



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA



Pollonein-LAAO: uma nova L-aminoácido oxidase isolada de *Bothrops moojeni* induz estresse oxidativo em células tumorais de próstata e células endoteliais interferindo na progressão tumoral *in vitro*

Discente: Lorena Polloni

Orientadora: Profa. Dra. Veridiana de Melo Rodrigues Ávila

Co-orientadora: Dra. Tássia Rafaella Costa

Co-orientadora: Dra. Daiana Silva Lopes

UBERLÂNDIA – MG

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Aos vinte e quatro dias do mês de janeiro de dois mil e vinte e três, às 14:30 horas, reuniu-se via web conferência pela Plataforma *Cisco Webex*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Dr. Andreimar Martins Soares, Dr^a. Juliana Pavan Zuliani, Dr^a. Vivian Alonso Goulart, Dr^a. Renata Santos Rodrigues e Dr^a. Veridiana de Melo Rodrigues Ávila, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Dr^a. Veridiana de Melo Rodrigues Ávila apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

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Discente: Lorena Polloni

COMISSÃO EXAMINADORA

Presidente: Dra. Veridiana de Melo Rodrigues Ávila

Examinadores: Dr. Andreimar Martins Soares

Dra. Juliana Pavan Zuliani

Dra. Renata Santos Rodrigues

Dra. Vivian Alonso Goulart

Data da Defesa: 24/01/2023

Profª. Dra. Veridiana de Melo Rodrigues Ávila

Dedico aqueles que sempre me ensinou que o esforço, a dedicação e a determinação transformam o impossível em possível, meus amados pais, Aparecida Genoveva Silingardi Polloni e Luiz Sergio Polloni.

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“Nas horas difíceis jamais abaixe a cabeça, porque a solução para o problema não está no chão, mas sim na sua determinação.”

Autor desconhecido

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LISTA DE ABREVIATURAS

APAF-1 – fator ativador de protease apoptótica
CAKS – cinases ativadoras de cinases dependentes de ciclinas
CDKS – cinases dependentes de ciclina
CDKN1A – gene que codifica a proteína p21
CKI – inibidores de cinases dependentes de ciclina
DISC – complexo de sinalização de morte
DNA – ácido desoxirribonucleico
E2F – gene que codifica fator de ligação ao promotor E2
EMT – transição epitelial-mesenquimal
EROs/ROS – espécies reativas de oxigênio
FAD – flavina adenina dinucleotídeo
FADD – proteína adaptadora do domínio de morte FAS
FAS-L – FAS ligante
H₂O₂ – peróxido de hidrogênio
HIF1- α – fator induzível por hipóxia 1 alfa
HUVECs – célula endotelial de veia umbilical humana
IAP – proteína inibidora de apoptose
MMP – metaloproteinases de matriz
MOMP – permeabilização da membrana externa mitocondrial
OMI – proteína mitocondrial requerida em altas temperaturas (HtrA2)
P53 – proteína supressora de tumor 53
PC-3 – célula de câncer de próstata humano
PTEN – fosfatase homóloga a tensina
RB1 – proteína do retinoblastoma
svLAAO – L-aminoácido oxidase de peçonha de serpente
TME – microambiente tumoral
TNF – fator de necrose tumoral
TRADD – proteína adaptadora do domínio de morte TNF
VEGF - fator de crescimento endotelial vascular
VEGFR – receptor do fator de crescimento endotelial vascular

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APRESENTAÇÃO

O câncer é um problema de saúde mundial devido sua alta incidência e mortalidade. Apesar dos grandes avanços na compreensão da biologia e genética tumoral, pouco foi convertido em práticas clínicas, e isso se deve ao fato das células tumorais apresentarem diversas características adaptativas em relação às células normais, as quais dificultam um tratamento bem-sucedido. Entretanto, essas mesmas características adaptativas fornecem oportunidades para o design de fármacos mais efetivas para o tratamento do câncer por serem específicas e indispensáveis para as células tumorais.

Dentre essas características pode-se destacar o estado pró-oxidativo das células tumorais que favorece seu desenvolvimento e progressão através da regulação da instabilidade genômica, inflamação, angiogênese, invasão e metástase. O estado pró-oxidativo é um fenômeno observado em diversos tipos de cânceres, o qual está relacionado com a ruptura do equilíbrio entre pró-oxidantes e antioxidantes através da alta produção e eliminação de espécies reativas de oxigênio (EROs), com intuito de manter os níveis de EROs abaixo do limite citotóxico. Em comparação às células normais, as células cancerosas têm níveis mais altos de EROs e, para não serem induzidas à morte celular, elas desenvolveram mecanismos que ativam uma resposta adaptativa a partir da regulação positiva da capacidade antioxidante.

Tendo em vista que as células tumorais apresentam altos níveis de EROs endógeno, o uso de agentes exógenos capazes de acumular EROs acima do limiar citotóxico pode desencadear morte celular tumoral. Visto que, a capacidade antioxidante das células tumorais já está ativada ao máximo para manter os elevados níveis de EROs abaixo do limiar citotóxico. Sendo assim, o aumento de EROs induzido por um agente exógeno pode levar à falha do sistema antioxidante adaptativo e, consequentemente, elevação dos níveis de EROs acima do limiar citotóxico, resultando em morte celular. Dessa maneira, a dicotomia das espécies reativas de oxigênio é um interessante alvo terapêutico para impedir o crescimento tumoral, porém o papel das EROs na homeostasia redox das células tumorais não é completamente compreendido.

As L-aminoácidos oxidases isoladas de peçonhas de serpentes (svLAAOs) vem despertando interesse dos pesquisadores devido às suas ações biológicas e farmacológicas. Diversos estudos demonstraram resultados promissores da eficácia antitumoral das svLAAOs devido sua capacidade de gerar peróxido de hidrogênio como subproduto de sua

reação enzimática, promovendo estresse oxidativo e morte celular por apoptose. Assim sendo, as svLAAOs são biomoléculas ativas que podem ser utilizadas em estudos que buscam investigar a influência do estresse oxidativo na progressão tumoral.

A presente tese foi dividida em três capítulos, sendo o capítulo I destinado para revisão bibliográfica sobre a epidemiologia e etiologia do câncer, bem como explicar a dicotomia das EROs na progressão tumoral; o capítulo II tem como objetivo mostrar os resultados alcançados, em forma de artigo, dos efeitos do estresse oxidativo induzido por uma nova L-aminoácido oxidase na proliferação e invasão em células humanas de câncer de próstata *in vitro*; e o capítulo III tem o intuito de demonstrar os resultados obtidos a partir de ensaios *in vitro*, em forma de artigo, dos efeitos do estresse oxidativo gerado pela nova L-aminoácido oxidase na angiogênese tumoral utilizando célula endotelial como modelo.

Capítulo I

1. INTRODUÇÃO

1.1 Epidemiologia do câncer

O câncer é considerado um dos principais problemas de saúde pública mundial devido à sua alta incidência, prevalência, e principalmente elevada mortalidade, sendo uma das quatro principais causas de morte prematura (antes dos 70 anos) na maioria dos países. De acordo com a Organização Mundial da Saúde (do inglês – WHO, *World Health Organization*), a incidência e a mortalidade por câncer vêm aumentando rapidamente no mundo, devido ao crescimento e envelhecimento populacional, mas também pela prevalência e distribuição de fatores de riscos de câncer que estão associados ao desenvolvimento socioeconômico (Bray et al., 2018; Sung et al. 2021). O envelhecimento populacional desempenha um papel importante na incidência de câncer, sendo que o risco de ser diagnosticado com câncer antes de 40 anos de idade é de aproximadamente 2%, porém aos 80 anos esse risco aumenta para 50% (Martincorena e Campbell, 2015).

Segundo a WHO, foram relatados em 2020 cerca de 19,3 milhões de novos casos de câncer ocasionando em, aproximadamente, 10,0 milhões de óbitos por câncer. Havendo uma estimativa de que para o ano de 2040 ocorra um aumento de 47%, em relação ao ano de 2020, de novos casos de câncer, sendo esperado cerca de 28,4 milhões de novos casos. Dentre os tipos de cânceres os mais diagnosticados na população masculina são o de pulmão (14,3%), seguido pelo câncer próstata (14,1%), colorretal (10,6%), estômago (7,1%) e fígado (6,3). Entretanto, o câncer responsável pela maior mortalidade nos homens é o de pulmão (21,5%) seguido pelos cânceres de fígado (10,5%), colorretal (9,3%), estômago (9,1%) e próstata (6,8%). Em contraste com a população masculina, os cânceres mais incidentes na população feminina são de mama (24,5%), seguido pelo colorretal (9,4%), pulmão (8,4%) e cervical (6,5%). Sendo o câncer de mama (15,5%) o que lidera os casos de morte por câncer entre as mulheres, seguido pelo de pulmão (13,7%), colorretal (9,5%) e cervical (7,7%) (Sung et al., 2021).

No Brasil, conforme os dados obtidos pelo Instituto Nacional de Câncer José Alencar Gomes da Silva (INCA), a estimativa para cada ano do triênio 2020-2022 foi de cerca de 625 mil novos casos de câncer. O tipo de câncer mais frequente na população masculina foi de próstata com cerca de 65.840 novos casos, o que corresponde a um risco estimado de 62,95 novos casos a cada 100 mil homens. Nas mulheres, o câncer mais incidente foi o de

mama, sendo estimado 66.280 novos casos, o que corresponde a um risco estimado de 61,61 novos casos a cada 100 mil mulheres (INCA, 2019).

Com base nessas estatísticas é notável que apesar dos grandes avanços no conhecimento da biologia e genética do câncer, pouco foi convertido em prática clínica, pois a erradicação ou controle da doença, ainda, não foi alcançado. Em outras palavras, a complexidade celular e características evolutivas do câncer fornecem tanto barreiras como oportunidades para um tratamento bem-sucedido, tornando assim, pesquisas nessa área de extrema relevância para conter essa doença (Greaves e Maley, 2012).

1.2 Etiologia do câncer

A oncogênese é um processo microevolutivo conduzido por mutações graduais que ocorrem em uma única célula somática normal seguida pela sua expansão clonal, fenômeno conhecido como evolução clonal. A evolução clonal é um processo de seleção natural que ocorre nas etapas iniciais do desenvolvimento tumoral, que é caracterizada pela competição entre os clones. Os clones selecionados durante a carcinogênese são aqueles que acumulam mutações que permitem sua sobrevivência e proliferação (Greaves e Maley, 2012). Essa seleção favorece a formação de uma população sub clonal heterogênea, ou seja, população de células geneticamente distintas (Burrell et al., 2013), permitindo que as células cancerosas sobrevivam em um microambiente tumoral inóspito, o qual apresenta baixo nível de oxigênio, escassez de nutrientes e com barreiras naturais ao crescimento impostas pelo tecido adjacente (Alberts et al., 2010).

As mutações associadas à evolução clonal podem ser classificadas como passageiras (do inglês, *passengers*) e condutoras (*drivers*). As mutações passageiras são consideradas neutras, visto que não conferem vantagens para proliferação tumoral. Desse modo, essas mutações não resultam em alterações fenotípicas ou em efeitos biológicos que favorecem a seleção de clones. Já as mutações condutoras são as mais relevantes e observadas nas neoplasias por estarem relacionadas com alterações em genes que regulam processos celulares, que são essenciais para aquisição de características que induzem a proliferação celular e o crescimento tumoral (Greaves e Maley, 2012; Martincorena e Campbell, 2015; Aparisi et al., 2019). Essas mutações estão relacionadas com a seleção positiva de clones, que por sua vez precisam acumular diversas mutações condutoras para promoverem o surgimento de um câncer (Martincorena e Campbell, 2015). Entretanto, as mutações

condutoras não são necessárias para a manutenção do câncer, mas sim foram essenciais em algum momento durante a evolução tumoral (Aparisi et al., 2019).

As mutações condutoras ocorrem em dois tipos de genes conhecidos como proto-oncogenes e genes supressores tumorais. As células normalmente possuem proto-oncogenes os quais exercem diversas funções como fatores de crescimento (p.ex. *SIS* e *FGF5* que codifica fator de crescimento derivado de plaquetas e fator de crescimento de fibroblasto-5, respectivamente), transdutores de sinal (p.ex. *RAS* e *CDK4*), fatores anti-apoptóticos (p.ex. *BCL2*) e fatores de transcrição nuclear (p.ex. *MYC*, *JUN*, *FOS*). Desse modo, são fundamentais na regulação da diferenciação e proliferação celular. Vale ressaltar que a expressão de proto-oncogenes é regulada pelas células de modo que, geralmente, apresentam baixos níveis de expressão, mas em eventos específicos, como crescimento e replicação celular, podem ser fortemente induzidos (Kontomanolis et al., 2020).

O motivo pelo qual as células exercem um controle rigoroso da expressão de proto-oncogenes é pelo fato desses genes serem capazes de induzir a proliferação descontrolada da célula, colaborando para o surgimento de um tumor (Shen et al., 2018). Entretanto, mutações podem ocorrer nos proto-oncogenes levando a sua ativação constitutiva, ou seja, transformando-os em oncogenes. O ganho de função dos oncogenes ocorre de forma dominante (Wang et al., 2018), a partir de diferentes alterações genéticas como translocação cromossômica, mutação pontual, amplificação gênica e incorporação da região promotora de um gene próximo ao proto-oncogene, levando a expressão desregulada do mesmo (Kontomanolis et al., 2020).

Os genes supressores tumorais (p.ex. *RBI*, *P53*) são considerados cruciais para homeostasia celular, sendo responsáveis pelo reparo do DNA, inibição da divisão celular e indução da apoptose (Wang et al., 2018; Kontomanolis et al., 2020), isto é, esses genes protegem as células e evitam o desenvolvimento tumoral. Diferentemente dos oncogenes, os genes supressores tumorais quando sofrem mutações são inativados, contribuindo para o surgimento de um tumor (Kontomanolis et al., 2020). A perda da função dos genes supressores tumorais é associada a eventos genéticos recessivos (Wang et al., 2018), ou seja, é necessário inativação dos dois alelos desses genes ou um evento dominante negativo para inativá-los permanentemente (Shen et al., 2018; Kontomanolis et al., 2020). A inativação desses genes é comumente associada a eventos citogenéticos como a microdeleção. Entretanto, a metilação da região promotora também contribui para a regulação dos genes supressores tumorais (Wang et al., 2018).

Mutações que favorecem o ganho de função de oncogenes e a perda de função de genes supressores tumorais são necessárias para que uma célula normal dê origem a uma célula cancerosa (Shen et al., 2018), visto que o acúmulo dessas alterações contribui para instabilidade genômica, podendo causar desregulação da divisão celular e desequilíbrio entre o crescimento e morte celular, favorecendo assim o surgimento do câncer (Shen, 2011). A instabilidade genômica é um fenômeno que facilita que as células malignas passem pelas barreiras estabelecidas pelo organismo e desenvolvam características do câncer (Andor et al., 2017). Todos os cânceres desenvolvem diversas características que são essenciais para atribuir às células tumorais vantagens consideradas adaptativas perante as células normais. Essas características são conhecidas na oncologia molecular como marcas do câncer (do inglês, *Hallmarks of cancer*) (Hanahan e Weinberg 2000; Hanahan e Weinberg, 2011).

As marcas do câncer podem ser divididas em dois grupos, sendo: i) alterações celulares intrínsecas que tornam a célula autônoma em uma determinada função, como sustentação da sinalização proliferativa, evasão de supressores de crescimento, resistência à morte celular programada, imortalidade replicativa e reprogramação do metabolismo energético; ii) alterações que dependem da comunicação das células tumorais com as células normais do microambiente tumoral, culminando na invasão das células cancerosas em tecidos adjacentes, sua disseminação e metástase. Sendo esses eventos associados às marcas de instabilidade genômica, inflamação promovida pelo tumor, evasão da destruição imune, indução de angiogênese e ativação de invasão e metástase (**Figura 1**) (Hanahan e Weinberg, 2011; Welch e Hurst, 2019).

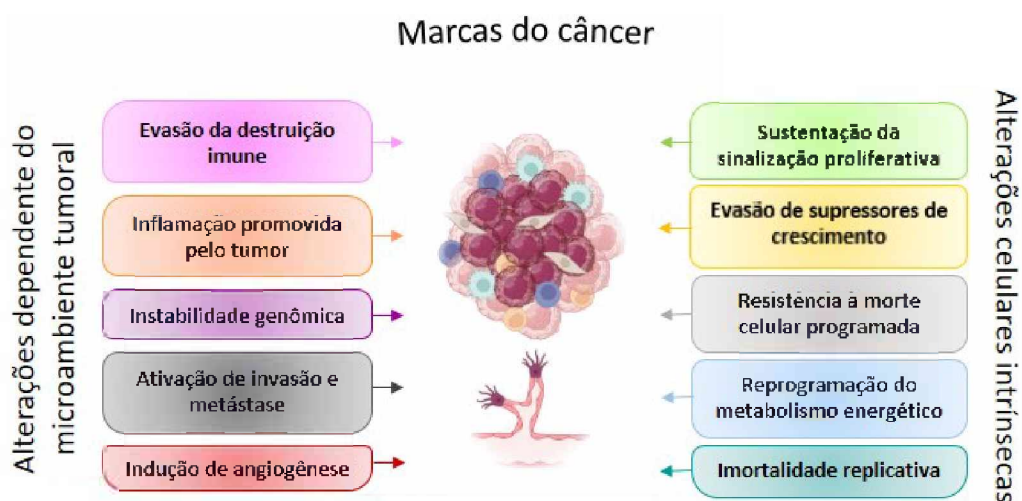


Figura 1. Marcas do câncer. Fonte: autoria própria, criado em BioRender.com

Como descrito até o momento, a iniciação tumoral é baseada em uma série de mutações que ocorrem em uma célula normal, resultando em hiperplasia, proliferação descontrolada e resistência à morte celular. Contudo, a progressão tumoral (invasão, disseminação e metastização) é fortemente influenciada pelo microambiente tumoral (TME). O TME é constituído pelas células tumorais, matriz extracelular, e células não-tumorais como células estromais (como fibroblastos, pericitos, células endoteliais), vasos sanguíneos e linfáticos, bem como células do sistema imune (linfócitos B e T, natural killer e macrófagos associados ao tumor) (Roma-Rodrigues et al., 2019). A comunicação entre as células tumorais e não-tumorais no TME é conduzida por uma rede dinâmica de citocinas, quimiocinas, fatores de crescimento e fatores inflamatórios que favorecem a progressão tumoral através da remodelagem da matriz extracelular (Balkwill et al., 2012). Além disso, as células tumorais utilizam as células não-tumorais como suporte de nutrientes e defesa contra a atividade do sistema imune, a partir da capacidade adquirida de recrutar, incorporar e reprogramar as células não-tumorais, contribuindo para o fenótipo tumoral (Jiang et al., 2015).

A interação entre as células do estroma e células cancerosas no microambiente tumoral leva a formação de novos vasos sanguíneos e linfáticos, aparecimento de regiões necróticas e metástase. Esses fenômenos estão interligados a hipóxia, estresse oxidativo e acidose em resposta às alterações do metabolismo das células cancerosas que está associado ao aumento do volume da massa tumoral e remodelagem do TME (Roma-Rodrigues et al., 2019). Desse modo, a progressão tumoral não depende apenas das mutações genéticas e alterações epigenéticas, mas também da rede bidirecional e dinâmica de interações entre as células do estroma e as células cancerosas dentro do microambiente tumoral (Jiang et al., 2015).

A compreensão dos mecanismos envolvidos na progressão tumoral permite o desenvolvimento de estratégias terapêuticas capazes de atuar em estágios evolutivos tumoral específicos, favorecendo assim o desenvolvimento de estratégias terapêuticas mais eficazes para o tratamento do câncer (Quail e Joyce, 2013; Roma-Rodrigues et al., 2019; Rafaeva e Erler, 2020; Neophytou et al., 2021a).

1.3 Desregulação da divisão celular e células tumorais

O ciclo celular eucariótico compreende duas fases sendo elas interfase (subdividida em G1, S e G2) e mitose (fase M) que consistem, basicamente, em períodos de replicação do DNA seguido da sua segregação em duas células-filhas geneticamente idênticas, respectivamente. Dessa maneira, a função básica do ciclo celular é de duplicar e segregar, com extrema precisão, o DNA genômico da célula parental para as células-filhas (Shen, 2011; Matthews, et al., 2021). A estabilidade genômica durante a divisão celular é mantida devido a existência de um rigoroso sistema de controle que regula a progressão da célula pelo ciclo celular. O principal componente do sistema de controle do ciclo celular são as enzimas denominadas como cinases dependentes de ciclinas (CDKs), as quais necessitam da interação com ciclinas para serem ativadas e, assim, exercerem sua função de fosforilar diversos alvos proteicos da maquinaria do ciclo celular (Morgan, 2007), podendo os mesmos serem ativados ou inativados (p.ex. Rb, p53, E2F, fosfatase Cdc25A, p27, CKI, entre outros) (Donjerkovic e Scott, 2000).

O controle da progressão do ciclo celular está correlacionado com a concentração de ciclinas, que por sua vez podem oscilar durante o ciclo celular através da sua regulação a nível gênico ou proteico (proteólise) (Morgan, 2007). É importante ressaltar que para estabilização e ativação completa do complexo CDKs-ciclina é necessário a fosforilação do mesmo por enzimas denominadas como cinases ativadoras de CDKs (CAKs). As CAKs têm como alvo o resíduo de treonina localizado próximo ao sítio ativo das CDKs, que é exposto após a mudança conformacional induzida pela ligação com ciclinas (Morgan, 2007; Pennycook e Barr, 2020).

O sistema de controle regula a transição das fases do ciclo celular em pontos de transição conhecidos como pontos de checagem (do inglês, *checkpoints*) (Morgan, 2007). O primeiro ponto de checagem G1 é considerado o ponto determinante o qual as células são direcionadas de maneira irreversível para divisão celular, sendo controlado pelos complexos CDK2-ciclina E e CDK4/CDK6-ciclina D, as quais sinalizam a transição da fase G1 para S. Essa transição ocorre somente se a célula ao final da fase G1 apresentar tamanho e disponibilidade de nutrientes adequados e integridade do DNA. O segundo ponto de checagem G2 verifica a integridade do DNA após sua replicação na fase S, garantindo assim, a fidelidade da replicação do DNA antes da sua divisão na fase M. Os complexos CDK2-ciclina A, CDK1-ciclina A e CDK1-ciclina B são responsáveis em direcionar a célula de G2

para mitose (fase M) (Bertoli et al., 2013; Kashyap et al., 2021). Por fim, o ponto de checagem do fuso mitótico é responsável pela verificação da ligação correta dos microtúbulos do fuso as cromátides irmãs, direcionando as células da metáfase para anáfase através da ativação do complexo promotor da anáfase/ciclossomo (APC/C) (Matthews, et al., 2021).

A atividade dos complexos CDKs-ciclinas durante o ciclo celular é regulada pelos inibidores de cinases dependentes de ciclinas (CKI). Existem duas classes de CKIs, a primeira composta pelas proteínas p21, p27 e p57 (membros da família Cip/Kip) que apresentam capacidade de se ligar e inibir todos os complexos de CDKs-ciclinas. Em contraste, o segundo grupo de proteínas membros da família INK4 (p15, p16, p18 e p19) são inibidores específicos dos monômeros CDK4 e CDK6, prevenindo a formação dos heterodímeros CDK4/CDK6-ciclina D (Donjerkovic e Scott, 2000; Malumbres, 2014; Pennycook e Barr, 2020). De modo geral, os CKIs da família Cip/Kip bloqueiam a progressão do ciclo celular através da inibição das CDKs presentes na transição entre as fase G1 e S e entre S e G2, mas dependendo da situação podem ativar as CDKs durante a fase G1. Entretanto, os CKIs da família INK4 são estritamente inibidores das CDKs presentes na fase G1 (Morgan, 2007).

Os pontos de checagem dependem de vias de sinalização que monitoram danos no DNA durante a interfase, perda da integridade do replissomo (estresse replicativo) durante a fase S e erros na formação dos fusos mitóticos durante a fase M, para regular transição entre as fases do ciclo celular. Sendo assim, os pontos de checagem são considerados cruciais para impedir o acúmulo e a propagação de erros genéticos durante a divisão celular (Kashyap et al., 2021; Matthews, et al., 2021), pois interrompem o ciclo celular em resposta a danos no DNA ou a replicação incompleta do DNA (Bertoli et al., 2013). Desse modo, alterações na funcionalidade dos sistemas de controle do ciclo celular podem resultar em modificações genéticas nas células-filhas, que por sua vez podem desencadear desregulação da divisão celular, desequilíbrio entre o crescimento e morte celular, e câncer (Shen, 2011).

A sustentação da sinalização proliferativa que impulsiona a divisão celular descontrolada é uma das características do câncer que é considerada essencial para o crescimento tumoral (Matthews, et al., 2021). Essa proliferação descontrolada é proveniente de mutações de ganho de função (ativação de oncogenes) e de perda de função (inibição de genes supressores tumorais, como p53 e Rb) (Kashyap et al., 2021), os quais impedem a saída das células tumorais do ciclo celular favorecendo sua divisão contínua (Matthews, et

al., 2021). Dessa maneira, podemos inferir que o câncer está fortemente correlacionado com a regulação da maquinaria responsável pela regulação do ciclo celular. Visto que produtos de oncogenes (como CDK 1, 2, 3, 4, 6 e ciclinas D, E e A) e genes supressores tumorais (como p53 e Rb) promovem ou inibem a progressão do ciclo celular, respectivamente (Gordon et al., 2018).

A proliferação desregulada característica das células tumorais está diretamente relacionada com mutações ou desregulação de proteínas envolvidas no sistema de controle do ciclo celular responsável pela transição da fase G1 para S. Visto que a decisão celular de transição da fase G1 para a fase S do ciclo celular é considerada crítica para o desenvolvimento normal das células, por ser o ponto crucial em que as células são direcionadas de maneira irreversível para entrar no ciclo celular, iniciar a replicação do DNA e se dividir, ou sair do ciclo celular e entrar em quiescência (fase G0), senescência ou morte celular (Rubin et al., 2020). Desse modo, alterações no sistema de controle da transição G1-S é considerado uma das características do câncer por permitir que as células progridam para a fase S de forma irrestrita (Bertoli et al., 2013; Rubin et al., 2020).

O evento mais crítico que marca a transição da fase G1 para S é a ativação do fator de transcrição E2F que estimula a expressão de genes que impulsionam a progressão irreversível do ciclo celular (Bertoli et al., 2013; Rubin et al., 2020). Essa capacidade do fator de transcrição E2F impulsionar a entrada das células na fase S é restringida pela proteína do retinoblastoma (Rb), que forma complexo repressivo com E2F nas regiões promotoras de genes essenciais para a transição da fase G1 para S (p.ex. *CDK2* – cinase dependente de ciclina 2; *CCNE1* – ciclina E) e para a replicação do DNA (p.ex. *POLA1* – DNA polimerase α) (Stevaux e Dyson, 2002; Engeland, 2022). Essa repressão é aliviada por fosforilação sucessivas, em sítios específicos presentes na estrutura da Rb, realizadas primariamente pelos complexos CDK4/6-ciclina D e mais tardiamente pelo heterodímero CDK2-ciclina E. A hiperfosforilação mediada pelas CDKs levam à mudanças conformacionais na estrutura da Rb desencadeando sua dissociação de E2F (Rubin et al., 2020). Desse modo, a Rb é considerada a reguladora central do ciclo celular, de modo que sua hiperfosforilação é um evento crucial que precede a síntese de DNA (Engeland, 2022).

Em casos de dano no DNA, a transição da fase G1 para S é interrompida através da ativação da transcrição de genes que codificam CKIs, ocasionando na inativação dos complexos CDKs-ciclinas e, conseqüentemente, a hipofosforilação de Rb e formação do complexo repressor Rb-E2F (Rubin et al., 2020; Engeland, 2022). O aprisionamento da

célula no ponto de checagem G1 é devido à sinalização de quebras na dupla fita do DNA, o qual gera a sinalização pela ataxia telangiectasia mutada de proteína cinase (ATM). O fator de transcrição p53 é um dos alvos fosforilados e ativados pela ATM (Matthews, et al., 2021), que então se liga a elementos presentes na região promotora do gene *CDKN1A* induzindo assim a codificação da proteína p21. A p21, uma das CKIs, inibe todos os heterodímeros de CDKs-ciclinas que estão envolvidos na hiperfosforilação da Rb. Em suma, todos esses eventos reprimem a transcrição de genes envolvidos na transição da fase G1 para S através da inibição da atividade do fator de transcrição E2F pela proteína Rb (Engeland, 2022). Dependendo da severidade do dano ao DNA, as células podem reentrar ou sair permanentemente do ciclo celular e serem induzidas à apoptose. Desse modo, na fase pré-replicativa (G1), se a célula não for destinada à morte por apoptose, a sua progressão pelo o ciclo celular pode ser interrompida de modo reversível (quiescência) ou irreversível (senescência), sendo todos esses eventos regulados pelo guardião do genoma, p53 (Matthews, et al., 2021; Engeland, 2022).

Devido a importância da proteína do retinoblastoma (Rb) e o fator de transcrição p53 no controle da transição do ciclo celular da fase G1 para S, eles são comumente encontrados mutados em diversos tipos de cânceres, estando assim, diretamente relacionados com o desenvolvimento tumoral (Bertoli et al., 2013; Gordon et al., 2018; Rubin et al., 2020; Engeland, 2022). Visto que esses supressores tumorais mutados comprometem a capacidade da célula de sair do ciclo celular na fase pré-replicativa em resposta a danos no DNA, favorecendo assim, o acúmulo e a propagação de erros genéticos durante a divisão celular (instabilidade genômica) e, o surgimento do câncer. É importante ressaltar que o sistema de controle dos outros pontos de checagem (estresse replicativo do DNA e formação do fuso mitótico) raramente são encontrados mutados nas células tumorais, pelo fato de serem considerados vitais para a progressão tumoral por prevenir níveis catastróficos de danos no DNA (Matthews, et al., 2021).

A dependência das células tumorais de regular o sistema de controle do ciclo celular oferece oportunidade terapêutica por apresentar mutações específicas que são indispensáveis para a progressão tumoral, mas dispensáveis para as células saudáveis (Matthews, et al., 2021).

