed at actual size.

Solid lines should not be broken up. Any lines in the graphic should be no smaller than 2 points wide.

Please note that saving a figure directly as an image file (JPEG, TIF) can greatly affect the resolution of your image. To avoid this, one option is to export the file as PDF, then convert into TIFF or EPS using a graphics software.

Format and Color Image Mode

The following formats are accepted: TIF/TIFF (.tif/.tiff), JPEG (.jpg), and EPS (.eps) (upon acceptance).

Images must be submitted in the color mode RGB.

Chemical Structures

Chemical structures should be prepared using ChemDraw or a similar program. If working with ChemDraw please use our Frontiers ChemDraw template. If working with another program please follow the guidelines given below:

Drawing settings: chain angle, 120° bond spacing, 18% width; fixed length, 14.4 pt; bold width, 2.0 pt; line width, 0.6 pt; margin width, 1.6 pt; hash spacing, 2.5 pt. Scale 100% Atom Label settings: font, Arial; size, 8 pt.

Assign all chemical compounds a bold, Arabic numeral in the order in which the compounds are presented in the manuscript text.

Table Requirements and Style Guidelines

Tables should be inserted at the end of the manuscript in an editable format. If you use a word processor, build your table in Word. If you use a LaTeX processor, build your table in LaTeX. An empty line should be left before and after the table.

Table captions must be placed immediately before the table. Captions should be preceded by the appropriate label, for example "Table 1." Please use only a single paragraph for the caption.

Kindly ensure that each table is mentioned in the text and in numerical order.

Please note that large tables covering several pages cannot be included in the final PDF for formatting reasons. These tables will be published as supplementary material.

Please note that tables which are not according to the guidelines will cause substantial delay during the production process.

Accessibility

Frontiers encourages authors to make the figures and visual elements of their articles accessible for the visually impaired. An effective use of color can help people with low visual acuity, or color blindness, understand all the content of an article.

These guidelines are easy to implement and are in accordance with the W3C Web Content Accessibility Guidelines (WCAG 2.1), the standard for web accessibility best practices.

Ensure sufficient contrast between text and its background

People who have low visual acuity or color blindness could find it difficult to read text with low contrast background color. Try using colors that provide maximum contrast.

WC3 recommends the following contrast ratio levels:

Level AA, contrast ratio of at least 4.5:1

Level AAA, contrast ratio of at least 7:1

Level	AA
Contast ratio 4.6:1	

Level	AA
Contast ratio 9.5:1	

You can verify the contrast ratio of your palette with these online ratio checkers:

WebAIM

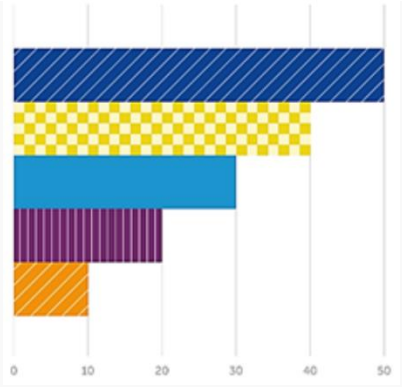
Color Safe

Avoid using red or green indicators

More than 99% of color-blind people have a red-green color vision deficiency.

Avoid using only color to communicate information

Elements with complex information like charts and graphs can be hard to read when only color is used to distinguish the data. Try to use other visual aspects to communicate information, such as shape, labels, and size. Incorporating patterns into the shape fills also make differences clearer; for an example please see below:



Supplementary Material

Data that are not of primary importance to the text, or which cannot be included in the article because they are too large or the current format does not permit it (such as videos, raw data traces, powerpoint presentations, etc.), can be uploaded as Supplementary Material during the submission procedure and will be displayed along with the published article. All supplementary files are deposited to Figshare for permanent storage and receive a DOI.

Supplementary Material is not typeset, so please ensure that all information is clearly presented without tracked changes/highlighted text/line numbers, and the appropriate caption is included in the file. To avoid discrepancies between the published article and the supplementary material, please do not add the title, author list, affiliations or correspondence in the supplementary files.

