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INSTITUTO DE CIÊNCIAS BIOMÉDICAS



PROGRAMA DE PÓS-GRADUAÇÃO EM IMUNOLOGIA E
PARASITOLOGIA APLICADAS

CAROLINA DE SOUZA GARDENGHI

**Avaliação *in vitro* dos efeitos antiparasitário, antitumoral e
antimicrobiano de proteínas extraídas e purificadas de
semente de *Cycas revoluta*.**

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Avaliação *in vitro* dos efeitos antiparasitário, antitumoral e antimicrobiano de proteínas extraídas e purificadas de semente de *Cycas revoluta*.

Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Imunologia e Parasitologia Aplicadas como parte da obtenção do título de Mestre.

Orientadora: Profa. Dra. Fernanda Maria Santiago



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*“Como um eco na floresta, outro
dia virá, como se nada tivesse
acontecido, a vida continua...”*

Bangtan Sonyeondan, 2020.

RESUMO

Cycas revoluta (Thunb.), pertencente à família Cycadaceae é uma gimnosperma nativa da China, é conhecida por seu grande apelo ornamental. Recentemente, a comunidade científica tem investigado seu potencial terapêutico contra uma variedade de patógenos. Este estudo foca na análise das proteínas presentes em suas sementes, com o objetivo de fornecer informações sobre as atividades biológicas dessas proteínas, com destaque para suas propriedades antiparasitárias, antitumorais e antimicrobianas. Primeiramente, a extração de proteínas das sementes de *C. revoluta* foi realizada por maceração e homogeneização com NaCl, seguida da purificação proteica em coluna de gel filtração Sephadex G-75. As proteínas foram então visualizadas por meio de eletroforese em SDS-Page, quantificadas pelo método BCA e analisadas por espectrometria de massa. Antes de realizar as atividades biológicas, foram feitos testes para avaliar a citotoxicidade das amostras, garantindo que dosagens seguras fossem usadas para células não tumorais e evitando possíveis vieses no estudo. O estudo inclui testes para avaliar o efeito antiparasitário, analisando a invasão e proliferação dos taquizoítos de *Toxoplasma gondii*. Além disso, avalia o efeito antitumoral em câncer de pulmão e próstata, com experimentos focados tanto no tratamento direto das células (MTT) quanto na prevenção (formação de colônias tumorais). Também são realizados testes para investigar a atividade antimicrobiana, determinando a dose mínima capaz de inibir o crescimento de *Escherichia coli*, *Staphylococcus aureus* e *Salmonella enterica*. O extrato bruto das sementes de *Cycas revoluta* e as quatro frações proteicas purificadas, denominadas Pico I (Proteína de fusão multifuncional - Glicina-tRNA sintetase – 113,7 kDa; Prostaglandina G/H sintase – 63,5 kDa; Quitinase A – 38,7 kDa), Pico II (Quitinase A – 38,7 kDa), Pico Associado (Quitinase A – 38,7 kDa; Hidrolase – 15,4 kDa), and Pico III (Hidrolase – 15,4 kDa), foram investigadas. Cada fração proteica apresentou resultados distintos em análise biológica. Na avaliação da atividade anti-*Toxoplasma gondii*, o Pico II foi particularmente notável por sua eficácia no tratamento do parasita e das células infectadas. No ensaio antitumoral, a análise MTT revelou que apenas o extrato bruto reduziu a viabilidade das células de câncer de próstata. No ensaio clonogênico, tanto o extrato bruto quanto o Pico Associado foram eficazes na redução da formação de colônias em células de câncer de próstata (PC3) e de pulmão (A549). Na avaliação antibacteriana, as frações com os menores valores de MIC foram o Pico III para *Escherichia coli* e *Staphylococcus aureus*, e o Pico II para *Salmonella enterica*. Em conclusão, as frações proteicas purificadas da *C. revoluta* apresentam um potencial terapêutico promissor. Novos estudos científicos sobre essa espécie são incentivados na busca de novas moléculas terapêuticas com grande potencial de aplicação.

Palavras-chave: *Cycas revoluta*, atividade biológica, sementes, frações proteicas.

ABSTRACT

Cycas revoluta (Thunb.), belonging to the Cycadaceae family, is a gymnosperm native to China, known for its significant ornamental appeal. Recently, the scientific community has been investigating its therapeutic potential against a variety of pathogens. This study focuses on analyzing the proteins present in its seeds, aiming to provide insights into their biological activities, particularly their antiparasitic, antitumor, and antimicrobial properties. Firstly, protein extraction from *C. revoluta* seeds was performed by maceration and homogenization with NaCl, followed by protein purification using a Sephadex G-75 gel filtration column. The proteins were then visualized via SDS-PAGE gel electrophoresis, quantified using the BCA method, and analyzed by mass spectrometry. Before assessing biological activities, cytotoxicity tests were conducted to ensure that safe dosages were used for non-tumor cells, thereby avoiding potential biases in the study. The study includes tests to evaluate the antiparasitic effect by analyzing the invasion and proliferation of *Toxoplasma gondii* tachyzoites. Additionally, it assesses the antitumor effect on lung and prostate cancer, with experiments focused on both direct treatment of cells (MTT) and prevention (tumor colony formation). Antimicrobial activity tests were also conducted to determine the minimum inhibitory concentration (MIC) required to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica*. The crude extract from *Cycas revoluta* seeds and the four purified protein fractions, designated as Peak I (Multifunctional fusion protein - Glycine-tRNA synthetase – 113,7 kDa; Prostaglandin G/H synthase – 63,5 kDa; Chitinase A – 38,7 kDa), Peak II (Chitinase A – 38,7 kDa), Associated Peak (Chitinase A – 38,7 kDa; Hydrolase – 15,4 kDa), and Peak III (Hydrolase – 15,4 kDa), were investigated. Each protein fraction exhibited distinct results in biological analysis. In the evaluation of anti-*Toxoplasma gondii* activity, Peak II was particularly notable for its efficacy in treating the parasite and infected cells. In the antitumor assay, the MTT analysis revealed that only the crude extract reduced the viability of prostate cancer cells. In the clonogenic assay, both the crude extract and the Associated Peak effectively reduced colony formation in prostate (PC3) and lung (A549) cancer cells. In the antibacterial evaluation, the fractions with the lowest MIC values were Peak III for *Escherichia coli* and *Staphylococcus aureus*, and Peak II for *Salmonella enterica*. In conclusion, the purified protein fractions of *C. revoluta* exhibit promising therapeutic potential. Further scientific studies on this species are encouraged in the search for new therapeutic molecules with significant application potential.

Keywords: *Cycas revoluta*, biological activity, seeds, protein fractions.

LISTA DE ABREVIACÕES E SIGLAS

A549 – linhagem de célula tumoral de pulmão
A2780 – linhagem de célula tumoral de ovário
AKT - proteína quinase-B
AR - receptor de andrógeno
Beas-2B – linhagem de célula de pulmão
CO₂ - Dióxido de carbono
C. revoluta – *Cycas revoluta*
CRPC - castration-resistant prostate câncer
DHFR - enzima diidrofolato redutase
DHPS - diidropteroato sintase
DNA – ácido desoxirribonucleico
E. coli - *Escherichia coli*
ERG – oncogene
GARS - Glycine-tRNA synthetase
GLOBOCAN - Observatório Global do Câncer
HFF – linhagem de célula fibroblástica humana
IARC - Agência Internacional de Pesquisa sobre o Câncer
MCF – linhagem de célula tumoral de mama
MDA-MB-231 – linhagem de célula tumoral de mama
MIC – concentração inibitória mínima
NaCl – cloreto de sódio
NIH - linhagem de célula fibroblástica de camundongo
NSCLC - non-small cell lung câncer
OMS - Organização Mundial da Saúde
PBS – solução salina tamponada com fosfato
PC3 – linhagem de célula tumoral de próstata
PGHS - Prostaglandin G/H synthase
Pnt-2 – linhagem de célula de próstata
pTEN - A fosfatase homóloga à tensina
PSA - antígeno específico da próstata
PYR – pitimetamina
PI3K - fosfatidilinositol 3-quinase

P53 – proteína citoplasmática com peso 53kDa

RBCs – células vermelhas do sangue

RB1 - gene de suscetibilidade ao retinoblastoma

S. aureus - *Staphylococcus aureus*

SCLC - small cell lung câncer

SDZ – sulfadiazina

S. entérica - *Salmonella entérica*

SKOV3 – linhagem de célula tumoral de ovário

T.gondii – *Toxoplasma gondii*

UHC - cobertura universal de saúde

UPEC - *Escherichia coli* uropatogênica

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CAPÍTULO 1

FUNDAMENTAÇÃO TEÓRICA

1. INTRODUÇÃO

1.1. Produtos naturais

O reino vegetal é um reservatório de compostos naturais bioativos. Na literatura, a grande presença de estudos relacionados a compostos naturais estão se concentrando em diversas espécies de plantas, já que se dedicam a busca de compostos naturais que possuem algumas atividades imunoestimulantes, antioxidantes, antiinflamatórias, anticancerígenas, neuro e hepatoprotetora (Ekiert & Szopa, 2020).

Produtos naturais são fontes de várias moléculas bioativas com estruturas distintas, grande diversidade química e propriedades terapêuticas. Atualmente, a pesquisa científica está cada vez mais focada na identificação e isolamento de novos compostos a partir de extratos naturais para descobrir novos medicamentos. Já foram relatadas inúmeras moléculas extraídas e sintetizadas a partir de produtos naturais que apresentaram efeitos antiprotozoários, anticancerígenos, antimicrobianos, antivirais, entre outros (Morais et al., 2020; Atanasov et al., 2021).

Pesquisas na literatura sobre o efeito protetor de compostos naturais com atividade contra protozoários incluem: oleorresinas de *Copaifera* spp. para *Toxoplasma gondii* (Teixeira et al., 2020); *Paraboeremia selaginellae* contra *T. gondii* (Mazzone et al., 2022); análise de produtos naturais com atividade antiparasitária contra *Trypanosoma cruzi* (Peres et al., 2021); compostos naturais com atividade anti-*Plasmodium falciparum* (Arnold et al., 2021); e plantas como fontes de agentes antimaláricos (Habibi et al., 2022).

Outros estudos que envolvem a análise da atividade antitumoral de compostos naturais incluem o mel Saudi Sidr, obtido da árvore *Ziziphus jujuba*, inibe o crescimento de células cancerígenas gástricas, colorretais, mamárias e pulmonares, causando apoptose e interrupção do ciclo celular (Matsuo, 2023); ácidos graxos isolados das folhas de *Oryza officinalis*, que suprimiram a viabilidade das células tumorais de pulmão H1299 (Kim, 2018); e saponinas triterpênicas extraídas das sementes de castanha-da-Índia (*Aesculus hippocastanum*), que apresentam atividade anticancerígena por induzirem a inibição do crescimento e apoptose no câncer de pâncreas (Yue et al., 2017).

Estudos que investigam o uso de extratos naturais no tratamento antibacteriano abrangem o tratamento profilático com extrato de chá verde (*Camellia sinensis*), rico em catequina, que demonstrou melhora na colite patogênica induzida pela bactéria *Escherichia coli* (Kim et al., 2021); e *Kalanchoe brasiliensis* (Flor-da-fortuna), utilizada como uma fonte natural

promissora de atividades antioxidantes e antibióticas contra a bactéria multirresistente *Salmonella* (Mayorga et al., 2019).

1.2. Gênero *Cycas*

As cicadáceas estão entre os grupos mais primitivos e antigos de plantas gimnospermas, existindo há mais de 200 milhões de anos, tendo sua origem antes do período Permiano e alcançando maior diversidade no período Jurássico-Cretáceo (Wang et al., 2019). Evolutivamente, compartilham características morfológicas com as samambaias, como a presença de espermatófitas com anterozóides. Nas Cycadaceae, os tubos polínicos liberam espermatozoides móveis para o óvulo, que é gerado no megagametófito semelhante a folhas. Uma característica chave para o sucesso da polinização é a secreção de fluido ovular, que possui alta concentração de carboidratos, servindo como recompensa para os insetos que polinizam a planta fêmea. As cicadáceas também compartilham características com as gimnospermas, como sementes nuas e ramificações dicotômicas. Devido a essas características são consideradas um grupo-chave para a compreensão da evolução inicial das plantas com sementes. As cicadáceas compreendem duas famílias, Cycadaceae e Zamiaceae, com 10 gêneros e 355 espécies identificadas e aceitas, sendo restritas a regiões tropicais e subtropicais (Xiao, Moller, 2015; Aderkas et al., 2022).

Cycas revoluta Thunb., pertencente à família Cycadaceae, é uma planta gimnosperma conhecida como “sagu-de-jardim” ou “palmeira-sagu”, nativa da Ásia e amplamente cultivada para fins ornamentais. Assim como outras plantas cicadáceas, a *C. revoluta* é dióica, podendo atingir até 3 metros de altura e apresentando folhas pinadas. A planta masculina produz cones solitários oblongo no ápice do broto, enquanto a planta feminina apresenta megasporófilos no cone solitário, que produzem sementes férteis (Figura 1), de cor amarelo-alaranjada (Li et al., 2023).

O cone masculino possui numerosos microsporófilos, cada um com 30 a 600 microsporângios, que liberam os grãos de pólen. Por outro lado, os megasporófilos das plantas femininas de *C. revoluta* apresentam de um a oito óvulos (7-10 mm de largura) em ambas as margens e na parte inferior. Durante a polinização, os cones femininos se abrem e emitem odor, fechando-se logo após a polinização. No período de fertilização, os óvulos chegam a medir 25-35 mm de largura, com coloração predominantemente alaranjada (Kono, Tobe, 2007).



Figura 1 - *Cycas revoluta*. (A) *C. revoluta* masculina; (B) *C. revoluta* feminina; (C) *C. revoluta* feminina representando o megasporófito com megásporo fértil, as setas indicam as sementes.. Fonte: Unesp Rio Claro (2004).

Plantas cicadáceas são apreciadas tanto pelo seu valor botânico e uso ornamental em jardins, quanto por suas aplicações alimentares (devido à sua rica fonte de amido) e medicinais em diversas culturas, embora seja necessário remover as toxinas antes do consumo. Na África, o comércio de medicamentos tradicionais coloca em risco a existência natural de cicadáceas, especialmente das espécies *Encephalartos* e *Stangeria eripus* (Primos et. al., 2012). Na Austrália e na Polinésia, sementes de plantas do gênero *Cycas* são utilizadas popularmente para tratar feridas, hemorroidas e outros distúrbios. Já pelos chineses, a espécie *C. revoluta* é usada para tratar certos tumores (Kowalska et. al., 1995).