1.4 Evasão da apoptose por células tumorais

A morte celular é considerada um processo crucial no controle do equilíbrio entre a proliferação e a eliminação de células, mantendo assim a homeostasia celular (Koff et al. 2015; Jan e Chaudhry, 2019). A apoptose é um mecanismo de indução de morte celular altamente regulada, que em resposta a uma sinalização exógena ou endógena desencadeia reprogramação genética a qual induz a eliminação de células indesejáveis ou desnecessárias. Dessa maneira, a apoptose atua tanto em células durante o desenvolvimento celular normal e morfogênese, quanto em células danificadas (Jan e Chaudhry, 2019; Westaby et al., 2021). Células apoptóticas apresentam algumas alterações em suas características morfológicas e bioquímicas. As alterações morfológicas incluem condensação da cromatina, fragmentação do DNA, redução do volume celular (picnose), formação de bolhas citoplasmáticas e corpos apoptóticos. E as principais alterações bioquímicas incluem a externalização de fosfatidilserina e a ativação de caspases (Wong, 2011; Jan e Chaudhry, 2019).

O mecanismo de indução de apoptose consiste em duas vias centrais: a extrínseca e a intrínseca. A via de apoptose extrínseca é ativada pela sinalização extracelular produzida por células T citotóxicas do sistema imune em resposta a células que foram infectadas ou sofreram algum dano (Pfeffer e Singh, 2018). A sinalização apoptótica de ligantes extracelulares como TNF (fator de necrose tumoral) e Fas-L (ligante de Fas), são reconhecidos pelo domínio extracelular de seus respectivos receptores, sendo eles receptor de TNF (TNFR1 e TNFR2) e receptor Fas, os quais estão localizados na membrana celular e são conhecidos como receptores de morte. Essa interação entre ligante-receptor de morte ocasiona uma mudança conformacional que induz o recrutamento de proteínas adaptadoras (TRADD – domínio de morte associado ao receptor TNF; FADD – domínio de morte associado a Fas) pela porção intracelular do receptor (Koff et al., 2015; Jan e Chaudhry, 2019; Carneiro e El-Deiry, 2020; Chota et al., 2021).

Desse modo, a interação ligante-receptor-proteína adaptadora, conhecido como complexo de sinalização de indução de morte (DISC), recruta caspases iniciadoras (pró-caspase -8 e -10), as quais são ativadas de modo que seus pró-domínios permanecem associados ao DISC, enquanto a caspase iniciadora ativa dissocia do DISC e inicia uma cascata de ativação de caspases executoras (caspases -3, -6 e -7) (Koff et al., 2015; Jan e Chaudhry, 2019; Carneiro e El-Deiry, 2020; Chota et al., 2021). Vale ressaltar que as caspases -8 e -10, também, podem clivar e ativar o fator pró-apoptótico BID que conecta a

via de apoptose extrínseca com a intrínseca através da ativação de Bax e Bak (Koff et al., 2015; Carneiro e El-Deiry, 2020; Westaby et al., 2021).

A via de apoptose intrínseca é desencadeada em resposta a estímulos internos como danos no DNA, estresse oxidativo, aumento na concentração de Ca^{2+} citosólico, privação de fatores de crescimento e citocinas (Wong, 2011; Pfeffer e Singh, 2018), os quais ocasionam a permeabilização da membrana externa mitocondrial (MOMP) e a liberação do citocromo *c* do espaço intermembranar mitocondrial para o citosol. A MOMP e a liberação do citocromo *c* é considerado o ponto determinante da indução da apoptose, ou seja, é o ponto que a célula é irreversivelmente direcionada para morte celular e pode ocorrer independente da ativação de caspases (Carneiro e El-Deiry, 2020). O extravasamento do citocromo *c* é regulado pela família BCL-2, a qual é composta por proteínas anti-apoptóticas (Bcl-2, Bcl-xL, Bcl-W, Mcl1, Bcl-B e Bcl-A1); pró-apoptóticas (Bax, Bak); e regulatórias (Bad, Bik, Bid, HrK, Bim, BMF, NOXA e PUMA) que inibem proteínas anti-apoptóticas e ativam as pró-apoptóticas (Koff et al., 2015; Chota et al., 2021).

Em suma, as proteínas anti-apoptóticas regulam a apoptose por meio da interação com proteínas pró-apoptóticas Bax e Bak, impedindo assim a liberação de citocromo *c*. Entretanto, mediante um estímulo apoptótico interno, o efeito das proteínas anti-apoptóticas é neutralizado pelas proteínas regulatórias, as quais se ligam diretamente a Bax-Bak, liberando-as da inibição. Subsequente a esse evento, Bax-Bak, os efetores centrais da apoptose intrínseca, sofrem mudanças conformacionais e oligomerização, culminando na formação de poros na membrana mitocondrial externa. Essa permeabilização desencadeia perda da função mitocondrial a qual induz morte celular a partir da liberação de proteínas intermembranar como citocromo *c*, SMAC (segundo ativador de caspases derivado da mitocôndria) e Omi. O citocromo *c*, por sua vez, estimula a formação do apoptossomo através da sua interação com APAF-1 (fator ativador de protease apoptótica) e pró-caspase-9. Dentro do apoptossomo a pró-caspase-9 é convertida em caspase-9 que adquire capacidade de ativar caspases executoras (caspases -3, -6 e -7). Além disso, Omi e SMAC inibem a atividade de inibidores endógenos de caspases como XIAP (inibidor de apoptose ligado ao X) e IAP (proteína inibidora da apoptose), respectivamente (Matsuura et al., 2016; Pfeffer e Singh, 2018; Carneiro e El-Deiry, 2020).

Apesar das vias de apoptose extrínseca e intrínseca serem induzidas em resposta a diferentes estímulos celular, ambas as vias culminam na ativação de caspases executoras (caspases -3, -6 e -7) (Matsuura et al., 2016), que são responsáveis pela indução da

degradação de proteínas cinases, proteínas do citoesqueleto, proteínas de reparo do DNA e da subunidade inibitória de endonucleases. Uma vez ativadas, as endonucleases degradam o material nuclear ocasionando a condensação da cromatina, formação de bolhas citoplasmáticas e corpos apoptóticos (Wong, 2011; Jan e Chaudhry, 2019). É importante destacar que ao final do processo de apoptose os restos celulares são reconhecidas e fagocitados por macrófagos, sendo esse processo mediado pela externalização do fosfolípido fosfatidilserina na membrana plasmática das células apoptóticas que funciona como um sinalizador de destruição celular (Kourtzelis et al., 2020).

Em contraste com as células normais que dependem da apoptose para manter a homeostasia celular, as células tumorais desenvolveram mecanismos que impedem a ativação das vias apoptóticas para sustentar danos no DNA, favorecendo assim, sua sobrevivência e eventualmente formação do câncer. Além disso, a capacidade de evitar a morte celular por apoptose é crucial durante a tumorigênese, visto que as células tumorais estão sob constante sinalização de estresse (instabilidade genômica, privação de nutrientes, hipóxia) o que estimularia a ativação da via apoptótica intrínseca (Fernald e Kurokawa, 2013; Westaby et al., 2021). Sendo assim, as células tumorais podem se adaptar para escapar desses estímulos de morte celular a partir de modificações da sua maquinaria intrínseca de apoptose, seja pela regulação positiva de fatores anti-apoptóticos, e/ou pela regulação negativa de fatores pró- apoptóticos da família BCL-2, ou, ainda, pela redução da atividade de caspases (Wong, 2011; Sharma et al., 2019; Westaby et al., 2021).

O fator de transcrição p53 modula a expressão de genes que induz a apoptose em resposta à sinalização de estresse celular. Devido esse fato, o gene *TP53* que codifica o supressor tumoral p53 é o mais frequente encontrado inativo ou mutado em diferentes tipos de cânceres, apresentando uma taxa de mutação em torno de 50%. Desse modo, genes como *BAX*, *PUMA*, *NOXA* e *APAF-1*, que são regulados positivamente pelo p53, são afetados em células tumorais, reduzindo assim a sinalização apoptótica intrínseca através da redução da probabilidade de oligomerização de Bax com Bak, da não formação do apoptossomo e da inibição da ativação de proteínas pró-apoptóticas (Fernald e Kurokawa, 2013; Sharma et al., 2019). Além da estratégia de reduzir a expressão de genes pró-apoptóticos, as células tumorais podem, também, aumentar a expressão de genes que codificam proteínas anti-apoptóticas, como no caso do gene *BCL-2* que através da translocação passa apresentar uma expressão constitutiva, e os genes *BCL-X* e *MCL-1* que são amplificados nas células tumorais (Fernald e Kurokawa, 2013).

A via fosfatidilinositol-3 cinase (PI₃K)/Akt é frequentemente hiperativada em células malignas e está envolvida na ativação de fatores anti-apoptóticos como Bcl-2, Bcl-xL e IAPs (Survivina e XIAP), através da fosforilação de CREB (proteína de ligação ao elemento de resposta ao AMP cíclico) e IκB cinase (IKK), regulador positivo de NFκB. Além disso, Akt fosforila diretamente a proteína pró-apoptótica Bad no resíduo de serina¹³⁶, dificultando assim sua interação com Bcl-xL, favorecendo sua função antiapoptótica. Outro substrato da Akt é a pró-caspase-9, que através da fosforilação do resíduo de serina¹⁹⁶ induz sua inativação. Vale ressaltar que as IAPs são frequentemente superexpressas em cânceres humanos auxiliando na evasão da apoptose, pelo fato de bloquear a atividade de caspases. As células tumorais regulam as IAPs, como a Survivina, a partir da ativação de vias que modulam a expressão do NFκB (Neophytou et al., 2021b).

Embora o mecanismo mais comum mutado nas células tumorais seja da via apoptótica intrínseca, as células tumorais também apresentam mutações na via apoptótica extrínseca, sendo essas alterações relacionadas com a diminuição da expressão de receptores de morte (Wong, 2011). Como descrito nessa seção, a apoptose depende da ativação de vias de sinalização distintas as quais são frequentemente desreguladas no câncer, de modo a favorecer a sobrevivência das células malignas no microambiente tumoral hostil. Dessa maneira, a busca por drogas que têm capacidade de restaurar as vias apoptóticas nas células tumorais, ou seja, induzir apoptose em células tumorais sem interferir na fisiologia das células não-tumorais, tem grande potencial de inibir a progressão tumoral. Sendo assim, a indução da apoptose em células tumorais é uma das estratégias terapêuticas mais desejadas para o tratamento do câncer (Wong, 2011; Chota et al., 2021).

1.5 Angiogênese tumoral

Durante o aumento do volume da massa tumoral os nutrientes e oxigênio que as células tumorais necessitam são adquiridos de vasos sanguíneos próximos. Essa fase avascular é predominante até que a massa tumoral inicial alcance um estágio caracterizado pelo balanço entre a proliferação e apoptose, restringindo assim seu crescimento até 1-2 mm³ (Zuazo-Gaztelu e Casanovas, 2018; Saman et al., 2020). Para superar esse estágio de latência, caracterizado por um microambiente com baixo suprimento de nutrientes e de hipóxia, e sustentar uma proliferação ilimitada, as células tumorais liberam fatores que estimulam a formação de sua própria rede vascular, isto é, formação de novos vasos

sanguíneo a partir de uma vasculatura pré-existente. Esse fenômeno é conhecido como angiogênese tumoral (Zuazo-Gaztelu e Casanovas, 2018; Teleanu et al., 2019; Al-Ostoot et al., 2021).

A angiogênese tumoral é induzida pelo desequilíbrio entre fatores estimulatórios (pró-angiogênicos) e inibitórios (anti-angiogênicos), prevalecendo o estado pró-angiogênico, fenômeno conhecido como “interruptor angiogênico” (do inglês – “*angiogenic switch*”). Os mediadores pró-angiogênicos incluem fator de crescimento de fibroblastos (FGF), fator estimulador de colônia de granulócitos (G-CSF), interleucina-8 (IL-8), fator de transformação do crescimento alfa e beta (TGF- α e TGF- β), fator de crescimento endotelial vascular (VEGF) (Saman et al., 2020), angiopoetina-2 (Ang-2) e fator de crescimento derivado de plaquetas (PDGF). O ambiente pró-angiogênico favorece a quebra da dormência das células endoteliais dos vasos sanguíneos, através da interação dos fatores angiogênicos com seus respectivos receptores presentes nas células endoteliais, os quais, por sua vez, desencadeiam sinalização intracelular que favorece a orquestra de todos os eventos essenciais da angiogênese (Teleanu et al., 2019; Parmar e Apte, 2021). Dentre os mediadores angiogênicos, o VEGF é conhecido como o principal regulador da angiogênese tumoral, no qual a ligação do VEGF em seu respectivo receptor (VEGFR-2), encontrado predominantemente em células endoteliais, desencadeia a proliferação, sobrevivência, reorganização do citoesqueleto (Teleanu et al., 2019), migração e formação de tubos de células endoteliais (Saman et al., 2020).

A iniciação da formação de novos vasos sanguíneos é caracterizada pela desestabilização das interações entre células endoteliais e periendothelias (por exemplo, pericitos), as quais mantém a vasculatura estabilizada. Uma vez que as células endoteliais perdem as suas junções, elas se tornam mais responsivas aos estímulos dos fatores pró-angiogênicos presentes no microambiente tumoral, possibilitando o brotamento, a proliferação e a migração das mesmas. A Ang-2 e o VEGF são considerados fatores essenciais para que ocorra a desestabilização das células endoteliais ativadas (Hall e Ran, 2010).

Durante a indução da angiogênese tumoral, ocorre a seleção da célula endotelial líder e das células seguidoras (do inglês, *tip cells* e *stalk cells*, respectivamente) através da regulação da via Notch-Delta devido a presença de VEGF (Hall e Ran, 2010; Lugano et al., 2020), prevenindo assim, uma migração desordenada das células endoteliais a favor do gradiente angiogênico (Teleanu et al., 2019). A célula líder adquire fenótipo invasivo, e

devido sua maior motilidade ela secreta enzimas proteolíticas (metaloproteinases de matriz - MMPs) que promovem a remodelagem da matriz extracelular através da ruptura da membrana basal (Lugano et al., 2020), permitindo sua migração a favor do gradiente de fatores pró-angiogênicos no microambiente tumoral (Jiang et al., 2020). As células endoteliais seguidoras são guiadas pela célula líder e formam brotamentos a partir do vaso sanguíneo pré-existente, desse modo apresentam um maior potencial proliferativo (Lugano et al., 2020).

Devido a constante sinalização de fatores pró-angiogênicos no microambiente tumoral as redes vasculares recém formadas não completam o processo de maturação, resultando em vasos malformados, desorganizados e de calibre heterogêneo (Lopes-Coelho et al., 2021). Por esse motivo, o fluxo sanguíneo dentro do parênquima tumoral é desigual, ocasionando em áreas de hipóxia persistente ou intermitente (Lugano et al., 2019). Vale ressaltar que os vasos sanguíneos presentes na massa tumoral são compostos primariamente por células endoteliais (Jiang et al., 2020), as quais sofrem constantemente interrupções em suas junções, e por esse motivo apresentam uma maior permeabilidade e pressão intersticial, aumentando a fragilidade dos vasos e o risco de hemorragia (Lugano et al., 2019; Lopes-Coelho et al., 2021).

É importante destacar que a angiogênese tumoral é essencial para a progressão tumoral, pois além de fornecer nutrientes, oxigênio e remover metabólitos provenientes do metabolismo das células tumorais, a formação de novos vasos facilita a disseminação das células cancerosas da massa tumoral inicial para regiões distantes, ou seja, a angiogênese contribui para o extravasamento e metastização das células tumorais (Zuazo-Gaztelu e Casanovas, 2018).

O papel fundamental da angiogênese na progressão e disseminação tumoral despertaram interesse para o desenvolvimento de novos compostos antiangiogênicos, no intuito de combinar com tratamentos preconizados para o câncer. Até o momento, tratamentos direcionados a um alvo único (moléculas estimulatórias ou inibitórias) na angiogênese tumoral têm se mostrado promissores. Entretanto, acredita-se que a monoterapia antiangiogênica é insuficiente para combater a sinalização pró-angiogênica das células cancerosas e do microambiente tumoral, devido ao surgimento de mecanismos de resistência relacionados ao uso de drogas antiangiogênicas. Desse modo, é necessário a compreensão da modulação da angiogênese tumoral de uma maneira mais integrada, para

assim desenvolver estratégias terapêuticas holísticas para melhorar a taxa de sobrevivência de pacientes com câncer (Teleanu et al., 2019; Saman et al., 2020).

1.6 Invasão e metástase tumoral

As células tumorais passam por diversas mudanças em sua fisiologia celular como autossuficiência na sinalização proliferativa, insensibilidade a sinais inibidores de crescimento, potencial replicativo ilimitado, evasão da apoptose, angiogênese e capacidade de invasão em tecidos adjacentes, no intuito de metastizar para locais distantes do corpo (Gkretsi e Stylianopoulos, 2018). A metástase é considerada a última manifestação da evolução de uma célula cancerosa para se tornar independente do hospedeiro. Em vista disso, é a causa primária da morbidade e mortalidade por câncer, sendo responsável por 90% dos casos de mortes dessa doença (Seyfried e Huysentruyt, 2013; Welch e Hurst, 2019).

O termo metástase é designado para o desenvolvimento de tumores secundários em regiões do corpo distante da massa tumoral inicial (Fares et al., 2020). Esse fenômeno compreende uma cascata de eventos onde as células cancerosas se destacam do tumor primário, intravasam no sistema circulatório ou linfático, evitam o ataque do sistema imunológico, extravasam em redes capilares, invadem e proliferam em órgãos secundários (Seyfried e Huysentruyt, 2013). Na etapa inicial, as células cancerosas se dissociam da massa tumoral original, reorganizam sua ligação à matriz extracelular e degradam a mesma para proporcionar sua invasão em tecidos adjacentes e/ou intravasar nos vasos sanguíneos (Gkretsi e Stylianopoulos, 2018). Esse processo é associado a mudanças morfológicas e bioquímicas reversíveis que permitem que a célula tumoral altere seu fenótipo epitelial para assumir um fenótipo mesenquimal, fenômeno este conhecido como transição epitelial-mesenquimal (EMT) (Jiang et al., 2015).

A EMT é caracterizada pela perda da polarização celular através da alteração do perfil de expressão de proteínas de junção como E-caderina, claudinas e zônula ocludente (ZO-1) e subsequente aumento da expressão de marcadores mesenquimais como N-caderina, vimentina e fibronectina, culminando na reorganização do citoesqueleto. A substituição da E-caderina pela N-caderina é uma característica marcante da EMT em diversos tipos de cânceres, sendo responsável pela maior invasividade e motilidade de células tumorais, isto é, aumentam o potencial metastático dessas células. De fato, alterações na E-caderina impactam na adesão célula-célula e célula-matriz extracelular, pelo fato, desta molécula de

adesão desenvolver um papel essencial na adesão de células e tecidos junto com outros membros do complexo de adesão (p.ex β -catequina) através da regulação da sinalização, transcrição e adesão celular (Jiang et al., 2015).

A regulação transcricional negativa da E-caderina é considerada o processo inicial da EMT, isso ocorre a partir da sinalização do fator de crescimento do hepatócito (HGF), fator de crescimento epidérmico (EGF) e fator de crescimento e transformação beta (TGF- β) que são responsáveis pela regulação positiva de fatores de transcrição que induzem a EMT como Snail, Slug e Twist. Os fatores de transcrição Snail e Slug estão envolvidos na regulação negativa da E-caderina, além disso, atuam como ativadores de outras moléculas efetoras na EMT, como a família de metaloproteinases de matriz (MMP) (Jiang et al., 2015). As MMP auxiliam no processo de invasão das células tumorais, pois desempenham papel essencial na degradação da matriz extracelular, permitindo a penetração das células tumorais no estroma subjacente (Welch e Hurst, 2019).

Durante o processo de invasão e migração, as células tumorais interagem fracamente com a matriz extracelular de modo a criar uma passagem entre a massa tumoral original e um compartimento de transporte como vasos sanguíneos ou linfáticos (Welch e Hurst, 2019). Ao atingir o compartimento de transporte, ocorre o intravasamento das células tumorais, ou seja, a entrada e disseminação das células cancerosas na corrente sanguínea. Esse processo pode ser ativo ou passivo (Gkretsi e Stylianopoulos, 2018), visto que o intravasamento precisa da degradação parcial da matriz extracelular e da membrana basal subjacente às células endoteliais. De maneira passiva, as células tumorais não rompem totalmente as junções endoteliais, mas sim projetam filopódios os quais facilitam sua passagem pelas células endoteliais em direção ao lúmen vascular. O processo de intravasamento passivo pode ser facilitado na presença de cavidades entre as junções endoteliais, fenômeno este observado na vasculatura anormal formada a partir da angiogênese tumoral. O intravasamento ativo ocorre a partir do auxílio de macrófagos, os quais são induzidos a secretar proteinases para facilitar a passagem das células cancerosas para o interior dos vasos sanguíneos (Welch e Hurst, 2019).

Ao adentrar na corrente sanguínea, as células tumorais circulantes (CTC) movem-se por meio de mecanismos de motilidade ativa ou passiva (empurradas junto com o fluxo do fluido). É importante ressaltar que das milhões de células que entram na vasculatura diariamente, menos de 0,01% consegue extravasar e colonizar regiões secundárias (Welch e Hurst, 2019). Esse baixo sucesso de colonização está associado à atividade do sistema imune

sobre as CTC. Por esse motivo, as CTC rapidamente associam-se às plaquetas, as quais funcionam como escudo para as células tumorais contra o reconhecimento e degradação pela Natural killer. Além disso, as plaquetas podem interagir com as células endoteliais tornando a vasculatura mais permeável e facilitando o processo de extravasamento (Lambert et al., 2017).

O extravasamento é um processo complexo que envolve interação ligante-receptor, quimiocinas e células circulantes não-tumorais, para facilitar a passagem das CTC pelas barreiras rígidas da membrana basal das células endoteliais. As integrinas são as responsáveis em determinar os locais em que o extravasamento e a subsequente colonização das CTC irão ocorrer (Fares et al., 2020). As células tumorais extravasantes encontram-se em um novo microambiente do qual é desprovido de células estromais familiares, fatores de crescimento e constituintes da matriz extracelular que anteriormente sustentavam sua sobrevivência no microambiente do tumor primário (Lambert et al., 2017). Desse modo, as células tumorais entram em dormência para facilitar a aclimação e subsequente disseminação e colonização das mesmas. O período de dormência interrompe a progressão tumoral após invasão em locais secundários para escapar do sistema imune, sendo assim, as células cancerosas regulam negativamente a expressão de moléculas de reconhecimento pelo sistema imunológico (Fares et al., 2020). A quebra da dormência ocorre após a aclimação das células tumorais associada a aquisição de mutações em resposta ao novo microambiente tumoral e subsequente indução da angiogênese tumoral, permitindo assim a progressão tumoral da metástase (Welch e Hurst, 2019).

A metástase, como descrita, é um processo complexo que necessita do desenvolvimento de tratamentos mais eficazes, para isso é necessário a compreensão dos processos que governam esse fenômeno do início ao fim (Fares et al., 2020).

1.7 Espécies reativas de oxigênio na regulação da progressão tumoral

As espécies reativas de oxigênio (EROs) são metabólitos parcialmente reduzidos provenientes do metabolismo aeróbico que apresentam alto potencial oxidante (Arfin et al., 2021). O alto potencial oxidante é justificado por esses metabólitos conterem oxigênio em sua estrutura que pode apresentar ou não elétron desemparelhado, sendo assim classificados como radicais livres ou não- radicais (Policastro et al., 2013). O ânion superóxido ($O_2^{\cdot-}$), radical hidroxila ($OH^{\cdot}$) são exemplos de radicais livres e o peróxido de hidrogênio (H_2O_2)

de não-radical (Harris e DeNicola, 2020). Dentre as espécies reativas de oxigênio, o H_2O_2 é o menos reativo e devido essa característica ele atua como segundo mensageiro de algumas vias redox-sensíveis que envolvem a transdução de sinais extracelulares e o controle da expressão gênica (Perillo et al., 2020).

A maior fonte de EROs intracelular são as mitocôndrias, sendo gerados pela fosforilação oxidativa, onde nos complexos I e III pode ocorrer um vazamento de elétrons os quais reduzem parcialmente o oxigênio molecular dando origem a EROs, mais especificamente $\text{O}_2^{\bullet-}$ que é convertido em H_2O_2 pela célula. Vale destacar que cerca de 0,15-2% do oxigênio consumido são convertidos em espécies reativas de oxigênio pela mitocôndria (Policastro et al., 2013; Aggarwal et al., 2019). As EROs também podem ser geradas por fontes extrínsecas como radiação, toxinas pró-oxidantes ambientais, compostos químicos (tabaco e álcool) e algumas drogas quimioterápicas (Aggarwal et al., 2019).

As células fazem um controle rigoroso para manter os níveis fisiológicos das espécies reativas de oxigênio através de antioxidantes que são responsáveis pela transformação de EROs em moléculas estáveis e menos prejudiciais ao organismo (Arfin et al., 2021). Desse modo, o sistema de defesa antioxidante é essencial para o balanço entre a produção e a neutralização das EROs, conhecido como equilíbrio de redução-oxidação (redox) celular. Os antioxidantes podem ser classificados em enzimáticos como a catalase (CAT), glutathione peroxidase (GPx), superóxido dismutase (SOD), tioredoxina (Trx), peroxirredoxina (Prx), entre outros (Wang et al., 2021); e em não-enzimáticos como bilirrubina, melatonina, coenzima Q, ferritina, ácido úrico, flavonoides, β -carotenoides, entre outros. O equilíbrio entre níveis de EROs e antioxidantes (equilíbrio redox) é fundamental para manter a homeostasia celular (Aggarwal et al., 2019).

A ruptura do equilíbrio redox, isto é, maior produção de EROs e redução na atividade de antioxidantes (estresse oxidativo), favorece o surgimento de patologias como doença cardiovascular, diabetes, doença neurodegenerativa e câncer (Hayes et al., 2020). O estresse oxidativo vem sendo considerado uma das principais razões do desenvolvimento e progressão tumoral, uma vez que as EROs atuam em diferentes marcas do câncer como a instabilidade genômica, inflamação, angiogênese, invasão e metástase (Aggarwal et al., 2019; Perillo et al., 2020; Arfin et al., 2021). As células cancerosas são caracterizadas pela produção de níveis mais elevados de EROs em comparação às células normais que as originam. Esse estresse oxidativo crônico nas células tumorais é influenciado por diversos fatores como alterações genéticas, desregulação de enzimas antioxidantes, disfunção

mitocondrial, metabolismo anormal (Efeito Warburg), hiperproliferação e aquisição do fenótipo metastático (Policastro et al., 2013).

Os elevados níveis de EROs nas células tumorais levam a modificação de macromoléculas celulares como o DNA, lipídeos e proteínas. Essas alterações interferem na sinalização celular de vias redox-sensíveis a partir da modificação de cinases, fosfatases e fatores de transcrição nuclear (Aggarwal et al., 2019). A regulação pela sinalização redox ocorre a partir da oxidação de grupos tióis presentes nas cadeias laterais de resíduos de cisteína, levando a modificações pós-traducionais que podem ativar, modificar ou inativar a função da proteína. A oxidação nos resíduos de cisteína pode ser reversível ou irreversível dependendo dos níveis de espécies reativas de oxigênio disponíveis para realizar a oxidação, isto é, quanto maior for a concentração de EROs maior as chances de ocorrer oxidação irreversível nos resíduos de cisteína (Shi e Dansen, 2020).

O aumento nos níveis de EROs em células cancerosas, como do H_2O_2 , ativa vias de sinalização como a via da fosfatidilinositol-3-cinase (PI3k)/proteína cinase B (Akt)/, da proteína alvo da rapamicina em mamíferos (mTOR), através da oxidação de resíduos de cisteína e consequente inativação da fosfatase homóloga a tensina (PTEN), levando ao aumento da expressão de VEGF. Como dito anteriormente, o VEGF é um importante fator de crescimento que estimula a permeabilidade, proliferação, migração e formação de estruturas tubulares das células endoteliais, ou seja, é essencial para a angiogênese tumoral. Além disso, as EROs são capazes de estabilizar e ativar o HIF-1 α , um fator de transcrição redox-sensível responsável pelo aumento da expressão de VEGF e VEGFR. Deste modo, HIF-1 α e VEGF podem ser considerados um dos principais componentes regulatórios na relação entre EROs e angiogênese tumoral (Aggarwal et al., 2019; Arfin et al., 2021).

As EROs exercem um papel importante na migração e invasão de células cancerosas, a partir da regulação positiva de diversos fatores de transcrição como NF- κ B (fator de transcrição nuclear kappa B), Twist e Snail; de metaloproteinases como MMP-9 e MMP2; e de citocinas e quimiocinas como TGF- β . Esses componentes estão relacionados com a transição epitelial-mesenquimal (EMT), e por esse motivo as EROs são consideradas a maior causa da EMT. Vale ressaltar que diversas vias reguladas pelas EROs que envolvem a EMT servem de suporte para a sobrevivência e proliferação das células endoteliais como, por exemplo, a ativação de HIF-1 α (Aggarwal et al., 2019) e o aumento da expressão do VEGF (Saman et al., 2020). Além disso, o estresse oxidativo induz mudanças epigenéticas na região

promotora da E-caderina mediante o aumento da expressão do Snail, favorecendo assim o processo de metastização tumoral (Aggarwal et al., 2019).

É importante destacar que as EROs desempenham tanto sinalização pró-tumorigênica (sobrevivência, proliferação, evasão da apoptose, angiogênese e metástase) quanto anti-tumorigênica (parada do ciclo celular, senescência e morte celular). A sinalização da supressão tumoral das EROs ocorre quando seus níveis ultrapassam o limiar citotóxico das células cancerosas, ocasionando na indução da morte dessas células (Wang et al., 2021). Devido essa ambiguidade das EROs na regulação da progressão tumoral, o uso de agentes exógenos que levam a indução da produção de EROs em níveis acima do limite citotóxico das células tumorais, vêm sendo uma alternativa interessante para o tratamento do câncer. Visto que o desequilíbrio redox nas células tumorais induz a sinalização anti-tumorigênica pelas EROs (Kim et al., 2019).