The Supplementary Material can be uploaded as Data Sheet (Word, Excel, CSV, CDX, FASTA, PDF or Zip files), Presentation (PowerPoint, PDF or Zip files), Image (CDX, EPS, JPEG, PDF, PNG or TIF/TIFF), Table (Word, Excel, CSV or PDF), Audio (MP3, WAV or WMA) or Video (AVI, DIVX, FLV, MOV, MP4, MPEG, MPG or WMV).

For Supplementary Material templates (LaTeX and Word), see our Supplementary Material templates.

References

All citations in the text, figures or tables must be in the reference list and vice-versa.

The names of the first six authors followed by et al. and the DOI (when available) should be provided.

The reference list should only include articles that are published or accepted.

Unpublished data, submitted manuscripts or personal communications should be cited within the text only, for the article types that allow such inclusions.

For accepted but unpublished works use "in press" instead of page numbers.

Data sets that have been deposited to an online repository should be included in the reference list. Include the version and unique identifier when available.

Personal communications should be documented by a letter of permission.

Website URLs should be included as footnotes.

Any inclusion of verbatim text must be contained in quotation marks and clearly reference the original source.

Preprints can be cited as long as a DOI or archive URL is available, and the citation clearly mentions that the contribution is a preprint. If a peer-reviewed journal publication for the same preprint exists, the official journal publication is the preferred source. See the Preprints section for more information.

Science, Engineering and Humanities Journals

In-text Citations

For works by a single author, include the surname, followed by the year.

For works by two authors, include both surnames, followed by the year.

For works by more than two authors, include only the surname of the first author followed by et al., followed by the year.

For Humanities and Social Sciences articles, include the page numbers.

Reference List

ARTICLE IN A PRINT JOURNAL

Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell. 5, 163-172.

ARTICLE IN AN ONLINE JOURNAL

Tahimic, C.G.T., Wang, Y., Bikle, D.D. (2013). Anabolic effects of IGF-1 signaling on the skeleton. Front. Endocrinol. 4:6. doi: 10.3389/fendo.2013.00006

ARTICLE OR CHAPTER IN A BOOK

Sorenson, P. W., and Caprio, J. C. (1998). "Chemoreception," in The Physiology of Fishes, ed. D. H. Evans (Boca Raton, FL: CRC Press), 375-405.

BOOK

Cowan, W. M., Jessell, T. M., and Zipursky, S. L. (1997). Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press.

ABSTRACT

Hendricks, J., Applebaum, R., and Kunkel, S. (2010). A world apart? Bridging the gap between theory and applied social gerontology. Gerontologist 50, 284-293. Abstract retrieved from Abstracts in Social Gerontology database. (Accession No. 50360869)

WEBSITE

World Health Organization. (2018). E. coli. https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

PATENT

Marshall, S. P. (2000). Method and apparatus for eye tracking and monitoring pupil dilation to evaluate cognitive activity. U.S. Patent No 6,090,051. Washington, DC: U.S. Patent and Trademark Office.

DATA

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular Tolls for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

THESES AND DISSERTATIONS

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master's thesis]. [Chicago (IL)]: University of Chicago

PREPRINT

Smith, J. (2008). Title of the document. Preprint repository name [Preprint]. Available at: https://persistent-url (Accessed March 15, 2018).

Resources

Chicago Manual of Style

Frontiers Science Endnote Style

Frontiers Science, Engineering and Humanities Bibstyle

Health, Physics, and Mathematics Journals

4.2.1.In-text Citations

Please apply the Vancouver system for in-text citations.

In-text citations should be numbered consecutively in order of appearance in the text—identified by Arabic numerals in the parenthesis for Health articles and in square brackets for Physics and Mathematics articles.

4.2.2. Reference List

ARTICLE IN A PRINT JOURNAL

Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. Mol Cell (2000) 5:163-72.

ARTICLE IN AN ONLINE JOURNAL

Tahimic CGT, Wang Y, Bikle DD. Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol (2013) 4:6. doi: 10.3389/fendo.2013.00006

ARTICLE OR CHAPTER IN A BOOK

Sorenson PW, Caprio JC. "Chemoreception,". In: Evans DH, editor. The Physiology of Fishes. Boca Raton, FL: CRC Press (1998). p. 375-405.