Espécies de plantas da família Cycadaceae (gênero *Cycas*) têm sido estudadas por apresentarem uma rica fonte de flavonóides, ácidos fenólicos, esteróis e ácidos graxos que exibem atividades biológicas. Fração de acetato de etilo extraída das folhas de *Cycas rumphii* apresentou propriedades anti-*Toxoplasma gondii*, inibindo a invasão de taquizoitos da cepa RH nas células hospedeiras, cerca de IC_{50} de $3,51 \pm 0,2 \mu\text{g/ml}$ (El-Seadawy et. al., 2022). Um componente isolado extraído das suas folhas e caule de *C. revoluta*, sendo eles flavonoides, apresentou atividade anti-câncer de pulmão (inibindo a metástase de células A549 em 80% no período de 24h em $128 \mu\text{mol/L}$) e sua lectina demonstrou atividade contra câncer gástrico, em células MGC803 e HGC27 (com uma inibição na viabilidade celular de 60% em ambas as células a $300 \mu\text{g/ml}$) (Wang et. al., 2018; Cui et. al., 2019). Extrato metanólico de *Cycas tuarsii* exibiu atividade antibacteriana para *Pseudomonas aeruginosa*, com um MIC de $16\text{--}128 \mu\text{g/mL}$ (Binsuawaidan et. al., 2022). Os extratos clorofórmicos de folhas de *C. revoluta* exibiram

atividade antibacteriana contra *Staphylococcus aureus* (CI₅₀ valores de 3,9, 9,7 e 8,2 µM) e *S. aureus* resistente à meticilina (IC₅₀ valores de 5,9, 12,5 e 11,5 µM). (Moawad, 2010).

1.3. Toxoplasmose

1.3.1 Doença

A toxoplasmose é uma doença zoonótica causada pelo parasita intracelular obrigatório *Toxoplasma gondii*, pertencente ao filo Apicomplexa. Esta doença tem grande importância médica e veterinária e é transmitida por diversas vias. A toxoplasmose possui distribuição geográfica mundial, com altas taxas de prevalência sorológica. No entanto, casos graves são observados em recém-nascidos e indivíduos imunodeprimidos, incluindo receptores de órgãos, gestantes, pacientes HIV positivos (Vírus da Imunodeficiência Humana) e indivíduos em tratamento quimioterápico. A sintomatologia da toxoplasmose varia conforme o estado imunológico do paciente, podendo ser assintomática ou causar problemas neurológicos em imunodeprimidos e danos irreversíveis ao feto ou abortos em grávidas infectadas durante a gestação. A principal forma de detecção da doença é por meio de diagnóstico laboratorial, sendo os mais comuns utilizados ELISA e PCR (Rey, 2008; Neves, 2011; Lopes, Berto, 2012).

O protozoário *Toxoplasma gondii* foi descoberto em 1908 por Nicolle e Manceaux em um roedor do norte da África conhecido como gondi. Simultaneamente, o parasita foi identificado em um coelho no Brasil por Splendore (Rey, 2008). O nome *Toxoplasma* deriva de sua morfologia arqueada (*toxos* = arco; *plasma* = molde), enquanto *gondii* faz referência ao roedor em que foi encontrado. De vida intracelular obrigatória, o *T. gondii* possui várias formas infectantes, incluindo taquizoítos, bradizoítos e esporozoítos. A infecção atinge animais de sangue quente, com os felinos sendo os hospedeiros definitivos e aves e mamíferos (incluindo os humanos) atuando como hospedeiros intermediários (Schnell, 2011).

1.3.2 Ciclo de vida e formas de transmissão

Três estágios de desenvolvimento do *T. gondii* podem infectar as células durante seu ciclo de vida: taquizoítos (forma de multiplicação rápida encontrada nas infecções agudas), bradizoítos (forma de multiplicação lenta presente na infecção crônica, originando cistos teciduais) e esporozoítos (presentes apenas no hospedeiro definitivo durante a reprodução sexuada do parasita e liberados nos oocistos nas fezes dos felinos), representados na figura 2 (Dubey, Lindsay, Speer, 1998; Tong et al., 2021). A transmissão da toxoplasmose ocorre de duas formas principais. A transmissão horizontal ocorre pela ingestão oral de oocistos liberados no ambiente pelas fezes dos gatos ou de cistos teciduais presentes em carnes cruas ou

malcozidas, ingeridas pelos hospedeiros intermediários. A transmissão vertical ocorre de forma transplacentária, com os taquizoítos sendo passados da mãe para o feto (Dubey, 2012).

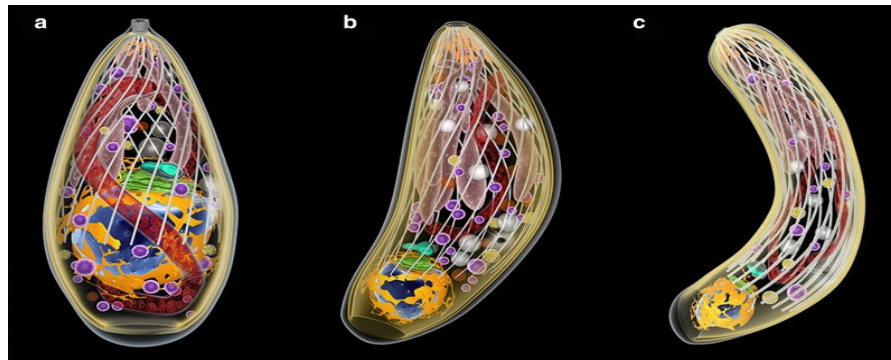


Figura 2 – Formas infectantes *Toxoplasma gondii*. (A) Taquizoíto; (B) Bradizoíto; (C) Esporozoíto. Núcleo (representado de azul), o qual é circundado pelo retículo endoplasmático rugoso (amarelo). Complexo de Golgi está representado acima do núcleo (verde) e o apicoplasto (verde água). Presença de uma única mitocôndria (vermelha), granulos densos (magenta) e de amilopectina (brancos). Complexo apical é composto pelo conóide cilíndrico, por um conjunto de microtúbulos dispostos de forma espiral, organela secretora e a presença de anéis polares. Fonte: Attias et. al. (2020).

Geralmente, os gatos jovens são infectados pela primeira vez ao ingerirem cistos teciduais (contendo bradizoítos) de *T. gondii* ao se alimentarem de pássaros e/ou roedores infectados (representado na figura 3). As enzimas digestivas no intestino do gato quebram a parede desses cistos, liberando os bradizoítos, que invadem as células epiteliais do intestino, permitindo ao parasita se desenvolver nas células hospedeiras. A parte sexuada do ciclo do *T. gondii* ocorre no intestino delgado do gato (hospedeiro definitivo), comumente no íleo. Nesse local, os microgametas masculinos fecundam os macrogametas femininos, resultando na formação de um zigoto e na formação de uma parede de oocisto ao redor do parasita. As células epiteliais contendo os oocistos se rompem, liberando-os no intestino do gato, de onde são excretados nas fezes. Esses oocistos não são infecciosos para outros hospedeiros até que sejam esporulados, um processo que ocorre em alguns dias fora do corpo do gato. Cada oocisto contém dois esporocistos, que por sua vez contêm quatro esporozoítos. Uma característica dos oocistos é possuírem uma casca externa resistente, permitindo que sobrevivam por longos períodos no ambiente. Assim que esporulados, os oocistos se tornam infecciosos para novos hospedeiros, que podem ser infectados ao consumirem alimentos ou água contaminada. Um gato infectado pode liberar milhões de oocistos durante uma a duas semanas após a infecção primária pelo parasita (Dubey, 2012; Lelu et al., 2012; Innes et al., 2019).

Nos hospedeiros intermediários, a transmissão do *T. gondii* ocorre através do consumo de oocistos em alimentos (como frutas e verduras) ou água contaminada, e pela ingestão de carne crua ou malcozida de animais persistentemente infectados. Após a ingestão dos oocistos, os esporozoítos são liberados e invadem os enterócitos e células do epitélio do íleo, transformando-se em taquizoítos (estágio de multiplicação rápida do parasita). Os taquizoítos então invadem e se multiplicam dentro das células hospedeiras, formando um vacúolo parasitóforo. Um estágio crucial no ciclo celular do *T. gondii* ocorre quando os taquizoítos se convertem em bradizoítos (estágio de multiplicação lenta), permitindo que o parasita escape da resposta imunológica do hospedeiro. Dentro de uma célula infectada, os bradizoítos modificam o vacúolo parasitóforo para formar a parede de um cisto tecidual. Os bradizoítos se multiplicam lentamente dentro desses cistos e podem persistir durante a vida do hospedeiro, podendo ser encontrados no cérebro, coração, olhos e músculos esqueléticos, permanecendo ocultos no hospedeiro (Dubey, 2012; Innes et al., 2019).

Uma terceira via de transmissão é a transplacentária. Se uma infecção primária ocorrer durante a gravidez, os taquizoítos de *T. gondii* podem ser transportados através da circulação para a placenta, permitindo que o parasita infecte as células placentárias e se espalhe para as células do feto durante o seu desenvolvimento, causando toxoplasmose congênita. Além de afetar humanos, a toxoplasmose congênita pode ocorrer em ovinos e caprinos, levando ao aborto e à mortalidade neonatal, sendo uma grande preocupação médica veterinária (Innes et al., 2019).

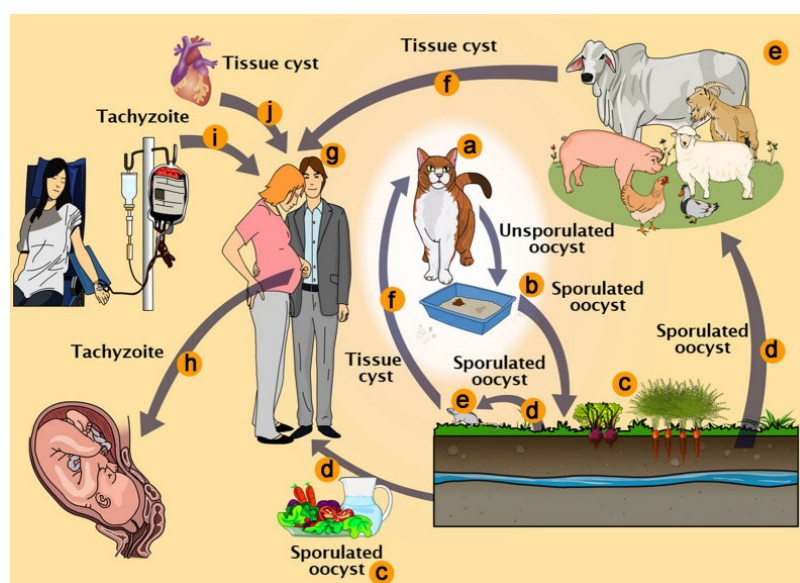


Figura 3 – Ciclo biológico *Toxoplasma gondii*. (A) Hospedeiro definitivo (gato); (B) Oocistos não esporulados encontrados nas fezes do gato; (C) Alimentos contaminados com os oocistos

já esporulados; (D) Oocistos presentes nos vegetais ou água podendo ser ingeridos por hospedeiros intermediários; (E) Hospedeiros intermediário (exemplo: bovinos, ovinos, aves e suínos); (F) Ingestão de cistos teciduais por carne crua; (G) Hospedeiros intermediários (humanos); (H) Taquizoítos transmitidos por meio da placenta para o feto (toxoplasmose congênita); (I e J) Transmissão por transfusão de sangue e transplante de órgãos. Fonte: Attias et. al. (2020).

1.3.3 Tratamento

Atualmente, os tratamentos para toxoplasmose, devido ao seu modo de ação, apenas controlam a infecção aguda causada por taquizoítos e as reativações da doença. Em indivíduos imunocomprometidos, a mortalidade pode ser elevada se o tratamento da toxoplasmose for adiado. O tratamento convencional envolve a combinação de dois medicamentos: pirimetamina (PYR) e sulfadiazina (SDZ). Além disso, esses medicamentos podem ser combinados com ácido folínico (leucovorina) ou clindamicina (CLD) (Elsheika, Marra, Zhu, 2020).

As terapias atuais são eficazes apenas contra os taquizoítos, pois estes são vulneráveis enquanto se replicam rapidamente. Em contraste, os bradizoítos, que se replicam lentamente, não são vulneráveis aos mesmos tratamentos. O número de bradizoítos aumenta ao se replicarem, com cada cisto tecidual podendo conter de 100 a 500 parasitas ao longo de 1 a 2 meses, enquanto os taquizoítos se replicam a cada 6 a 8 horas. A pirimetamina (PYR) é capaz de eliminar os taquizoítos em cultura durante a noite; no entanto, uma exposição de apenas 4 horas ao medicamento não inibe a replicação do parasita, sendo necessária uma exposição contínua para melhor eficácia. A parede fortemente glicosilada do cisto tecidual não impede fisicamente a ação da PYR, sugerindo que o estado semi-adormecido dos bradizoítos seja o responsável pela falha no tratamento. O tratamento com clindamicina (CLD) tem sido utilizado como alternativa para a toxoplasmose aguda, pois possui a capacidade de inibir a síntese proteica na organela vital do apicoplasto (Barrett et al., 2019; Elsheika, Marra, Zhu, 2020).

A pirimetamina (PYR) atua como um antagonista do ácido fólico, inibindo a enzima diidrofolato redutase (DHFR), o que bloqueia a síntese de purinas e pirimidinas, essenciais para a síntese do DNA e a multiplicação celular. Quando combinada com a sulfadiazina (SDZ), a PYR interfere na síntese de ácido fólico do *T. gondii* ao inibir competitivamente a diidropteroato sintase (DHPS). A combinação de PYR com ácido fólico não pode ser usada durante o primeiro trimestre de gravidez, pois a pirimetamina possui um alto potencial teratogênico. Além disso, foram relatadas várias complicações graves, como agranulocitose, síndrome de Stevens-Johnson, necrólise epidérmica e hepática, embora sejam raras. Portanto,

é necessário pesquisar novos tratamentos que sejam eficazes e apresentem menos ou nenhum efeito colateral (Elsheika, Marra, Zhu, 2020; Silva et al., 2021; Shamaa, Powell, Benmerzouga, 2021).

Estudos com plantas têm sido significativos na busca por tratamentos para toxoplasmose aguda. Extratos de sementes de *Jatropha curcas* (planta da família Euphorbiaceae) purificados em cromatografia Sephadex G-75, demonstraram atividade contra taquizoítos de *T. gondii*, interferindo na sua invasão em células hospedeiras, resultando em apenas 26,2% de células infectadas em um tratamento com 3 mg/ml (Soares et al., 2015). Ácidos graxos purificados por cromatografia gasosa também mostraram efeitos anti-*Toxoplasma gondii*, diminuindo a viabilidade parasitária a quase 100% em 200 µg/ml (Atolani et al., 2019). Além disso, extratos de sementes de *Alyssum homolocarpum* (8,06 µg/ml), *Lepidium perfoliatum* (2,59 µg/ml), *Lepidium sativum* (0,74 µg/ml) e partes aéreas de *Nasturtium officinale* (0,78 µg/ml) e *Capsella bursa-pastoris* (0,65 µg/ml) apresentaram concentração inibitória de 50% da proliferação de taquizoítos de *T. gondii* em células hospedeiras infectadas (Montazeri et. al., 2020).