1.8 Sinalização anti-tumorigênica de EROs: uma abordagem terapêutica

As espécies reativas de oxigênio (EROs) contribuem para o surgimento de diversas características do câncer como a instabilidade genômica e ativação de vias de sinalização de sobrevivência, proliferação, evasão da apoptose, angiogênese e metástase. Devido seu papel crucial na tumorigênese, as células tumorais apresentam elevados níveis de EROs intracelular, sendo esse estado pró-oxidante tolerado através do aumento da capacidade antioxidante das células malignas. Sendo assim, a regulação positiva da capacidade antioxidante é fundamental para manter os níveis de EROs abaixo do limiar citotóxico das células tumorais (PolICASTRO et al., 2013).

As células tumorais se adaptaram a elevados níveis endógenos de EROs devido à capacidade dos mesmos de ativar o fator de transcrição NRF2 (Fator nuclear eritróide 2 relacionado ao fator 2). Em condições basais, os níveis de NRF2 são restringidos devido sua associação a KEAP1 (Proteína 1 associada a ECH semelhante a Kelch), a qual proporciona a ubiquitinação e subsequente degradação proteossomal de NRF2. Entretanto, em condições de estresse oxidativo ocorre o acúmulo do NRF2 a partir de modificações em resíduos de cisteína da KEAP1 que inviabiliza a ubiquitinação e degradação do NRF2. A expressão constitutiva do NRF2 nas células cancerosas promove a transcrição de diversos genes do sistema antioxidante, incluindo genes relacionados à via antioxidante de glutathione (GSH) e tiorredoxina (Trx). Interessantemente, o acúmulo de NRF2 é comum em vários tipos de

cânceres, evidenciando que o aumento da defesa antioxidante é essencial para diferentes estágios do processo tumorigênico (Harris e DeNicola, 2020).

Tendo em vista que as células cancerosas apresentam altos níveis de EROs endógeno, o uso de agentes exógenos capazes de induzir um nível ainda mais alto de EROs pode desencadear morte celular (Tan et al., 2018), ou seja, a indução de estresse oxidativo através do acúmulo excessivo de EROs, excedendo o limiar citotóxico, pode matar seletivamente as células tumorais (Arggarwal et al., 2019; Kim et al., 2019; Arfin et al., 2021). Essa seletividade para as células malignas pode ser justificada pela reduzida capacidade de neutralizar EROs de origem exógena do sistema antioxidante tumoral quando comparado com as células normais (Policastro et al., 2013; Wang et al., 2021). Por esse motivo, as células tumorais são mais vulneráveis ao uso de agentes pró-oxidantes exógenos, sendo esse fato observado por diversos estudos que demonstram que elevadas concentrações de EROs tem habilidade de induzir parada no ciclo celular, senescência e morte das células malignas (Arggarwal et al., 2019; Kim et al., 2019; Arfin et al., 2021).

Elevados níveis de EROs podem desencadear sinalização de morte celular por apoptose tanto pela via extrínseca quanto pela via intrínseca. As EROs estimulam a via apoptótica extrínseca através da aceleração da ubiquitinação e degradação proteossomal da proteína inibitória de FLICE celular (c-Flip), a qual é considerada um fator anti-apoptótico por impedir a formação do complexo de sinalização de morte (DISC) através da competição pela ligação a proteína adaptadora (FADD ou TRADD) com a pró-caspase 8. Desse modo, a degradação de c-Flip pelas EROs proporcionam a formação de DISC e consequentemente indução da apoptose das células cancerosas. Em relação à via intrínseca, as EROs atuam aumentando a permeabilização da membrana externa mitocondrial por meio da regulação da família Bcl-2 que induzem a liberação do citocromo *c*. O citocromo *c* quando liberado no citosol desencadeia uma cascata de sinalização que induz a apoptose das células tumorais (Wang et al., 2021).

A dicotomia das espécies reativas de oxigênio é um interessante alvo terapêutico para impedir o crescimento tumoral através da inibição da inflamação, angiogênese e metástase. Devido as EROs possuírem diversos alvos moleculares nas células, suas atividades são reguladas por diferentes vias de sinalização. Desse modo, é essencial pesquisas para tentar entender o papel, ainda, enigmático das EROs na homeostasia redox das células tumorais, para assim desenvolver um tratamento antitumoral mais eficaz (Aggarwal et al., 2019). Visto que, apesar dos avanços no tratamento para o câncer, diferentes estratégias ainda são

necessárias para complementar ou ser utilizadas como alternativa aos tratamentos existentes (Tan et al., 2018).

1.9 Potencial biotecnológico de L-aminoácido oxidase de peçonha de serpentes (svLAAO) no estudo e no tratamento do câncer

As peçonhas de serpentes são compostas por uma rica mistura de biomoléculas ativas, as quais apresentam grande valor medicinal. Além disso, essas toxinas ofídicas são amplamente utilizadas em pesquisas como ferramentas biotecnológicas em estudos de biologia celular, fornecendo assim informações de mecanismos de toxicidade que podem ser úteis para o desenvolvimento de estratégias terapêuticas mais eficientes. Dentre as toxinas presentes nas peçonhas de serpentes, as L-aminoácido oxidases (svLAAOs) são amplamente distribuídas nas famílias Viperidae, Elapidae e Crotalidae (Guo et al., 2012; Tan et al., 2018). A exemplo, a espécie *Bothrops moojeni* da família Viperidae apresenta cerca de 4,2-5,2% do conteúdo proteico da sua peçonha de LAAOs (Amorim et al., 2018). Vale ressaltar que na mesma peçonha de serpente pode ser encontrada isoformas da mesma LAAO, as quais podem ser ácidas, neutras ou básicas. E, devido a diferença de carga essas isoformas podem apresentar diferentes efeitos farmacológicos (Izidoro et al., 2014).

As L-aminoácido oxidases de peçonhas de serpentes (EC 1.4.3.2) são flavoenzimas homodiméricas, em que cada subunidade faz uma ligação não covalente com FAD (flavina adenina dinucleotídeo) ou FMN (flavina mononucleotídeo), sendo que o cofator FAD é o mais comumente encontrado em svLAAOs, o qual é responsável pela cor amarela de muitas peçonhas de serpentes (Guo et al., 2012). As svLAAOs possuem massa molecular variando de 120 a 150 kDa na forma dimérica e 50 a 70 kDa na forma monomérica, e ponto isoelétrico (pI) de cerca de 4,4 a 8,12 (Costa et al., 2014).

Em relação a sua estrutura, as svLAAOs apresentam três domínios estruturais bem conservados, sendo eles o domínio de ligação ao FAD, o domínio de ligação ao substrato e o domínio helicoidal. A maior diferença nas estruturas das svLAAOs são encontradas nos resíduos de aminoácidos presentes nas regiões de *loops* que conferem a essas enzimas diferentes graus de especificidade à seus substratos (aminoácidos), pelo fato de alterar o volume da cavidade, a profundidade, e a distribuição de carga do sítio ativo (Ullah, 2020). Entretanto, a maioria das svLAAOs demonstram especificidade relativamente alta para

aminoácidos hidrofóbicos (L-fenilalanina, L-metionina, L-leucina, L-isoleucina) (Guo et al., 2012).

O mecanismo catalítico das svLAAOs é composto por uma meia-reação redutora e uma meia-reação oxidativa. O ciclo catalítico se inicia com a desprotonação do grupo α -amino do aminoácido pelo resíduo His²³³ localizado no sítio ativo de svLAAOs, ocasionando na transferência de elétrons do nitrogênio alfa para o carbono alfa. Essa modificação torna o aminoácido mais ativo, impulsionando a transferência do íon hidreto do carbono alfa para o cofator FAD, reduzindo-o, e, conseqüentemente, levando a formação de um iminoácido. O iminoácido sofre hidrólise não-enzimática liberando α -cetoácido e amônia. Concomitante a meia-reação redutora, outra meia-reação oxidativa ocorre completando o ciclo catalítico com a reoxidação de FADH₂ em FAD pelo oxigênio molecular (O₂), produzindo assim peróxido de hidrogênio (H₂O₂) (Costa et al., 2014; Hiu e Yap, 2020; Ullah, 2020).

As L-aminoácido oxidases são consideradas o agente apoptótico mais potente presente nas peçonhas de serpentes. Além disso, são responsáveis por causar edema, agregação/inibição de plaquetas, hemorragia e atividade anticoagulante durante processo fisiológico e patológico do envenenamento. Entretanto, as svLAAOs vêm despertando interesse dos pesquisadores devido seu grande potencial farmacológico, sendo observado em baixas concentrações efeitos antiviral, antifúngico, antiparasitário, antimicrobiano e antitumoral (Izidoro et al., 2014; Ullah, 2020).

Em relação ao efeito antitumoral, diversos estudos demonstraram que svLAAOs exibem uma potente atividade citotóxica em diferentes linhagens tumorais (Hiu e Yap, 2020), acompanhada de efeito citotóxico menos expressivo em células não-tumorais (Lee et al., 2014; Costa et al., 2018; Abdelkafi-Koubaa et al., 2021; Nikodijevic et al., 2021). Apesar do mecanismo pelo qual as svLAAOs exercem sua atividade citotóxica não estar totalmente elucidada, atribui esse efeito ao H₂O₂ liberado como subproduto da reação enzimática das svLAAOs (Tan et al., 2018; Mahfouz et al., 2021). Essa observação levou a hipótese de que o efeito citotóxico das svLAAOs possa estar relacionada com a presença de regiões glicosiladas na superfície das enzimas, visto que o canal responsável pela liberação de H₂O₂ para o exterior da superfície da enzima está localizado próximo de regiões glicosiladas. Por esse motivo, acredita-se que as svLAAOs direcionam a liberação de altas concentrações de H₂O₂ em uma determinada região da superfície celular devido a porção glicano apresentar capacidade de interagir com a superfície celular (Tan et al., 2018; Mahfouz et al., 2021). Essa hipótese pode justificar a diferença no mecanismo de indução de morte celular entre

peróxido de hidrogênio exógeno e o peróxido de hidrogênio gerado por svLAAO (Suhr e Kim, 1999).

A seletividade observadas em diversos estudos das svLAAOs perante as linhagens tumorais pode ser explicada, parcialmente, pela maior afinidade das svLAAOs com a superfície de células tumorais quando comparado com as células não-tumorais (Costa et al., 2018; Abdelkafi-Koubaa et al., 2016), sugerindo que as células tumorais apresentam regiões com propriedades físico-químicas que favorece a interação com as svLAAOs. Além disso, as células tumorais são mais vulneráveis a aumentos de níveis de EROs, desse modo, indutores exógenos de EROs como svLAAOs apresentam grande potencial de induzir a morte seletiva de células tumorais (Tan et al., 2018).

O efeito citotóxico das svLAAOs sobre células tumorais está relacionado com a sua capacidade de gerar estresse oxidativo, através do acúmulo de H_2O_2 , ativando a morte celular por apoptose tanto pela via intrínseca quanto pela via extrínseca, devido a regulação positiva da expressão de Bax, caspase-9, caspase-3 e caspase-8 e redução da expressão de Bcl-2 (Hiu e Yap, 2020; Mahfouz et al., 2021). Além disso, as svLAAOs induzem parada do ciclo celular, sendo visualizado aprisionamento das células tumorais em G0/G1 e redução de células nas fases S e G2/M (Zhang e Wu, 2008; Paiva et al., 2011; Costa et al., 2017). Outro efeito interessante recentemente observado foi a modulação da expressão gênica e proteica de PGP1 (glicoproteína-P) nas linhagens celulares de câncer de cólon humano (SW-480 e HT-29) após tratamento com LAAO obtida da peçonha de *Crotalus adamanteus*, demonstrando o potencial dessa enzima de reduzir a expressão do transportador comumente expresso em células tumorais que desenvolvem resistência na terapia antitumoral (Nikodijevic et al., 2021).

Como descrito até o momento, diversos estudos demonstraram resultados promissores da eficácia tumoral das svLAAOs, devido sua capacidade de controlar a liberação de H_2O_2 em células tumorais, gerando estresse oxidativo e morte celular por apoptose. Atualmente, três L-aminoácido oxidases foram isoladas da peçonha de *Bothrops moojeni*, sendo elas BmooLAAO-I (66 kDa) que demonstrou efeito antitumoral em células de Ehrlich de carcinoma ascítico (Stábeli et al., 2007) e em células humanas de leucemia (HL-60, HL-60.Bcr-Abl, K562-S e K562-R) (Burin et al., 2020); BmooLAAO-II (46,6 kDa) que apresentou efeito leishmanicida (Barbosa et al., 2020); e BmLAAO (58 kDa) que foi capaz de inibir a angiogênese *in vitro* e *in vivo* (Bhat et al., 2021). Diante desses prévios resultados, o presente trabalho propôs expandir a análise dos efeitos farmacológicos de

svLAAOs e trazer informações sobre o mecanismo de ação do estresse oxidativo gerado por uma nova L-aminoácido oxidase da peçonha de *Bothrops moojeni* (Pollonein-LAAO) em diferentes etapas da progressão tumoral (proliferação, invasão e angiogênese), utilizando as linhagens de câncer de próstata humana (PC-3) e endotelial de veia umbilical humana (HUVEC). Visto que o uso de agentes que inibem diversos mecanismos relacionados com a progressão tumoral é considerado crucial para o desenvolvimento de novos agentes terapêuticos e, conseqüentemente, um tratamento mais efetivo do câncer.

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Objetivos

3. OBJETIVOS

3.1 Objetivo geral

O presente trabalho teve como objetivo investigar os efeitos do estresse oxidativo de uma nova L-aminoácido oxidase de peçonha de *Bothrops moojeni* (Pollonein-LAAO) na proliferação e invasão de células de câncer de próstata humano (PC-3) e na angiogênese tumoral em células endoteliais de veia umbilical humana (HUVECs) a partir de ensaios *in vitro*.

3.2 Objetivos específicos

- Isolar e sequenciar a Pollonein-LAAO a partir da peçonha de *Bothrops moojeni*.
- Avaliar a citotoxicidade da Pollonein-LAAO sobre células de câncer de próstata humano (PC-3) na presença ou ausência de catalase.
- Mensurar os níveis de espécies reativas de oxigênio (EROs) intracelular induzidos pela Pollonein-LAAO na célula PC-3.
- Verificar o efeito antiproliferativo da Pollonein-LAAO, bem como sua interferência no ciclo celular e na expressão de genes envolvidos no controle da progressão do ciclo celular em células PC-3.
- Avaliar a indução de morte celular por apoptose e/ou necrose, bem como a expressão de genes envolvidos nas vias intrínseca e extrínseca apoptótica em células PC-3.
- Verificar a capacidade da Pollonein-LAAO de provocar alterações no potencial mitocondrial em células PC-3.
- Avaliar a atividade citotóxica da Pollonein-LAAO em modelo de esferóide tumoral tridimensional.
- Verificar a capacidade da Pollonein-LAAO de impedir processos críticos envolvidos durante a invasão de células tumorais por meio de ensaios de adesão em diferentes componentes da matriz extracelular, migração e invasão em células PC-3 na presença ou ausência de catalase; bem como a expressão de genes e proteínas envolvidas em vias de sinalização no processo de invasão.
- Avaliar a atividade citotóxica e antiproliferativa da Pollonein-LAAO, bem como sua capacidade de gerar EROs em células endotelial de veia umbilical humana (HUVECs) na presença ou ausência de catalase.

- Verificar a capacidade da Pollonein-LAAO de interferir em etapas cruciais da angiogênese tumoral através de ensaios de adesão em diferentes componentes da matriz extracelular, migração e invasão em HUVECs na presença e ausência de catalase; bem como avaliar a expressão de genes e proteínas envolvidas na sinalização da angiogênese tumoral.
- Analisar as alterações no citoesqueleto induzidas pela Pollonein-LAAO nas HUVECs na presença e ausência de catalase.
- Avaliar a capacidade da Pollonein-LAAO de inibir a formação de vasos endoteliais *in vitro*, e quantificar os níveis de VEGF presentes no sobrenadante.
- Verificar o efeito da Pollonein-LAAO em modelo de co-cultura de vasos endoteliais e esferóide (PC-3) *in vitro*.

Capítulo II

Oxidative stress induced by a new L-amino acid oxidase from *Bothrops moojeni* snake venom (Pollonein-LAAO) impairs prostate cancer cell survival and invasion

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Abstract

Cancer cells produce abnormal levels of reactive oxygen species (ROS) that contribute to their progression. Due to the importance of ROS in promoting malignant phenotype, it has been widely accepted that augmenting intracellular ROS levels above threshold can trigger cancer cell death. In this framework, we hypothesized that the change in ROS concentration of prostate cancer cells (PC-3) could impair critical steps of cancer cell proliferation and invasion. Therefore, the purpose of this study was to evaluate the use of a new L-amino acid oxidase obtained from *Bothrops moojeni* venom (Pollonein-LAAO) as a ROS-based anti-tumorigenic agent. Our results demonstrated that Pollonein-LAAO was cytotoxic to PC-3 cells in two-dimensional ($IC_{50}=2.4\text{ }\mu\text{g/mL}$) and three-dimensional (tumor spheroid) *in vitro* assays. Pollonein-LAAO was able to increase the intracellular ROS generation that culminates in cell death from apoptosis by both intrinsic and extrinsic pathways due to the up-regulation of *TP53*, *BAX*, *BAD*, *TNFRSF10B* and *CASP8*. Additionally, Pollonein-LAAO reduced mitochondrial membrane potential and impaired cell cycle progression by causing G0/G1 phase to delay, due to the up-regulation of *CDKN1A* and the down-regulation of the expression of *CDK2* and *E2F*. Interestingly, Pollonein-LAAO inhibited PC-3 cell migration, invasion and adhesion in collagen IV and fibronectin. Those results may be explained by the ability of Pollonein-LAAO to down-regulate genes that participate in critical steps of the cellular invasion process (*SNAIL1*, *VIM*, *MMP2*, *ITGA2*, *ITGAV* and *ITGB3*). Furthermore, the Pollonein-LAAO effects were abolished in the presence of catalase. Altogether, ROS generation by Pollonein-LAAO affected several malignant phenotypes of PC-3 cell.

Keywords: AlphaVbeta3, Apoptosis, G0/G1 arrest, p53, ROS, tumor spheroid

1. Introduction

Reactive oxygen species (ROS) are a by-product of aerobic metabolism that have been associated with cancer, since the accumulation of ectopic ROS can promote protumorigenic signaling that regulates cancer cell proliferation, survival and growth, angiogenesis and metastasis [1,2]. In fact, cancer cells increase their ROS production due to uncontrolled metabolic processes during hyperproliferation; thus, cancer cells have higher basal ROS levels than do their counterpart cells [3], and subsequently elevated antioxidant levels to maintain tolerated levels of ROS below the toxic threshold [2,4].

In line with this, cancer cells have increased pro-oxidant status and, hence, augment intracellular ROS levels by exogenous ROS-modulating agents in turn elevating ROS levels above the toxic threshold, resulting in cell death. Therefore, ROS have been considered to play a dual role in cancer development [2,4,5], in which excess ROS cellular levels may be deleterious and trigger oxidative stress [5,6]. On account of the importance of ROS in promoting malignant phenotype, the disruption of reduction-oxidation balance has been reported to be one of the most crucial reasons underlying cancer development, its progression and metastasis. Therefore, the dual role of ROS is an interesting therapeutic target that should be exploited, as ROS and its redox state in malignancies are still enigmatic [7].

As cancer cells are inherently vulnerable to increases in intracellular ROS levels, exogenous ROS inducers such as snake venom L-amino acid oxidase (svLAAOs) are interesting bioactive molecules to be investigated. Due to the fact that svLAAOs can catalyze a stereospecific oxidative deamination of L-amino acids to their corresponding α -keto acids with the release of ammonia and hydrogen peroxide (H_2O_2) [8,9], H_2O_2 released by svLAAO activity has been proposed to be the main mechanism of their biological activities [9,10]. In fact, H_2O_2 is capable of diffusing across the cell membrane and mitochondria, which can affect multiple cellular signaling events, leading to cell and organelles damage [9,11].

The effects of LAAO purified from *Bothrops moojeni* snake venom on the growth, survival and invasion of prostate cancer cells have not yet been studied in detail. In this context, the current study sought to improve our understanding of the oxidative stress produced by Pollonein-LAAO, a novel L-amino acid oxidase from *B. moojeni*, in human prostate cancer cell line.

2. Material and Methods

2.1 Snake venom collection and Pollonein-LAAO purification

The crude venom was collected from *Bothrops moojeni* snake kept at the Venom Bank at Fiocruz Rondônia/UNIR, Porto Velho, RO, under local government authorization license number: CGEN/AFCAB61; SISBIO 64385-1; IBAMA n° 27131-3 and CGEN/CNPq 010627/2011-1. This work was registered in the National Management System of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number A2C2534. A new L-amino acid oxidase was purified from *B. moojeni* snake venom, determined as Pollonein-LAAO, with the three-step chromatographic method using molecular exclusion, hydrophobic interaction and affinity columns [12], as previously reported with some modifications. The columns used for the three chromatographic steps were HiPrep Sephacryl S-300 column (16/60, GE Healthcare, USA); HiTrap Phenyl-Sepharose 6 Fast Flow (FF) column (High sub, 16/10, GE Healthcare, USA) and HiTrap Benzamidine High-Performance column (GE Healthcare, USA), which were operating in ÄKTAprime plus FPLC system (GE Healthcare). The homogeneity of Pollonein-LAAO was determined by reverse-phase chromatography (RP-HPLC) using C18 column (46 x 250 mm; Thermo Fisher Scientific). The isolated and purified Pollonein-LAAO was stored at 4 °C.

Pollonein-LAAO relative molecular mass (M_r) was estimated by 12.5% (w/v) SDS-PAGE (Laemmli, 1970). Samples were heated at 100 °C for 5 min and ran under reducing conditions (5% β -mercaptoethanol) in parallel with molecular weight markers. The gel was stained with Coomassie Brilliant Blue R-250. The M_r was estimated by interpolation from a linear logarithmic plot of relative molecular mass *versus* migration distance [13]. The molecular weight markers used were: β -galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp98l (25.0 kDa), β -lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) (Thermo Fisher Scientific, USA).

2.1.1 Determination of protein concentration and L-amino acid oxidase activity

Protein concentration of Pollonein-LAAO and chromatographic fractions were determined by Bradford assay using Bradford reagent (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. Bovine serum albumin was used as standard (Thermo Fisher Scientific, USA). LAAO activity was determined using the method reported by [14], with some modifications. Briefly, Pollonein-LAAO or the chromatographic fractions (2 μ g) were incubated for 30 min, at room temperature, with 5 mM of L-leucine,

0.1 M Tris-HCl buffer pH 7.2, 1 U/mL horseradish peroxidase (Sigma-Aldrich, Brazil), and 2 mM *o*-phenylenediamine (Sigma-Aldrich, Brazil). The reaction was stopped with 10% of citric acid (v/v) and absorbance was recorded by spectrophotometer at 490 nm. The absorbance values were used to calculate the LAAO specific activity in U/mg/min, which is the amount of hydrogen peroxide (H₂O₂, μ mol) formed per minute per mg of protein. The amount of H₂O₂ produced was quantified from a standard curve of H₂O₂ concentration expressed in nmol/min. Noteworthy, before performing any biological assay, the L-amino acid oxidase activity was assessed to assure the enzymatic activity of Pollonein-LAAO.

2.1.2 Sequencing of Pollonein-LAAO by mass spectrometry

The sequencing of Pollonein-LAAO was performed by mass spectrometry. Briefly, 100 μ g of lyophilized toxin was dissolved into 20 μ L of 50 mM NH₄HCO₃ pH 7.8 and an aliquot of 10 μ g was applied for the next steps. The sample was reduced with 2 μ L of 500 mM dithiothreitol (DTT) for 40 min at 56 °C under shaking at 650 rpm. Next, an alkylation with 3 μ L of 500 mM iodoacetamide was performed for 30 min at room temperature in the dark. The reaction was quenched with 2 μ L of 500 mM DTT for 10 min at room temperature in the dark. For the digestion, we applied trypsin in two consecutive steps at 37°C. The first one, at a ratio of 1:50 (trypsin:protein) with an overnight incubation and shaking at 650 rpm followed by a second step, was performed with a ratio of 1:100 with incubation for 3h. The digestion was quenched by the addition of TFA at a final concentration of 10%. The digested sample was dried on speed vacuum and desalted on ZipTip pipette tips with C18 resin with elution with 20 μ L of 0.1% TFA/ACN (50/50). Thereafter, the digested peptides were analyzed by the LC-MS/MS performed on an Acquity M-Class UPLC (Waters) hyphenated to a Q Exactive (Thermo Scientific), in nanoelectrospray positive ion mode.

The sequencing was performed using Peaks Studio X software [15] against database of “L amino acid oxidase” from *Bothrops moojeni* downloaded from NCBI in February of 2022, which comprises 3 sequences (AAR31183.1, ATU85535.1 and Q6TGQ8.2). For the database search, we applied the following parameters: methionine oxidation (modification variable), cysteine carbamidomethylation (fixed modification) and trypsin as an enzyme (maximum missed cleavages=3). Parent mass error was set at 5 ppm while the fragment mass error tolerance was fixed at 0.015 Da. The false discovery rate (FDR) selected was 0.1% and mutation ion intensity $\geq$ 5%. In addition, unique peptide $\geq$ 1 considering the significant peptides and only peptides with $-10\lg P > 20$ were used to detect the proteins

from the database. For the analysis, we used the SPIDER algorithm. The multiple sequence alignment was assessed by Clustal Omega software with BmooLAAO-I (ATU85535.1) [16] and BmooLAAO-II [17] primary sequences.

2.2 Cell line and culture conditions

The human prostate adenocarcinoma cell (PC-3) was obtained from American Type Culture Collection (CRL-1435) and maintained routinely in RPMI 1640 medium (Sigma, Brazil) supplemented with 10% fetal bovine serum (FBS; Cultilab, Brazil), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, Brazil) – complete RPMI 1640 medium – at 37 °C in a humidified 5% CO₂ atmosphere. For spheroid formation, human fibroblast cell (HFF-1) was obtained from American Type Culture Collection (SCRC-1041) and maintained in the same conditions described for PC-3 cell.

2.3 Cell viability assay

PC-3 cell (2×10^4 cells/well) was seeded in 96-well microplates containing complete RPMI 1640 medium for 24 h at 37 °C in 5% CO₂ incubator. Next, cells were incubated with different concentrations of Pollonein-LAAO (ranging from 0.09 to 50 µg/mL) in presence of catalase (150 U/mL, Sigma, Brazil) or not for 24 h. Non-treated cells were considered as a control group. The cell viability was assessed using MTT solution (5 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Sigma, Brazil) for 3 h and formazan crystals formed were dissolved by a solution containing 10% SDS and 0.01 N HCl in phosphate buffered saline (PBS) for 18 h. The optical density was determined at 570 nm in a plate reader (Multiskan GO, Thermo Scientific, USA). The half-maximal inhibitory concentration (IC₅₀) was calculated from the concentration-response curve using GraphPad prism software version 6.0 (Inc., CA, USA).

2.4 Measurement of reactive oxygen species (ROS)

To quantify intracellular ROS generation, PC-3 cell (1.0×10^5 cells/well) was seeded in 24-well microplates containing complete RPMI 1640 medium for 24 h at 37 °C in 5% CO₂ incubator. Cells were incubated with different concentrations of Pollonein-LAAO (0.09, 0.19, 0.39, 0.78 and 3.125 µg/mL) for 4 h. Non-treated cells were considered as a control group. Afterwards, the wells were gently washed with phosphate buffered saline (PBS1x) and incubated with H₂DCF-DA fluorescent dye solution (10 µM of 2',7'-

dichlorodihydrofluorescein diacetate; Sigma, Brazil) for 30 min at 37 °C under darkness. Finally, the wells were gently washed, cells were harvested and the quantified fluorescence intensity was determined by flow cytometer (CytoFLEX - Beckman Coulter, USA) and 10,000 events were acquired per sample. The data were obtained using Kaluza software version 1.5.

2.5 Cell cycle analysis

PC-3 cell (5×10^5 cells/well) was seeded in 12 wells containing complete RPMI 1640 medium and incubated for 24 h at 37 °C in 5% CO₂ incubator. Cells were incubated with complete RPMI 1640 medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.19, 0.78 and 3.125 µg/mL) for 24 h. After treatment, cells were harvested, fixed in 70% (v/v) ice-cold ethanol and kept overnight at 4 °C. Then, cells were incubated with RNase A (2 µg/mL, Merck, Brazil) and propidium iodide (PI) (10 µg/mL, Sigma, Brazil) for 45 min at 37 °C in the dark. Cell distribution across the cell cycle was determined immediately by flow cytometer (CytoFLEX - Beckman Coulter, USA) and 10,000 events were acquired per sample. The data were obtained using Kaluza software version 1.5.

2.6 Apoptosis measurement by annexin V-PE staining

Apoptosis was assessed by an FITC annexin V apoptosis detection kit (BD, USA), according to the manufacturer's instruction. Briefly, PC-3 cell (1.0×10^5 cells/well) was seeded in 24-well containing complete RPMI 1640 medium and incubated for 24 h at 37 °C in 5% CO₂ incubator. Next, cells were incubated with complete RPMI 1640 medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.19, 0.78 and 3.125 µg/mL) for 24 h. After incubation, cells were resuspended in a binding buffer containing FITC-conjugated annexin V and propidium iodide for 15 min. All of the samples were analyzed immediately by flow cytometer (CytoFLEX - Beckman Coulter, USA) and 10,000 events were acquired per sample. Analyses were performed by Kaluza software version 1.5.

2.7 Spheroid viability assay

PC-3 and HFF-1 cells (5000 cells/well from each type) were added onto agarose coated 96-well microplates containing complete RPMI 1640 medium at 37 °C in 5% CO₂

incubator [18]. After 4 days, spheroids were formed and, then, treated with different concentrations of Pollonein-LAAO (0.19, 0.78 and 3.125 $\mu\text{g/mL}$) for 24 h. Non-treated spheroids were considered as a control group. Thereafter, spheroids were stained with PI (1 mg/mL, Sigma, Brazil) and Hoechst 33258 (0.9 mg/mL, Sigma, Brazil) for 15 min, as previously described [19-21]. Fluorescence imaging was performed by using EVOS inverted microscope (Thermo Fisher Scientific, USA). The cell spheroid size was evaluated by ImageJ software [22].