BOOK

Cowan WM, Jessell TM, Zipursky SL. Molecular and Cellular Approaches to Neural Development. New York: Oxford University Press (1997). 345 p.

ABSTRACT

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, editor. Genetic Programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3–5; Kinsdale, Ireland. Berlin: Springer (2002). p. 182–91.

WEBSITE

World Health Organization. E. coli (2018). https://www.who.int/news-room/fact-sheets/detail/e-coli [Accessed March 15, 2018].

PATENT

Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible Endoscopic Grasping and Cutting Device and Positioning Toll Assembly. United States patent US 20020103498 (2002).

DATA

Perdiguero P, Venturas M, Cervera MT, Gil L, Collada C. Data from: Massive sequencing of Ulms minor's transcriptome provides new molecular Tolls for a genus under the constant threat of Dutch elm disease. Dryad Digital Repository. (2015) http://dx.doi.org/10.5061/dryad.ps837

THESES AND DISSERTATIONS

Smith, J. (2008) Post-structuralist discourse relative to phenomological pursuits in the deconstructivist arena. [dissertation/master’s thesis]. [Chicago (IL)]: University of Chicago

PREPRINT

Smith, J. Title of the document. Preprint repository name [Preprint] (2008). Available at: https://persistent-url (Accessed March 15, 2018).

4.2.3. Resources

Citing Medicine

Frontiers Health Endnote Style

Frontiers Health and Physics Bibstyle

ANEXO B – Normas de publicação do periódico *PLoS One*

Submission Guidelines

Copyediting manuscripts

Prior to submission, authors who believe their manuscripts would benefit from professional editing are encouraged to use language-editing and copyediting services. Obtaining this service is the responsibility of the author, and should be done before initial submission. These services can be found on the web using search terms like "scientific editing service" or "manuscript editing service."

Submissions are not copyedited before publication. Submissions that do not meet the *PLOS ONE* publication criterion for language standards may be rejected.

Manuscript Organization

Manuscripts should be organized as follows. Instructions for each element appear below the list.

Beginning section	*The following elements are required, in order:* Title page: List title, authors, and affiliations as first page of manuscript Abstract Introduction
Middle section	*The following elements can be renamed as needed and presented in any order:* Materials and Methods Results Discussion Conclusions (optional)
Ending section	*The following elements are required, in order:* Acknowledgments References Supporting information captions (if applicable)
Other elements	• Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately. • Tables are inserted immediately after the first paragraph in which they are cited. • Supporting information files are uploaded separately.

Please refer to our downloadable sample files to ensure that your submission meets our formatting requirements:

- Download sample title, author list, and affiliations page (PDF)
- Download sample manuscript body (PDF)

Viewing Figures and Supporting Information in the compiled submission PDF

The compiled submission PDF includes low-resolution preview images of the figures after the reference list. The function of these previews is to allow you to download the entire submission as quickly as possible. Click the link at the top of each preview page to download a high-resolution version of each figure. Links to download Supporting Information files are also available after the reference list.

Parts of a Submission

Title

Include a full title and a short title for the manuscript.

Title	Length	Guidelines	Examples
Full title	250 characters	Specific, descriptive, concise, and comprehensible to readers outside the field	Impact of cigarette smoke exposure on innate immunity: A *Caenorhabditis elegans* model Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial
Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

Author list

Authorship requirements

All authors must meet the criteria for authorship as outlined in the authorship policy. Those who contributed to the work but do not meet the criteria for authorship can be mentioned in the Acknowledgments. Read more about Acknowledgments.

The corresponding author must provide an ORCID iD at the time of submission by entering it in the user profile in the submission system. Read more about ORCID.

Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

- First name (or initials, if used)
- Middle name (or initials, if used)
- Last name (surname, family name)

Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled "current address." At a minimum, the address must include the author's current institution, city, and country.

If an author has multiple affiliations, enter all affiliations on the title page only. In the submission system, enter only the preferred or primary affiliation. Author affiliations will be listed in the typeset PDF article in the same order that authors are listed in the submission.