1.4. Câncer

Segundo estimativas da Agência Internacional de Pesquisa sobre o Câncer (IARC) da Organização Mundial da Saúde (OMS), em 2022 houve 20 milhões de novos casos de câncer e cerca de 9,7 milhões de mortes. A pesquisa da IARC sobre a cobertura universal de saúde (UHC) e o câncer revelou que apenas 39% dos países avaliados cobriam os aspectos básicos do tratamento do câncer. (Organização Pan-americana da Saúde, 2024).

O câncer pode se desenvolver em qualquer órgão ou estrutura do corpo, caracterizado pelo crescimento anormal de células. Tumores dependem da expressão de oncogenes mutantes únicos para poderem promover seu crescimento e a sobrevivência do tumor (Denisenko et. al., 2018). O tratamento convencional geralmente envolve a remoção cirúrgica do tumor ou nódulo, quimioterapia ou radioterapia. As células cancerosas apresentam mutações que levam à expressão desregulada de oncogenes e à alteração da expressão gênica. Pesquisas recentes sobre o câncer têm se concentrado cada vez mais na prevenção da evasão imunológica, o que tem impulsionado os avanços na imunoterapia e em terapias a partir de produtos naturais. Este campo tem mostrado melhorias significativas no tratamento do câncer (Roy & Saikia, 2016).

A literatura já destaca a ligação de compostos naturais na inibição e proliferação de células cancerosas, com ênfase nos compostos obtidos a partir de plantas. Efeitos citotóxicos, antiproliferativos e antimetastáticos do extrato de *Allium autumnale* (Amaryllidaceae) foram

observados em linhagens de células tumorais humanas MCF-7 e MDA-MB-231 (Isbilen et al., 2018). Extratos etanólicos de *Solanum lyratum* Thunb (fruta-do-lobo) controlam a proliferação de células de câncer de ovário nas linhagens A2780 e SKOV3 (Zhang et al., 2021). Em um estudo, as interações moleculares entre um extrato de *Citrus limetta* (limão) e um composto de lecitina de soja em 62,93 µg/ml demonstraram uma diminuição significativa (IC₅₀) na morte de células tumorais de mama MDA-MB-231 (Taghizadeh et al., 2022). Sesquiterpenoides extraídos das folhas de *Chimonanthus nitens* a 100µM apresentaram efeito citotóxico em células de câncer de mama MDA-MB-231 (Guo et al., 2023).

1.4.1 Câncer de próstata

De acordo com a GLOBOCAN (Observatório Global do Câncer), o câncer de próstata ocupou o quarto lugar em incidência mundial no ano de 2022, com aproximadamente 1.467.854 novos casos e ocupou em oitavo no *ranking* de mortalidade, com 397.430 mortes. O câncer de próstata é diagnosticado por meio de biópsia e análise do órgão, como exame retal digital, ressonância magnética, testes de antígenos específicos da próstata e exames de saúde. Considerado uma doença heterogênea, o câncer de próstata pode ser influenciado por fatores genéticos, ambientais e comportamentais, como idade, obesidade, entre outros (Sutera et. al., 2023).

A grande maioria dos pacientes com câncer de próstata progride para o estado de resistência à castração (CRPC, da sigla em inglês castration-resistant prostate cancer). Cerca de 10 a 20% dos cânceres de próstata evoluem para CRPC dentro de 5 anos, e 84% dos casos recém-diagnosticados de CRPC apresentam metástase (Crawford et al., 2017). Genes que participam da via androgênica e do metabolismo da testosterona são candidatos à predisposição ao câncer de próstata (Bluemn & Nelson, 2012). Pesquisas destacam a importância da perda de genes supressores de tumor, como p53 e pTEN, bem como do gene ERG, na progressão e na resistência terapêutica do câncer de próstata em estágio avançado. A proteína p53 tem um papel crucial na manutenção do ciclo celular em resposta aos danos ao DNA e à expressão de oncogenes. A perda do gene pTEN promove a progressão do câncer de próstata para CRPC através das vias PI3K (fosfatidilinositol 3-quinase) e AKT (proteína quinase-B) (Wade & Kyprianou, 2018).

As opções de tratamento para um paciente com câncer de próstata dependem da natureza do tumor, do nível de antígeno específico da próstata (PSA), do grau e do estágio do tumor. Por exemplo, para câncer de próstata de baixo risco, recomenda-se a remoção da próstata e dos tecidos próximos ao órgão, juntamente com a radioterapia. Já para cânceres que se espalham

além da próstata, a terapia de privação de andrógenos (terapia hormonal) é recomendada. No entanto, esses tratamentos estão associados a efeitos colaterais graves e resistência ao tratamento, o que destaca a necessidade de descobrir novos agentes quimioterápicos que sejam econômicos, tenham poucos efeitos colaterais e apresentem maior eficácia (Termini et al., 2020; Sekhoacha et al., 2022).

Uma das linhagens de células de câncer de próstata mais utilizadas na pesquisa científica é a PC3, derivada de linfonodos e metástases ósseas. Essa linhagem não expressa receptor de andrógeno (AR) nem antígeno específico de próstata (PSA). A PC3, como uma forma agressiva de câncer de próstata, tem sido usada em pesquisas tanto para tumores dependentes de andrógenos quanto para tumores resistentes à castração (Tai et al., 2011).

Estudos que analisam os efeitos antitumorais de produtos naturais contra tumores de próstata incluem: o isolamento de Lupeol das raízes de *Bowdichia virgilioides* (Fabaceae), que apresenta atividade citotóxica na linhagem de células PC3 (apresentando uma concentração inibitória de 50% nas células tumorais em 1.78 µg/ml) (Victor et al., 2017); sulforodamina B extraída de *Cecropia pachystachya*, que mostrou efeitos citotóxicos sobre a linhagem celular PC3, apresentando uma diminuição de 50% nas células em 70,85 µg/ml (Rosa et al., 2020); e extratos de acetato de etila de *Linum numidicum* e *Linum trigynum*, que possuem capacidade anticancerígena contra células de tumor de próstata PC3, diminuindo aproximadamente 100% da viabilidade celular ao tratamento de 72h em 1000 µg/ml (Mouna, 2022). Além disso, efeitos antiproliferativos e apoptóticos foram observados na linhagem PC3 a partir de quatro extratos de polpas de Murici (*Byrsonima crassifolia* Kunth e *Byrsonima verbascifolia* (L.) e Taperebá (*Spondias mombin* L.) nas concentrações de 5 a 20mg/ml (Paim et al., 2024).

1.4.2 Câncer de pulmão

De acordo com a GLOBOCAN (Observatório Global do Câncer), o câncer de pulmão ocupou o primeiro lugar em incidência mundial no ano de 2022, com aproximadamente 2.480.301 novos casos e 1.817.172 mortes. Os dois subtipos principais de câncer de pulmão são: o carcinoma pulmonar de células pequenas (SCLC, do inglês small cell lung cancer), responsável por 15% dos casos e fortemente associado ao tabagismo, e o carcinoma pulmonar de células não pequenas (NSCLC, do inglês non-small cell lung cancer), representando 85% dos casos (Li et al., 2021).

Para um tratamento e prognóstico eficaz, é crucial identificar o estágio do tumor. A taxa de sobrevivência do câncer de pulmão em 5 anos é de cerca de 19% (Brett et al., 2020). A baixa taxa de sobrevivência ao câncer de pulmão é devida ao fato de mais de 50% dos pacientes com

carcinoma pulmonar de células não pequenas serem diagnosticados em estágio metastático. A taxa de sobrevivência relativa em 5 anos é de 90% para o estágio 1 da doença, mas cai para 10% quando o paciente com NSCLC está no estágio 4. Pacientes com carcinoma pulmonar de células pequenas apresentam uma taxa de sobrevida relativa em 5 anos de cerca de 30% (Pirker, 2020). O câncer de pulmão requer monitoramento contínuo devido às tendências de crescimento populacional relacionadas à poluição, ao envelhecimento da população e aos comportamentos de tabagismo (Cheng et al., 2016).

O SCLC e o NSCLC podem ser diferenciados morfológicamente por critérios da microscopia óptica. O SCLC possui uma maior proporção de cromatina nuclear e citoplasmática finamente granular, ausência de nucléolos e um formato fusiforme comum (Travis, 2014). O SCLC é caracterizado por uma boa resposta inicial à quimioterapia e radiação, mas apresenta uma proliferação agressiva e uma alta taxa de metástase (Wang et al., 2020). As alterações genéticas mais notáveis em pacientes com SCLC incluem a perda dos supressores de tumor p53, a perda do gene de suscetibilidade ao retinoblastoma (RB1) e a amplificação do MYC (Semenova et al., 2015). A proteína p53 desempenha um papel crucial na estabilidade genômica, apoptose e supressão da angiogênese. O supressor tumoral p53 é ativado em resposta ao estresse celular causado por danos ao DNA, hipóxia e senescência, induzindo a parada do ciclo celular e a apoptose (Carvajal & Manfredi, 2013). Outro supressor tumoral, o RB1, encontrado em cerca de 65% dos casos de SCLC, é essencial para a regulação do ciclo celular, retardando a transição da fase G1 para a fase S. Sua depleção pode levar à ativação de fatores de transcrição que aumentam as propriedades de pluripotência, resultando em uma reprogramação mais agressiva das células e na tumorigênese (Kareta et al., 2015). A amplificação de mutações nos genes da família MYC (incluindo MYC, MYCL, MYCN) ocorre em cerca de 20% dos casos de SCLC. Essa amplificação está associada à progressão do tumor e à tolerância à quimioterapia. As proteínas da família MYC são fatores de transcrição que ativam a expressão de uma série de genes envolvidos na proliferação celular e progressão do ciclo celular (Wang et al., 2020).

Os tumores classificados como NSCLC (carcinoma pulmonar de células não pequenas) apresentam uma ampla variedade de alterações genômicas, incluindo uma alta taxa de mutações somáticas, como rearranjos cromossômicos que resultam em várias fusões de proteínas oncogênicas, sendo mais comuns as mutações nos genes p53 e LRP1B. O adenocarcinoma (ADC) é um subtipo de NSCLC caracterizado por uma genômica complexa com vários tipos de alterações somáticas, incluindo substituições, inserções, deleções e mutações no local de splicing (Wang et al., 2020; Alam et al., 2023). O adenocarcinoma é o tipo mais comum de

câncer de pulmão, representando cerca de 40% dos casos, e é um dos tumores mais agressivos e fatais. Sua alta resistência à radioterapia e quimioterapia convencional continua sendo um grande desafio para a eficácia do tratamento. As mutações detectadas nesse tipo de câncer de pulmão incluem alterações no receptor do fator de crescimento epidérmico (EGFR) e no receptor de tirosina-quinase transmembrana (RTK). Mutações no EGFR, localizadas próximas à fenda de ATP do domínio tirosina-quinase (TK), resultam em um aumento da ativação do receptor, atuando como condutores oncogênicos e estimulando o crescimento do tumor (Denisenko et al., 2018). Outro subtipo comum encontrado em NSCLC é o carcinoma de células escamosas, responsável por 30% de todos os casos de câncer de pulmão de células não pequenas (Niu et al., 2023).

Pesquisas na área de oncologia têm sido conduzidas para compreender a biologia do NSCLC, visando encontrar melhores opções terapêuticas para este tipo de câncer. Em 1973, Giard e colaboradores desenvolveram uma série de linhagens celulares derivadas de tumores humanos, entre elas a linhagem A549, um adenocarcinoma coletado de um tumor sólido de um paciente masculino durante uma resseção cirúrgica. Devido às suas boas características em cultura de células, essa linhagem é um modelo atrativo para pesquisas que estudam o metabolismo e os mecanismos de diferentes substâncias anti-NSCLC (Gao et al., 2017).

Vários estudos abordam os efeitos antitumorais em tumores de pulmão. Em um estudo de meta-análise, um conjunto de dados de 58 cardenolídeos provenientes de plantas foi compilado, relatando sua atividade antiproliferativa na linhagem de células tumorais de pulmão A549 (Meneses-Sagrero et al., 2020). Extratos de *Caesalpinia sappan* demonstraram ser agentes anticancerígenos contra a linhagem A549, inibindo seu crescimento em 45,19% a 1704 µg/ml em 24 h de tratamento (Widodo et al., 2022). Lignanas isoladas da planta *Euphorbia hirta* L. foram avaliadas quanto à proliferação de linhagens de células cancerígenas, incluindo a linhagem de tumor de pulmão A549, inibindo 50% da viabilidade celular em 8,5 µM (Zhang et al., 2020). A síntese de compostos derivados de produtos naturais demonstrou eficácia na atividade antitumoral, desencadeando apoptose em células A549, com IC₅₀ em 0,34 µM (Hou, 2024).

1.5 Bactérias

1.5.1 *Escherichia coli*

Escherichia coli é uma bactéria gram-negativa da família Enterobacteriaceae. Cepas comensais de *E. coli* coexistem em mutualismo com os humanos sem causar efeitos adversos; contudo, em indivíduos imunocomprometidos, podem transpor barreiras gastrointestinais e

causar distúrbios como diarreia e doenças extraintestinais (Pakbin, Bruck, Rossen, 2021). Foram descritos oito patótipos de *E. coli*: seis patótipos intestinais (enteropatogênica, enterohemorrágica, enterotoxigênica, enteroagregativa, enteroinvasiva e difusamente aderente) e dois associados a infecções extraintestinais, denominados *E. coli* uropatogênica e *E. coli* associada à meningite. Esses patótipos patogênicos são responsáveis por surtos de diarreia com sangue, diarreia infantil, cistite, meningite e pielonefrite (Liu et al., 2020; Braz; Melchior, Moreira, 2020).

E. coli uropatogênica (UPEC) é responsável por 80 a 95% dos casos de infecções do trato urinário e 27% dos casos de sepse. O tratamento das infecções por UPEC geralmente envolve o uso de antibióticos β -lactâmicos e não β -lactâmicos, como fluoroquinolonas, sulfametoxazol-trimetoprima e aminoglicosídeos. Bactérias da família Enterobacteriaceae, produtoras de β -lactamases de espectro estendido, incluindo a *E. coli*, são frequentemente resistentes a antibióticos não β -lactâmicos. Portanto, os carbapenêmicos são recomendados para o tratamento dessas infecções. A disseminação global da resistência a cefalosporinas e aos carbapenêmicos, mediada pelas β -lactamases, fez com que esses microrganismos se tornassem resistentes a muitos antibióticos, tornando-os ineficazes (Terlizzi, Griboaud, Maffei, 2017; Guetierrez, Baño, 2019; Walker et al., 2022).