2.8 Cell adhesion assay

In order to verify Pollonein-LAAO-treated PC-3 cell adhesion in different extracellular matrix (ECM) coatings, PC-3 cell (2×10^4 cell/ well) was pre-incubated with complete RPMI 1640 medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) for 1 h at 37 °C in 5% CO₂ incubator. Alternatively, cells were co-treated with catalase (150 U/mL, Sigma, Brazil) in complete RPMI medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$), incubated as described above. Subsequently, cells were seeded into 96-well microplates previously coated with collagen IV (10 $\mu\text{g/mL}$, Sigma, Brazil), fibronectin (5 $\mu\text{g/mL}$, Gibco, USA) or Matrigel™ (1:10, Corning, USA) and blocked with 1% of bovine serum albumin (BSA) for 1 hour. After 3 h of incubation, the unattached cells were removed by gentle washes with PBS, and the adherent cells were measured by MTT assay as described previously (section 2.3).

2.9 Detection of $\alpha 2 \beta 1$ integrin by immunofluorescence assay

PC-3 cell (1.5×10^5 cells) was cultured on glass coverslips (13 mm) for 24 h. After adhesion, cells were incubated with complete RPMI 1640 medium in absence (control group) or presence of Pollonein-LAAO at 0.19 $\mu\text{g/mL}$ for 24 h. Afterwards, cells were fixed in 4% paraformaldehyde for 10 min, followed by permeabilization with 0.1% PBS-Tween 20 solution and blocked with 1% BSA, 0.3 M glycine for 1 h [23]. Then, cells were labeled with anti-human $\alpha 2$ (CD49b), anti-human $\beta 1$ (CD29) fluorescein monoclonal antibodies (Ambriex, Brazil) at a concentration of 1:50 (v/v) and nuclei were stained with blue-fluorescent TO-PRO-3 (ThermoFisher Scientific, USA) for 30 min in the dark at room temperature. Coverslips were mounted on glass slides and then PC-3 cell was imaged in a

Zeiss LSM 510 Meta confocal microscope at 40x magnification and the fluorescence intensity was measured with the Image J software.

2.10 Scratch wound-healing assay

PC-3 cell (2×10^5 cells/well) was seeded in 96-well microplates containing complete RPMI 1640 medium and incubated for 24 h at 37 °C in 5% CO₂ incubator. After reaching confluence, an artificial wound was created by scratching the cell monolayer with a 200 µL pipette tip. The monolayer was washed with PBS to remove unattached cells. Subsequently, cells were incubated with complete RPMI 1640 medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) in presence of catalase (150 U/mL, Sigma, Brazil) or not for 24 h. To evaluate the wound-healing process, an inverted microscope (Nikon Eclipse TS100) was used to photograph the monolayer immediately after scratching ($t = 0$ h) and upon 24 h of treatment ($t = 24$ h). The cell migration on the created scratching edge was measured with the Image J software and the percentage of wound closure was obtained by the following equation: % wound closure = $((A_{t0h} - A_{t24h}) / A_{t0h}) \times 100$, where “ A_{t0h} ” is the area of wound measured after scratching and “ A_{t24h} ” is the area of wound measured 24 h after scratching [24,25].

2.11 Transwell migration assay

Cell migration assay was performed using a ThinCert™, 8.0-µm pore (Greiner Bio-one®, Brazil). PC-3 cell (1×10^5 cells/well) was pre-incubated with RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) for 1 h at 37 °C in 5% CO₂ incubator. Alternatively, cells were co-treated with catalase (150 U/mL, Sigma, Brazil) in RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) for 1 h at 37 °C in 5% CO₂ incubator. Afterwards, cells were inserted into the upper chamber, which were immersed in complete RPMI 1640 medium. Cells were allowed to migrate for 24 h at 37 °C. The negative control was considered as the transwell inserted on RPMI 1640-FBS free medium, and the positive control as the transwell inserted on complete RPMI 1640 medium, both incubated as described above. The non-migrating cells were removed from the upper chamber with a cotton swab and the migrating cells were stained with Panotic Kit (Laborclin, Brazil). Cell

migration was quantified by counting cells under a microscope (Nikon Eclipse TS100) at 10x magnification.

2.12 Cell invasion assay

Cell invasion assay was performed using a ThinCert™, 8.0-µm pore (Greiner Bio-one®, Brazil) pre-coated with Matrigel™ (Corning, USA). Briefly, PC-3 cell (1×10^5 cells/well) was pre-incubated with RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) for 1 h at 37 °C in 5% CO₂ incubator. Alternatively, cells were co-treated with catalase (150 U/mL, Sigma, Brazil) in RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) for 1 h at 37 °C in 5% CO₂ incubator. Thereafter, cells were inserted into the upper chamber, which were immersed on wells containing complete RPMI 1640 medium, and cell invasion was allowed to occur for 24 h at 37 °C. The negative control was considered as the transwell inserted on RPMI 1640-FBS free medium and the positive control as the transwell inserted on complete RPMI 1640 medium, both incubated as described above. The non-invading cells were removed from the upper chamber with a cotton swab and the invading cells were stained with Panotic Kit (Laborclin, Brazil). Cell invasion was quantified by counting cells under a microscope (Nikon Eclipse TS100) at 10x magnification.

2.13 Analysis of gene expression by quantitative reverse transcription polymerase chain reaction (RT-qPCR)

PC-3 cell (2×10^6 cells) was incubated with complete RPMI 1640 medium in absence (control group) or presence of Pollonein-LAAO at 0.19 µg/mL or 0.78 µg/mL for 24 h, to evaluate the expression of genes correlated with antitumoral and antimetastatic pathways, respectively. After treatment, cells were harvested and processed in accordance with the guidelines of the RNA extraction Kit (PureLink™ RNA Mini Kit, Thermo Fisher Scientific, USA). The RNA concentration and quality were measured using a spectrophotometer (Biodrop µLite, Nova Analítica, Brazil) and all samples complied with the pre-established ratios (A260/280 and A260/A230). After quantification of the obtained RNAs, the samples were processed for synthesis of cDNAs by High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The expression of *TP53*, *CDKN1A*, *CDK2*, *E2F*, *BAX*, *BAD*, *BCL2*, *CASP8*, *TNFRSF10B*, *ITGA2*, *ITGAV*, *ITGB1*, *ITGB3* and *MMP2* were made

through the Human Cancer Pathway Primer Library, following the manufacturer's instructions (GO-GenOne; Brazil; <http://www.genone.com.br/>). In addition, the expression of *SNAIL* (NM_005985) and *VIM* (NM_003380) were measured using validated primers from IDTDNA (Sintese Biotecnologia, Brazil). Quantitative PCRs were carried out in triplicate using GoTaq® qPCR Master Mix (Promega, USA). Relative gene expression was assessed by comparing the values of cycle threshold (Ct) between the treated group and the respective control group by using the $2^{-\Delta\Delta C_t}$ method [26]. Data were normalized to the expression of the endogenous control β -glucuronidase (*GUSB*), a housekeeping gene.

2.14 Statistical analysis

The experiments were carried out in triplicate and the results are expressed as means $\pm$ standard error of the means (S.E.M). The significance differences between treatments and control were analyzed by One-way ANOVA, followed by the Bonferroni's post-test. Mann-Whiney's test was used to compare the differences between two groups. All statistical analyses were performed using the GraphPad Prism Software version 6.0 (Inc., CA, USA). Differences were considered as statistically significant if * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$.

3. Results

3.1 Isolation and structure characterization of Pollonein-LAAO

A new L-aminoacid oxidase was isolated from *Bothrops moojeni* crude venom by three-chromatographic steps, named as Pollonein-LAAO. **Fig. 1** illustrates the yielded fractions obtained from the molecular exclusion, the hydrophobic interaction and the affinity chromatographies, whose LAAO-active fractions were S1 (**Fig. 1A**), Ph1 (**Fig. 1B**) and Bz2 (**Fig. 1C**). The SDS-PAGE analysis under reducing conditions of the LAAO-active fractions revealed that Pollonein-LAAO (Bz2 fraction) was successfully isolated and showed molecular weight estimated (M_r) of 62.89, which was calculated by relative migration distance (R_f) of the molecular mass standard bands and the LAAO band on SDS-PAGE (**Fig. 1D**). Analysis of Pollonein-LAAO homogeneity by reversed-phase HPLC demonstrated two different peaks corresponding to FAD cofactor and the polypeptide structure, Pollonein-LAAO (**Fig. 1E**). The purification process described herein yielded approximately 1.2 mg of Pollonein-LAAO from the crude venom (200 mg) that displayed LAAO activity of 3832.62 U.mg/min.

Upon sequence analysis (PEAKS software), the best match was shown to be for an L-amino acid oxidase from *B. moojeni* venom named BmooLAAO-I (ATU85535.1). Considering BmooLAAO-I as a template, Pollonein-LAAO showed a -10lgP of 694.45 and obtained 235 peptides, 359 *de novo* tags and 611 mass spectra. Pollonein-LAAO sequenced 397 amino acid residues from 502 of the template, totaling 79% of coverage.

The primary sequence obtained by mass spectrometry represented in the **Fig. 2** demonstrated that Pollonein-LAAO has six mutations sites, with only one conservative modification in the position 159 observed, where E (glutamic acid) was substituted for D (aspartic acid), both polar acid amino acids. The other five mutations were non-conservative: position 170, the polar uncharged amino acid S (serine) was replaced by basic polar R (arginine); for the position 182, a non-polar A (alanine) amino acid was mutated to a basic polar R; position 273, V (valine), a non-polar amino acid is substituted for R, a polar basic amino acid; in the position 297, the polar acid E is replaced by a non-polar A; and finally in the position 364, the polar uncharged amino acid S is replaced by a basic polar R. These punctual modifications reinforce that Pollonein-LAAO is a new isoform of LAAO from *B. moojeni* venom. The multiple sequence alignment (data not shown) enabled identification of a high identity of Pollonein-LAAO with BmooLAAO-I (98.74%) and BmooLAAO-II (92.16%).

3.2 Pollonein-LAAO reduces cells viability and increases intracellular reactive oxygen species (ROS) in PC-3 cell

The cytotoxic potential of Pollonein-LAAO against human cancer prostate cell (PC-3) was determined by the MTT assay. Pollonein-LAAO decreased PC-3 cell viability by around 40% at the highest concentrations tested (ranging from 3.125 to 50 µg/mL) after 24 h, displaying a concentration necessary to inhibit 50% of PC-3 cell viability (IC₅₀) of 2.4 µg/mL. Cells cotreated with catalase (150 U/mL) prevented the demise of the treated cells; however, it promoted cell growth at concentrations ranging from 0.09 to 12.5 µg/mL in comparison to the control (untreated cells) (**Fig. 3A**). Moreover, in order to assume that the cytotoxicity induced by Pollonein-LAAO is related to increased intracellular ROS generation, PC-3 cell was incubated with the H₂DCF-DA fluorescent probe and analyzed through flow cytometry. As depicted in **Fig. 3B**, Pollonein-LAAO measured a significantly elevated intracellular ROS at all concentrations when compared to control (untreated cells).

3.3 Pollonein-LAAO induces apoptosis in PC-3 cells

PC-3 cells incubated with Pollonein-LAAO became apoptotic in a concentration-dependent manner, since the apoptotic population increased from 8% in the control to 13%, 45% and 77% at 0.19, 0.78 and 3.125 $\mu\text{g/mL}$, respectively. Furthermore, the population of necrotic cells increased in PC-3 cell incubated with Pollonein-LAAO at 0.78 and 3.125 $\mu\text{g/mL}$ when compared to the control group (4% and 6%, respectively), while the control group presented a small population of necrotic cells (0.02%). Therefore, the prevalent mechanism to induce death in PC-3 cells after incubation with Pollonein-LAAO is apoptosis (**Fig. 4A,B**). Based on this, the expression of some genes involved in apoptosis cell death signaling pathway was analyzed. Pollonein-LAAO (0.78 $\mu\text{g/mL}$) up-regulated *TP53*, *BAX*, *BAD*, *TNFRSF10B* and *CASP8* while down-regulated *BCL2* gene expression (**Fig. 4C**).

3.4 Pollonein-LAAO causes alterations in the mitochondrial membrane potential of PC-3 cells

The next step was to investigate the changes in the mitochondrial membrane potential in PC-3 cells since Pollonein-LAAO induced cell death by apoptosis. This was achieved by labelling the cells with rhodamine 123 (Rh123), a cationic fluorescent probe that accumulates within mitochondria with polarized membranes. PC-3 cells treated with 0.19 $\mu\text{g/mL}$ of Pollonein-LAAO did not change the mitochondrial membrane potential. Two cell populations were identified with a different fluorescence intensity in PC-3 cells incubated with 0.78 $\mu\text{g/mL}$ of Pollonein-LAAO, indicating a small change in the mitochondrial membrane potential. On the contrary, at the concentration of 3.125 $\mu\text{g/mL}$ most of the cells presented a change in mitochondrial membrane potential, represented by the reduced fluorescent emission of Rh123 (**Fig. 5A, B**).

3.5 Pollonein-LAAO induces PC-3 cell arrest at the G0/G1 phase of the cell cycle

PC-3 cells displayed irregular distribution in different phases of the cell cycle compared to the distribution of control cells (untreated cells), with accumulation of cells in the G0/G1 phase from 61% in the control to 72% at 0.19 $\mu\text{g/mL}$ of Pollonein-LAAO. An increased concentration of Pollonein-LAAO leads to lower rates in G0/G1 at 0.78 and 3.125 $\mu\text{g/mL}$ (56% and 15%, respectively) with an increase in the population of subG1 cells at 0.78 and 3.125 $\mu\text{g/mL}$ (28% and 79%, respectively) and accompanied by corresponding reduction of cells in the S- and G2/M-phases (**Fig. 6A,B**). The expression of some genes

involved in the cell cycle control signaling pathways was analyzed. Pollonein-LAAO (0.78 µg/mL) up-regulated *CDKN1A*, whereas down-regulated *CDK2* and *E2F* expression (**Fig. 6C**).

3.6 Pollonein-LAAO induces cytotoxicity to multicellular tumor spheroid (MCTS)

To verify MCTS viability after Pollonein-LAAO treatment for 24 h, a fluorescence assay was assessed, where the spheroid area and the fluorescence intensity of necrotic core were measured using the ImageJ software. As observed in the control group, the PC-3 cell formed a tight aggregation of spheroids in the presence of HFF-1 cells with a characteristically necrotic and hypoxic core (in red, stained with PI) surrounded by an outer layer of actively proliferating cells (in blue, stained with Hoechst 33258). After Pollonein-LAAO treatment, the tumor spheroid area slightly increased at 0.78 µg/mL, while significantly increased at 3.125 µg/mL in comparison to the control (from 32 cm² to 38 cm²) (**Figs. 7A, B**). Interestingly, the MCTS treated with 3.125 µg/mL of Pollonein-LAAO showed a loose aggregation compared to the control spheroid. Moreover, the spheroid showed an augmented area of necrotic core (in red) and displayed disaggregated cells that were stained with PI when compared to the control (**Figs. 7A, C**). These findings suggest that Pollonein-LAAO is cytotoxic to PC-3 cells both in a monolayer culture and tumor spheroid model.

3.7 Pollonein-LAAO inhibits critical cell invasion steps via oxidative stress

3.7.1 Pollonein-LAAO decreases PC-3 adhesion to different extracellular matrix (ECM) components and modulates $\alpha 2\beta 1$ and $\alpha V\beta 3$ integrin expression

Since Pollonein-LAAO affects cell cycle progression and induces cell death by apoptosis in PC-3 cells, we decided to investigate whether Pollonein-LAAO could interfere with tumor cell adhesion, migration and invasion, three crucial events in the cell invasion process. Pollonein-LAAO inhibited the adhesion of PC-3 cells to different components of the extracellular matrix (ECM) in all concentrations. Compared to the control, Pollonein-LAAO-treated PC-3 cells reduced collagen IV, fibronectin and matrigel attachment by about 33%, 42% and 54% at concentrations of 0.09, 0.19 and 0.39 µg/mL, respectively. Pollonein-LAAO effects on cell adhesion were eliminated when catalase was also incubated with PC-3 cells, reinforcing that the major source of ROS induced by this new L-aminoacid oxidase is the hydrogen peroxide (**Figs. 8A-D**).

Furthermore, expressions of some genes were analyzed, such as *ITGA2*, *ITGB1*, *ITGAV* and *ITGB3*, which encode collagen IV and fibronectin binding integrins ($\alpha_2\beta_1$ and $\alpha_V\beta_3$). Pollonein-LAAO (0.19 $\mu\text{g/mL}$) significantly down-regulated *ITGA2*, *ITGAV* and *ITGB3*, and unlike up-regulated *ITGB1* (**Fig. 9A**). To validate this result, $\alpha_2\beta_1$ integrin content in PC-3 cells was assessed by immunofluorescence. Both α_2 (in green) and β_1 (in red) subunits are expressed in untreated cells (control group); however, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) treatment decreased the α_2 subunit and increased β_1 subunit compared to the control. The presence of yellow regions suggests the co-localization of these two subunits (**Fig. 9B**). Quantitative analysis exhibited a significant increase in β_1 subunit fluorescence intensity (**Fig. 9C**) and a decrease in α_2 subunit fluorescence intensity (**Fig. 9D**) compared to the control.

3.7.2 Pollonein-LAAO affects PC-3 cell motility and invasiveness

Pollonein-LAAO affected the motility of PC-3 cells in two different migration models, transwells and wound healing. Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) inhibited the migration of PC-3 cells through a transwell compared to the positive control. The number of migrated PC-3 cells was 349 in positive control, reducing to around 205, 5 and 0 cells at 0.09, 0.19 and 0.39 $\mu\text{g/mL}$ of Pollonein-LAAO, respectively (**Figs. 10A, C**). In the scratch assay, Pollonein-LAAO impaired the scratch closure of PC-3 cells in 56% and 64% at 0.19 and 0.39 $\mu\text{g/mL}$, respectively, compared to control (untreated cells) (**Figs. 11A, B**). In addition, PC-3 cells lost their invasive capacity if incubated with Pollonein-LAAO at all tested concentrations in the matrigel transwell assay. The number of invading cells reduced from 724 cells in the positive control to approximately 438, 2 and 1 cells at 0.09, 0.19 and 0.39 $\mu\text{g/mL}$, respectively, compared to the positive control (**Figs. 10B, D**).

In order to verify whether the observed effects were associated with peroxide hydrogen production induced by Pollonein-LAAO, PC-3 cells were co-incubated with catalase (150 U/mL). The antioxidant effects of catalase alter the oxidative effects of Pollonein-LAAO, maintaining the invasiveness of PC-3 cells. As a result, the catalase cotreatment abolished the effects caused by Pollonein-LAAO in PC-3 cell migration and invasion (**Fig. 10, Fig. 11**). Analysis of genes involved in critical steps of the metastasis processes revealed that Pollonein-LAAO (0.19 $\mu\text{g/mL}$) was able to down-regulate *SNAIL*, *VIM* and *MMP-2* expression compared to the control (untreated-cells) (**Fig. 11C**).

4. Discussion

Snake venoms are an invaluable source of bioactive molecules that can be used as pharmacological tools to elucidate human disease mechanisms, to uncover new therapeutic targets and to develop new life-saving drugs [27,28]. In this context, this is the first report of a new L-amino acid oxidase (LAAO) purified from *Bothrops moojeni* venom, named as Pollonein-LAAO, and its effects in prostate cancer cell progression evaluated by *in vitro* assays.

Pollonein-LAAO was isolated in three chromatographic steps and represented 0.6 % of the soluble venom protein, i.e., the fractionation of 200 mg of *B. moojeni* venom yielded about 1.2 mg of Pollonein-LAAO. Under reducing condition, Pollonein-LAAO revealed a relative molecular mass of 62.89. Snake venom L-amino acid oxidases (svLAAO) are glycoprotein with molecular mass of 120-150 kDa under dimeric form and 50-70 kDa in monomeric form [14,29,30]. The molecular mass of other LAAOs obtained from *B. moojeni* venom, as reported in earlier studies, ranged from 46.6 kDa to 64.8 kDa [17,31,32]. This variation in molecular mass may be related to the sites of glycosylation since svLAAOs are considered to be glycoproteins that contain about 3-4% carbohydrates in their structure, and in some cases, the content may be up to 12% of their total molecular mass [30,33,34].

Since Pollonein-LAAO displayed a different molecular mass from other LAAOs purified from *B. moojeni* venom, we decided to perform mass spectrometry to elucidate the Pollonein-LAAO sequence. As a result, Pollonein-LAAO showed the best match with BmooLAAO-I (ATU85535.1) with 79% of amino acid residue coverage. Furthermore, Pollonein-LAAO has six mutation sites in comparison to BmooLAAO-I sequence, reinforcing that Pollonein-LAAO is a new isoform of LAAO from *B. moojeni* venom. In addition, Pollonein-LAAO shared high identity with BmooLAAO-I and BmooLAAO-II primary sequence (>90%). It is noteworthy that amino acid sequence alignment analysis indicates a high degree of sequence identity among svLAAOs (>84%) [30].

L-amino acid oxidase is considered as the most potent apoptotic agent in snake venom due to their cytotoxicity that has been studied for decades [9,30]. The most accepted assumption is that accumulated hydrogen peroxide (H₂O₂), a reactive oxygen species (ROS), generated as a by-product from svLAAOs catalytic activity, plays a major role in triggering cell death through the induction of oxidative stress in cancer cells [9,10]. In this sense, we herein investigated the H₂O₂ effects induced by Pollonein-LAAO in prostate cancer cell

growth and progression and, for the first time, its effects in the invasion process, assessed by *in vitro* assays.

Pollonein-LAAO exerts cytotoxicity against human prostate cancer cells (PC-3) that is associated with its ability to increase intracellular ROS generation since cotreatment with catalase, a potent antioxidant that exhibits catalytic activity by converting H_2O_2 in H_2O and O_2 [35], completely abolished Pollonein-LAAO-induced cytotoxicity effect. As the deregulated production of ROS contributes to promote several characteristics of malignant phenotype, consequently, cancer cells have an increased pro-oxidant status that enhances their vulnerability to elevated ROS levels generated by exogenous ROS inducers beyond the cytotoxic threshold leading to cell death [4,7,11]. Our results are consistent with previous studies reporting that several svLAAOs exhibited cytotoxic effects against different tumor cell lines such as BjussuLAAO-II in human breast adenocarcinoma ($\text{IC}_{50}= 1.8 \mu\text{g/mL}$) [12], BmooLAAO-I in human promyelocytic leukemia cells ($\text{IC}_{50}= 0.038 \mu\text{g/mL}$) [36], CC-LAAO in human glioblastoma ($\text{IC}_{50}= 0.3 \mu\text{g/mL}$) [37], LAAO from *Crotalus atrox* venom in human hepatocellular carcinoma ($\text{IC}_{50}= 3.7 \mu\text{g/mL}$) [38] and LAAO from *Crotalus adamanteus* venom in human colorectal carcinoma ($\text{IC}_{50}= 2.88 \mu\text{g/mL}$) [39].

The acceleration of accumulative ROS over the cytotoxic threshold disrupts cellular reduction-oxidation (redox) homeostasis and causes severe cellular damage leading to cancer cell death [3]. In fact, Pollonein-LAAO induced apoptosis in a concentration-dependent manner, which may be associated with its ability to up-regulate the tumor suppressor gene *TP53* that codes for tumor suppressor protein 53 (p53). The transcription factor p53 activation in response to oxidizing conditions may also occur regardless of DNA damage through redox signaling, which from a therapeutic perspective, would provide a means to activate p53 without inducing collateral DNA damage and mutation [40].

The stabilized p53 regulates apoptosis transcriptionally by down-regulating pro-apoptotic genes such as Bcl-2, Bcl-XL, IAPs and survivin, and upregulating pro-apoptotic genes that are essential to induce an intrinsic pathway of apoptosis as Bax, Bid, Puma, Noxa and Apaf-1, but also, extrinsic pro-apoptotic factors like Fas, FasL, DR-4 and DR-5. Indeed, apoptosis induced by H_2O_2 has been associated with increased protein expression of p53, Noxa, Puma and Bax [6]. As illustrated, Pollonein-LAAO ($0.78 \mu\text{g/mL}$) was able to modulate the expression of Bcl-2 family members by increasing the pro-apoptotic genes *BAX* and *BAD* and decreasing the anti-apoptotic *BCL2* that codes Bax, Bad and Bcl-2 proteins, respectively. In response to cell death stimuli, Bad binds and inhibits the anti-

apoptotic proteins like Bcl-2, Bcl-W and Bcl-XL that, in turn, allow the oligomerization of two pro-apoptotic proteins, Bax and Bak, which cause mitochondrial outer membrane permeabilization (MOMP) and subsequent release of cytochrome *c* leading to the initiation of caspase-mediated apoptosis [41-43].

The apoptosis process was apparent in both intrinsic and extrinsic pathways of apoptosis since Pollonein-LAAO (0.78 µg/mL) also up-regulated the gene expression of the tumor necrosis factor receptor (*TNFRSF10B* also known as *DR5*), a cell surface death receptor that when activated is typically involved in the cleavage and activation of caspase-8, which is in accordance with our results of up-regulation of *CASP8*. These findings are in accordance with Mukherjee et al. [44] and Costa et al. [45], who observed that svLAAOs were able to activate extrinsic and intrinsic pathways of apoptosis. It is noteworthy that cancer cells are dependent on dysfunctional apoptotic processes for their survival [42,46]. Thus, the capacity of Pollonein-LAAO to restore the apoptotic signaling pathways gives it the potential to eliminate cancer cells.

Apoptosis can be triggered by DNA damage, oxidative stress, growth factor deprivation and mitochondrial membrane potential collapse [47]. The mitochondrial membrane potential can be assessed by using cationic probes like Rh123, whose uptake by mitochondria is proportional to the driving force; in other words, Rh123 only enters mitochondria with intact membrane potential. Thus, when membrane potential is lost, the dye is leached out of the mitochondria and its accumulation induces fluorescence quenching that is correlated with mitochondrial membrane potential [48,49]. The reduction of dye fluorescence is probably due to its aggregation or stacking after accumulation [48]. Our results demonstrated that the Rh123 fluorescence quenching rate increased in Pollonein-LAAO-treated PC-3 cells, which is correlated with the loss of the mitochondrial membrane potential. The same results were obtained with Rusvinoxidase, an LAAO isolated from *Daboia russelii russelii* venom [44]. In fact, ROS such as H₂O₂ can trigger apoptosis through stimulation of mitochondrial permeability transition pore (MPTP) opening through oxidative modifications of MPTP components like cyclophilin D, leading to the collapse of mitochondrial membrane potential, mitochondrial translocation of Bax, Bad and cytochrome *c* leakage [6].

As mentioned, Pollonein-LAAO increased *TP53* gene expression that can act as a regulator of cellular proliferation [42] by induction of apoptosis and cell cycle arrest [50]. In line with this, Pollonein-LAAO prevented the continuous proliferation of PC-3 cell by

blocking cell cycle entry in the replicative S phase, which may be associated with the up-regulation of the expression of genes involved in cell cycle-control G1-to-S transition pathway including *CDKN1A* and down-regulation of *CDK2* and *E2F* expression. The stabilized p53 binds elements to the *CDKN1A* promoter and activates the codification of the cyclin-dependent kinase inhibitor p21. The p21 protein then inhibits all cyclin-CDK2 complexes that are involved in the hyper-phosphorylation of Rb. Thus, hypo-phosphorylated Rb successively forms complexes with the E2F transcription factor that down-regulates the transcription of genes promoting G1-to-S transition. Taken together, this sequence of events prevents the cyclin E/A-CDK2 activity blocking cell cycle progression in the G1 phase [50,51].

It is noteworthy that tumor growth depends on continued cell proliferation that is strongly interconnected with defective cell cycle checkpoint, which compromises mainly the ability of tumor cells to exit the cell cycle [51]. Commonly, the G1-S transition control machinery is found mutated in cancer cells, since abrogation of the G1-S control point allows cells to enter the cell cycle and progress into S phase in an unrestrained fashion, regardless of signals from the environment [52].

Upon the antiproliferative effects, Pollonein-LAAO (0.78 and 3.125 $\mu\text{g/mL}$) increased subG1 population that has been considered by some authors as evidence of apoptosis initiation [25,53-55]. It is attempting to speculate that these results propose that G0/G1-phase shifted into subG1 as a consequence of increased accumulation of ROS levels generated by Pollonein-LAAO, which induced irreparable damage and then initiated programmed cell death [3,40]. Consistent with this idea, our data demonstrated that Pollonein-LAAO induces apoptosis cell death in PC-3 cells in all concentrations assessed. Few studies have suggested that svLAAOs can selectively bind to the cancer cell surface, which allows the release of high local concentration of hydrogen peroxide, leading to apoptosis cell death [9,56]. Furthermore, the delivery mode of hydrogen peroxide might be crucial, since exogenous hydrogen peroxide induces a distinct cell death process in comparison with svLAAO-induced cell death [57]. Extensive studies have revealed that svLAAOs trigger apoptosis cell death on cancer cells [36-38,44,45,58,59].

In view of the fact that Pollonein-LAAO affects prostate tumor cells proliferation and induces apoptosis cell death in two-dimensional (2D) conventional *in vitro* assays, its effects in three-dimensional (3D) multicellular tumor spheroid (MCTS) were investigated. As a result, Pollonein-LAAO (0.78 $\mu\text{g/mL}$) was cytotoxic against MCTS by means of

decreased spheroid area after 24 h of treatment. Furthermore, cell-to-cell interaction was disrupted due to the cytotoxicity of Pollonein-LAAO at 3.125 $\mu\text{g/mL}$, since Pollonein-LAAO-treated spheroids displayed a loose aggregation form, suggesting that the adhesion molecules were affected since it was observed that PC-3 cells lost the ability to aggregate into the sphere [60]. Spheroids have typical mass transport limitations that limit the diffusion of nutrients, oxygen, metabolic waste and even drugs. This occurs due to their unique features as cellular organization and density and chemical gradients (oxygen, nutrients, pH), thus displaying close physiological similarities to *in vivo* solid tumors. Therefore, tumor spheroids have been considered as a promisor tool to evaluate and predict drug efficacy *in vitro* in comparison to 2D culture models [20,60-62].