Author names will be published exactly as they appear in the manuscript file. Please double-check the information carefully to make sure it is correct.

Corresponding author

The submitting author is automatically designated as the corresponding author in the submission system. The corresponding author is the primary contact for the journal office and the only author able to view or change the manuscript while it is under editorial consideration.

The corresponding author role may be transferred to another coauthor. However, note that transferring the corresponding author role also transfers access to the manuscript. (To designate a new corresponding author while the manuscript is still under consideration, watch the video tutorial below.)

Only one corresponding author can be designated in the submission system, but this does not restrict the number of corresponding authors that may be listed on the article in the event of publication. Whoever is designated as a corresponding author on the title page of the manuscript file will be listed as such upon publication. Include an email address for each corresponding author listed on the title page of the manuscript.

 **How to select a new corresponding author in Editorial Manager**

Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include its name in the manuscript byline. Do not add it to the author list in the submission system. You may include the full list of members in the Acknowledgments or in a supporting information file.

PubMed only indexes individual consortium or group author members listed in the article byline. If included, these individuals must qualify for authorship according to our criteria.

Read the group authorship policy.

Author contributions

Provide at minimum one contribution for each author in the submission system. Use the CRediT taxonomy to describe each contribution. Read the policy and the full list of roles.

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and we expect that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission.

Cover letter

Upload a cover letter as a separate file in the online system. The length limit is 1 page.

The cover letter should include the following information:

- Summarize the study's contribution to the scientific literature
- Relate the study to previously published work
- Specify the type of article (for example, research article, systematic review, meta-analysis, clinical trial)
- Describe any prior interactions with PLOS regarding the submitted manuscript
- Suggest appropriate Academic Editors to handle your manuscript (see the full list of Academic Editors)
- List any opposed reviewers

IMPORTANT: Do not include requests to reduce or waive publication fees in the cover letter. This information will be entered separately in the online submission system.

Read about publication fee assistance.

Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

 Download our sample title, author list, and affiliations page (PDF)

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

Protocol documents for clinical trials, observational studies, and other **non-laboratory** investigations may be uploaded as supporting information. We recommend depositing **laboratory protocols** at protocols.io. Read detailed instructions for depositing and sharing your laboratory protocols.

Human or animal subjects and/or tissue or field sampling

Methods sections describing research using human or animal subjects and/or tissue or field sampling must include required ethics statements. For details, consult the reporting guidelines for specific study types.

Data

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to "data not shown."

Cell lines

Methods sections describing research using cell lines must state the origin of the cell lines used. See the reporting guidelines for cell line research.

Laboratory protocols

To enhance the reproducibility of your results, we recommend and encourage you to deposit laboratory protocols in protocols.io, where protocols can be assigned their own persistent digital object identifiers (DOIs).

To include a link to a protocol in your article:

1. Describe your step-by-step protocol on protocols.io
2. Select **Get DOI** to issue your protocol a persistent digital object identifier (DOI)
3. Include the DOI link in the Methods section of your manuscript using the following format provided by protocols.io: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]

At this stage, your protocol is only visible to those with the link. This allows editors and reviewers to consult your protocol when evaluating the manuscript. You can make your protocols public at any time by selecting **Publish** on the protocols.io site. Any referenced protocol(s) will automatically be made public when your article is published.

New taxon names

Methods sections of manuscripts adding new zoological, botanical, or fungal taxon names to the literature must follow the guidelines for new taxon names.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

PLOS journals publicly acknowledge the indispensable efforts of our editors and reviewers on an annual basis. To ensure equitable recognition and avoid any appearance of partiality, do not include editors or peer reviewers—named or unnamed—in the Acknowledgments.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management Toll, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	**Format**
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005. Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.
Accepted, unpublished articles	Same as published articles, but substitute "Forthcoming" for page numbers or DOI.
Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.