1.5.2 *Staphylococcus aureus*

Staphylococcus aureus é uma bactéria gram-positiva amplamente envolvida em doenças humanas e está atualmente associada a um grupo de bactérias caracterizadas por resistência a múltiplos medicamentos (Bonikowska, 2022). *S. aureus* é um dos principais patógenos causadores de infecções comunitárias e hospitalares, podendo provocar desde infecções leves de pele e tecidos moles, como furúnculos, abscessos e infecções em feridas, até infecções graves como endocardite infecciosa, osteomielite, bacteremia e pneumonia fatal (Cheung, Bae, Otto, 2021).

A infecção sistêmica por *S. aureus* depende da ruptura da camada protetora epitelial do indivíduo. Por exemplo, infecções de pele podem ocorrer a partir de pequenos arranhões no tecido epitelial. Outras vias de infecção pelo patógeno incluem ambientes hospitalares, onde há contaminação de dispositivos médicos internos. Além disso, pode ocorrer intoxicação alimentar quando alimentos estão contaminados com enterotoxinas estafilocócicas, representando um caso especial de infecção aguda por *S. aureus*. O patógeno também pode aproveitar condições predisponentes do indivíduo ou danos primários causados por outros patógenos (Cheung, Bae, Otto, 2021).

As infecções por *S. aureus* são uma das mais frequentes em hospitais e estão associadas a alta mortalidade, tornando-se uma preocupação significativa para a saúde pública (Mansour et. al., 2021). Essas infecções são particularmente problemáticas devido à resistência à meticilina (MRSA). A resistência desse patógeno aos antibióticos beta-lactâmicos tradicionais, como a penicilina e seus derivados, é comum, e a vancomicina tem sido o último recurso para tratar infecções por *S. aureus* resistentes à meticilina. No entanto, já existem cepas que adquiriram resistência intermediária à vancomicina. Portanto, a busca e análise de novos tratamentos continuam sendo uma prioridade na pesquisa para o desenvolvimento de medicamentos contra *S. aureus* (Guo et. al., 2020).

1.5.3 *Salmonella enterica*

Salmonella enterica é uma bactéria gram-negativa causadora da salmonelose, que inclui mais de dois mil sorovares distintos. Esta diversidade permite que o patógeno evolua para sobreviver em uma ampla variedade de ambientes e hospedeiros. Clinicamente, a salmonelose pode causar febre entérica, gastroenterite, complicações extraintestinais e estado de portador crônico. No entanto, as manifestações clínicas variam de acordo com o estado imunológico do hospedeiro e os fatores específicos da patovariante de *Salmonella* que está causando a infecção (Pulford et. al., 2019). *S. enterica* adaptou-se a vários hospedeiros, especialmente galinhas, tornando as aves e seus produtos, como ovos, os principais veículos de infecção humana por *Salmonella*. Outras vias de infecção incluem animais de estimação, répteis, frutas, vegetais e transmissão entre humanos. Diversos fatores explicam a ampla gama de hospedeiros do patógeno, incluindo a aquisição de genes por meio da transferência horizontal de genes com fagos, plasmídeos e transposons (Foley et. al., 2017).

Cepas resistentes do patógeno são comumente encontradas em fontes alimentares de origem animal. No entanto, a gestão inadequada de agentes antimicrobianos tem promovido a resistência da *Salmonella* a quinolonas, fluoroquinolonas e cefalosporinas de terceira geração, medicamentos clinicamente significativos. Os mecanismos pelos quais a *Salmonella* desenvolve resistência a antimicrobianos incluem a produção de enzimas que degradam a permeabilidade celular aos antibióticos, a ativação de bombas de efluxo antimicrobiano e a produção de β -lactamase, que degrada a estrutura química do antimicrobiano (Andino, Hanning, 2015).

Pesquisas vem sendo desenvolvidas para avaliar o efeito bactericida em compostos e moléculas extraídas em produtos naturais. O óleo de *Melaleuca alternifolia* possui efeito bactericida contra *E. coli* e *S. aureus*, apresentando concentração mínima inibitória (MIC) de 4

$\mu\text{g/ml}$ e $8 \mu\text{g/ml}$, respectivamente (Yasin et al., 2021). As folhas de *Astragalus membranaceus*, tradicionalmente usadas como chá na tradição chinesa, contêm componentes bioativos com funções antibacterianas contra *E. coli*, *S. aureus* e *Salmonella*, apresentando uma zona de inibição de $8.15 \pm 1.02 \text{ mm}$ para *E. coli*, $7.34 \pm 0.16 \text{ mm}$ em *S. aureus* $9.11 \pm 0.30 \text{ mm}$ em *Salmonella*, todos em $600 \mu\text{g/ml}$ (Samuel et. al., 2021). Extratos hidroetanólicos das folhas de *Kalanchoe brasiliensis* Cambess. foram analisados quanto ao modo de ação contra cinco cepas de *S. aureus* e cinco cepas clínicas de *Salmonella enterica*, apresentando MIC de $5000 \mu\text{g/ml}$ (Santos et al., 2019). Extratos de castanha (*Castanea sativa*) e da madeira de quebracho (*Schinopsis lorentzii*) foram avaliados quanto à atividade antibacteriana (concentração inibitória mínima) contra *Salmonella Gallinarum* e *Salmonella enteritidis* de 2 a 4 mg/ml (Casanova, 2021). Um composto natural de *Streptomyces* foi analisado quanto à ação antivirulência contra *Salmonella enterica*, inibindo 63% da viabilidade bacteriana em $5 \mu\text{g/ml}$ (Bruna et al., 2022).

2 OBJETIVOS

2.1 Objetivo geral

O objetivo geral consiste em investigar o potencial terapêutico *in vitro* das proteínas extraídas da semente de *Cycas revoluta*, a fim de analisar atividades antiparasitária, antitumoral e antimicrobiana.

2.2 Objetivos específicos

- Extrair as proteínas presentes na semente de *Cycas revoluta*;
- Purificar proteínas isoladas da semente de *Cycas revoluta* em coluna de Gel filtração, Sephadex G-75;
- Caracterizar as frações proteicas por Espectrometria de massa.
- Determinar a concentração citotóxica *in vitro* das proteínas de *C. revoluta* para posteriormente analisar o uso terapêutico;
- Avaliar atividades antiparasitária frente ao *Toxoplasma gondii*, antitumoral em células tumorais de próstata e de pulmão, e antimicrobiana para *E. coli*, *S. aureus* e *Salmonella enterica*, em ensaios *in vitro*.

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CAPÍTULO 2

ARTIGO CIENTÍFICO

Protein effects from *Cycas revoluta* seeds on antiparasitic, antitumor, and antimicrobial activity

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ABSTRACT**Ethnopharmacological Relevance**

Plants belonging to the Cycadaceae family are known for their significant ornamental, nutritional, and medicinal value in various cultures. In African countries, species from this family are threatened due to their extensive use in the traditional medicine trade. In Australia and Polynesia, seeds of plants from the genus *Cycas* are popularly used to treat wounds and hemorrhoids, and in China, the species *Cycas revoluta* is used to treat tumors. Consequently, the scientific community has recently been investigating the therapeutic potential of *C. revoluta* (Thunb.) against a variety of pathogens and the present study focuses on the analysis of proteins found in its seeds.

Study Objective

This study aims to provide information on the biological activities of the proteins present in *C. revoluta* seeds, with a specific focus on analyzing their anti-tumor, anti-parasitic, and anti-microbial properties.

Materials and Methods

Firstly, protein extraction from *C. revoluta* seeds was performed by maceration and homogenization with NaCl, followed by protein purification using a Sephadex G-75 gel filtration column. The proteins were then visualized via SDS-PAGE electrophoresis, quantified using the BCA method, and analyzed by mass spectrometry. Before assessing biological activities, cytotoxicity tests were conducted to ensure that safe dosages were used for non-tumor

cells, thereby avoiding potential biases in the study. The study includes tests to evaluate the antiparasitic effect by analyzing the invasion and proliferation of *Toxoplasma gondii* tachyzoites. Additionally, it assesses the antitumor effect on lung and prostate cancer, with experiments focused on both direct treatment of cells (MTT) and prevention (tumor colony formation). Antimicrobial activity tests were also conducted to determine the minimum inhibitory concentration (MIC) required to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enterica*.

Results

The crude extract from *Cycas revoluta* seeds and the four purified protein fractions, designated as Peak I (Multifunctional fusion protein - Glycine-tRNA synthetase – 113,7 kDa; Prostaglandin G/H synthase – 63,5 kDa; Chitinase A – 38,7 kDa), Peak II (Chitinase A – 38,7 kDa), Associated Peak (Chitinase A – 38,7 kDa; Hydrolase – 15,4 kDa), and Peak III (Hydrolase – 15,4 kDa), were investigated. Each protein fraction exhibited distinct results in biological analysis. In the evaluation of anti-*Toxoplasma gondii* activity, Peak II was particularly notable for its efficacy in treating the parasite and infected cells. In the antitumor assay, the MTT analysis revealed that only the crude extract reduced the viability of prostate cancer cells. In the clonogenic assay, both the crude extract and the Associated Peak effectively reduced colony formation in prostate (PC3) and lung (A549) cancer cells. In the antibacterial evaluation, the fractions with the lowest MIC values were Peak III for *Escherichia coli* and *Staphylococcus aureus*, and Peak II for *Salmonella enterica*.

Conclusions

In conclusion, the purified protein fractions from *C. revoluta* show promising therapeutic potential. New scientific studies on this species are encouraged in the search for new therapeutic molecules with great potential for application.

ARTICLE INFO

Keywords: *Cycas revoluta*, biological activity, seeds, protein fractions.

1. Introduction

Isolates of a diversity of compounds and bioactive molecules present in natural products have attracted the scientific community's attention due to their diverse biological activities. Notable examples include honey extracted from the *Ziziphus jujubas* tree, which has demonstrated the ability to inhibit the growth of colorectal, breast, and lung cancer cells (Qanach, 2023). In addition, the fatty acid isolated from the leaves of *Oryza officinalis* has shown efficacy in suppressing the viability of lung tumor cells (Yang, 2014). Studies have also revealed that oleoresins from *Copaifera spp.* have potential against the parasite *Toxoplasma gondii* (Teixeira et al., 2020), while *Paraboeremia selaginellae* has also shown activity against *T. gondii* (Mazzone et. al., 2022). Research has also revealed analyses of natural products with antiparasitic activity against *Trypanosoma cruzi* (Peres et. al., 2021), anti-*Plasmodium falciparum* action through natural compounds (Arnold et. al., 2021), identification of plants as sources of antimalarial agents (Habibi et. al., 2022), and the use of ginger essential oil against *Escherichia coli* and *Staphylococcus aureus* (Wang et. al., 2020). Additionally, isolates of fungal pathogens from plants have demonstrated antimicrobial activity against *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *E. coli*, *Mycobacterium abscessus* and *M. marinum* (Cadelis, et. al., 2023).

Cycad plants are appreciated both for their botanical value and ornamental use in gardens, as well as for their food (due to their rich source of starch) and medicinal applications in various cultures, although it is necessary to remove the toxins before consumption. In Africa, the trade in traditional medicines endangers the natural existence of cycads, especially the *Encephalartos* and *Stangeria eripus* species (Primos et. al., 2012). In Australia and Polynesia, seeds from plants of the genus *Cycas* are popularly used to treat wounds, hemorrhoids and other disorders. The Chinese use the species *Cycas revoluta* to treat tumors (Kowalska et. al., 1995). Plants belonging to the Cycadaceae family, especially the *Cycas* genus, have exhibit various biological activities. An example is the ethyl acetate fraction extracted from the leaves of *Cycas rumphii*, which has demonstrated anti-*Toxoplasma gondii* properties (El-Seadawy, et al., 2022). In addition, antibacterial activity has been observed in the methanolic extract of *Cycas thouarsii* (Binsuawaidan et al., 2022).

C. revoluta Thunb., belonging to the Cycadaceae family and popularly known as “garden sago”, is a gymnosperm plant. Recent research has highlighted the remarkable immunomodulatory potential of this species, evidenced by an isolated component extracted from its leaves and stems. The flavonoid compounds of *C. revoluta* have shown activity against lung cancer, while the lectin fraction derived from its leaves has shown efficacy against gastric

cancer (Wang et al., 2018; Cui et al., 2019). Given that the protein fraction derived from the seeds of *C. revoluta* has not yet been addressed in the literature and considering the diversity of biological activities found in plants of the same genus, this study aims to investigate whether the protein extract from the seeds of *C. revoluta* has potential activity against lung and prostate cancer, as well as against protozoa belonging to the phylum Apicomplexa, *T. gondii*, and the bacteria *E. coli*, *S. aureus* and *Salmonella enterica*.

2. Material and methods

2.1 Obtaining and extracting the material

Seeds of *C. revoluta*, were collected in the Federal University of Uberlândia, (-18.885205683268236S, -48.25978351429416W, geographical coordinates), Minas Gerais State, Brazil in 2023. The proteins from these seeds were obtained by extraction in saline solution (0.95% NaCl). To carry out the extraction, the seeds were removed from the raw plant using a scalpel. The material was then macerated until it was powdered. For every 30 grams of powdered material, 100 ml of 0.95% NaCl was added, ensuring that the material was completely immersed. The whole mixture was homogenized for 24 hours for complete protein extraction. After stirring, all the material was centrifuged at 5000 rpm for 30 minutes at 4°C, the resulting pellet was discarded and the supernatant was dialyzed in distilled water 3 times over 24 hours.

2.2 Protein purification and dosage

After dialysis, the crude extract from the seeds of *C. revoluta* was subjected to protein fractionation on a Sephadex G-75 gel-filtration column (140 cm height x 15 mm diameter) (Sigma-Aldrich), previously equilibrated with phosphate-buffered saline (PBS). Elution was carried out using the same buffer at a flow rate 15ml/h (Bollag, 1994). To assess the electrophoretic profile of the proteins in the fractions collected, a 12% SDS-Page was run and subsequently stained with Coomassie Blue (Laemmli, 1970), resulting in the separation of the fractions into distinct peaks. The molecular weight of each protein fraction (kDa), as determined by the gel, was measured using ImageLab 4.0 software. Finally, the purified proteins were measured by the BCA (Bicinchoninic acid assay) method, using bovine serum albumin (BSA) as a standard curve, and followed by recording the absorbance at 570nm.