As outlined above, Pollonein-LAAO was able to reduce the MCTS that mimics the *in vivo* primary tumor mass, so we decided to evaluate Pollonein-LAAO effects in critical steps of metastasis such as cellular adhesion, migration and invasion. In tumor invasion, three crucial processes are required to form metastasis that include adhesion, extracellular matrix (ECM) reorganization and motility. Upon adhesion, in cancer derived from epithelium, reduction in cell-cell adhesion is one of the key factors to lead the dissociation of cancer cells from the primary tumor and, consequently, enhances metastatic dissemination to secondary locations [63]. One central process that enables these steps is the epithelial-mesenchymal transition (EMT), which is orchestrated and coordinated by a series of master EMT-inducing transcription factors such as Snail, Slug, Twist and Zeb1 [64].

In this sense, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) decreased the gene expression of *SNAIL* that encodes the transcription factor Snail. The transcription factor Snail can mediate EMT and promote metastasis through the down-regulation of cell adhesion molecules, epithelial markers, such as E-cadherin, claudin, occludin and zona occluden-1 by binding in their promoter region [65-67]. The Snail expression is associated with tumor malignancy since it appears to enforce a mesenchymal phenotype by promoting the expression of mesenchymal genes like vimentin, fibronectin and N-cadherin, and, thus, high *SNAIL* expression has been correlated with cancer aggressiveness and poor prognosis [65,68,69]. Interestingly, Pollonein-LAAO was able to down-regulate mesenchymal marker gene expression such as *VIM* (that encodes vimentin). Vimentin is a cytoskeletal protein that supports mechanical integrity of the migratory machinery, generation of directional force, focal adhesion modulation and extracellular attachment. Thus, loss of vimentin decreases migration and

mesenchymal cellular phenotype [70,71]. Taken together, these results can explain, in part, the effects of Pollonein-LAAO in the inhibition of PC-3 cell migration and invasion.

Pollonein-LAAO was able to down-regulate *VIM* gene expression that is correlated with reduced focal adhesion, which explains, at least in part, the reduction in PC-3 cell attachment to different ECM proteins components (collagen IV, fibronectin and matrigel) in a similar way. In accordance with this finding, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) can also inhibit cell-to-matrix adhesion through the down-regulation of gene expression of integrin subunits such as *ITGA2*, *ITGAV* and *ITGB3* and decrease the α_2 integrin expression in PC-3 by immunofluorescence quantification. The cell-matrix adhesion occurs through transmembrane glycoproteins known as integrins, which form focal adhesion between cancer cells and ECM enhancing their migratory and metastatic capabilities [63,72]. The $\alpha_2\beta_1$ is a well-known receptor for collagen that is highly expressed in prostate cancer cells, which has been implicated in prostate cancer progression since its activation/phosphorylation can active multiple signal transduction pathways that may support tumor cell invasion and metastasis [73,74]. Integrin $\alpha V\beta_3$ mediates cell adhesion and migration on different ECM proteins, including vitronectin, fibronectin, fibrinogen, laminin, collagen, and others [75].

Similar to $\alpha_2\beta_1$, $\alpha V\beta_3$ is associated with the growth, survival, invasion and metastasis of different tumor cells [72]. Furthermore, bone is the most frequent metastatic site for prostate cancer and, hence, reducing the expression of $\alpha V\beta_3$ and $\alpha_2\beta_1$ have been reported to inhibit growth of tumor in bone and increases apoptosis of prostate tumor cells [76,77]. Of note, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) up-regulated both gene and protein expression of integrin β_1 , which may be explained as a compensatory expression of β_3 , since the expression levels of β_1 integrin and β_3 integrin are associated [72].

Extracellular matrix (ECM) disassembly is a crucial step in the invasive and metastatic processes of cancer cells, which are regulated by matrix metalloproteinases (MMPs) [78]. Indeed, MMPs can induce cell migration through cleavage of cell-matrix or cell-cell receptors, removing sites of adhesion, exposing new building sites and releasing chemoattractants from ECM [79]. The analysis revealed that Pollonein-LAAO down-regulates *MMP2* gene expression, a member of the MMP gelatinase family whose expression has been extensively reported in prostate cancer, since elevated MMP-2 levels have been associated with larger tumor size [80], tumor angiogenesis and metastasis [81]. Moreover,

MMP-2 is overexpressed in prostate cancer tissues in comparison to benign prostatic hyperplasia; thus, MMP-2 expression has a prognostic value [82].

5. Conclusion

In the present study, a new L-aminoacid oxidase named as Pollonein-LAAO was isolated from *Bothrops moojeni* venom. Herein, Pollonein-LAAO induced ROS production in PC-3 cells causing oxidative stress that culminates in the reduction of PC-3 cell proliferation by means of a delay of the G0/G1 cell cycle phase, apoptosis and inhibition of cellular adhesion, migration and invasion, which are critical processes involved in cancer cell invasion. According to these data, Pollonein-LAAO modulated genes that are associated with intrinsic and extrinsic apoptosis pathways (*TP53*, *BAX*, *BAD*, *BCL2*, *TNFRSF10B* and *CASP8*), cell cycle control G1-to-S transition (*CDKN1A*, *CDK2* and *E2F*) and epithelial-mesenchymal transition (*SNAIL*, *VIM*, *MMP2*, *ITGA2*, *ITGAV* and *ITGB3*).

Altogether, these results demonstrate that oxidative stress plays an intriguing role in the mechanism underlying prostate cancer progression, since Pollonein-LAAO affected several malignant phenotypes. To this end, Pollonein-LAAO is an intriguing ROS-based agent that can be used as a biotechnological tool to enhance the current understanding of cancer treatment strategies.

CRedit authorship contribution statement

Lorena Polloni: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Tássia Rafaella Costa:** Conceptualization, Investigation, Writing – Review & Editing, Supervision. **Lorena Pinheiro Moraes:** Investigation. **Bruna Cristina Borges:** Formal analysis, Investigation, Writing – Review & Editing. **Samuel Cota Teixeira:** Resources, Investigation, Writing – Review & Editing. **Thales Alves de Melo Fernandes:** Formal analysis, Investigation. **Lucas Ian Veloso Correia:** Investigation, Writing – Review & Editing. **Luciana Machado Bastos:** Resources, Writing – Review & Editing. **Fernanda Gobbi Amorim:** Formal analysis, Resources, Investigation, Writing – Review & Editing. **Loïc Quinton:** Resources, Investigation, Writing – Review & Editing. **Andreimar Martins Soares:** Resources, Writing – Review & Editing. **Marcelo José Barbosa Silva:** Resources, Writing – Review & Editing. **Eloisa Amália Vieira Ferro:** Resources, Writing – Review & Editing. **Daiana Silva Lopes:** Conceptualization, Writing – Review & Editing. **Veridiana de Melo**

Rodrigues Ávila: Conceptualization, Writing – Review & Editing, Supervision, Project administration.

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Declaration of competing interest

All authors declare that they have no conflict of interest.

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Figures and Captions

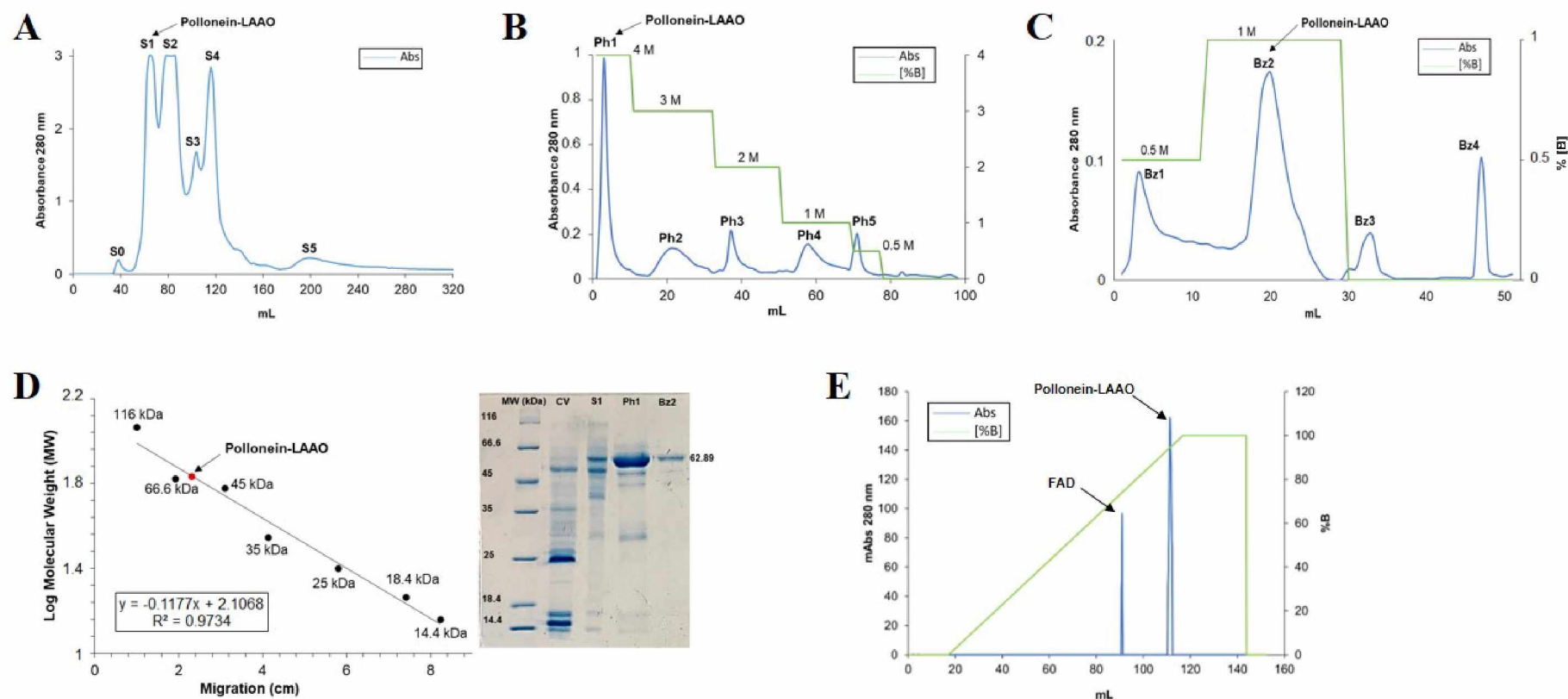


Figure 1. Purification of Pollonein-LAAO from *Bothrops moojeni* snake venom. (A) Chromatography profile of the crude venom (200 mg). (B) Chromatographic profile of the subfraction S1 (27.4 mg). (C) Chromatographic profile of the subfraction Ph1 (4.3 mg). The green line (%B) corresponds to the concentration of NaCl in the elution buffer. (D) Molecular mass estimation of Pollonein-LAAO based on its migration (cm) through the SDS-PAGE (12.5%). Inset: SDS-PAGE (12.5%) under reducing conditions stained with Coomassie Brilliant Blue R-350 showing molecular mass standards, crude venom (CV) and chromatographic fractions S1, Ph1 and Bz2 (Pollonein-LAAO). (E) Chromatographic profile of subfraction Bz2 (300 µg) on a C18 reversed-phase HPLC column. The green line (%B) corresponds to the concentration of acetonitrile.

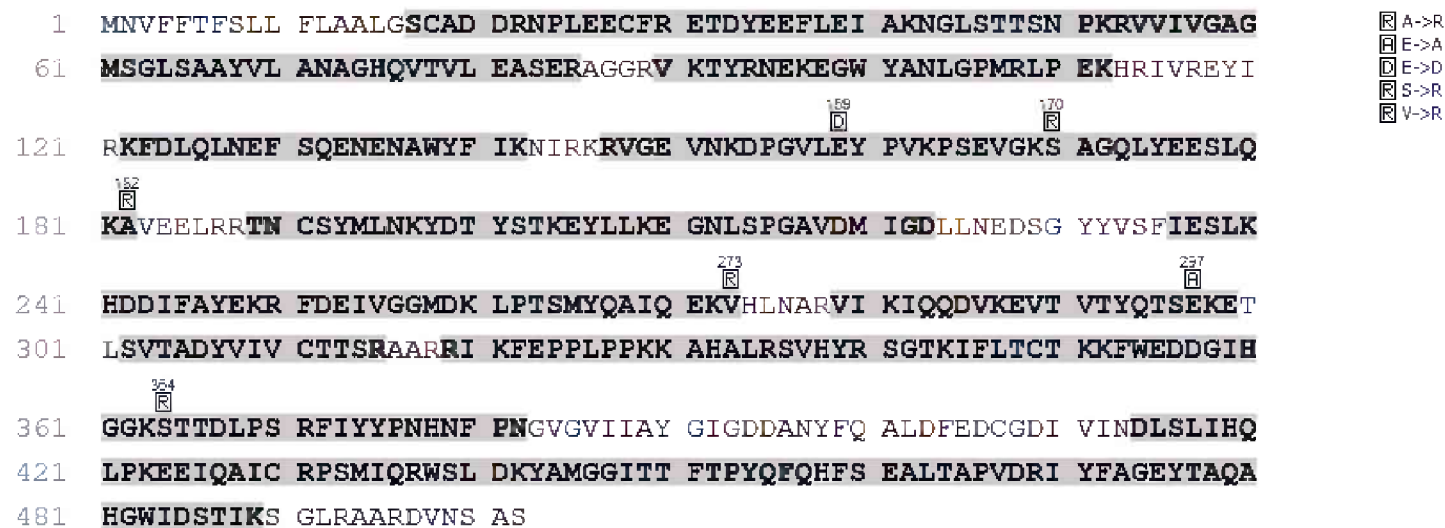


Figure 2. *De novo* sequencing of the Pollonein-LAAO using the L-amino acid oxidase from *Bothrops moojeni* (ATU85535.1) as a **template**. The amino acid residues sequenced by mass spectrometry are represented in gray. The white boxes represent the mutations, where E (glutamic acid) was substituted for D (aspartic acid); S (serine) was replaced by basic polar R (arginine); A (alanine) was replaced R; and V (valine) was substituted for R. Only confident mutations are displayed as icons above the protein sequence.

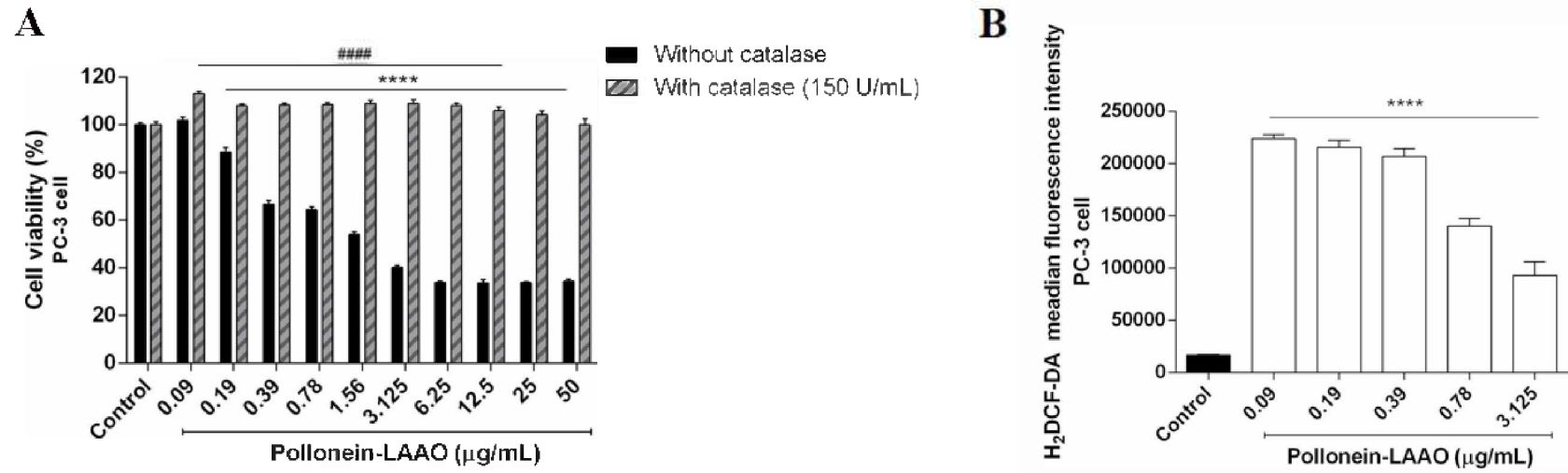


Figure 3. Pollonein-LAAO induces cytotoxicity and generates intracellular reactive oxygen species (ROS) in PC-3 cells. (A) Graph representing viability of the cells treated with different concentrations of Pollonein-LAAO (ranging from 0.09 to 50 µg/mL) in human prostate cancer cell (PC-3) after 24 h incubation with or without catalase (150 U/mL) determined by MTT. **(B)** Representative graph of changes in ROS levels in PC-3 cells measured by flow cytometry. Cells were treated with different concentrations of Pollonein-LAAO (0.09, 0.19, 0.39, 0.78 and 3.125 µg/mL) for 4 h and stained with the probe H₂DCF-DA. **** $p < 0.0001$ compared to the control (non-treated cells) and ##### $p < 0.0001$ compared to the control with catalase.

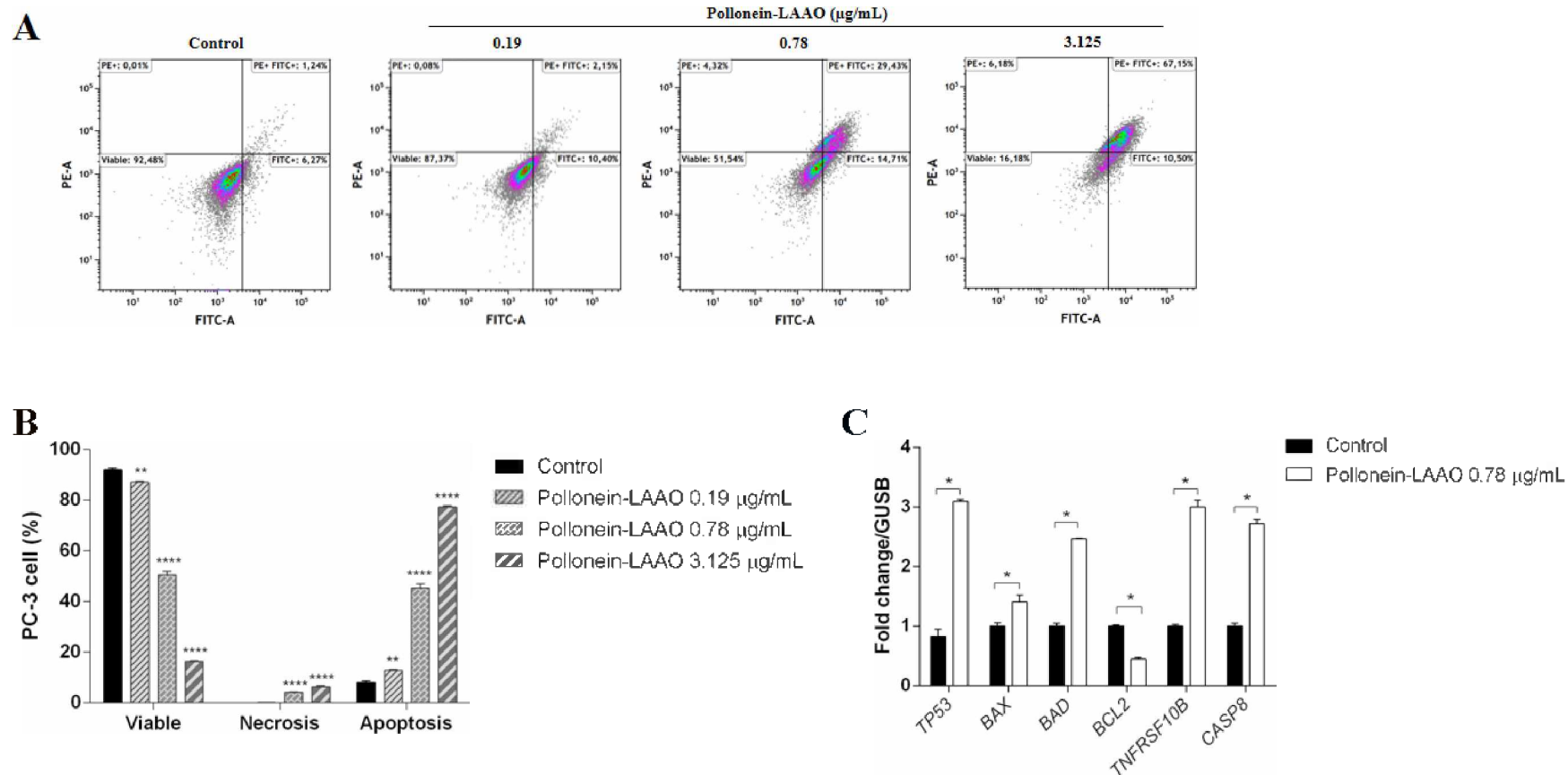


Figure 4. Pollonein-LAAO induces apoptosis and modulates expression of both intrinsic and extrinsic apoptotic genes in PC-3 cells. (A) Dot plot graphs demonstrating the distribution of PC-3 cells after Pollonein-LAAO (0.19, 0.78 and 3.125 $\mu\text{g/mL}$) treatment for 24 h, according to staining or not of annexin V (FITC+) and propidium iodide (PE+), performed by flow cytometry. **(B)** Representative graph of the percentage of PC-3 cells in apoptosis (FITC+ and PE+ FITC+ quadrants) and necrosis (PE+ quadrant). **(C)** Graph representing the gene expression profile in PC-3 cells treated with Pollonein-LAAO (0.78 $\mu\text{g/mL}$) for 24h. The gene expression was determined by quantitative reverse PCR. *GUSB* was used as a reference gene. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ compared to the control (non-treated cells).

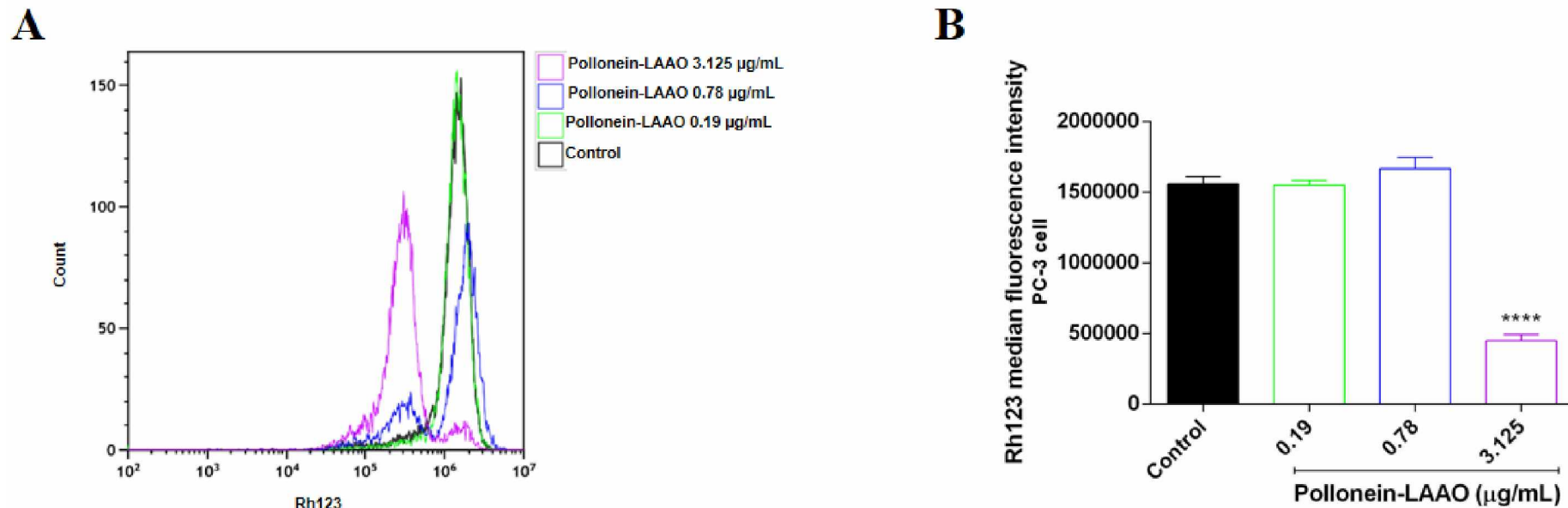


Figure 5. Pollonein-LAAO induces mitochondrial damage in PC-3 cells. (A) Histogram demonstrating the fluorescence intensity of Rh123 in control cells (non-treated cells, black line) cells treated with 0.19 $\mu\text{g/mL}$ (green line), 0.78 $\mu\text{g/mL}$ (blue line) and 3.125 $\mu\text{g/mL}$ (purple line) of Pollonein-LAAO. **(B)** Graph representing the quantification of the median fluorescence intensity obtained by flow cytometry. **** $p < 0.0001$ compared to the control (non-treated cells).

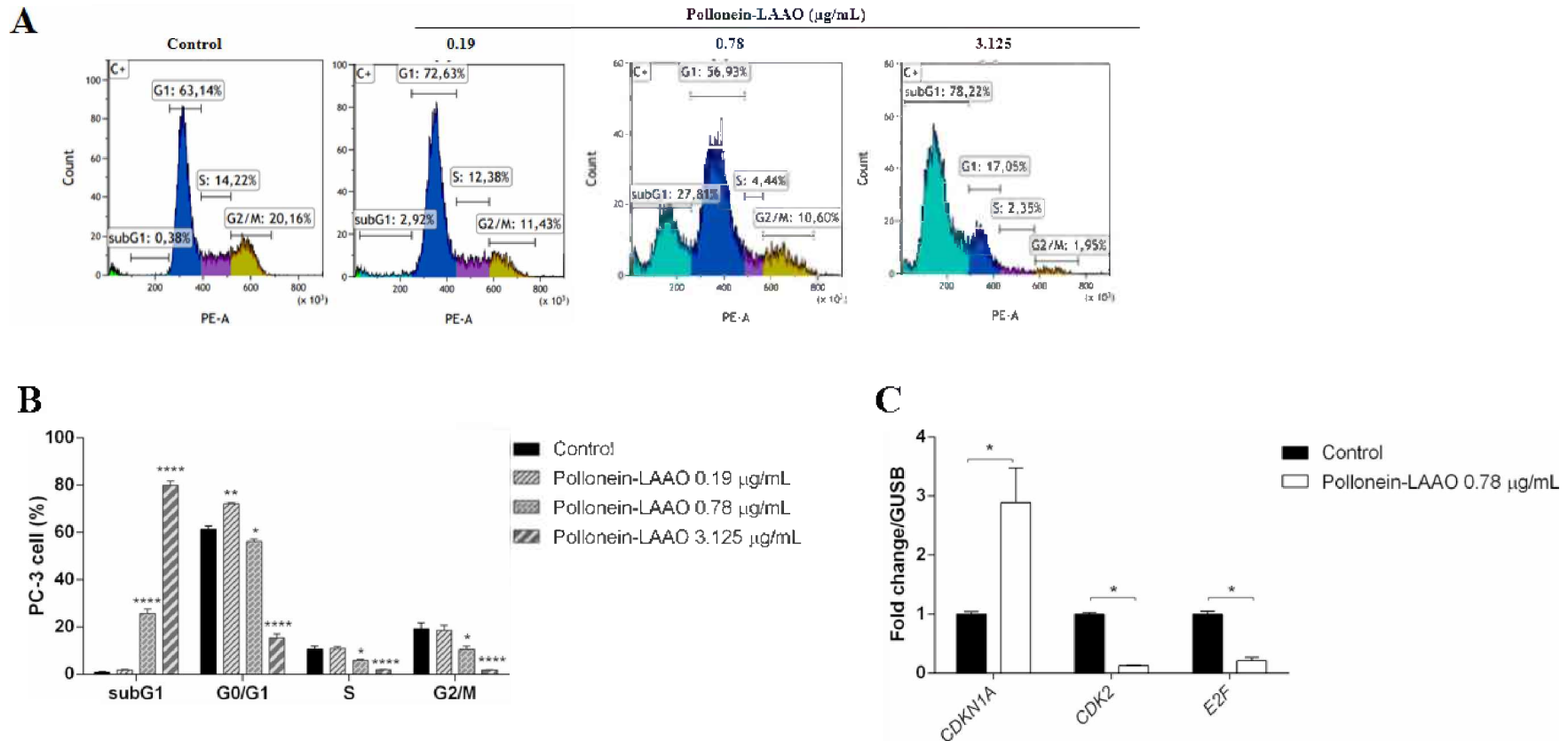


Figure 6. Pollonein-LAAO causes cell cycle delay in G0/G1 phase with upsurge of subG1 population and modulates the expression of genes involved in cell cycle-control signaling pathway in PC-3 cells. (A) Histograms illustrating the distribution of PC-3 cells in the phases of the cell cycle after Pollonein-LAAO (0.19, 0.78 and 3.125 $\mu\text{g/mL}$) treatment for 24 h, according to DNA content after PI staining, assessed by flow cytometry. **(B)** Representative graph of the percentage of PC-3 cells in each cell cycle phase. **(C)** Graph representing the gene expression profile in PC-3 cells treated with Pollonein-LAAO (0.78 $\mu\text{g/mL}$) for 24h. The gene expression was determined by quantitative reverse PCR. *GUSB* was used as a reference gene. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ compared to the control (non-treated cells).