Source	Format
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity. arXiv:1403.3301v1 [Preprint]. 2014 [cited 2014 March 17]. Available from: https://128.84.21.199/abs/1403.3301v1 Kording KP, Mensh B. Ten simple rules for structuring papers. BioRxiv [Preprint]. 2016 bioRxiv 088278 [posted 2016 Nov 28; revised 2016 Dec 14; revised 2016 Dec 15; cited 2017 Feb 9]: [12 p.]. Available from: https://www.biorxiv.org/content/10.1101/088278v5 doi: 10.1101/088278
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 2014 Jan 29 [Cited 2014 March 17]. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 20 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order at first appearance in the manuscript file.

Read the guidelines for figures and requirements for reporting blot and gel results.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and “Figure” abbreviated to “Fig” (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of “Fig 1” must refer to a figure file named “Fig1.tif”).
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order).

Do not submit your tables in separate files.

Tables require a label (e.g., "Table 1") and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

Statistical reporting

Manuscripts submitted to *PLOS ONE* are expected to report statistical methods in sufficient detail for others to replicate the analysis performed. Ensure that results are rigorously reported in accordance with community standards and that the statistical methods employed are appropriate for the study design.

Consult the following resources for additional guidance:

- SAMPL guidelines, for general guidance on statistical reporting
- *PLOS ONE* guidelines, for clinical trials requirements
- *PLOS ONE* guidelines, for systematic review and meta-analysis requirements
- EQUATOR, for specific reporting guidelines for a range of other study types

Reporting of statistical methods

In the methods, include a section on statistical analysis that reports a detailed description of the statistical methods. In this section:

- List the name and version of any software package used, alongside any relevant references
- Describe the technical details or procedures required to reproduce the analysis
- Provide the repository identifier for any code used in the analysis (See our code-sharing policy.)

Statistical reporting guidelines:

- Identify research design and independent variables as being between- or within-subjects
- For pre-processed data:
    - Describe any analysis carried out to confirm the data meets the assumptions of the analysis performed (e.g. linearity, co-linearity, normality of the distribution).
    - If data were transformed include this information, with a reason for doing so and a description of the transformation performed
- Provide details of how outliers were treated and your analysis, both with the full dataset and with the outliers removed
- If relevant, describe how missing/excluded data were handled
- Define the threshold for significance (alpha)

- If appropriate, provide sample sizes, along with a description of how they were determined. If a sample size calculation was performed, specify the inputs for power, effect size and alpha. Where relevant, report the number of independent replications for each experiment.
- For analyses of variance (ANOVAs), detail any post hoc tests that were performed
- Include details of any corrections applied to account for multiple comparisons. If corrections were not applied, include a justification for not doing so
- Describe all options for statistical procedures. For example, if t-tests were performed, state whether these were one- or two-tailed. Include details of the type of t-test conducted (e.g. one sample, within-/between-subjects).
- For step-wise multiple regression analyses:
    - Report the alpha level used
    - Discuss whether the variables were assessed for collinearity and interaction
    - Describe the variable selection process by which the final model was developed (e.g., forward-stepwise; best subset). See SAMPL guidelines.
- For Bayesian analysis explain the choice of prior trial probabilities and how they were selected. Markov chain Monte Carlo settings should be reported.

Reporting of statistical results

Results must be rigorously and appropriately reported, in keeping with community standards.

- **Units of measurement.** Clearly define measurement units in all tables and figures.
- **Properties of distribution.** It should be clear from the text which measures of variance (standard deviation, standard error of the mean, confidence intervals) and central tendency (mean, median) are being presented.
- **Regression analyses.** Include the full results of any regression analysis performed as a supplementary file. Include all estimated regression coefficients, their standard error, p-values, and confidence intervals, as well as the measures of goodness of fit.
- **Reporting parameters.** Test statistics (F/t/r) and associated degrees of freedom should be provided. Effect sizes and confidence intervals should be reported where appropriate. If percentages are provided, the numerator and denominator should also be given.
- **P-values.** Report exact p-values for all values greater than or equal to 0.001. P-values less than 0.001 may be expressed as $p < 0.001$, or as exponentials in studies of genetic associations.
- **Displaying data in plots.** Format plots so that they accurately depict the sample distribution. 3D effects in plots can bias and hinder interpretation of values, so avoid them in cases where regular plots are sufficient to display the data.