2.3 Analysis of protein fractions by mass spectrometry

The protein fractions from the *C. revoluta* seeds, purified using Sephadex G-75, were

analyzed by direct infusion mass spectrometry (MS) employing a high-resolution Q-TOF mass spectrometer (Agilent model 6520B) (Kopylov et. al., 2014), and Phyre2 was used for molecular modeling (Kelley et al., 2015).

2.4 Cells and Parasites Culture

Non-tumor cell lines: mouse embryo fibroblast cells (ATCC:3T3-NIH CRL 1658); human lung cells (Beas-2B); human prostate cells (Pnt-2) and human cancer cell lines, specifically a lung cancer cell (A549) and a prostate cancer cell (PC3), were grown in the Gibco Roswell Park Memorial Institute 1640 medium (RPMI). RH strain of *T. gondii* was maintained in human foreskin fibroblast (HFF) cells cultured in Dulbecco's Modified Eagle's medium (DMEM). All culture media were supplemented with 5% Fetal Bovine Serum (Gibco), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, at 37°C in a 5% CO₂ atmosphere, as previously described (Li, et., 2009; Sohn et.al., 2011).

2.5 Cytotoxicity assessment

2.5.1 Hemolysis

Red blood cells (RBCs) from Balb/c mice were collected at the Rodent Bioterium Network (Rebir-UFU), and approved by the Ethics Committee. After collection, the RBCs were washed in a 0.95% NaCl solution and centrifuged at 400g for 5 minutes. The RBCs were adjusted to a concentration of 0.5%. In a 96-well V-bottom plate, the crude extract of *C. revoluta* and its purified protein fractions were serially diluted in 0.95% NaCl (starting at a concentration of 300 µg/ml up to 0.58 µg/ml). Then 50 µl of 0.5% red blood cells were added to all the wells and incubated for 60 minutes at 37°C. The negative control consisted only of red blood cells with NaCl, while the cell death control used 1% Triton X-100 and red blood cells. After incubation, the plate was centrifuged at 500g for 10 minutes, and only the supernatant was removed for reading on the spectrophotometer to quantify the cells. The samples were considered cytotoxic when hemolysis occurred.

2.5.2 Evaluation of cellular viability of NIH/3T3 fibroblasts, Beas-2B and Pnt-2 non-tumor cell

To assess the cytotoxicity of the proteins of interest in non-tumor cells, the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) colorimetric test was carried out. Initially, NIH, Beas-2B, and Pnt-2 cells were seeded in a 96-well plate, adjusted to a concentration of 1×10^4 cells/well in a complete RPMI medium, and incubated for 24 hours at

37°C, 5% CO₂, and 95% humidity. Subsequently, the crude extract and the purified protein fractions from the *C. revoluta* seed were added individually to the cells, starting at a concentration of 300 µg/ml and diluting serially until 0.1 µg/ml was reached. After this addition, the cells were incubated for 24 hours. As a negative control, the cells were treated with PBS. After the incubation interval, an MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich Co, USA) was added in the proportion of 10 µl/well, along with 90 µl of RPMI medium containing 10% fetal bovine serum. The plates were incubated for 4 hours. Afterward, the plate was centrifuged at 500g for 10 minutes at 37°C, and 200 µl/well of a solution of 10% SDS (Sodium Dodecyl Sulphate) and 50% DMF (dimethylformamide) was added to solubilize the crystals formed. After 24 hours, the solubilized crystals were read in a spectrophotometer at a wavelength of 570 nm (Garn et al., 1994).

2.5.3 Antiparasite Activity

2.5.3.1 Plaque assay

To assess the invasion capacity of the RH strain of *Toxoplasma gondii*, and consequently cause cell lysis, HFF cells were distributed in 24-well plates, at a density of 1×10^5 cells per well, and incubated for 24 hours. The plate assay was conducted in two different ways: in one plate, the parasites were treated with the desired samples of *C. revoluta* seeds and stirred constantly for 45 minutes at room temperature. As a control, the parasites were treated only with an incomplete DMEM medium. After treatment, the parasites were centrifuged, washed, and adjusted to a concentration of 100 parasites/well, thus infecting the confluent HFF monolayer and remained incubated for approximately 5 days at 37°C with 5% CO₂. In another 24-well plate, the cells of the HFF monolayer were infected with around 100 parasites/well for 2 hours and then treated with the protein fractions of *C. revoluta*. As a control, the infected cells were treated with 10% DMEM medium and incubated for 5 days at 37°C and 5% CO₂. At the end of this period, the wells were washed with PBS, fixed with ice-cold 100% methanol for 10 minutes, and stained with 1% crystal violet for visualization under an optical microscope. The plate count was quantified from the samples, and the plate assays were carried out in triplicate.

2.5.3.2 Invasion and Proliferation Assay

As an additional experiment designed to assess the ability of *T. gondii* tachyzoites to invade and proliferate, HFF cells were distributed in 24-well plates at a density of 1×10^5 cells per well and incubated for 24 hours. The *T. gondii* tachyzoites were treated with the desired

samples of *C. revoluta* seed and stirred constantly for 45 minutes at room temperature. As a control, the parasites were treated only with an incomplete RPMI medium. After the treatment period, the parasites were centrifuged, washed, and adjusted to a concentration of 2×10^5 parasites/well. The cells were then incubated for 24 hours in the presence of HFF-1 monolayers at 37°C and 5% CO₂. At the end of this period, the wells were washed with PBS, fixed with ice-cold methanol for 10 minutes, and stained with rapid panoptic for visualization in optical microscopes, followed by counting. In 100 randomly selected cells, the proportion of cells invaded by the parasite was determined. Within these 100 infected cells, the number of parasites present in the vacuoles in each cell was counted, followed by obtaining the average of this count (Mota et. al., 2019).

2.5.3.3 Antitumor Activity

2.5.3.4 MTT assay

To assess the anti-tumor activity of the proteins present in the crude extract and purified fractions of *C. revoluta*, the PC3 and A549 cell lines were used, using the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) method, as previously described in this study. However, the treatment was carried out at concentrations that did not show cytotoxicity for non-tumor cells, these being 150 µg/ml to 1.17 µg/ml.

2.5.3.5 Clonogenic assay

The colony assay was carried out to analyze the survival of PC3 and A549 tumor cells, which provides information on the proliferation and colony-forming potential of cells when exposed to treatment with *C. revoluta* proteins. The cells were plated in a 24-well plate at a ratio of 200 cells/well and were subsequently treated with the proteins at concentrations of 150 µg/ml and 75 µg/ml. The experiment was carried out in duplicate. As a control, the cells were treated with a complete RPMI medium and were kept at 37°C, 5% CO₂, and 95% humidity for 7 days. After this period, the reaction was stopped by adding ice-cold methanol for 5 minutes, then washing the wells 3 times with PBS and staining with 1% crystal violet to visualize the colonies. Counting was carried out using an optical microscope and ImageJ software.

2.5.4 Antibacterial Activity

2.5.4.1 Bacteria

The bacteria used in this study were obtained from the American Type Culture Collection (ATCC). *Escherichia coli* (ATCC 25923), *Staphylococcus aureus* (ATCC 25922), and

Salmonella enterica (Typhimurium ATCC 14028) were kept in the Clinical Bacteriology Laboratory of the Federal University of Uberlândia at 20°C, in 15% glycerol solution.

2.5.4.2 Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) is determined by the lowest concentration of the tested sample capable of inhibiting bacterial growth. The crude extract and protein fractions of the *C. revoluta* seed were tested for *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Salmonella enterica* serovar Typhimurium (ATCC 14028). In a sterile 96-well plate (TPP Techno Plastic Products AG, Trasadinger, 7 166 Schaffhausen, Switzerland), 100ul of the *C. revoluta* seed protein samples (crude and peaks) were added, which were diluted to concentrations of 150 µg/ml, 75 µg/ml, 37.5 µg/ml, 18.75 µg/ml, 9.37 µg/ml, 4.68 µg/ml, and 2.34 µg/ml, and 100ul of Schaedker broth (Difco) and a bacterial suspension containing 1×10^5 colony-forming units per milliliter (CFU/ml) were added.

A control for the bacteria and a control to check the sterility of the broth and samples were used as reaction controls. The plate was then incubated at 37°C, 5% CO₂ for 24 hours. To reveal bacterial growth, 30ul of 0.02% resazurin (Sigma) was used and incubated for 60 minutes. The results were analyzed according to the color of the wells, with blue showing no bacterial growth and pink showing the presence of bacterial growth. The lowest concentration in which the well of the plate remained colored blue was used to determine the MIC.

2.6 Animals

The management of blood collected, and euthanasia protocols of the animals were approved by the Animal Research Ethics Committee of the Federal University of Uberlândia, Brazil (CEUA/UFU 109/16).

2.7 Statistic analysis

Results are shown as means $\pm$ standard errors of the mean (SEM) of at least 3 independent experiments. Cell viability analysis used ANOVA and Dunnett's post-test, of antitumor activity used ANOVA for MTT assays and Two-way ANOVA for available colony formation. For investigated antiparasite activity One-way ANOVA multicomparison, and ANOVA was followed by Kruskal-Wallis. All analyses were performed using GraphPad Prism Software 9.0 (GraphPad Software, Inc., San Diego, CA, USA).

3 Results and Discussion

3.1 Protein purification and dosage

The sample of crude *C. revoluta* seed extract showed an average protein concentration of 3.9 mg/ml. For the samples obtained after the Sephadex G-75 gel filtration column, the chromatogram showed the presence of four protein fractions, called Peak I, Peak II, Associated Peak, and Peak III (Figure 1A).

The SDS-PAGE electrophoretic profile showed that the crude extract had peptide components ranging from 160 to 15 kDa (Figure 1B, lane 1). Peak I showed three predominant proteins of 113,7, 63,5 and 38,7 kDa (Figure 1B, lane 2), for Peak II one protein of 38,7 kDa were predominant (Figure 1B, lane 3), for Associated Peak the predominant proteins were both proteins found in Peak II and Peak III, of 38,7 and 15,4 kDa (Figure 1B, lane 4) and Peak III a protein of 15,4 kDa (Figure 1B, lane 5).

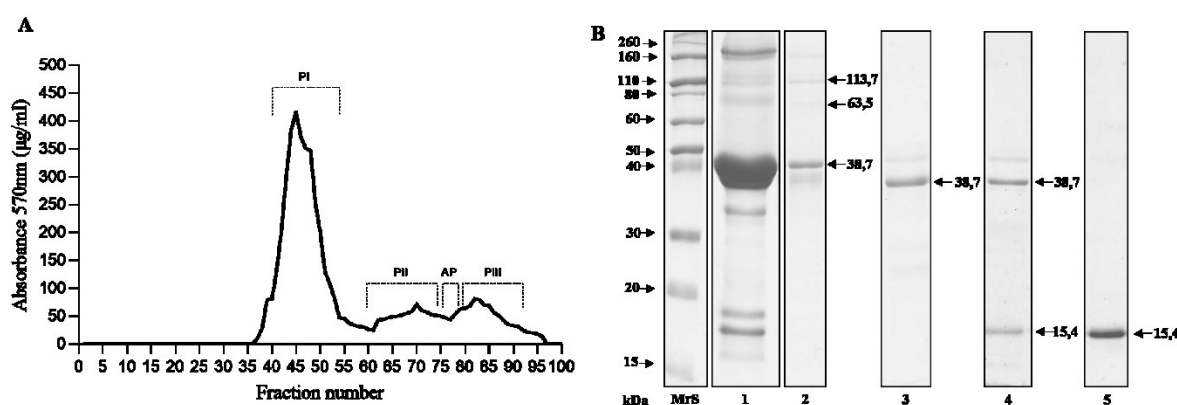


Figure 1. Chromatographic and electrophoretic profile. (A) Chromatographic profile of *C. revoluta* seed on Sephadex G-75 gel filtration column; Reading at 570nm carried out in OD by BCA protein assay. Peak I (PI); Peak II (PII); Associated Peak (AP); Peak III (PIII). (B) Electrophoretic profile of the protein fractions of *C. revoluta* seed purified on a Sephadex G-75 gel filtration column. 12% SDS-polyacrylamide gel stained with Coomassie Blue G-250. MrS: molecular weight marker, 1: crude extract of *C. revoluta* seed; 2: Peak I; 3: Peak II; 4: Associated Peak; 5: Peak III.

Some authors in the literature have used similar gel filtration column protocols to purify and separate proteins extracted from plant seeds. Oliveira et al. (2021) used Sephadex G-50 to purify a lectin from the seed of *Collaea speciosa* by a single chromatographic step, where the dimeric protein, composed of 25 kDa monomers, was analyzed for its physicochemical properties. In another study, they used a Sephadex G-15 column to purify hydrolyzed complexes of proteins extracted from chickpea seeds, to evaluate their inhibition of A549 lung tumor cells (Mukhamedov et al., 2021).

Hong and colleagues (2015) purified a lectin extracted from Chinese leek seeds using a Sephadex G-50 gel filtration column, obtaining a protein with a molecular weight of 23.6 kDa

for characterization. In another study, they extracted and purified a protein called F3, with 35 kDa, from the seeds of *Borreia hispida*, using a Sephadex G-100 gel filtration column. The protein produced a single protein peak and demonstrated antiproliferative and apoptotic activity in lung cancer (A549) and cervical cancer (HeLa) cells (Rupachandra & Sarada, 2014).

3.2 Analysis of protein fractions by Q-TOF/MS

The mass spectrometry revealed the proteins sequence of the *C. revoluta* fractions and these were then analyzed in comparison with the NCBI database (Table 1) and molecular modeling by Phyre2 (Figure S1).

Peak I revealed three distinct proteins: multifunctional fusion protein - Glycine-tRNA synthetase; Prostaglandin G/H synthetase 2 and Chitinase A.

Glycine-tRNA synthetase (GARS) is an aminoacyl-tRNA synthetase, an essential enzyme in protein synthesis, as it binds glycine amino acids to their cognate tRNAs for the process of protein translation. There is considerable evidence that this protein is related to tumorigenesis processes, correlating its overexpression with the prognosis of individuals with breast, lung, liver, kidney and prostate cancer (Sung et al., 2022; Kish et al., 2023).

Prostaglandin G/H synthase (PGHS) is an important enzyme in bone metabolism, which limits the rate of conversion of arachidonic acid, released from membranes, into prostanoids (Okada et al., 2000). PGHS has two isoforms (PGHS-1 and PGHS-2). The PGHS-2 isoform is related to its overexpression in human osteosarcoma cells (Min et al., 1998).