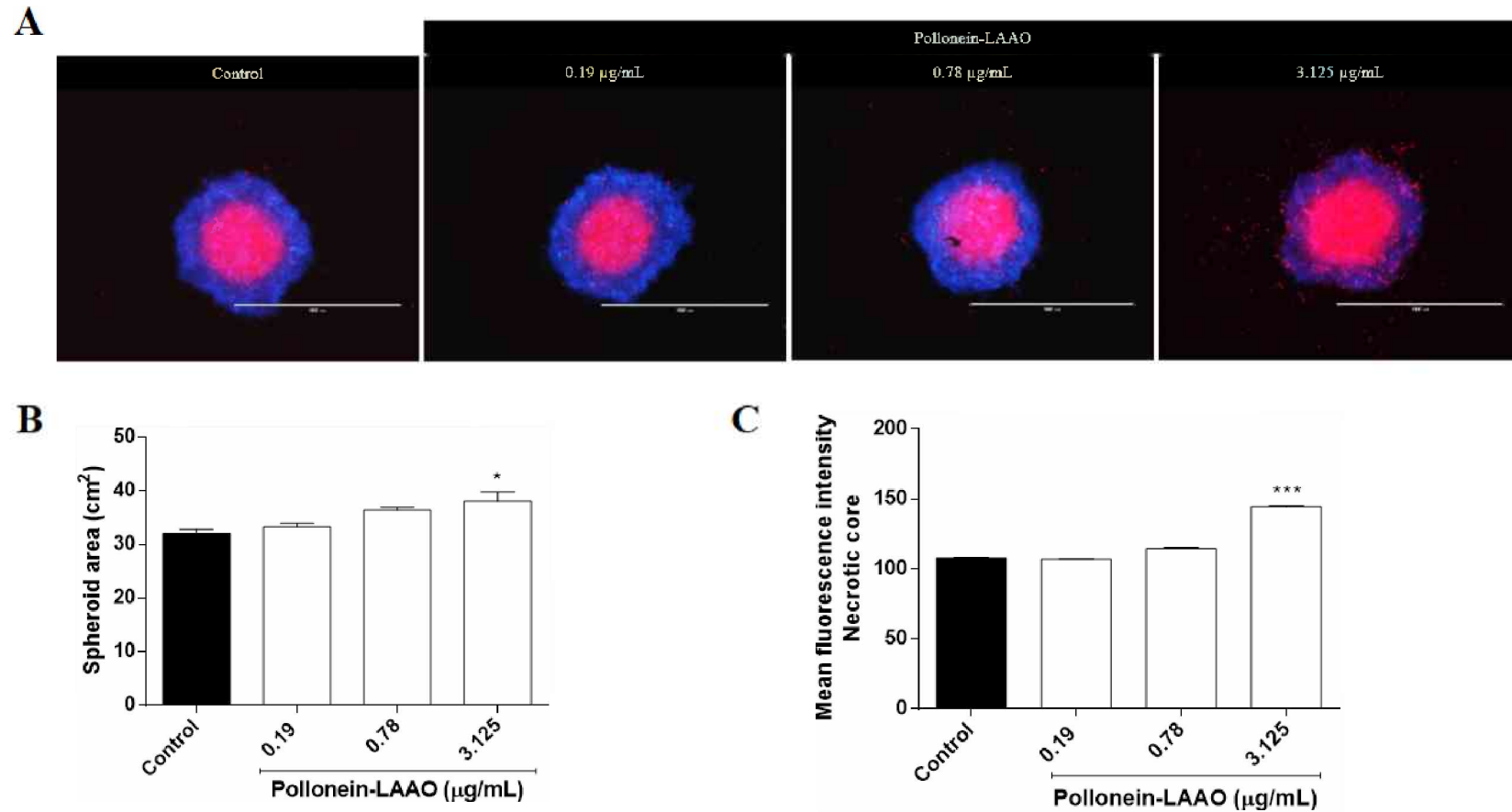


Figure 7. Pollonein-LAAO induces cytotoxicity in multicellular tumor spheroid (MCTS). (A) Photomicrography of the structure of MCTS after Pollonein-LAAO (0.19, 0.78 and 3.125 µg/mL) treatment for 24 h. MCTS is organized in two main layers, including a proliferative outer layer (in blue, stained with Hoechst 33258) and necrotic core (in red, stained with propidium iodide). Scale bars: 1,000 µm. Graphs representing the (B) MCTS area and (C) Mean fluorescence intensity of necrotic core obtained by ImageJ measurement. * $p < 0.05$ and *** $p < 0.001$ compared to the control (non-treated spheroids).

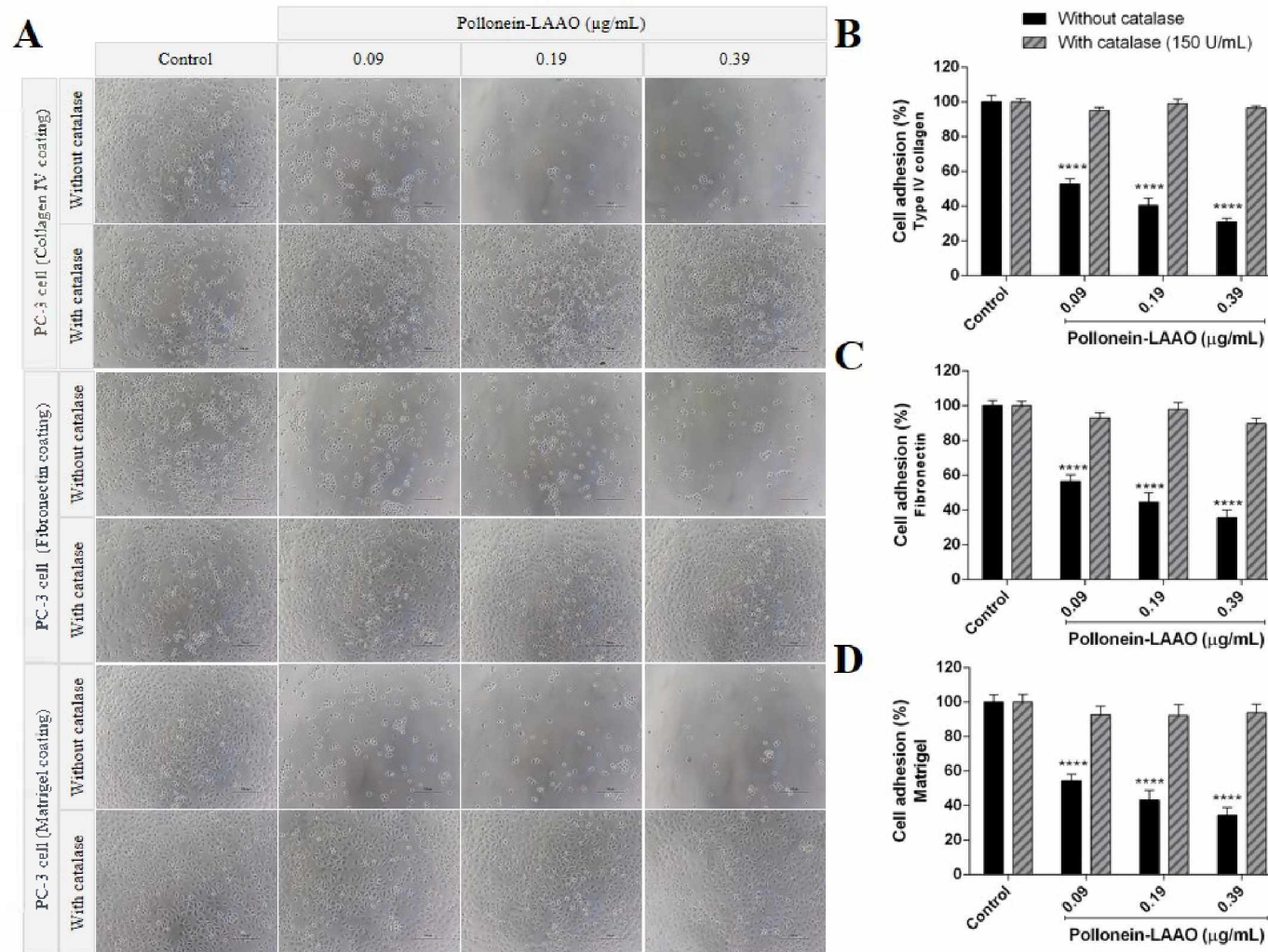


Figure 8. Pollonein-LAAO inhibits PC-3 cell adhesion in different extracellular matrix components. (A) Representative photomicrography of PC-3 cell adhesion in wells precoated with collagen IV, fibronectin and matrigel. Scale bars: 200 μm . Graph representing the percentage of PC-3 cell adhesion after Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) treatment in presence or not of catalase (150 U/mL) for 4 h on (B) collagen IV, (C) fibronectin and (D) matrigel, assessed by MTT assay. **** $p < 0.0001$ compared to the control (untreated cells).

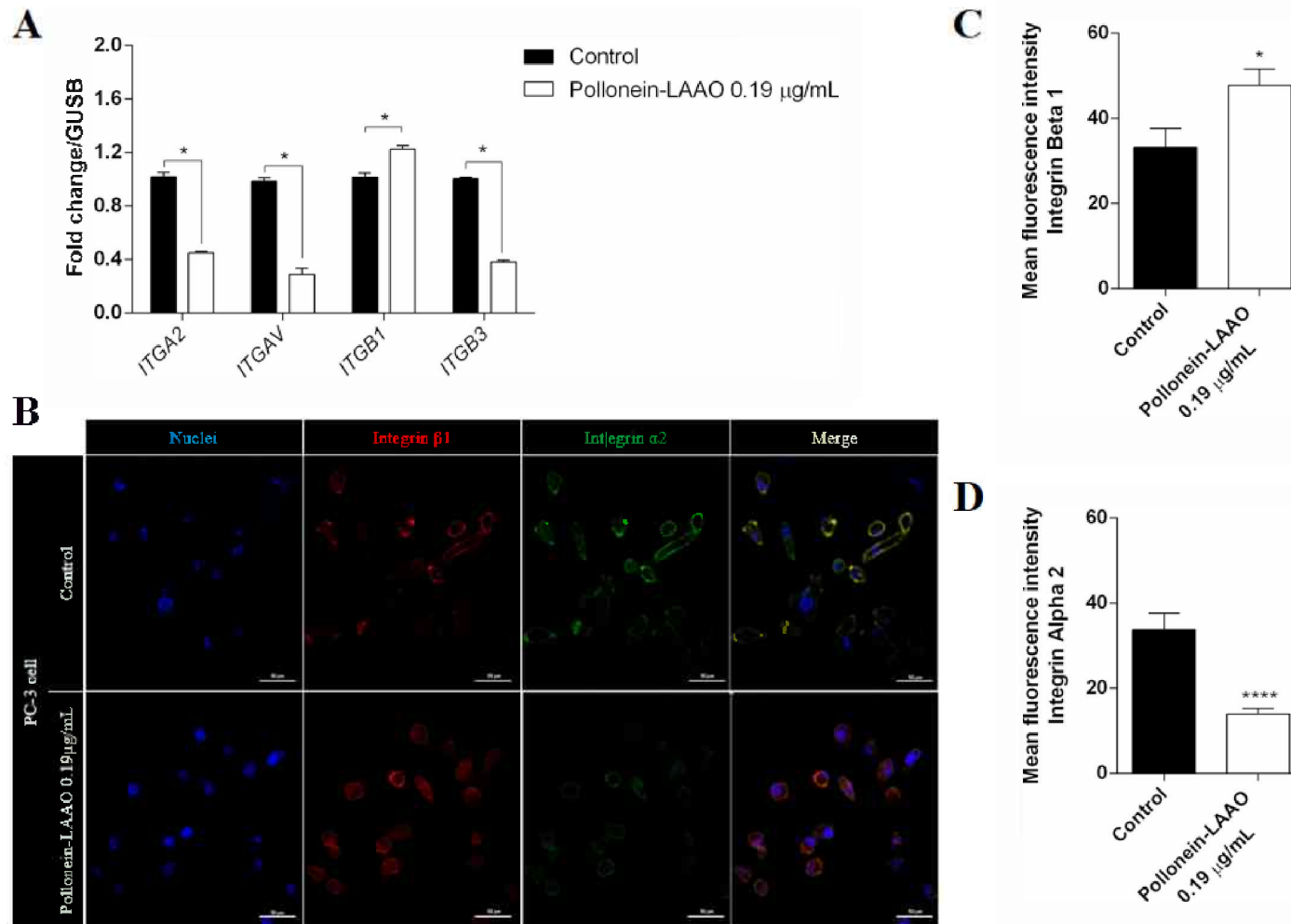


Figure 9. Pollonein-LAAO decreases integrin subunit expression in PC-3 cells. (A) Integrin subunits of gene expression profile in PC-3 cells treated with Pollonein-LAAO (0.19 µg/mL) for 24h. The gene expression was determined by quantitative reverse PCR. *GUSB* was used as a reference gene. (B) Representative photomicrography of immunofluorescence detection of α_2 (in green) and β_1 (in red) in PC-3 cells after Pollonein-LAAO treatment. Nuclei were stained with TO-PRO (in blue). Scale bars: 50 µm. Representative graphs of (C) β_1 and (D) α_2 integrin subunits fluorescence intensity measured by ImageJ software. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ compared to the control (untreated cells).

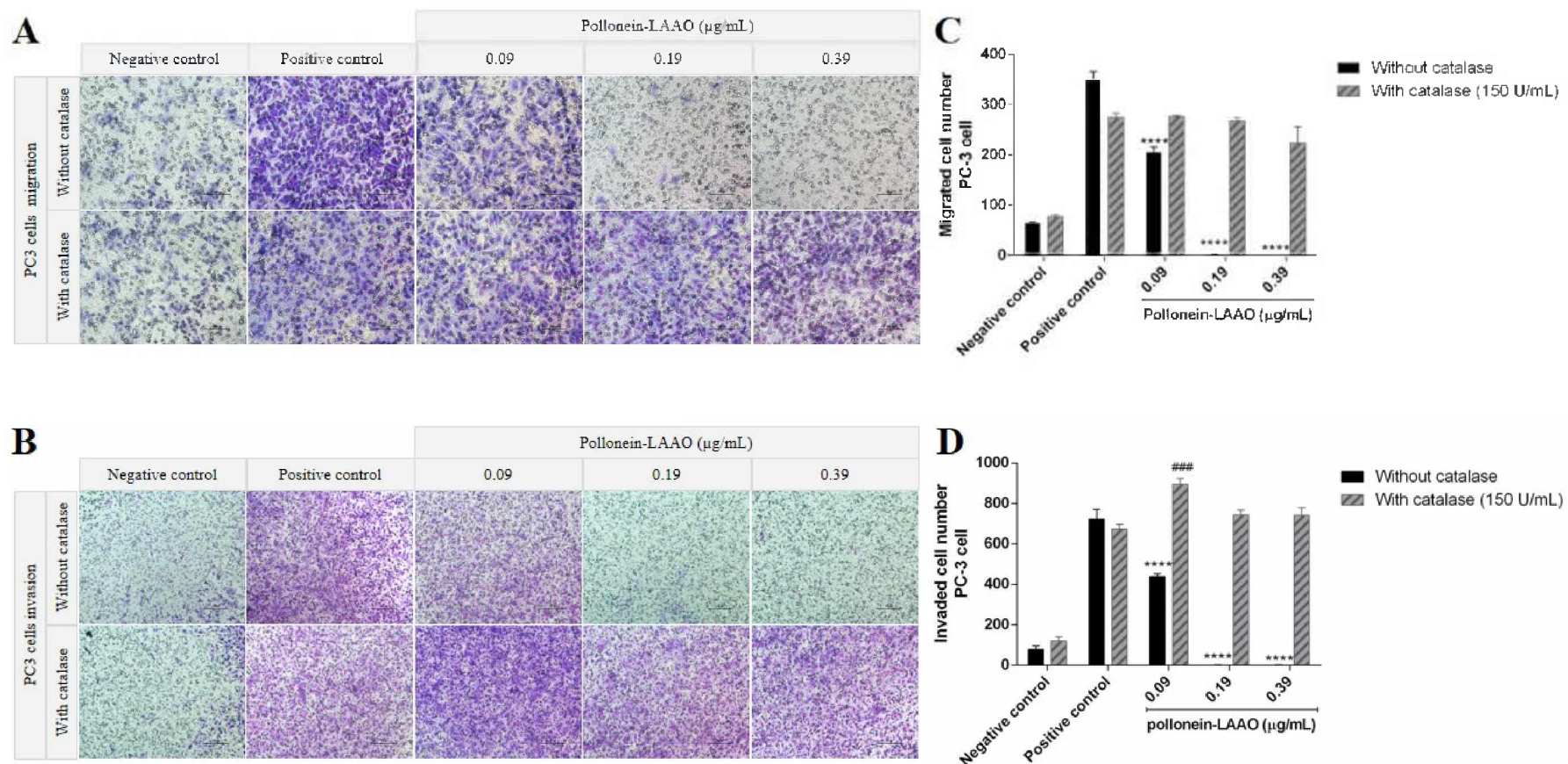


Figure 10. Pollonein-LAAO inhibits PC-3 cell migration and invasion in the transwell model. Photomicrography representing PC-3 cell (A) migration and (B) invasion after Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) treatment in the presence or not of catalase (150 U/mL) for 24 h. Untreated cells incubated with (positive control) or without (negative control) 10% of fetal bovine serum (FBS). Cells were stained with hematoxylin-panotic. 20x and 10x magnification in migration and invasion assay, respectively. Scale bars: 200 μm . Quantitative graphs of (C) migrated and (D) invasion cells. Six sites per group were randomly chosen for manual cell count. **** $p < 0.0001$ compared to positive control. ##### $p < 0.0001$ compared to positive control with catalase.

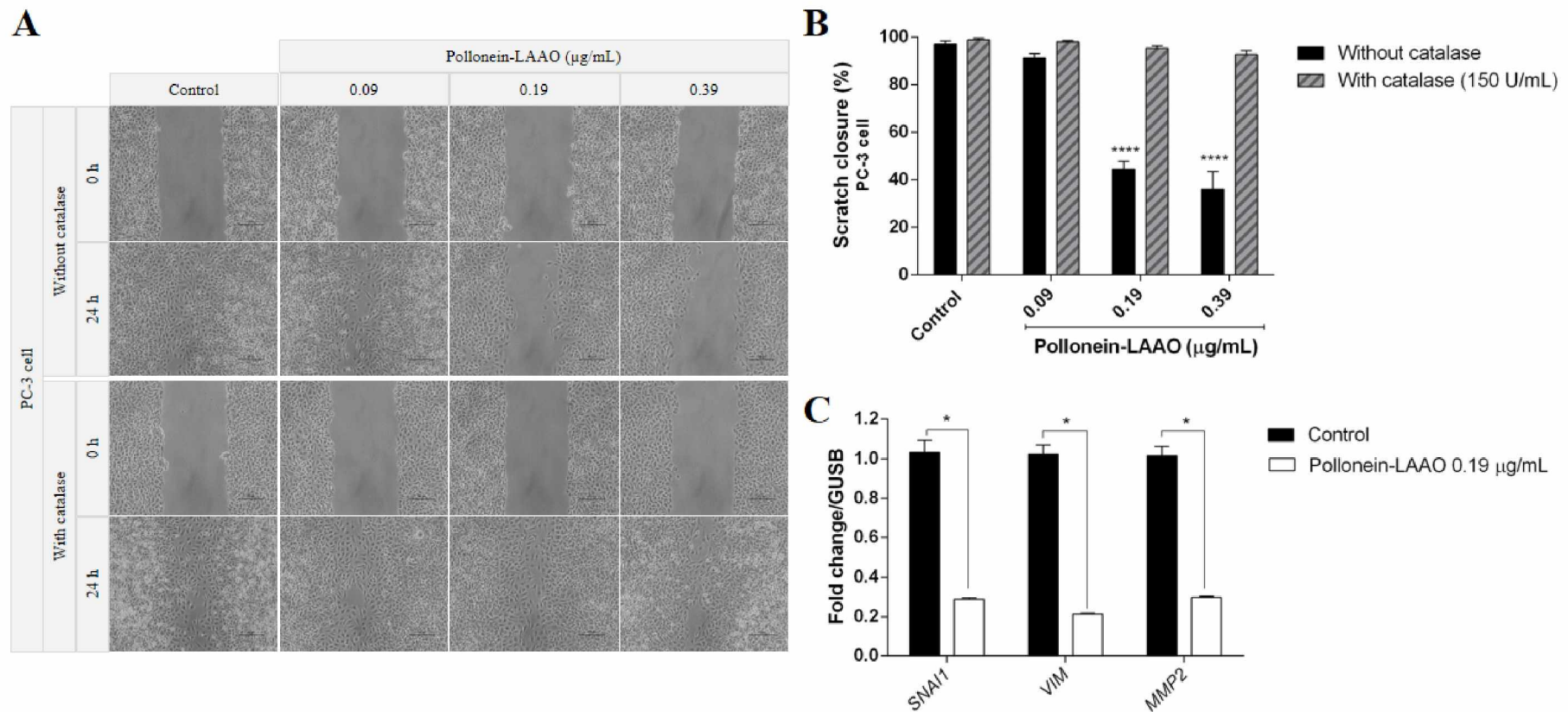


Figure 11. Pollonein-LAAO inhibits PC-3 cell migration in wound healing model and modulates genes involved in the invasion process. (A) Photomicrography representing the scratching edge made with a 200- μL pipette tip (0 h) and scratching edge closure (24 h) after Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) treatment in the presence or not of catalase (150 U/mL) for 24 h. Magnification 10x. Scale bars: 200 μm . (B) Percentage of wound closure (migrated cells) was obtained by ImageJ software. (C) Epithelial-mesenchymal transition gene expression profile in PC-3 cells treated with Pollonein-LAAO (0.19 $\mu\text{g/mL}$) for 24h. The gene expression was determined by quantitative reverse PCR. *GUSB* was used as a reference gene. * $p < 0.05$ and **** $p < 0.0001$ compared to the control (untreated cells).

Capítulo III

Insights of oxidative stress induced by Pollonein-LAAO a L-amino acid oxidase from *Bothrops moojeni* venom on *in vitro* tumor angiogenesis

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Abstract

Tumor angiogenesis is known to play a crucial role in tumor growth and spread being considered a powerful therapeutic target. Reactive oxygen species (ROS) is a critical regulator of tumor angiogenesis, however, their effects are concentration dependent. In this framework, it is unclear how ROS-induced oxidative stress influences tumor angiogenesis. We hypothesized that the change in endothelial cells concentration of ROS could impair critical steps of tumor angiogenesis. Therefore, the purpose of this study was to evaluate the use of Pollonein-LAAO, a L-amino acid oxidase from *Bothrops moojeni* venom as a ROS-based anti-angiogenic agent. Our results demonstrated that Pollonein-LAAO increased intracellular ROS levels that culminate in reduction of endothelial capillary-tube formation in matrigel model and inhibition of critical angiogenesis steps such as migration, invasion and adhesion. Moreover, Pollonein-LAAO exhibited significant anti-angiogenic properties in 3D co-culture models. Those results may be explained to Pollonein-LAAO negatively modulates expression of genes that participate in critical steps of tumor angiogenesis (*VEGFA*, *ANGPT-2*, *MMP-2* and *MMP-9*). Interestingly, Pollonein-LAAO decreased VEGF and $\alpha_2\beta_1$ protein expression and reduced formation of actin fiber stress. Furthermore, ROS inhibition by catalase abolished Pollonein-LAAO effects. This is the first report demonstrating the anti-angiogenic effects of oxidative stress via ROS generation by Pollonein-LAAO.

Keywords: Alpha2beta1, MMP, ROS, VEGF, 3D co-culture

1. Introduction

Tumor angiogenesis is a multistep process of new and abnormal blood vessels network formation from a pre-existing vasculature [1,2]. On account of its fundamental role in tumor growth, invasion and metastasis, tumor angiogenesis is considered a limiting factor of cancer progression. Indeed, solid tumors in absence of vascular support grow to a maximum of 1–2 mm³ in diameter before they stop growing and become necrotic or even apoptotic [1,3].

Given the importance of angiogenesis in tumor growth and spread, anti-angiogenic clinical approaches have been exploited. However, the current anti-angiogenic therapy seems to be ineffective as a monotherapy, and resistance is a common event in cancer patients [2]. Thus, highlighting the necessity for a better understanding of the underlying molecular mechanisms of tumor angiogenesis [4] that may create possibilities to develop drugs that targets multiple pathways in tumor angiogenesis simultaneously, both in tumor and endothelial cells [5].

In this framework, emerging studies have demonstrated that the disruption of reduction-oxidation (redox) by free radicals, predominantly reactive oxygen species (ROS), mediate various growth-related responses including tumor angiogenesis, through a ROS-dependent mechanism of HIF1- α stabilization [5-8]. Nonetheless, it is noteworthy mentioning that ROS plays opposite roles in tumorigenesis according to their different concentrations, distribution and duration, though the mechanisms remain insufficiently understood [9].

Understanding the full spectrum of ROS regulation of tumor angiogenesis signaling and their mediator is crucial to pave the way for new lines of intervention in cancer treatment [4]. In this context, snake venom L-amino acid oxidases (svLAAOs) are interesting bioactive molecules to investigate the ROS effects in tumorigenesis, since svLAAOs are able to

catalyze a stereospecific oxidative deamination of L-amino acids to their corresponding α -keto acids with the liberation of ammonia and hydrogen peroxide (H_2O_2) [10]. Accumulating evidence illustrates that svLAAOs display cytotoxicity against different tumor cell lines, with minor effects on normal cells, by increasing the intracellular ROS levels that trigger DNA fragmentation, cell cycle arrest and apoptosis. Then, svLAAOs are very promising molecules that could be used as potential drugs or as templates for developing new agents [11-13].

Despite the promising cytotoxic effects of svLAAOs in cancer cell lines, few studies have reported their potential activity in endothelial cells that correlates with tumor growth and invasion by means of induction of tumor angiogenesis [14,15]. In line with this, our previous work demonstrated that Pollonein-LAAO, a L-amino acid oxidase from *Bothrops moojeni* venom was able to impair critical processes of prostate cancer cell proliferation and invasion through ROS generation (Data not published). Thus, the current study sought to improve our understanding of the oxidative stress produced by Pollonein-LAAO in critical steps of angiogenesis in *in vitro* assays using endothelial cells as model.

2. Material and Methods

2.1 Snake venom collection and Pollonein-LAAO purification

The crude venom was collected from *Bothrops moojeni* snake kept at the Venom Bank at Fiocruz Rondônia/UNIR, Porto Velho, RO, under local government authorization license number: CGEN/AFCAB61; SISBIO 64385-1; IBAMA nº 27131-3 and CGEN/CNPq 010627/2011-1. This work was registered in the National Management System of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the number A2C2534. The L-amino acid oxidase was purified from *B. moojeni* snake venom (Pollonein-LAAO) as previously reported (Polloni et al., 2023) with the three-step chromatographic method using

molecular exclusion, hydrophobic interaction and affinity columns. The homogeneity of Pollonein-LAAO was determined by reverse-phase chromatography (RP-HPLC) using the C18 column. The isolated and purified Pollonein-LAAO was stored at 4 °C.

2.2 Cell line and culture conditions

The human umbilical vein endothelial cell (HUVEC) was obtained from American Type Culture Collection (CRL-1730) and maintained routinely in RPMI 1640 medium (Sigma, Brazil) supplemented with 10% fetal bovine serum (FBS; Cultilab, Brazil), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, Brazil) – complete RPMI 1640 medium – at 37 °C in a humidified 5% CO₂ atmosphere. For spheroid formation, human prostate adenocarcinoma cell (PC-3) and human fibroblast cell (HFF-1) were obtained from American Type Culture Collection (CRL-1435 and SCRC-1041, respectively) and maintained in the same conditions described for HUVECs.

2.3 Cell viability assay

HUVEC (2×10^4 cells/well) was seeded in 96-well microplates containing complete RPMI 1640 medium for 24 h at 37 °C in 5% CO₂ incubator. Next, cells were incubated with different concentrations of Pollonein-LAAO (ranging from 0.09 to 50 µg/mL) in presence of catalase (150 U/mL, Sigma, Brazil) or not for 24 h. Non-treated cells were considered as a control group. The cell viability was assessed using MTT solution (5 mg/mL of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Sigma, Brazil) for 3 h and formazan crystals formed were dissolved by a solution containing 10% SDS and 0.01 N HCl in phosphate buffered saline (PBS) for 18 h. The optical density was determined at 570 nm in a plate reader (Multiskan GO, Thermo Scientific, USA).

2.4 Measurement of reactive oxygen species (ROS)

As previously reported [17], the ROS quantification assay was performed with some modification. In order to quantify total intracellular ROS, HUVEC (2×10^4 cells/well) was seeded in 96-black well microplates (Corning, USA) containing complete RPMI 1640 medium for 24 h at 37 °C in 5% CO₂ incubator. Next, cells were incubated with different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) in presence of catalase (150 U/mL, Sigma, Brazil) or not for 4 h. Non-treated cells were considered as a control group. Subsequently, the treatments were removed and wells were gently washed with phosphate buffered saline (PBS). The cellular ROS was determined using H₂DCF-DA fluorescent dye solution (10 µM of 2',7'-dichlorodihydrofluorescein diacetate; Sigma, Brazil) for 30 min at 37 °C under darkness. The quantified fluorescence intensity was determined at an excitation wavelength of 485 nm and an emission wavelength of 530 nm in a plate reader (GloMax®, Promega, Brazil).

2.5 Endothelial cell tube formation assay and vascular endothelial growth factor (VEGF) quantification

Briefly, 50 µL of ice-cold Matrigel™ (Corning, USA) was added into the 96-well plate and incubated at 37 °C for 30 min to polymerize. After, HUVEC (1×10^5 cells/well) was exposed to complete RPMI 1640 medium supplemented with basic fibroblast growth factor (bFGF, 30 ng/mL, Sigma, Brazil) in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) in presence of catalase (150 U/mL, Sigma, Brazil) or not and seeded on the solidified Matrigel. The plate was incubated for 6 h to allow the tubulogenesis and capillary-like structures were observed in an inverted microscope (Nikon Eclipse TS100) and photographed at 10x magnification. All analyses were performed using the Angiogenesis Analyzer plugin for ImageJ software. Additionally,

the supernatants from this assay were collected to quantify VEGF levels by a commercial CBA kit to solubilize proteins (CBA human soluble protein master buffer kit and CBA human VEGF flex set, BD, USA), according to the manufacturer's protocol. The samples were assessed by the Cytoflex flow cytometer (Beckman Coulter, United States).