- **Open data.** As explained in PLOS's Data Policy, be sure to make individual data points, underlying graphs and summary statistics available at the time of publication. Data can be deposited in a repository or included within the Supporting Information files.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

See instructions on providing underlying data to support blot and gel results

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at full submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

Striking image

You can choose to upload a "Striking Image" that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

If no striking image is uploaded, we will designate a figure from the submission as the striking image.

Striking images should not contain potentially identifying images of people. Read our policy on identifying information.

The PLOS licenses and copyright policy also applies to striking images.

Additional Information Requested at Submission

Financial Disclosure Statement

This information should describe sources of funding that have supported the work. It is important to gather these details prior to submission because your financial disclosure statement cannot be changed after initial submission without journal approval. If your manuscript is published, your statement will appear in the Funding section of the article.

Enter this statement in the Financial Disclosure section of the submission form. Do not include it in your manuscript file.

The statement should include:

- Specific grant numbers
- Initials of authors who received each award
- Full names of commercial companies that funded the study or authors
- Initials of authors who received salary or other funding from commercial companies
- URLs to sponsors' websites

Also state whether any sponsors or funders (other than the named authors) played any role in:

- Study design
- Data collection and analysis
- Decision to publish
- Preparation of the manuscript

If they had no role in the research, include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript."

If the study was unfunded, include this sentence as the Financial Disclosure statement: "The author(s) received no specific funding for this work."

Read our policy on disclosure of funding sources.

Competing interests

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

Read our policy on competing interests.

Manuscripts disputing published work

For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite a signed review by the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

Related manuscripts

Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into "parts." Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion.

Read our policies on related manuscripts.

Preprints

PLOS encourages authors to post preprints as a way to accelerate the dissemination of research and supports authors who wish to share their work early and receive feedback before formal peer review. Deposition of manuscripts with preprint servers does not impact consideration of the manuscript at any PLOS journal.

Authors posting on bioRxiv or medRxiv may submit directly to relevant PLOS journals through the direct transfer to journal service.

Authors submitting manuscripts in the life sciences to *PLOS ONE* may opt-in to post their work on bioRxiv during the *PLOS ONE* initial submission process.

Read more about preprints.

Learn how to post a preprint to bioRxiv during *PLOS ONE* initial submission.

Guidelines for Specific Study Types

Registered Reports

Submission and format requirements for Registered Report Protocols and Registered Reports are similar to those for a regular submission and may be specific to your study type. For instance, if your Registered Report Protocol submission is about a Clinical Trial or a Systematic Review, follow the appropriate guidelines.

For Registered Report Protocols:

- Provide enough methodological detail to make the study reproducible and replicable
- Confirm that data will be made available upon study completion in keeping with the PLOS Data policy
- Include ethical approval or waivers, if applicable

- Preliminary or pilot data may be included, but only if necessary to support the feasibility of the study or as a proof of principle
- For meta-analyses or a Clinical Trials, consider using the protocol-specific reporting guidelines PRISMA-P or SPIRIT respectively

For more guidance on format and presentation of a protocol, consult the sample template hosted by the Open Science Framework. Discipline-specific and study-specific templates are also available.

If data need to be collected, modified or processed specifically for your study, or if participants need to be recruited specifically for your study, then it should occur only after your Registered Report Protocol is accepted for publication.

For Registered Report Research Articles:

- Report the results of all planned analyses and, if relevant, detail and justify all deviations from the protocol.
- The manuscript may also contain exploratory, unplanned analyses.

Read more about Registered Report framework.

Human subjects research

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form from the Downloads and Translations page. More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME
- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- **The name of the approving institutional review board or equivalent committee(s)**. If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- **Whether informed consent was written or oral**. If informed consent was oral, it must be stated in the manuscript:
    - Why written consent could not be obtained
    - That the Institutional Review Board (IRB) approved use of oral consent
    - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal, which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

Clinical trials

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- Provide the registry name and number in the methods section of the manuscript
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's “Fig 1”

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The methods section must include the name of the registry, the registry number, and the URL of your trial in the registry database for each location in which the trial is registered.