Chitinases are hydrolytic enzymes widely distributed in fungi, bacteria, insects and plants. They play an important role in the degradation of chitinous residues to produce oligosaccharides and N-acetylglucosamine. Chitinases cleave the β -(1,4)-glycosidic bonds in chitin and have examined biological characteristics such as antimicrobial (Xie et al., 2021) and antimalarial activity (Shahabuddin et al., 1993).

Peak II also presented the same Chitinase A protein found in peak I and according to the polyacrylamide gel, it suggests that this protein is a dimer.

Peak III showed the presence of the Hydrolase protein.

Hydrolases are a family of alpha/beta hydrolase enzymes that belong to one of the largest groups of enzymes with diverse catalytic functions. Members of this family are acetylcholinesterase, lipase, diene lactone hydrolase, thioesterase, proline, haloperoxidase, and among others. These enzymes have an efficient nucleophilic-his-acid catalytic triad to operate on substrates of different chemical compositions in various biological contexts. Studies have linked hydrolases to inhibitors of acyl-protein thioesterase in *Toxoplasma gondii* and

Plasmodium falciparum (Kemp et al., 2013) and the relationship of hydrolases in the biosynthesis and maintenance of the cell envelope of *Mycobacterium tuberculosis* (Johnson, 2017).

Multifunctional fusion protein (Glycine-tRNA synthetase)	Peak I	113.7	MPDFLELVGTEELPANFLSDAIVQWRSRIPQILTANSLSNDAVEVYGTFRRLAVLIKGLPQQQPDRE EHRGPPAQAAFKDQATPAAYGFAKKQGVLDALQRPDKGDFVFNKKTGPRVADILTELVF QWFEPLGKRLMRWGDGDKVFSRVRWLVALLDDAVLPIELVNGSETVKSDRISHGHRVLPKPI TINQATDYVDILRSQYVYVDDHIERSTTQKEVHAANHLNGYTEIVPDLKEVTNLVWSPSPVVG KFDSDFNLPTFVITTVMSHQRYFTVTQENIKELLNPTITVSNQDPAKSDIAKGNERVIRARAD QOFFYNADIAKPLESLPQLEKVTQEDLGSRAKVNRIVNAEQIATQLGLSATDSQNIQRAALLC KADLVSNQMVYFPELQGMGQKYALASGEPAVATAFEHVLPRGADDIIPITLTGRVYGLADRLD TLVSIFGLGIPTOSSDPFALRRAANAIVNTWAANLGINLDNLLQQTAAANFAQEYSKEPQGLVTTI QEFFLQRIKILLQEEKEIDYDLVNAVIGENDPEYTERALQDLLDVRDRALYLQQIRNDGTLDKIYET VNRSTRLAAGQDLDTKLEPAALIRPELFQKSEKAFYDAIVELVPQTQTAQQRNRYQLLVALEK IAPTVSNFFDGADSVLMDTNPEIKCNRLNLLGLLRNHARVLADFGAIVKNL	100
Chitinase A	Peak I and Peak II	38.7	MERLHLCIAGLLITSLCTYPAAASQNALKVGFWPAYSVSEFPSPKINSRLFTHLYYAFELNAPITEV RVPPGSEKTAEDFTPTVRRLNPSVKTLISIOGGGSEVRDNFAKLSNDASARQRFVKSSIALARRYGF HOLDLDYEYEPQLEMENFVKLVSELTAAIRIEARTSGKPRILLTEAVYTHQKLPFWVEVTEYFPVQ FIAAGLDWVNVIMAYDFHGSWENFTGAAPALRDPNSKFTASVGIESFLAAGMPEKLVGLIPLFGRS WLLKNNNEVGIGAPVAGPVDGALSFSIQNTIRGGAREVFDITTVSAYAYKDNVWVGYDNQQ SVALKVQYAKEKRLGGYTFWSVNDIDAILPKIASDTWGGTNLEDIKWPVSNRLTA	100

Table 1. List of proteins identified of *C. revoluta*, with molecular weight, sequencing and confidence percentage

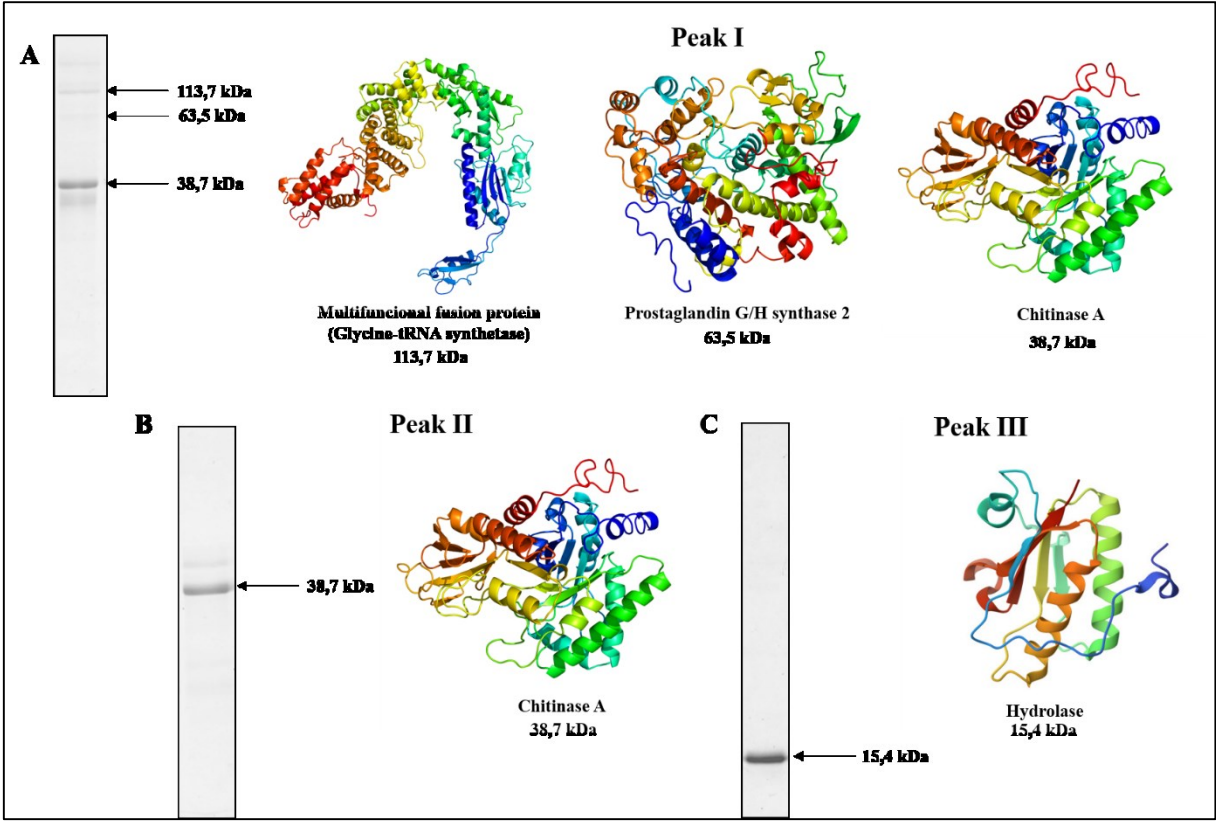


Figure S1 | Predicted Structural model from *C. revoluta*: (A) Proteins of Peak I.; (B) Peak II; (C) Peak III.

3.3 Cytotoxicity Evaluation

To assess the cytotoxicity of the samples in red blood cells, the immunohemolysis test was carried out. The test was conducted using the crude extract of the *C. revoluta* seed and the protein fractions obtained by gel filtration, and the hemolytic activity was checked at different concentrations, starting at 300 µg/ml. It was observed that the samples obtained little hemolytic activity at the concentrations analyzed, since at the highest concentration analyzed, cell viability was above 75%. (Figure 2A). The immunohemolysis test is a crucial tool in assessing the toxicity of natural compounds. It allows the detection of damage to red blood cells, indicative of hemolysis, and is one of the first experiments to test which concentration of a sample is toxic to red blood cells. In a study conducted by Chen and collaborators (2017), cytotoxicity was examined using the hemolysis assay, using various compounds derived from saponin D extracted from *Pulsatilla chinensis* roots, it was found that some of these derivatives showed no hemolytic activity ($HD_{50} > 500 \mu M$). This result suggests that both the structure of the sugar chain and the C-28 COOH group may influence the hemolytic toxicity of these compounds. In another study, the hemolysis assay was used to determine the concentration at which the methanolic extract of *Mentha spicata* leaves induces the lysis of red blood cells; this test aimed to establish safe concentrations for the subsequent investigation of its antispasmodic and antimalarial effects (Chatterjee, et. al., 2022).

The MTT test is also used to assess the cytotoxicity of samples, and to establish safe concentrations for subsequent biological analysis experiments. In this study, in addition to assessing cytotoxicity in red blood cells, its toxicity was also investigated in NIH-3T3 fibroblast cells, using the same concentration as the hemolysis assay (300 µg/ml). It was observed that Peak II and Associated Peak were significantly cytotoxic in NIH fibroblasts at a concentration of 300 µg/ml, $p < 0.05$, with almost 93% and 90% cell death, respectively. (Figure 2B). Subsequently, to assess the antitumor potential in lung and prostate cancer cells, MTT assays were carried out on non-tumor cells from these organs, starting at a concentration of 150 µg/ml, due to the toxicity observed of the protein fractions in NIH cells at a concentration of 300 µg/ml. The viability of normal lung cells (Beas-2B), and normal prostate cells (Pnt-2) in the presence of different concentrations of crude *C. revoluta* seeds and protein fractions demonstrated cell viability was over 100% for all samples when placed at 150 µg/ml (Figure 2C and 2D). Based on the data described, the remaining experiments were carried out at a concentration of 150 µg/ml for both the crude extract and the protein fractions of *C. revoluta*, ensuring that the results were not compromised by the cytotoxic effect.

El-Mekkawy and colleagues (2021) investigated the cytotoxicity of the methanolic extract of *Moringa stenopetala* leaves on normal skin fibroblast cells (BJ1). The crude methanolic

extract (E-1) and its fractions (Fr-1 to Fr-6), all at a concentration of 100 $\mu\text{g/ml}$, were tested. Of all the samples, only the Fr-5 fraction showed toxicity to normal cells at this concentration, leading to its exclusion from subsequent experiments. In another study, the MTT method was used to assess the cytotoxicity of an ethanolic extract of *Aphania senegalensis* leaves on kidney cells, revealing a toxic effect at 9.99 $\mu\text{g/ml}$. This analysis was conducted as part of the preparation to investigate the antimicrobial activity of the extract in subsequent studies. (Fall et. al., 2017).

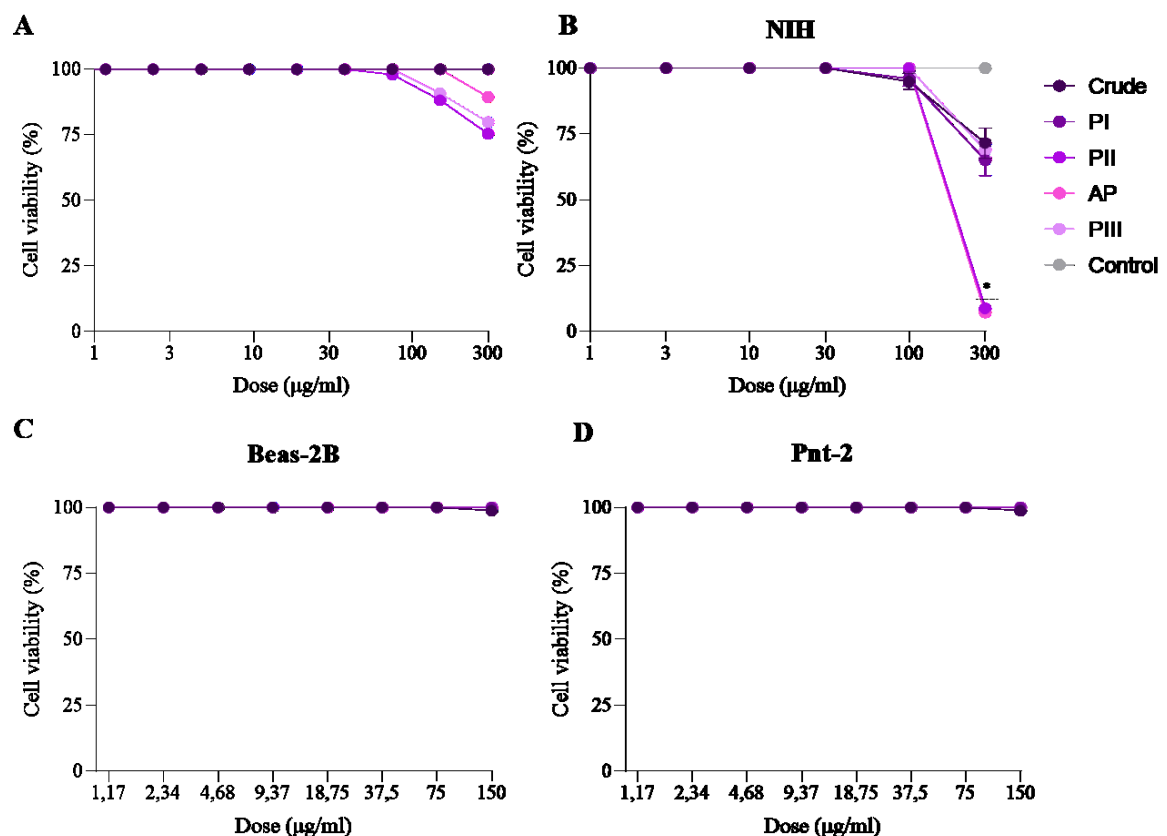


Figure 2. Cytotoxicity analysis (A) RBCs were incubated with different concentrations (0.58 to 300 $\mu\text{g/ml}$) of the crude extract of *C. revoluta* seeds and its fractions (peaks). The absorbance was measured at 540 nm and the results (mean $\pm$ SD) are reported as the percentage concentrations of crude *C. revoluta* (0.1 to 300 $\mu\text{g/mL}$) for 24 hours. (C) Beas-2B cells were cultured at concentrations of 1.17 to 150 $\mu\text{g/mL}$ for 24 hours. (D) Pnt-2. Both results were expressed as the percentage of viable cells in the control. Using ANOVA for independent samples and done in triplicate. The value of * $p < 0.05$.