2.6 Cell adhesion assay

In order to evaluate HUVEC adhesion in different extracellular matrix (ECM) coatings, cells (2×10^4 cell/ well) were pre-incubated with complete RPMI 1640 medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) for 1 h at 37 °C in 5% CO₂ incubator. Alternatively, cells were co-treated with catalase (150 U/mL, Sigma, Brazil) in complete RPMI medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$), incubated as described above. Thereafter, cells were seeded into 96-well microplates previously coated with collagen IV (10 $\mu\text{g/mL}$, Sigma, Brazil), fibronectin (5 $\mu\text{g/mL}$, Gibco, USA) or Matrigel™ (1:10, Corning, USA) and blocked with 1% of bovine serum albumin (BSA) for 1 hour. After 3 h of incubation at 37 °C in 5% CO₂ incubator, the unattached cells were removed by gentle washes with PBS1x, and the adherent cells were measured by MTT assay as described previously (section 2.3).

2.7 Detection of $\alpha 2\beta 1$ integrin by immunofluorescence

HUVEC (1.5×10^5 cells) was cultured on glass coverslips for 24 h. After adhesion, cells were incubated with complete RPMI 1640 medium in absence (control group) or presence of Pollonein-LAAO at 0.19 $\mu\text{g/mL}$ for 24 h. Afterward, cells were fixed in 4% paraformaldehyde for 10 min, followed by permeabilization with 0.1% PBS-Tween 20 solution and blocked with 1% BSA, 0.3 M glycine for 1 h [18]. Subsequently, cells were

labeled with anti-human $\alpha 2$ (CD49b), anti-human $\beta 1$ (CD29) fluorescein monoclonal antibodies (Ambriex, Brazil) at a concentration of 1:50 (v/v) and nuclei were stained with blue-fluorescent TO-PRO-3 (ThermoFisher Scientific, USA). HUVEC was imaged in a Zeiss LSM 510 Meta confocal microscope at 40x magnification and the fluorescence intensity was measured with the Image J software.

2.8 Actin cytoskeleton analysis

HUVEC (1.5×10^5 cells) was cultured on glass coverslips for 24 h. After adhesion, cells were incubated with complete RPMI 1640 medium in absence (control group) or presence of Pollonein-LAAO at 0.19 $\mu\text{g/mL}$ for 24 h. Alternatively, cells were co-treated with catalase (150 U/mL, Sigma, Brazil) in complete RPMI medium in absence (control group) or presence of Pollonein-LAAO (0.19 $\mu\text{g/mL}$) for 24 h. Next, cells were fixed in 4% paraformaldehyde for 30 min and permeabilized with 0.1% saponin. Afterwhile, cells were stained with fluorescent phalloidin conjugate (1:50, Phalloidin-Atto 565, Sigma, Brazil) and blue-fluorescent TO-PRO-3 (1:500, ThermoFisher Scientific, USA) for 1 h. HUVEC was imaged in a Zeiss LSM 510 Meta confocal microscope at 40x magnification and the actin fluorescence intensity were manually measured with the ImageJ software.

2.9 Scratch wound-healing assay

HUVEC (2×10^5 cells/well) was seeded in 96-well microplates containing complete RPMI 1640 medium and incubated for 24 h at 37 °C in 5% CO₂ incubator. After reaching confluence, an artificial wound was created by scratching the cell monolayer with a 200 μL pipette tip. The monolayer was washed with PBS to remove cell debris and unattached cells. Then, cells were incubated with complete RPMI 1640 medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) in

presence of catalase (150 U/mL, Sigma, Brazil) or not for 24 h. To evaluate the wound-healing process, an inverted microscope (Nikon Eclipse TS100) was used to photograph the monolayer immediately after scratching ($t = 0$ h) and upon 24 h of treatment ($t = 24$ h). The cell migration on the created scratching edge was measured with the Image J software and the percentage of wound closure was obtained by the following equation: % wound closure = $((At0h - At24h)/At0h) * 100$, where “At0h” is the area of wound measured after scratching and “At24h” is the area of wound measured 24 h after scratching [19,20].

2.10 Transwell migration assay

Cell migration assay was performed using a ThinCert™, 8.0- μ m pore (Greiner Bio-one®, Brazil). HUVECs (1×10^5 cells/well) pre-incubated with RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 μ g/mL) for 1 h at 37 °C in 5% CO₂ incubator. Alternatively, cells were co-treated with catalase (150 U/mL, Sigma, Brazil) in RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 μ g/mL) for 1 h at 37 °C in 5% CO₂ incubator. Afterwards, cells were inserted into the upper chamber, which were immersed in complete RPMI 1640 medium. Cells were allowed to migrate for 24 h at 37 °C. The negative control was considered as the transwell inserted on RPMI 1640-FBS free medium, and the positive control as the transwell inserted on complete RPMI 1640 medium, both incubated as described above. The non-migrating cells were removed from the upper chamber with a cotton swab and the migrating cells were stained with Panotic Kit (Loboclin, Brazil). Cell migration was quantified by counting cells under a microscope (Nikon Eclipse TS100) at 10x magnification.

2.11 Cell invasion assay

Cell invasion assay was performed using a ThinCert™, 8.0-μm pore (Greiner Bio-one®, Brazil) pre-coated with Matrigel™ (Corning, USA). Briefly, HUVECs (1x10⁵ cells/well) were pre-incubated with RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 μg/mL) for 1 h at 37 °C in 5% CO₂ incubator. Alternatively, cells were co-treated with catalase (150 U/mL, Sigma, Brazil) in RPMI 1640-FBS free medium in absence (control group) or presence of different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 μg/mL) for 1 h at 37 °C in 5% CO₂ incubator. Then, cells were inserted into the upper chamber, which were immersed on wells containing complete RPMI 1640 medium, and cell invasion was allowed to occur for 24 h at 37 °C. The negative control was considered as the transwell inserted on RPMI 1640-FBS free medium and the positive control as the transwell inserted on complete RPMI 1640 medium, both incubated as described above. The non-invading cells were removed from the upper chamber with a cotton swab and the invading cells were stained with Panotic Kit (Laborclin, Brazil). Cell invasion was quantified by counting cells under a microscope (Nikon Eclipse TS100) at 10x magnification.

2.12 Three-dimensional (3D) co-culture model of tumor angiogenesis

For 3D co-culture experiments, prostate cancer cell (PC-3) and fibroblast cell (HFF-1) aggregation were induced by growing cell suspensions in agarose-coated 96 well plates, as previously published [21]. After 4 days, the multicellular spheroids were formed, and then, five pre-formed multicellular spheroids (prostate cells:fibroblast cells) were added to pre-formed HUVEC tube-like structures, as previously reported [22]. The 3D co-culture was incubated with complete RPMI 1640 medium in absence (control group) or presence of

different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) for 24 h. After treatment, 3D co-culture was photographed at 10x magnification in an inverted microscope (Nikon Eclipse TS100). All analyses were performed with the Angiogenesis Analyzer plugin for ImageJ software.

2.13 Analysis of gene expression by quantitative reverse transcription polymerase chain reaction (RT-qPCR)

HUVEC (3×10^6 cells) was incubated with complete RPMI 1640 medium in absence (control group) or presence of Pollonein-LAAO at 0.19 $\mu\text{g/mL}$ for 24 h. After treatment, cells were harvested and processed in accordance with the guidelines of the RNA extraction Kit (PureLink™ RNA Mini Kit, Thermo Fisher Scientific, USA). The RNA concentration and quality were measured using a spectrophotometer (Biodrop μLite , Nova Analítica, Brazil) and all samples complied with the pre-established ratios (A_{260}/A_{280} and A_{260}/A_{230}). After quantification of the obtained RNAs, the samples were processed for synthesis of cDNAs by High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The expression of *ITGA2*, *ITGAV*, *ITGB1*, *ITGB3*, *MMP2*, *MMP9*, *VEGFA* and *ANGPT2* were made through the Human Cancer Pathway Primer Library, following the manufacturer's instructions (GO-GenOne; Brazil; <http://www.genone.com.br/>). Quantitative PCRs were carried out in triplicate using Master Mix – Sybr Green (LCG Biotecnologia, Brazil). Relative gene expression were assessed by comparing the values of cycle threshold (Ct) between treated group and the respective control group by using the $2^{-\Delta\Delta C_t}$ method [23]. Data were normalized using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene.

2.14 Statistical analysis

The experiments were carried out in triplicates and the results are expressed as means $\pm$ standard error of the means (S.E.M). The significance differences between treatments and controls were analyzed by One-way ANOVA, followed by the Bonferroni's post-test. Mann-Whiney's test was used to compare the differences between two groups. All statistical analyses were performed using the GraphPad Prism Software version 6.0 (Inc., CA, USA). Differences were considered as statistically significant if $*p<0.05$; $**p<0.01$; $***p<0.001$ and $****p<0.0001$.

3. Results

3.1 Pollonein-LAAO decreases endothelial cell viability and increases intracellular reactive oxygen species (ROS) generation in HUVECs

Firstly, we investigated the cytotoxic potential of Pollonein-LAAO in human umbilical vein endothelial cell (HUVEC) by MTT assay. Pollonein-LAAO decreased HUVEC viability by around 44% at the highest concentrations tested (from 3.125 to 50 $\mu\text{g/mL}$) after 24 h, displaying a concentration necessary to inhibit 50% of HUVEC viability (IC_{50}) of 4.6 $\mu\text{g/mL}$. Cells cotreated with catalase (150 U/mL) prevented the demise of the treated cells, however, it promoted cell growth in all concentrations tested compared to the control (untreated cells) (**Fig. 1A**). Moreover, in order to assume that antiangiogenic effects induced by Pollonein-LAAO is related to increased intracellular ROS generation, HUVEC was incubated with the $\text{H}_2\text{DCF-DA}$ fluorescent probe and analyzed through a plate reader. As depicted in **Fig. 1B**, Pollonein-LAAO measured a significantly elevated intracellular ROS levels at 0.19 and 0.39 $\mu\text{g/mL}$ compared to the control (untreated cells), while cells cotreated with catalase (150 U/mL) prevented intracellular ROS levels increasing by the

Pollonein-LAAO treatment, reinforcing that the major source of ROS induced by this L-amino acid oxidase is the hydrogen peroxide.

3.2 Pollonein-LAAO inhibits HUVECs tube formation and decreases angiogenic factors expression

We further addressed whether the oxidative stress induced by Pollonein-LAAO was able to impair tubulogenesis in cell tube formation assay. As depicted in **Fig. 2A**, HUVEC stimulated with bFGF (30 ng/mL) formed on matrigel the typical honeycomb-like mesh that simulates endothelial tubes *in vitro*, producing a high number of capillary-like structures (control group). However, Pollonein-LAAO impaired tube-like structures formation induced by bFGF on matrigel at 0.19 and 0.39 $\mu\text{g/mL}$, by means of reduction of the number of junctions and the number of meshes (**Fig. 2B**). A less effective inhibition of tube-like structures formation was caused by Pollonein-LAAO-treated cells in presence of catalase at all concentrations assessed (**Figs. 2A, B**). Furthermore, expressions of some pro-angiogenic genes were analyzed, such as *VEGFA* and *ANGPT2*, which encode vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang-2). Pollonein-LAAO (0.19 $\mu\text{g/mL}$) significantly down-regulated both angiogenic factors expression compared to the control (**Fig. 2C**). To validate this result, VEGF content in HUVECs supernatants collected from cell tube formation assay was assessed. Pollonein-LAAO (0.19 $\mu\text{g/mL}$) was able to reduce VEGF levels from 95 pg/mL in the control to 36 pg/mL (**Fig. 2D**).

3.3 Pollonein-LAAO decreases HUVECs adhesion to different extracellular matrix (ECM) components, promotes actin cytoskeleton alterations and reduces α_2 integrin subunit expression

Since Pollonein-LAAO affects tubulogenesis, we decided to investigate whether Pollonein-LAAO could interfere with HUVEC cell adhesion, migration and invasion, three events associated with the tumor angiogenesis process. Pollonein-LAAO inhibited the adhesion of HUVECs to different components of the extracellular matrix (ECM) in all concentrations. Compared to the control, Pollonein-LAAO-treated HUVECs reduced collagen IV, fibronectin and matrigel attachment by about 25%, 34% and 66% at concentrations of 0.09, 0.19 and 0.39 $\mu\text{g/mL}$, respectively. Co-treatment with catalase (150 U/mL) eliminated Pollonein-LAAO effects on HUVEC adhesion (**Figs. 3A-D**). Analysis of genes that encode integrins revealed that Pollonein-LAAO (0.19 $\mu\text{g/mL}$) down-regulated *ITGA2* and unlike up-regulated *ITGB1*, which encode collagen IV binding integrins ($\alpha_2\beta_1$) (**Fig. 4A**). To corroborate with this finding, $\alpha_2\beta_1$ integrin content in HUVECs was performed by immunofluorescence. Both α_2 (green) and β_1 (red) subunits are expressed in untreated cells (control group); however, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) treatment decreased the α_2 subunit and increased β_1 subunit compared to the control. The presence of yellow regions suggests co-localization of these two subunits (**Fig. 4C**). Quantitative analysis exhibited a significant increase in β_1 subunit fluorescence intensity (**Fig. 4C**) and a decrease in α_2 subunit fluorescence intensity compared to the control (**Fig. 4D**).

Curiously, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) did not affect the *ITGAV* and *ITGB3* expression (**Fig. 4A**). Thus, the actin cytoskeleton remodeling in HUVECs was analyzed by immunofluorescence. Our results revealed that phalloidin labeling showed changes in cytoskeletal assembly in response to Pollonein-LAAO (0.19 $\mu\text{g/mL}$) treatment, in which f-actin stress fibers formation were homogeneously reduced when compared to the control.

Quantitative analysis demonstrated a significant decrease in fluorescence intensity when compared to the control. Moreover, Pollonein-LAAO effects on f-actin stress fibers formation were eliminated when catalase (150 U/mL) was also incubated with HUVECs (**Figs. 5A-C**).

3.4 Pollonein-LAAO affects HUVECs migration and invasion modulates MMP-2 and MMP-9 gene expressions

Pollonein-LAAO affected motility of HUVECs in two different migration models, transwells and wound healing. As a result, Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) inhibited the migration of HUVECs through a transwell compared to the positive control. The number of migrated HUVECs was 298 cells in positive control, reducing to around 270, 19 and 15 cells at 0.09, 0.19 and 0.39 $\mu\text{g/mL}$, respectively (**Figs. 6A, C**). In the wound healing assay, Pollonein-LAAO impaired the scratch closure of HUVECs in 21% and 26% at 0.19 and 0.39 $\mu\text{g/mL}$ respectively, compared to control (untreated cells) (**Figs. 7A, B**). In addition, HUVECs lost their invasive capacity if incubated with Pollonein-LAAO at all tested concentrations in the matrigel transwell assay. The number of invading cells reduced from 433 cells in the positive control to approximately 379, 43 and 3 cells at 0.09, 0.19 and 0.39 $\mu\text{g/mL}$, respectively, compared to the positive control (**Figs. 6B, D**).

In order to verify whether the observed effects were associated with oxidative stress induced by Pollonein-LAAO, HUVECs were co-treated with catalase (150 U/mL). The antioxidant effects of catalase maintained the motility and invasiveness of HUVECs, since the co-treatment allowed HUVECs migration and invasion, as illustrated in **Fig. 6** and **Fig. 7**. Furthermore, expressions of some matrix metalloproteinases (MMP) genes were analyzed, such as *MMP-2* and *MMP-9*. Pollonein-LAAO (0.19 $\mu\text{g/mL}$) significantly down-

regulated both *MMP-2* and *MMP-9* expression compared to the control (untreated cells) (**Fig. 7C**).

3.5 Pollonein-LAAO disrupts capillary-like structures in co-culture model and decreases the tumor spheroid area

Finally, we develop a co-culture model that simulates the conditions of tumor angiogenesis *in vitro*. As shown in **Fig. 8A**, in the control images, HUVEC formed irregular tube capillary-like structures induced by bFGF on matrigel in presence of multicellular tumor spheroids (MCTS). However, Pollonein-LAAO in all concentrations abolished the stimulatory effect of bFGF, impairing the tube capillary-like structures formation since it was observed that the angiogenic parameters assessed such as number of meshes, number of junctions and total segments length decreased compared to the control (**Fig. 8B-D**). Interestingly, MCTS area significantly decreased at 0.19 and 0.39 $\mu\text{g/mL}$ of Pollonein-LAAO compared to the control (**Fig. 8E**).

4. Discussion

Tumor angiogenesis is known as one of the hallmarks of cancer, which plays a key role in tumorigenesis [24]. Thus, a better understanding of the mechanisms underlying tumor angiogenesis is crucial for further investigation of potential targets in alternative angiogenic signaling pathways to weaken tumor development [2]. In this context, we investigated whether the oxidative stress induced by Pollonein-LAAO, a L-amino acid oxidase from *Botrops moojeni* venom, was able to impair critical steps of angiogenesis on *in vitro* assays.

Accumulating evidences demonstrates that L-amino acid oxidases from snake venom (svLAAOs) biological effects in different cell lines is due to their ability to produce hydrogen peroxide (H_2O_2), which leads to disruption of cell physiology [13,25-27]. In fact,

Pollonein-LAAO exerts cytotoxicity against HUVECs that is associated with its ability to increase reactive oxygen species (ROS) generation, resulting in oxidative stress in endothelial cells (ECs). To validate this hypothesis we performed our experiments in presence of catalase, a potent antioxidant that exhibits catalytic activity by converting H_2O_2 in H_2O and O_2 [9]. We noticed that the co-treatment with catalase eliminated the Pollonein-LAAO-induced cytotoxic effect and ROS generation in ECs, reinforcing that the major source of ROS induced by this L-amino acid oxidase is H_2O_2 and its accumulation leads to oxidative stress damage. Pollonein-LAAO may interact with cell surface of ECs to allow the release of a high local concentration of H_2O_2 [13,25,26], whereas in presence of catalase the produced H_2O_2 is rapidly neutralized, thus abolishing its effects in ECs. Bhat et al [15] reported that LAAO isolated from *Bothrops moojeni* venom (BmLAAO) and from *Bothrops atrox* venom (BaLAAO) significantly reduced HUVECs viability even in low concentrations.

In consideration of Pollonein-LAAO may induce oxidative stress in HUVECs, by means of increasing intracellular H_2O_2 accumulation, we performed different assays to verify its potential anti-angiogenic ability. It is noteworthy that angiogenesis onset is triggered by a transformation of quiescent vessels to actively sprouting vessels through a phenomenon known as “angiogenic switch”. This process is activated by imbalance between endogenous angiogenic inducers and inhibitors more towards a pro-angiogenic outcome, due to the conditions of tumor microenvironment like hypoxia, lack of nutrients and inflammation [28,29]. Interestingly, the present study demonstrated that the oxidative stress induced by Pollonein-LAAO (0.19 $\mu\text{g/mL}$) reduced vascular endothelial growth factor (VEGF) in both gene and protein expression. VEGF is considered the major pro-angiogenic growth factor, which acts binding to its respective receptor on ECs that activates a series of

downstream pathways involved in endothelial cell survival and proliferation, cytoskeleton rearrangement and vascular permeability [30].

Several studies demonstrate that VEGF increases angiopoietin-2 (Ang-2) expression, which is a destabilization factor that makes vessels more responsive to the actions of other angiogenic mediators. Thus, Ang-2 is up-regulated together with VEGF in sites of angiogenic sprouting, whereas the dual VEGF and Ang-2 blockage has been associated with vascular regression and tumor growth suppression [29,31-33]. Curiously, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) negatively regulated *ANGPT-2* gene expression, suggesting that Pollonein-LAAO-generated ROS may impair the destabilization of pre-existing vessel leading to inhibition of tube-like structures formation on matrigel. Since the destabilization of tight endothelial cell-to-cell interactions is crucial to initiate the formation of new blood vessels, VEGF and Ang-2 are thought to be responsible for this critical event [28].

Additionally, Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) impaired the induction of endothelial cell migration/invasion. These effects may be associated with Pollonein-LAAO abilities to negatively regulate *VEGFA* and *ANGPT-2* expression, since these cytokines are major promoters of actin-based motility. It is known that in presence of VEGF, Ang-2 displays endothelial cell migration, proliferation and sprouting [34]. We also observed that Pollonein-LAAO (0.19 $\mu\text{g/mL}$) reduced actin stress fibers formation in HUVECs. Stress fibers are large bundle actin filaments that extend across cell's diameter and are anchored at one or both ends by focal adhesions that mediates cell traction toward the leading edge during migration [34,35].

Actin stress fibers work in close cooperation with integrin-based adhesion and the extracellular matrix (ECM), once integrins mediate the connection between cytoskeleton and surrounding ECM [36,37]. In this context, Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) similarly reduced the HUVECs attachment to different ECM protein components (collagen

IV, fibronectin and matrigel). It is attempting to speculate that these results might be correlated with reduced actin stress fibers formation after Pollonein-LAAO treatment, since to fully mature the integrin-based adhesion requires actin stress fiber-mediated tension [36]. Consistent with this idea, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) did not change the gene expression of fibronectin-binding integrin $\alpha\text{V}\beta_3$, however, Pollonein-LAAO significantly reduced HUVECs adhesion to fibronectin coating.

Upon integrin binding, Pollonein-LAAO (0.19 $\mu\text{g/mL}$) significantly down-regulated *ITGA2* and decreased the α_2 integrin expression in HUVECs by immunofluorescence quantification. Integrin α_2 forms heterodimers exclusively with β_1 generating the $\alpha_2\beta_1$ receptor, which the collagen recognition occurs mainly at the alpha subunit [38], thus, the inhibitory effect of Pollonein-LAAO in HUVECs adhesion to collagen IV coating could be also associated with decreased α_2 integrin expression. Furthermore, studies have been reported that VEGF potently induces $\alpha_2\beta_1$ integrin expression, through induction of mRNAs encoding the α_2 subunit, which displays a proangiogenic signaling pathway. Moreover, an inhibitory antibody against α_2 subunit has been reported to partially inhibit ECs adhesion to collagen, migration and tube formation [39-41]. Accordingly, our results demonstrated that the oxidative stress induced by Pollonein-LAAO (0.19 $\mu\text{g/mL}$) was able to decrease VEGF and α_2 subunit expression, which corroborates with our findings that even with β_1 integrin overexpression Pollonein-LAAO was able to decrease HUVECs cell migration, proliferation and tubulogenesis. Of note, β_1 integrin overexpression has been correlated with antiangiogenic resistance [42].

In view of angiogenesis involves migration/invasion of ECs into surrounding stroma, matrix metalloproteinases (MMPs) are critically important for degradation of the basement membrane surrounding endothelial cells in a vessel. Moreover, MMPs released by activated endothelial cells trigger the release of angiogenic growth factors that were stored within the

matrix [43,44]. The gelatinases *MMP-2* and *MMP-9* expression were down-regulated after Pollonein-LAAO (0.19 $\mu\text{g/mL}$) treatment, evidencing our previous results that the Pollonein-LAAO-induced oxidative stress inhibits different steps of angiogenesis. Several studies demonstrated that *MMP-2* and *MMP-9* knockout decreased tumor-induced angiogenesis [44,45]. Further, among MMPs, *MMP-9* appears to strongly contribute to the release of ECM-sequestered VEGF. Thus, genetic depletion of *MMP-9* abrogated tumor growth and development of new blood vessels [46].

As tumor-associated neovasculature formation depends upon up regulation of pro-angiogenic mediators within the tumor microenvironment these vessels are considerably different. Tumor blood vessels are mostly immature with disrupted ECs junctions. Hence, tumor blood vessels are highly fragile and permeable which facilitates tumor cells diffusion into the interstitial space [2,30]. Consequently, these features were observed in our 3D co-culture model that may explain the significant effect of Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) in tumor endothelial vessels formation in comparison to cell tube formation assay. Curiously, Pollonein-LAAO (0.19 and 0.39 $\mu\text{g/mL}$) in 3D co-culture also decreased the PC-3 spheroid area that is a desired characteristic to prevent tumor development by regulating different hallmarks of cancer. It is noteworthy to mention that *in vitro* 3D cultures might fill the gap between conventional 2D *in vitro* testing and animal models, since 3D cultures possess several *in vivo* features that allow them to better investigate the pathobiology of human cancers [47]. Moreover, 3D *in vitro* models have been adopted as preclinical tools for studying tumor behavior and drug response [21].

5. Conclusion

In the present study Pollonein-LAAO, a L-amino acid from *Bothrops moojeni*, inhibited critical angiogenesis steps such as migration/invasion, adhesion and tube vessel

formation by down-regulating of *VEGFA*, *ANGPT-2*, $\alpha 2\beta 1$ integrin and *MMP-2* and *MMP-9* expression. In addition, Pollonein-LAAO decreased the formation of actin stress fiber. Interestingly, Pollonein-LAAO inhibited tube vessel formation and tumor growth in 3D culture models. Altogether, these results bring more information about the complexity role of oxidative stress during tumor progression since Pollonein-LAAO may acts in both tumor survival and angiogenesis signaling pathways. In this context, additional mechanisms by which Pollonein-LAAO affects tumor angiogenesis process need to be thoroughly evaluated in further studies, due to svLAAOs biotechnological activities as potential therapeutic agents.

CRedit authorship contribution statement

Lorena Polloni: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Tássia Rafaella Costa:** Conceptualization, Investigation, Writing – Review & Editing, Supervision. **Lorena Pinheiro Morais:** Investigation. **Bruna Cristina Borges:** Formal analysis, Investigation, Writing – Review & Editing. **Samuel Cota Teixeira:** Resources, Investigation, Writing – Review & Editing. **Thales Alves de Melo Fernandes:** Formal analysis, Investigation. **Lucas Ian Veloso Correia:** Investigation, Writing – Review & Editing. **Luciana Machado Bastos:** Resources, Writing – Review & Editing. **Andreimar Martins Soares:** Resources, Writing – Review & Editing. **Marcelo José Barbosa Silva:** Resources, Writing – Review & Editing. **Eloisa Amália Vieira Ferro:** Resources, Writing – Review & Editing. **Daiana Silva Lopes:** Conceptualization, Writing – Review & Editing. **Veridiana de Melo Rodrigues Ávila:** Conceptualization, Writing – Review & Editing, Supervision, Project administration.

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Declaration of competing interest

All authors declare that they have no conflict of interest.

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Figures and Captions

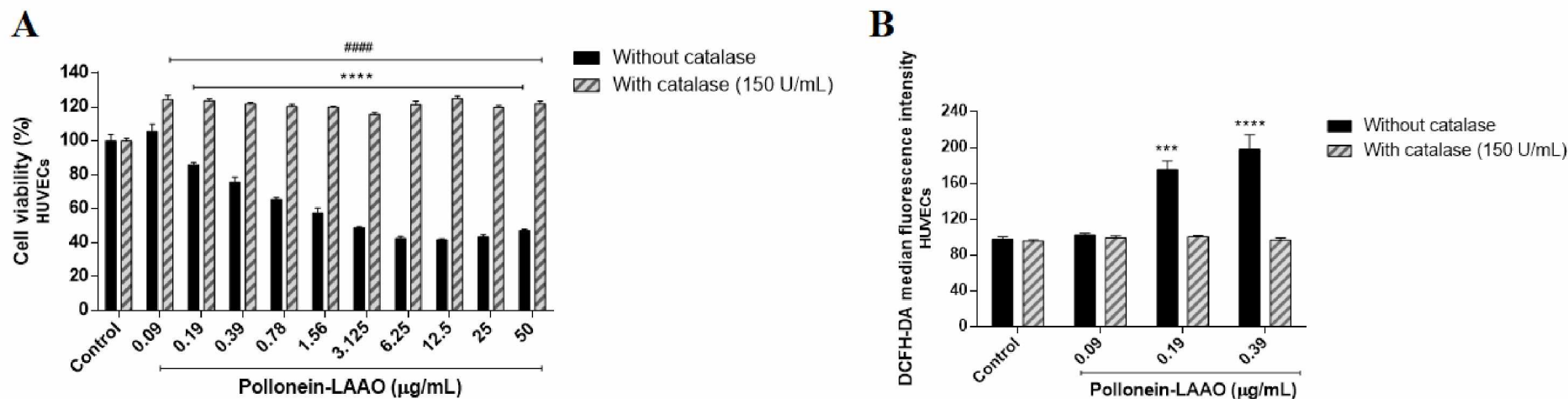


Figure 1. Pollonein-LAAO induces cytotoxicity and generates intracellular reactive oxygen species (ROS) in HUVECs. (A) Graph representing viability of the cells treated with different concentrations of Pollonein-LAAO (ranging from 0.09 to 50 µg/mL) in presence or not of catalase (150 U/mL) for 24 h, determined by MTT. **(B)** Representative graph of changes in ROS levels in HUVECs measured by microplate reader. Cells were treated with different concentrations of Pollonein-LAAO (0.09, 0.19 and 0.39 µg/mL) in presence or not of catalase (150 U/mL) for 4 h and stained with the probe DCFH-DA. *** $p < 0.001$ and **** $p < 0.0001$ compared to the control (non-treated cells) and ##### $p < 0.0001$ compared to the control with catalase.