Animal research

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

Manuscripts reporting animal research must state in the Methods section:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s).
- Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why. Provide any relevant regulations under which the study is exempt from the requirement for approval.
- Relevant details of steps taken to ameliorate animal suffering.

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Authors should always state the organism(s) studied in the Abstract. Where the study may be confused as pertaining to clinical research, authors should also state the animal model in the title.

To maximize reproducibility and potential for re-use of data, we encourage authors to follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information.

Non-human primates

Manuscripts describing research involving non-human primates must report details of husbandry and animal welfare in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research*, including:

- Information about housing, feeding, and environmental enrichment.
- Steps taken to minimize suffering, including use of anesthesia and method of sacrifice, if appropriate.

Random source animals

Manuscripts describing studies that use random source (e.g. Class B dealer-sourced in the USA), shelter, or stray animals will be subject to additional scrutiny and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Unacceptable euthanasia methods and anesthetic agents

Manuscripts reporting use of a euthanasia method(s) classified as unacceptable by the American Veterinary Medical Association or use of an anesthesia method(s) that is widely prohibited (e.g., chloral hydrate, ether, chloroform) must include at the time of initial submission, scientific justification for use in the specific study design, as well as confirmation of approval for specific use from their animal research ethics committee. These manuscripts may be subject to additional ethics considerations prior to publication.

Humane endpoints

Manuscripts reporting studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, must comprehensively report details of study design, rationale for the approach, and methodology, including consideration of humane endpoints. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality.

Definition of a humane endpoint

A humane endpoint is a predefined experimental endpoint at which animals are euthanized when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention ("death as an endpoint"), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. Please see the NC3Rs guidelines for more information. Additional discussion of humane endpoints can be found in this article: Nuno H. Franco, Margarida Correia-Neves, I. Anna S. Olsson (2012) How "Humane" Is Your Endpoint? — Refining the Science-Driven Approach for Termination of Animal Studies of Chronic Infection. PLoS Pathog 8(1): e1002399 doi.org/10.1371/journal.ppat.1002399.

Full details of humane endpoints use must be reported for a study to be reproducible and for the results to be accurately interpreted.

For studies in which death of an animal is an outcome or a planned experimental endpoint, authors should include the following information in the Methods section of the manuscript:

- The specific criteria (i.e. humane endpoints) used to determine when animals should be euthanized.

- The duration of the experiment.
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals.
- How frequently animal health and behavior were monitored.
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions.

If humane endpoints were not used, the manuscript should report:

- A scientific justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered.
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design.

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use. Read the policy.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

All necessary permits were obtained for the described study, which complied with all relevant regulations.

If no permits were required, please include the following statement:

No permits were required for the described study, which complied with all relevant regulations.

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses must include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study

and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants' experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.

See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For *de novo* (new) cell lines, including those given to the researchers as a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND

- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

Blots and gels

Please review *PLOS ONE's* requirements for reporting blot and gel results and providing the underlying raw images.

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to initial submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

Methods, software, databases, and Tolls

PLOS ONE will consider submissions that present new methods, software, databases, or Tolls as the primary focus of the manuscript if they meet the following criteria:

Utility

The Toll must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online Tolls, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation

Submissions presenting methods, software, databases, or Tolls must demonstrate that the new Toll achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new Toll is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability

If the manuscript's primary purpose is the description of new software or a new software package, this

software must be open source, deposited in an appropriate archive, and conform to the Open Source Definition. If the manuscript mainly describes a database, this database must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. In these cases, authors should provide a direct link to the deposited software or the database hosting site from within the paper. If the primary focus of a manuscript is the presentation of a new Toll, such as a newly developed or modified questionnaire or scale, it should be openly available under a license no more restrictive than CC BY.

Software submissions

Manuscripts whose primary purpose is the description of new software must provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

Read the PLOS policy on sharing materials and software.

New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

Anochetus boltoni *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called "Nomenclatural Acts":

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Solanum aspersum S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

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Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

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Hymenogaster huthii. Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

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