3.4 Antiparasitic activity

To investigate the anti-parasitic effect, the plaque assay evaluates the ability of the *T. gondii* parasite to invade, proliferate and migrate in host cells and, as a consequence, form lysed plaques. Firstly, the protective effect was examined by observing how the *T. gondii* tachyzoites behave after a pre-treatment before infection of the host cells. The crude extract of *C. revoluta*, Pico II and Pico III showed significant results at the highest dose of protein tested, 150 µg/ml. The crude extract showed 20% lysis formation at this concentration. In comparison, Peak II and Peak III recorded 1.69% and 5% lysis, respectively, compared to the positive control (DMEM and RH WT), which resulted in 59 lysis plates (considered 100% reaction). Associated Peak at the highest concentration (150 µg/ml) showed 59.3% lysis plaque formation. Both Peak II at 75 µg/ml and Peak III at 75 µg/ml resulted in 55% and 60% lysis, respectively (Figure 3A). It should be noted that the crude extract, Peak 2 and Peak 3 at the highest concentrations tested were the most effective in reducing lysis formation in HFF host cells.

On the other hand, when analyzing the antiparasitic effect by treating already infected HFF host cells, it can be seen that the overall effect is less significant than when treating the parasite. Notably, Peak II at 150 µg/ml continues to have a high percentage of inhibition in the proliferation and migration of the parasite in the host cells, with 17% lysis formation compared to the positive control, which resulted in 60 lysis plates (100% of the reaction). Peak III at 150 µg/ml showed approximately 38% lysis plaque formation, also standing out when treating the infected cells, while the other samples recorded results equal to or greater than 50% lysis formation (Figure 3B).

It is important to note that, in both investigations mentioned above, Pico II at 75 µg/ml resulted in more lysis formation than observed in the control of the two reactions, suggesting the need for future research into why it favours infection and proliferation of the parasite. As a negative control, only the host cells were treated with DMEM medium supplemented with 10% fetal bovine serum. Representative images of the formation of lysis plaques are present in the pre-treatment of tachyzoites (Figure 3C) and in the treatment of infected cells (Figure 3D).

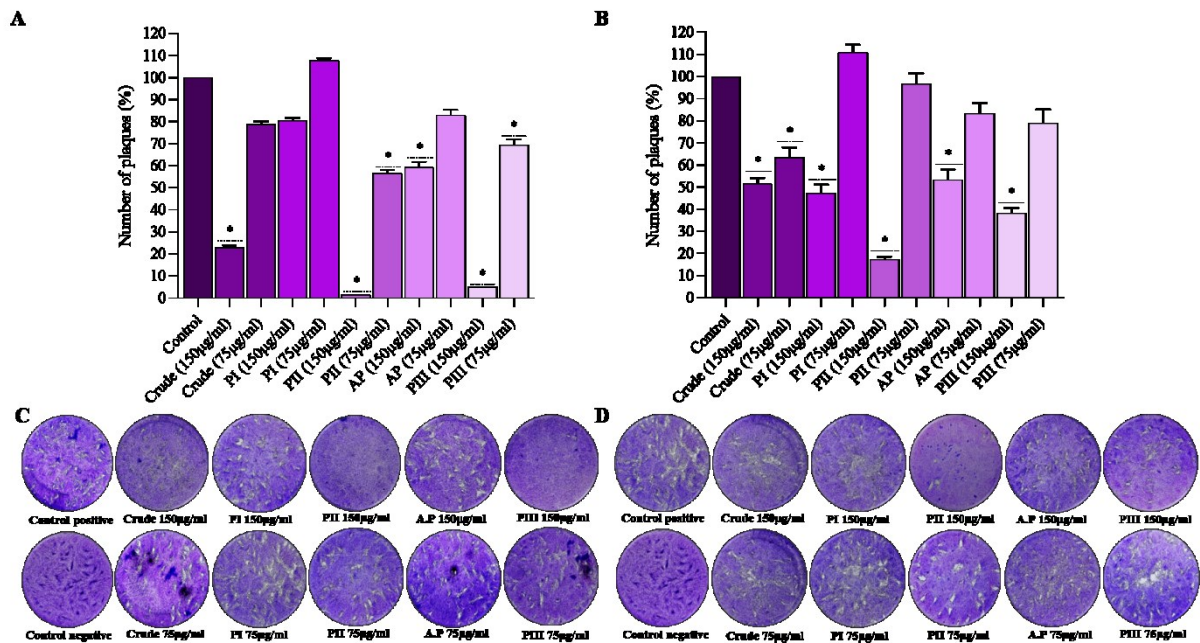


Figure 3. Effects of treatment with crude *C. revoluta* seeds and protein fractions on lysis formation by *T. gondii* strain RH WT. Plaque assay was carried out for 5 days with increasing concentrations of the crude and protein fractions of *C. revoluta* seeds (150 µg/mL and 75 µg/mL). (A) Treatment of tachyzoites before host cell infection. (B) Treatment of infected host cells. Data are expressed as the mean ± standard error of three independent experiments in triplicate, analyzed by One-way ANOVA followed by Kruskal-Wallis. P-value < 0.05.

Jiang et al. (2022) demonstrated the effect of piceatanol, a natural analogue of resveratrol and found mainly in passion fruit (*Passiflora edulis Sims*), blueberries, grapes, sugar cane, white tea and rhubarb, on acute toxoplasmosis infection. In this *in vitro* study, using the plaque assay, they analyzed the proliferation of tachyzoites and observed a significant reduction of 98.9% in the formation of lysis plaques. Other study demonstrated that the essential oil of *Lavandula angustifolia* exhibited anti-*T. gondii* activity at both analyzed concentrations of the natural compound (3,34 mg/ml and 0,83mg/ml). These results suggest that the RH strain may be inhibited from lysis formation when compared to the untreated group (Yao, et.al., 2021). Lipopeptides derived from lycosin-1 (an antimicrobial peptide) were analyzed for their effects on the invasion and proliferation of *Toxoplasma gondii* tachyzoites in host cells using a plaque assay. Infected human foreskin fibroblasts (HFF) were incubated with lycosin-I at 10 µM for 24 hours, resulting in a 55% decrease in tachyzoite proliferation rate (Liu et al., 2023).

To reassess the ability of RH strain *T. gondii* tachyzoites to invade (Figure 4A) and proliferate (Figure 4B) in host cells following treatment with *C. revoluta* protein samples, an invasion and proliferation assay was performed. As a control, the parasites were exposed only

to an incomplete DMEM medium before infecting HFF host cells. It was observed that treatment with the crude extract resulted in an invasion rate of 30% at a concentration of 150 $\mu\text{g/ml}$ and 60% at 75 $\mu\text{g/ml}$, reducing the parasite's ability to invade host cells by 70% and 40%, respectively, compared to the control. Upon analyzing the treatment with Peak I, it was found that the protein fraction at a concentration of 150 $\mu\text{g/ml}$ reduced parasite invasion by 70% (with only 30% of cells invaded). However, at a concentration of 75 $\mu\text{g/ml}$, Peak I showed a higher percentage of infected cells compared to the control. Peak II, at a concentration of 150 $\mu\text{g/ml}$, showed only 20% of the tachyzoites' capacity to invade cells, whereas at 75 $\mu\text{g/ml}$, the invasion capacity was 50%. The associated Peak showed 40% of cells invaded by *T. gondii* at a concentration of 150 $\mu\text{g/ml}$ and 65% at 75 $\mu\text{g/ml}$. Finally, Peak III exhibited 20% invasion at 150 $\mu\text{g/ml}$ and 65% at 75 $\mu\text{g/ml}$. Figure 5B represents the efficiency of parasite proliferation. The control showed 10 to 12 tachyzoites within the parasitophorous vacuole in 100 cells. The crude extract, at both concentrations analyzed, showed 6 to 8 parasites per vacuole. Peak I showed 4 to 8 parasites at a concentration of 150 $\mu\text{g/ml}$ and 10 to 12 parasites per vacuole at 75 $\mu\text{g/ml}$, matching the control. Peak II, at a concentration of 150 $\mu\text{g/ml}$, showed about 2 to 4 parasites per vacuole and about 4 to 6 parasites per vacuole at 75 $\mu\text{g/ml}$. The associated Peak and Peak III, at both concentrations, showed about 6 to 8 parasites per vacuole.

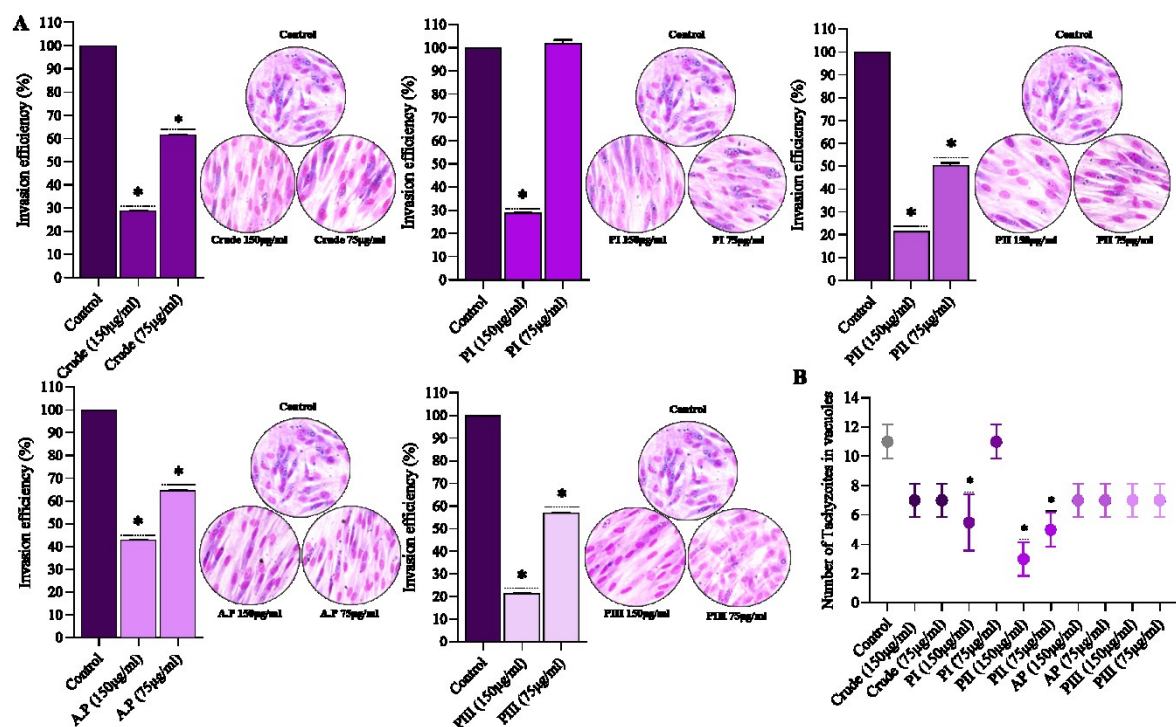


Figure 4 - Effects of treatment with the crude extract of *C. revoluta* and its purified protein fractions on the invasion and proliferation assay. The invasion and proliferation assay was carried out for 24 hours with increasing concentrations of the crude and protein fractions of *C. revoluta* seeds (150 $\mu\text{g/mL}$ and 75 $\mu\text{g/mL}$). (A)

Invasion analysis of the tachyzoites of the RH strain of *T. gondii* (average percentage of 100 invaded host cells). **(B)** Proliferation analysis of the tachyzoites of the RH strain of *T. gondii* (average number of tachyzoites present in the vacuoles in 100 host cells). Data are expressed as the mean $\pm$ standard error of three independent experiments in triplicate, analyzed by ANOVA multicomparison. P-value < 0.001.

In studies with other natural compounds against the invasion and proliferation of the RH strain of *T. gondii*, it was observed that Licarin B (isolated from *Amygdalus communis*, *Piper nigrum*, and *Lonicera japonica*) at 200 $\mu\text{g/ml}$ exhibited no parasitophorous vacuoles (100% antiparasitic effect) (Zhang, et. al., 2021). The natural phenolic compound piceatanol (100 μM) did not inhibit the intracellular invasion of *T. gondii* tachyzoites, but showed a statistically significant inhibition of parasite proliferation (98,9%), interfering with parasite replication (Jiang, et. al., 2022). Another study evaluated the susceptibility of *T. gondii* to the ethanolic extract of *Tinospora crispa*, demonstrating efficacy against parasite invasion and proliferation, in a 24-hour treatment, the extract caused a 73.47% reduction in parasite invasion of the host cell and a 53.65% reduction in tachyzoite replication (Sharif, et. al., 2019).

According to the parasite assays, the best treatment results for the purified protein fractions were: in the plaque assay, both in the pre-treatment of tachyzoites and the treatment of already infected host cells, the PII and PIII peaks stood out. In the invasion and proliferation test, both peaks PII and PIII also showed a lower percentage of invasion, indicating that the proteins involved in the anti-parasitic process are Chitinase A (present in Peak II) and Hydrolase (present in Peak III). In the literature, it is already known that chitinase induces macrophage activation, manifested by the production of high levels of pro-inflammatory cytokines. This enzyme may influence the pathogenesis of *T. gondii* infection, as it is located in the cytoplasm of the apical region of tachyzoites, suggesting its involvement in the parasite's invasion process (Almeida et al., 2015). *In vivo* studies suggested that high expression of chitinase is capable of reducing the formation of *T. gondii* tissue cysts (Nance et al., 2012). Knowing that chitinase has an anti-parasitic effect, we can understand that Peak I also showed good results when analyzed at its highest protein concentration. This is due to the presence of the Chitinase A protein, as previously observed in mass spectrometry, demonstrating its action in inhibiting parasite invasion.

3.5 Evaluation of the antitumor effect

In the context of assessing antitumor activity on the PC3 tumor cell line, only the crude extract of *C. revoluta* showed a statistically significant effect with cell mortality of 58% at a concentration of 150 $\mu\text{g/ml}$, $p < 0.05$ (Figure 5A). On the other hand, when analyzing the effect

on A549 tumor cells, none of the samples showed a significant anti-tumor effect at the concentrations analyzed (Figure 5B).