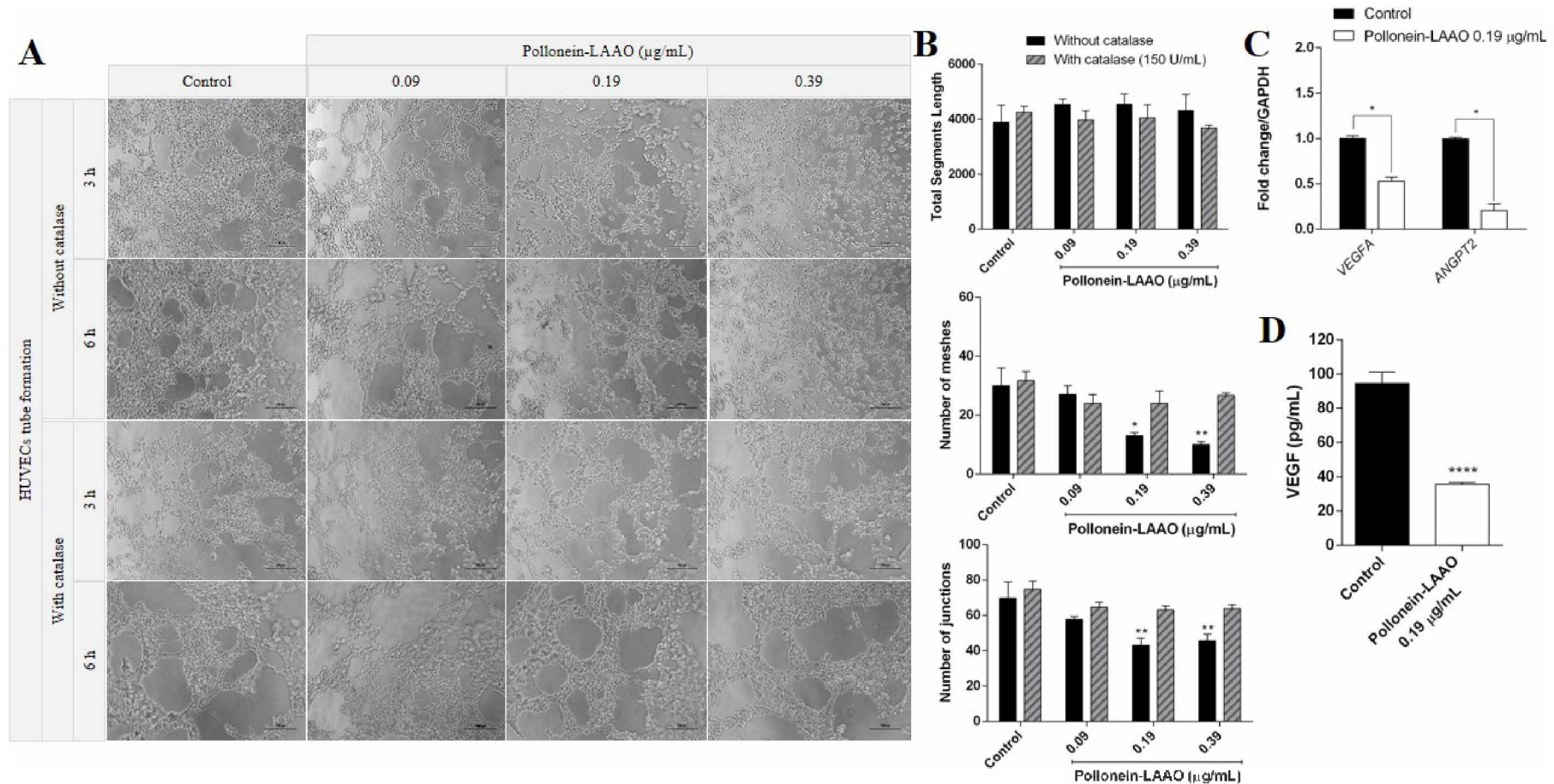


Figure 2. Pollonein-LAAO inhibits HUVECs tube formation and decreases angiogenic gene expression. (A) Representative photomicrography of HUVECs tube formation after Pollonein-LAAO treatment in presence or not of catalase (150 U/mL) for 6 h. Scale bars: 200 μm . (B) Quantitative graph of Number of meshes, Number of junctions and Total segment length determined by Angiogenesis analyzer software. (C) Expression profile of angiogenic genes determined by quantitative reverse PCR. *GAPDH* was used as a reference gene. (D) Graph representing the quantification of VEGF protein content obtained by matrigel endothelial vessels formation supernatants, after treatment with Pollonein-LAAO (0.19 $\mu\text{g/mL}$) for 6h. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$ compared to the control (untreated cells).

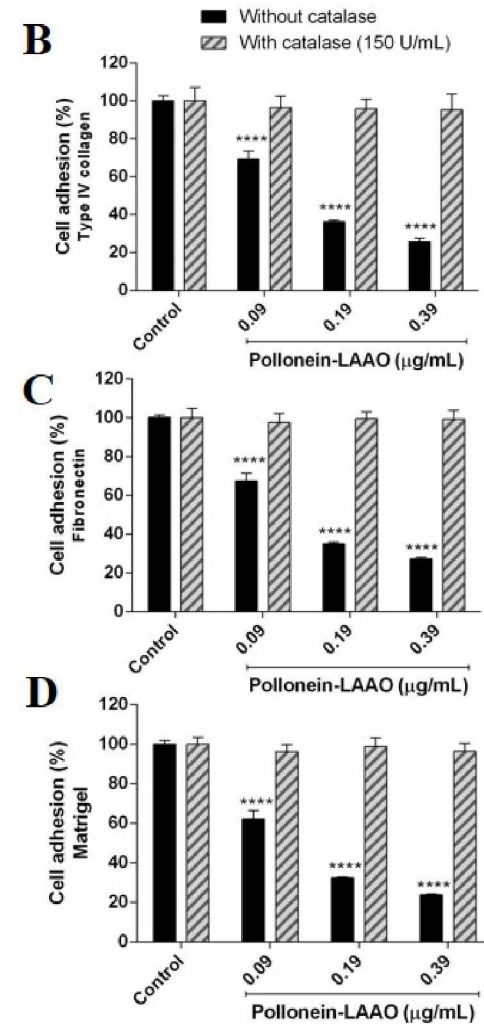
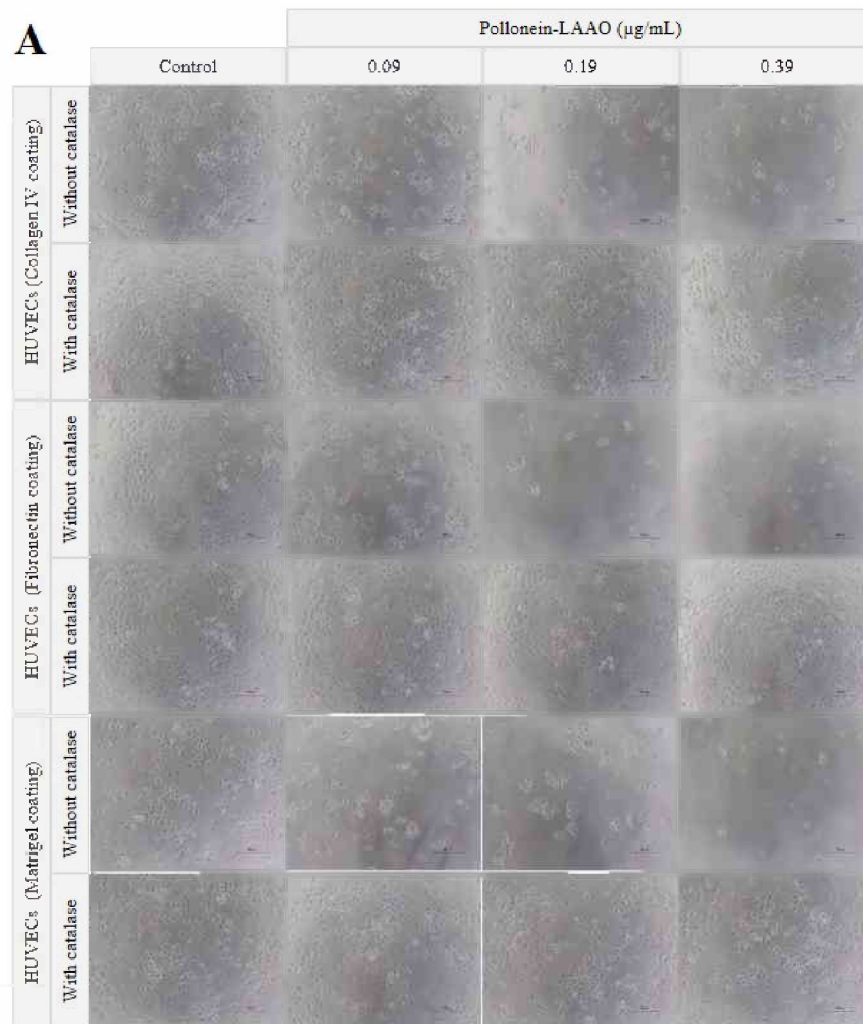


Figure 3. Pollonein-LAAO inhibits HUVECs adhesion in different substrates. (A) Representative photomicrography of HUVECs adhesion in wells precoated with collagen IV, fibronectin and matrigel. Scale bars: 200 μm . Percentage of HUVECs adhesion after Pollonein-LAAO (0.09, 0.19 and 0.39 $\mu\text{g/mL}$) treatment in presence or not of catalase (150 U/mL) in (B) collagen IV, (C) fibronectin and (D) matrigel, assessed by MTT assay. **** $p < 0.0001$ compared to the control (untreated cells).

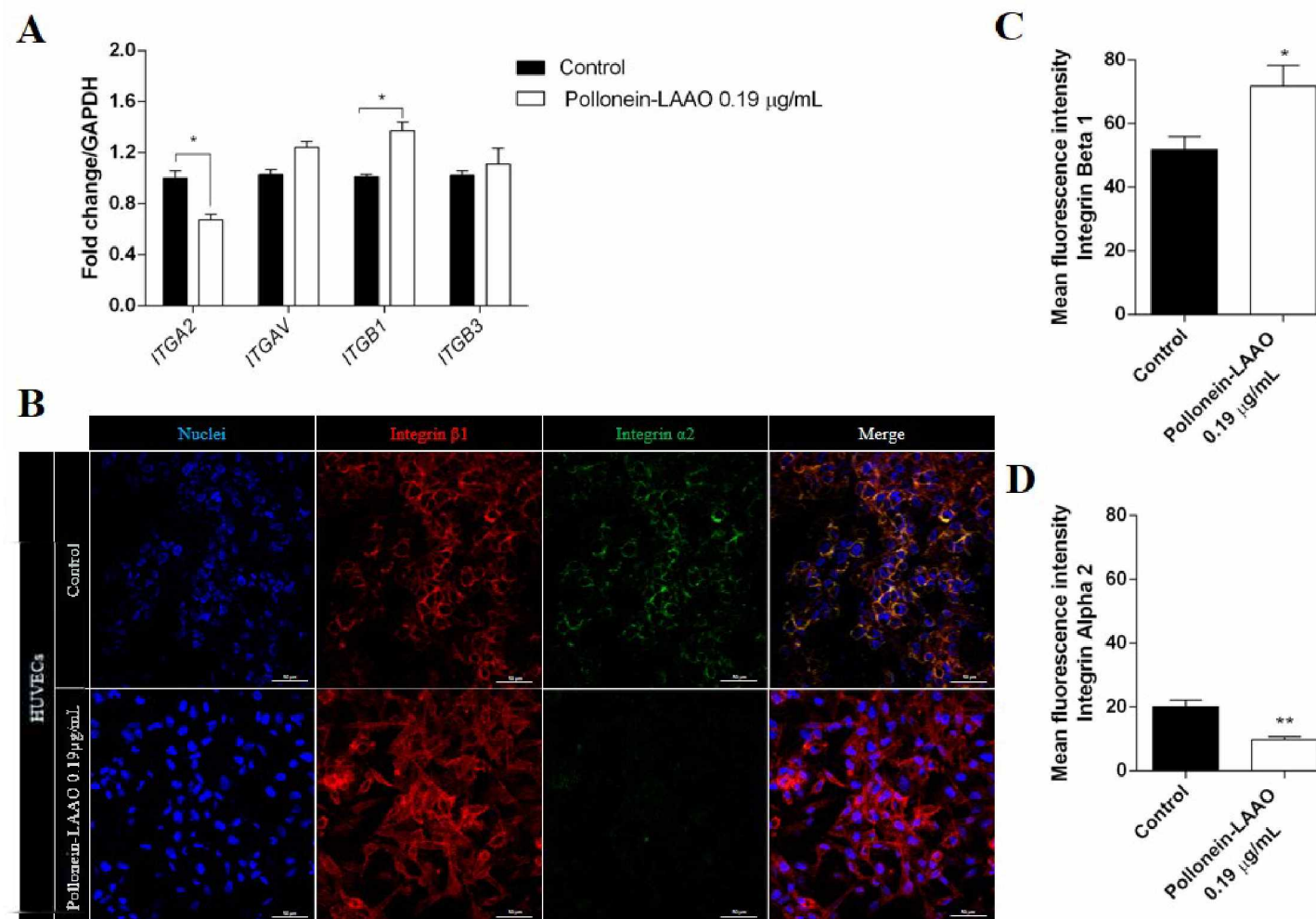


Figure 4. Pollonein-LAAO decreases α_2 integrin subunit both gene and protein expression in HUVECs. (A) Integrin subunits of gene expression profile in HUVECs treated with Pollonein-LAAO (0.19 µg/mL) for 24h. The gene expression was determined by quantitative reverse PCR. *GAPDH* was used as a reference gene. (B) Representative photomicrography of immunofluorescence detection of α_2 (in green) and β_1 (in red) in HUVECs after Pollonein-LAAO treatment. Nuclei were stained with TO-PRO (in blue). Magnification 40x. Scale bars: 50 µm. Representative graphs of (C) β_1 and (D) α_2 integrin subunits fluorescence intensity measured by ImageJ software. * $p < 0.05$ and ** $p < 0.01$ compared to the control (untreated cells).

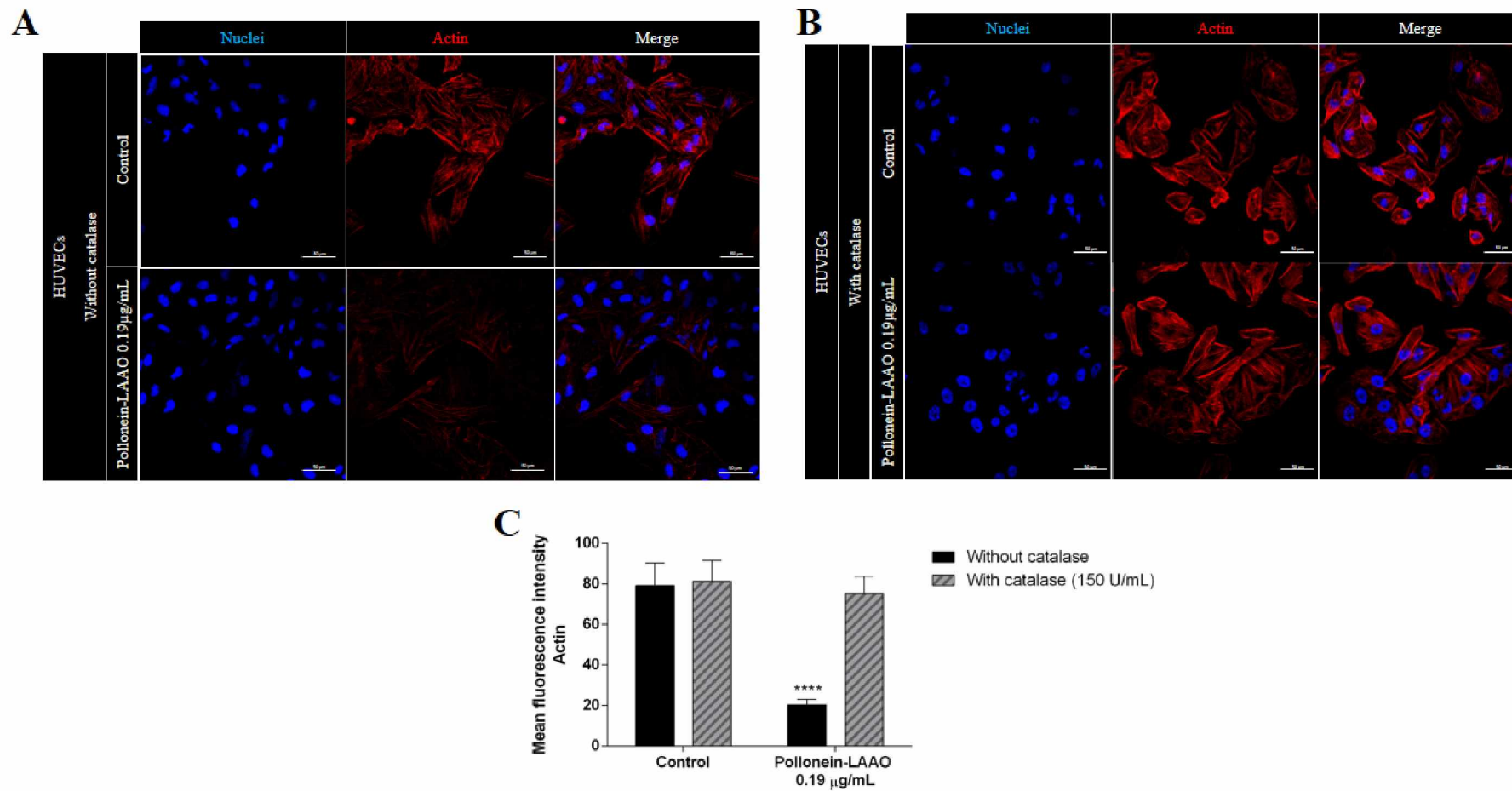


Figure 5. Pollonein-LAAO promotes actin cytoskeleton alterations in HUVECs. Representative photomicrography of HUVECs treated with **(A)** Pollonein-LAAO (0.19 µg/mL) and **(B)** Pollonein-LAAO (0.19 µg/mL) with catalase (150 U/mL) for 24 h, and then, actin cytoskeleton were stained with phalloidin (in red) and nuclei with TO-PRO (in blue). Magnification 40x. Scale bars: 50 µm. **(C)** Graph representing the actin fluorescence intensity measured by ImageJ software. **** $p < 0.0001$ compared to the control (untreated cells)

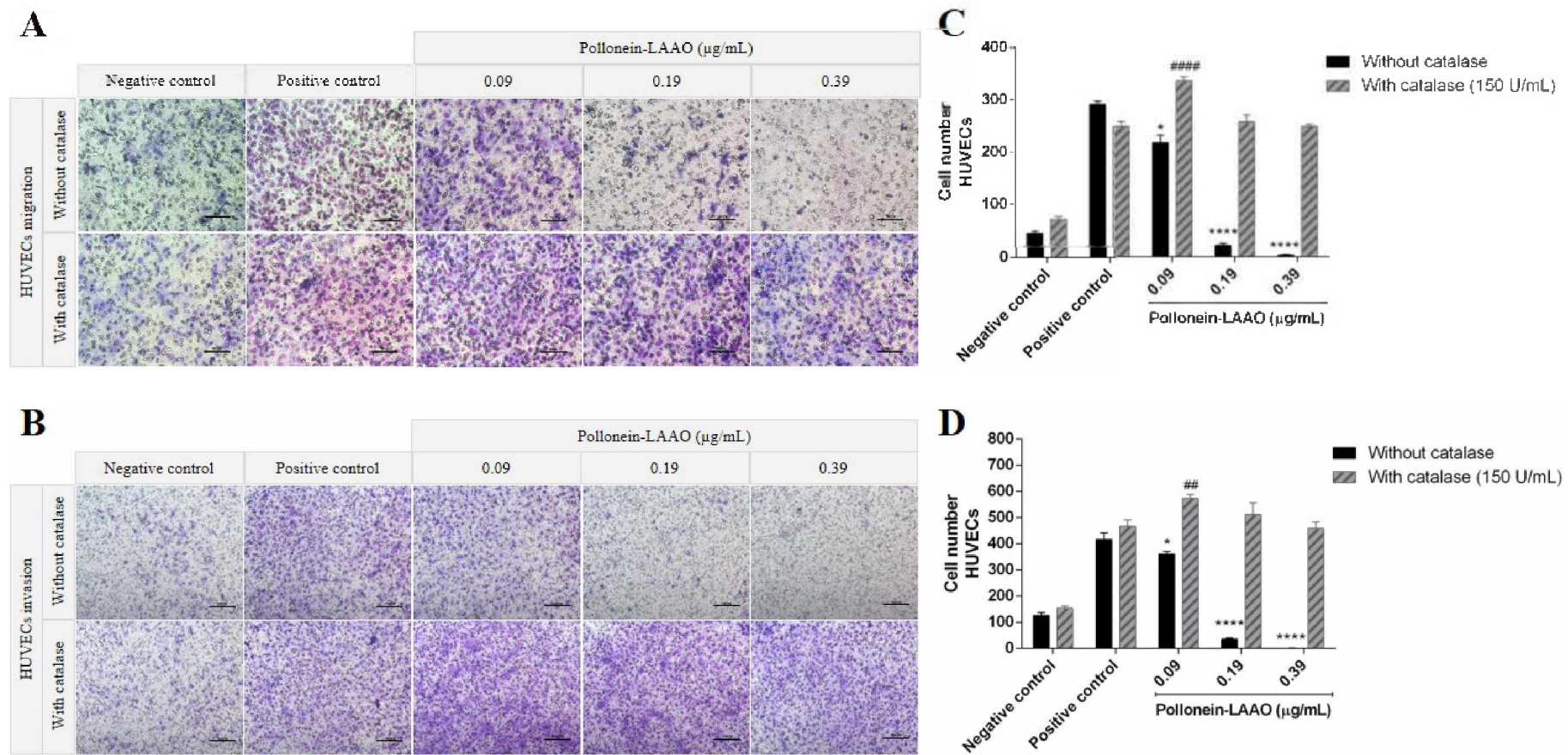


Figure 6. Pollonein-LAAO inhibits HUVECs migration and invasion in the transwell model. Photomicrography representing HUVECs (A) migration and (B) invasion after Pollonein-LAAO treatment in the presence or not of catalase (150 U/mL) for 24 h. Untreated cells incubated with (positive control) or without (negative control) 10% of fetal bovine serum (FBS). Cells were stained with hematoxylin-panotic. 20x and 10x magnification in migration and invasion assay, respectively. Scale bars: 200 μm. Quantitative graphs of (C) migrated and (D) invasion cells. Six sites per group were randomly chosen for manual cell counting. * $p < 0.05$ and **** $p < 0.0001$ compared to the positive control. ### $p < 0.01$ and #### $p < 0.0001$ compared to the positive control with catalase.

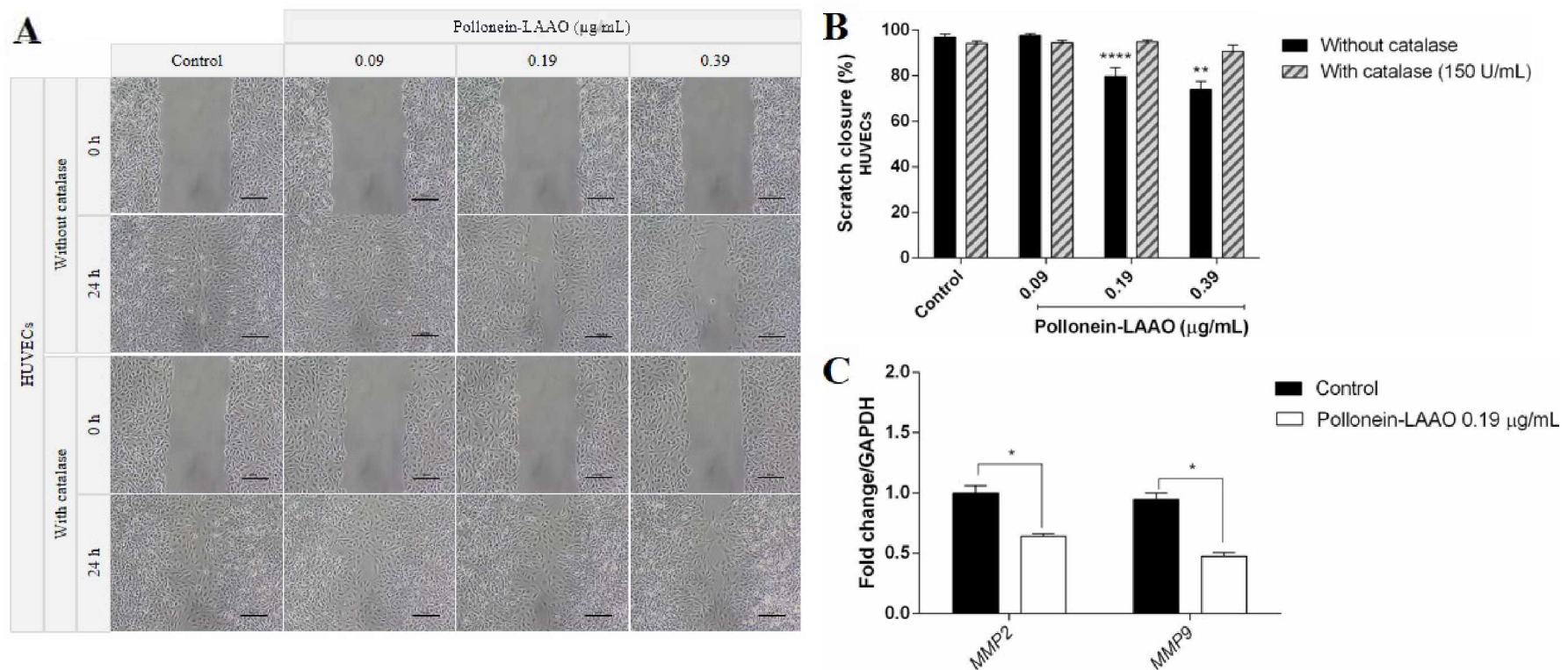


Figure 7. Pollonein-LAAO inhibits HUVECs migration in wound healing model and modulates matrix metalloproteinases gene expression. (A) Photomicrography representing the scratching edge made with a 200- μL pipette tip (0 h) and scratching edge closure (24 h) after Pollonein-LAAO treatment in the presence or not of catalase (150 U/mL) for 24 h. Scale bars: 200 μm . (B) Percentage of wound closure (migrated cells) obtained by ImageJ software. (C) Expression profile of matrix metalloproteinases genes were determined by quantitative reverse PCR. The results are presented as an average fold change in the expression of each gene in HUVECs after treatment with Pollonein-LAAO (0.19 $\mu\text{g/mL}$) for 24 h. Non-treated cells were used as control. *GAPDH* was used as a reference gene. * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$ compared to the control (untreated cells).

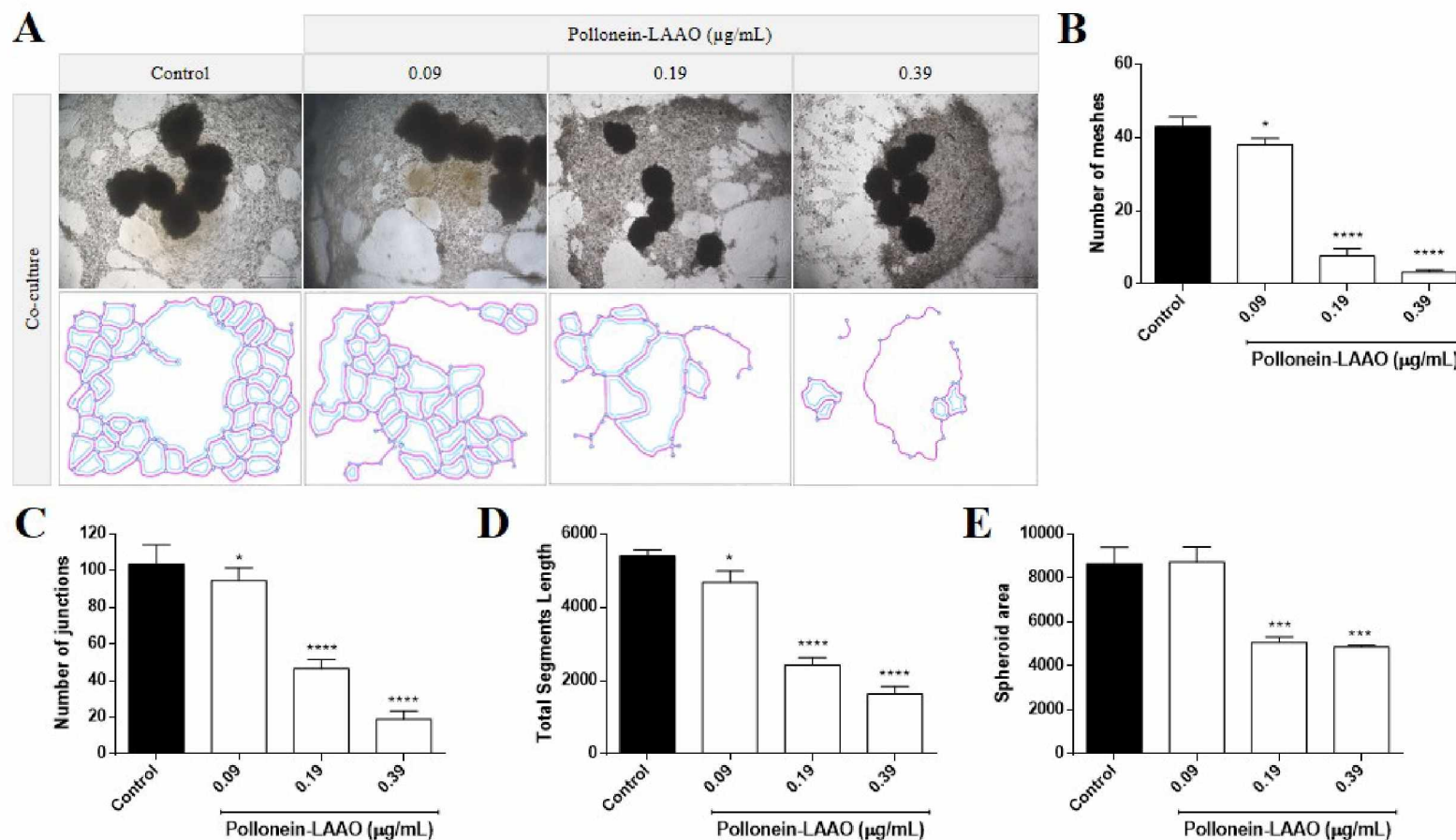


Figure 8. Pollonein-LAAO inhibits HUVECs tube formation in co-culture and decreases multicellular tumor spheroid area. (A) Representative photomicrography of HUVECs tube formation after Pollonein-LAAO treatment for 24 h in co-culture with multicellular tumor spheroid (PC-3 cells and HFF-1 cells). Representative maps of detection of Segments (magenta line), Junctions (blue circles) and Meshes (light blue line) obtained by Angiogenesis analyzer software. Scale bars: 200 μm . Quantitative graph of (B) Number of meshes, (C) Number of junctions, (D) Total segment length determined by Angiogenesis analyzer software, and (E) Spheroid area. * $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ compared to the control (untreated cells).