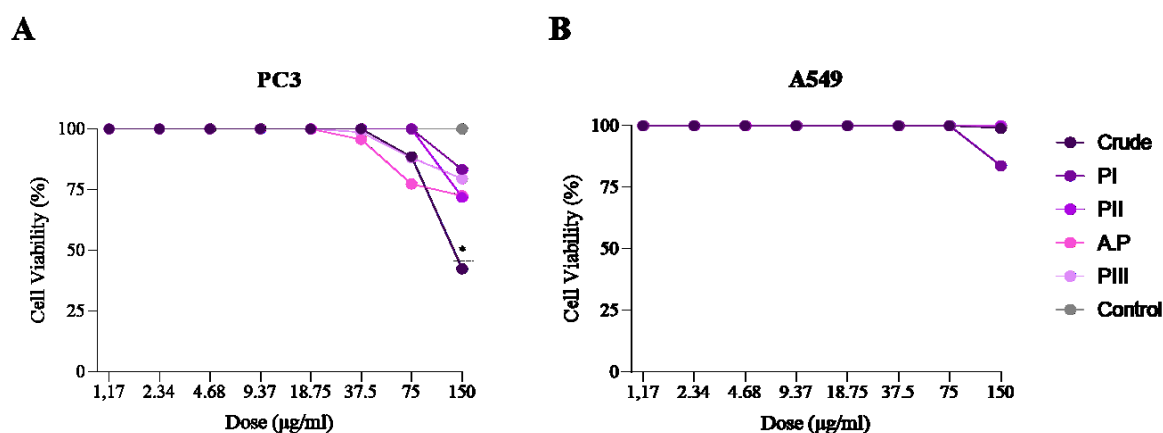


Figure 5. Effects of treatment with crude *C. revoluta* seeds and protein fractions on the inhibition of PC3 and A549 tumor cells. Cell viability was determined using the MTT assay. Tumor cells were treated for 24 hours with increasing concentrations of the crude and protein fractions of *C. revoluta* seeds (1.17, 2.34, 4.68, 9.37, 18.75, 37.5, 75 and 150 µg/mL). (A) PC3 cells. (B) A549 cells. Data are expressed as the mean ± standard error of three independent experiments in triplicate, analyzed by ANOVA. The asterisks * represent the p-value < 0.05.

The crude extract obtained from the seed of *C. revoluta* showed antitumor activity, being dose-dependent in the MTT assay on the prostate tumor line (PC3). In a study conducted by Cui and colleagues (2019), the effects of tumor inhibition by different doses of a *C. revoluta* leaf extract (5-fluorouracil) were investigated, revealing a significant reduction in the viability of gastric cancer cells (MGC-803, SGC-790, HGC-27, and SUN-5) in the MTT assay, the extract inhibited cell viability after 24 hours of treatment at 250 µg/ml. Another study also confirmed antitumor activity using the MTT assay on the HCT-8 colon cancer cell line, showing that the metabolic extracts of *C. revoluta* considerably reduced cell viability to 1000 µg/ml (Bera, et. al., 2018). The sotetsuflavone isolated from *C. revoluta*, using the HPLC column, also demonstrated a time-dependent inhibition of the growth of lung tumor cells, lineage A549, using the MTT assay (Wang et. al., 2018).

The extraction of protein fractions from chickpea seeds was analyzed against lung cancer, to analyze cellular inhibition against A549 lineage tumor cells using the MTT assay, which detected an inhibition in the fraction called CPH-V of approximately 80% at a concentration of 14.4ug/ml (Mukhamedov et al., 2021). Fraction F3 of a protein extract from the seeds of *B. hypida* was analyzed with a similar protocol for its cytotoxic effect on lung and cervical cancer cells, where dextrorubicin at 30ug/ml obtained 78% inhibition of the viability of A549 cells (lung tumor) and 61.38% inhibition of cell viability in HeLa cervical cancer cells

(Rupachandra & Sarada, 2014).

To investigate the inhibition of tumor cell proliferation and colony formation, the experiment lasted 7 days with the cells exposed to treatments with the protein fractions of *C. revoluta*. About the PC3 lineage, almost all the samples at concentrations of 150 and 75 µg/ml limited cell expansion, except for Peak III at 75 µg/ml, which showed no significant reduction in tumor growth. Compared to the control, which recorded an average of 27 colonies (considered 100%), the crude extract of *C. revoluta* seed, Peak II, and the Associated Peak stood out. The crude extract of *C. revoluta* seed showed a complete absence of tumor colonies at both concentrations analyzed (considered 100% inhibition of colony formation). Peak II and Associated Peak at 150 µg/ml showed averages of 5 colonies (representing 20% inhibition) and no colonies (100% inhibition), respectively (Figure 6A).

On the other hand, in A549 tumor cells, only the crude extract of *C. revoluta* seeds at a concentration of 150 µg/ml and Associated Peak at 150 and 75 µg/ml showed better results. The crude extract resulted in a total absence of colony formation, while Associated Peak showed no colonies at the highest concentration analyzed, representing a 100% antitumor effect compared to the control which showed 62 colonies (Figure 6B). At a 75 µg/ml concentration, Associated Peak showed only 2 colonies, demonstrating an antitumor effect of 98%. Representative images of colony formation are present for the PC3 (Figure 6C) and A549 (Figure 6D) line cells. The test was carried out in triplicate, considering a p-value < 0.05.

In both cell lines, the proteins extracted from *C. revoluta* seeds showed significant effects when the tumor cells were exposed to them for 7 consecutive days. The clonogenic assay suggests that the samples may have a predominantly preventative effect, inhibiting the proliferation of tumor cells, rather than a direct therapeutic effect observed in the MTT assays. Notably, the crude extract performed better at both concentrations tested, along with Peak II and Associated Peak at the highest concentration assessed (150 µg/ml) for the PC3 strain. For the A549 strain, the most notable results were obtained with the crude extract and Peak I at a concentration of 150 µg/ml and the Associated Peak at both concentrations analyzed. Cui and colleagues (2019) also investigated the inhibitory effect of exposing gastric cancer tumor cells to 5-fluorouracil, an extract of *C. revoluta*, for 9 days, obtaining effective prevention results (greater than 60%) against this cell line.

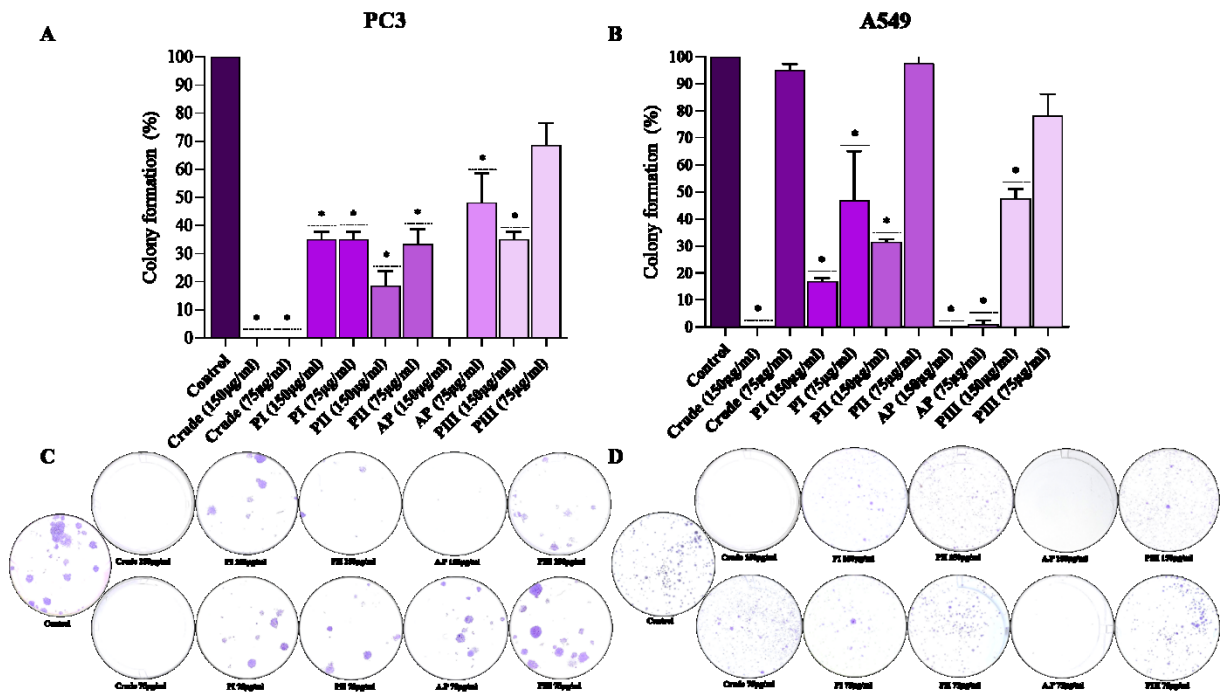


Figure 6. Effects of treatment with crude *C. revoluta* seeds and protein fractions on the clonogenic assay.

Tumor cells were treated for 7 days with increasing concentrations of the crude and protein fractions of *C. revoluta* seeds (150 µg/ml and 75 µg/ml). (A) PC3 cells. (B) A549 cells. (C) Representative figure of colonies formation. Data are expressed as the mean $\pm$ standard error of three independent experiments in triplicate, analyzed by Two-way ANOVA followed by Tuckey's test. P-value < 0.05.

Other studies employing the clonogenic assay to evaluate the antitumor effect revealed that methanolic extract of *C. revoluta* leaves inhibited the cell viability of gastric cancer cells (MGC-803, SGC-790, HGC-27 and SUN-5) by about 50% when treated at 250 µg/ml over 9 days (Cui et. al., 2019). Sotetsuflavone isolated from *C. revoluta* inhibited the invasion of lung tumor cells (A549) when treated at 128 µmol/ml (Wang et. al., 2018).

According to the antitumor tests, the antimetastatic effect observed in the colony assay for prostate (PC3) and lung (A549) tumor cells showed significant results for all the protein fractions. In the literature, it is already known that a protein compound derived from calixpirrole (Fraisinib at 10 µM), which acts as a ligand for the glycine-tRNA synthetase protein (protein present in Peak I), induced cell cycle arrest and caused apoptosis in A549 cells, with an inhibition rate of 57.4% (Toumia et al., 2023). In another study, glycyl-tRNA synthetase was shown to play an oncogenic role in prostate cancer, being associated with the induction of S-phase arrest of the cell cycle for cancer proliferation (Kish et al., 2023). Peptidases, enzymes belonging to the hydrolase class (protein present in Peak III), purified from *Eupenicillium javanicum* and *Myceliophthora thermophila*, decreased cell viability by around 40% in Cal27

cancer cells (Hamin, 2017). Chitinase A (protein present in Peak II) designated as Aconin, a similar antifungal protein from the seeds of *Acacia confusa*, inhibited the proliferation of breast cancer cells (MCF-7), showing an IC_{50} of $128 \pm 9 \mu M$ (Lam, Ng, 2010).

3.6 Antibacterial activity

Of the five *C. revoluta* seed protein samples tested at 150 to $2.34 \mu g/ml$, the best results for *E. coli* were the crude extract and Peak III with MICs equal to $4.68 \mu g/ml$. Peak I obtained an MIC of $9.37 \mu g/ml$ and Associated Peak $18.75 \mu g/ml$. The best result for *S. aureus* was Peak III with an MIC of $4.68 \mu g/ml$. The crude extract and Peak I obtained an MIC of $9.37 \mu g/ml$, while Associated Peak obtained an MIC of $37.5 \mu g/ml$. For both *E. coli* and *S. aureus* bacteria, Peak II did not inhibit the bacteria when tested at the highest concentration of $75 \mu g/ml$. For *Salmonella enterica* serovar Typhimurium bacteria all of the protein fractions and crude extract of *C. revoluta* demonstrated activity, the best result was crude extract which obtained a MIC of $4.68 \mu g/ml$, Peak II obtained a MIC of $9.37 \mu g/ml$, Peak I presented a MIC of $18.75 \mu g/ml$ and Associated Peak and Peak III obtained a MIC of $150 \mu g/ml$ (Table 2).

	<i>E. coli</i> ATCC 25922	<i>S. aureus</i> ATCC 25923	<i>Salmonella enterica</i> Typhimurium ATCC 14028
Samples ($\mu g/ml$)			
Crude	4,68	9,37	4,68
Peak I	9,37	9,37	18,75
Peak II	> 150	> 150	9,37
Associated Peak	18,75	37,5	75
Peak III	4,68	4,68	75

Table 2. Minimum inhibitory concentration (MIC) of the crude extract and seed peaks of *C. revoluta* against the pathogenic bacteria evaluated in the study.

Just as the crude extract of the seed of *C. revoluta* and its protein part purified on a Sephadex G-75 column showed activities against *E. coli*, *S. aureus*, and *Salmonella*, other extracts isolated from species of the same genus have also been evaluated; bioflavonoids isolated from the leaves of *C. circinalis* and *C. revoluta* showed antimicrobial activities against *S. aureus* (Moawad et al., 2010). Similarly, antimicrobial peptides isolated and purified from the seed of *C. revoluta*, using an HPLC column, showed the ability to inhibit the growth of phytopathogenic fungi (*Fusarium oxysporum*, *Geotrichum candidum*) presented a MIC of 7,8

µg/ml, 8,2 µg/ml and 8,0 µg/ml, and gram-positive (*Clavibacterium michiganensis*, *Curtobacterium flaccumfaciens*) MIC of 7,8 µg/ml and 8,0 µg/ml, and gram-negative bacteria (*Agrobacterium radiobacter*, *A. rizogenes*, *Erwinia carobora*) 7,3 µg/ml and 8,9 µg/ml (Yokoyama et al., 2008).

Regarding antimicrobial activity for the purified fractions of *C. revoluta*, Peak III demonstrated the greatest effect on *E. coli* and *S. aureus*, while Peak II was the most significant on *S. enterica*. *Acacia confusa* seeds have an antifungal protein similar to Chitinase A, analyzed in the Pico II protein fraction, which showed antifungal activity against *Mycosphaerella arachidicola*, *Fusarium oxysporum*, *Helminthosporium maydis* and *Valsa mali* (Lam, Ng, 2010). Purified chitinase from oat seed (*Avena chinensis*), obtained by ion exchange chromatography, exhibited potent inhibitory activity against the fungi *Panus conchatus* (MIC 1.05 µg/ml) and *Trichoderma reesei* (MIC 2.10 µg/ml) (Li et al., 2018). There have also been studies linking hydrolases (found in Pico III) with antibacterial activities, such as the lysozyme murein hydrolase from the Enterobacter phage myPSH1140, which showed strong antibacterial activity against gram-negative pathogens such as *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Citrobacter* sp. and *Acinetobacter baumannii* (with MICs from 0.007 to 0.5 mg/ml) (Ramesh et al., 2022).

4. Conclusions

Based on the results of the experiments carried out in this study, the biological activities of the crude extract and protein fractions of *C. revoluta* purified in Sephadex G-75 were identified for the first time. These activities include antitumor action against the PC3 and A549 cell lines, highlighting their ability to inhibit cell proliferation. Notably, a predominantly preventive efficacy was observed rather than a direct therapeutic effect. In addition, the samples demonstrated anti-parasitic properties against the *T. gondii* parasite, resulting in reduced invasion and subsequent intracellular proliferation compared to the control group. Inhibition of antimicrobial activity was also observed when *C. revoluta* seed proteins were challenged with three different types of bacteria: *E. coli*, *S. aureus*, and *Salmonella enterica*.

In conclusion, the purified protein fractions from *C. revoluta* show promising therapeutic potential. However, future researches are needed for a deeper understanding of the immunology and mechanisms underlying these approaches to explore their potential in the development of new therapies based on the Cycadaceae plant family.

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7. Conflict of interest

Nothing.

8. References

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