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"Ação de compostos fenólicos nas propriedades *in vitro* de células malignas humanas derivadas de adenocarcinomas de mama e de próstata"

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutor em Patologia.

Orientador: Prof. Dr. Deilson Elgui de Oliveira

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Epígrafe

(...)
Guarda sempre Ítaca em teu pensamento.
É teu destino aí chegar.
Mas não apresses absolutamente tua viagem.
É melhor que dure muitos anos
e que, já velho, ancores na ilha,
rico com tudo que ganhaste no caminho,
sem esperar que Ítaca te dê riqueza.
Ítaca deu-te a bela viagem.
Sem ela não te porias a caminho.
(...)

Fragmento do poema ÍTACA de Konstatino Kavafis
(Trad. Ísis Borges da Fonseca)

“Ação de compostos fenólicos nas propriedades *in vitro* de células malignas humanas derivadas de adenocarcinomas de mama e de próstata”

Sumário

Lista de abreviaturas

Definições conforme descritores de Ciências da Saúde (DECS; https://decs.bvsalud.org/).

Acrônimo	Definição por extenso	Em inglês (quando couber)
ACA	Ácido cafeico	*Caffeic acid*
ACI	Ácido cinâmico	*Cinnamic acid*
AFE	Ácido ferúlico	*Ferulic acid*
CAPE	Éster fenitil do ácido cafeico	*Caffeic Acid Phenitil Esters*
DL50	Dose letal 50	*Lethal Dose 50*
DMEM	Meio de Eagle modificado por Dulbecco	*Dulbecco´s Modified Eagle Medium*
DMSO	Dimetil Sulfóxido	*Dimethyl Sulfoxide*
DNA	Ácido desoxirribonucleico	*Deoxyribonucleic acid*
EMT	Transições epitelial-mesenquimal	*Epithelial-Mesenquimal Transitions*
FBS	Soro fetal bovino	*Fetal Bovine Serum*
IC50	Concentração inibitória 50	*Half maximal inhibitory concentration*
MMP-2	Metaloproteinase da matriz 2	*Matrix metalloproteinase-2*
MMP-9	Metaloproteinase da matriz 9	*Matrix metalloproteinase-2*
mRNA	RNA mensageiro	*RNA, Messenger*
MTT	brometo de 3-4,5-dimetil-tiazol-2-il-2,5-difeniltetrazólio	*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide*
PBS	Tampão fosfato salino	*phosphate buffered saline*
PCR	Reação em cadeia da polimerase	*Polymerase Chain Reaction*
STR	Repetições curtas em tandem	*Short tandem Repeats*

Lista de Figuras

Lista de Apêndices

Resumo

Os cânceres de mama e de próstata são doenças agressivas que constituem um importante problema de saúde pública em todo o mundo. Embora diversas drogas tenham sido desenvolvidas para o tratamento dessas doenças oncológicas, com frequência são observadas baixa seletividade para células neoplásicas, efeitos colaterais relevantes e resistência tumoral à quimioterapia, situações que motivam o desenvolvimento de novos fármacos com ação antineoplásica. Compostos fenólicos tem sido considerados potenciais candidatos a novos agentes antineoplásicos com base em estudos que sugerem ação citotóxica, citostática e antimetastática para essas substâncias. Entretanto, diversos estudos na literatura sobre o potencial efeito antineoplásico de compostos fenólicos apresentam inconsistências relevantes e seus possíveis mecanismo de ação não são adequadamente elucidados. No presente estudo, foi avaliado possíveis efeitos do tratamento de células malignas humanas derivadas de cânceres de mama e próstata com os ácidos cafeico (ACA), cinâmico (ACI) e ferúlico (AFE), compostos fenólicos obtidos de diferentes fontes naturais, tais como a própolis produzida por abelhas. Foram empregadas linhagens de células humanas com níveis diferentes de agressividade biológica, avaliadas quanto a viabilidade celular, taxas de migração e invasão *in vitro*. As células foram incubadas com os compostos fenólicos em concentrações finais de 25 - 800µM por 24, 48 e 72h. A viabilidade celular foi estimada pelo ensaio MTT e as taxas de migração e invasão celular foram avaliadas em ensaios com câmara de Boyden. Houve consistente redução da viabilidade celular em todas as linhagens expostas ao ACA (dose e tempo dependente), mas não foram observados efeitos com ACI e AFE nas mesmas condições. Os valores de IC_{50} para o tratamento com o ACA (72h) foram: 177 ± 15,3 µM para células MCF-7 e 260 ± 45,8 µM para MDA-MB-231 (ambas derivadas de adenocarcinomas de mama), e 90 ± 19,4 µM para LNCaP e 59,29 ± 5,3 µM para PC3 (derivadas de adenocarcinomas de próstata). Em concentrações não-citotóxicas, o tratamento com ACA reduziu significativamente a migração das células MCF-7 ($p<0.05$) e MDA-MB-231 ($p<0.0001$); ademais, a combinação dos ACA e ACI em concentrações não citotóxicas reduziram significativamente a inibição da migração de células PC3 ($p<0.05$) e a invasão de células MCF-7 ($p<0.05$), MDA-MB-231 ($p<0.001$), LNCaP ($p<0.001$) e PC3 ($p<0.0001$). Esses resultados indicam que o emprego de CA, isolado ou combinado com CIA, pode ser promissor para novos protocolos antineoplásicos dirigidos aos cânceres de mama e próstata.

Palavras-chave: tratamento do câncer, câncer de mama, câncer de próstata, compostos fenólicos, ácido cafeico, ácido cinâmico, ácido ferúlico, estudo pré-clínico.

Abstract

Breast and prostate cancers are aggressive diseases that constitute a major public health problem worldwide. Although several drugs have been developed for the treatment of these oncological diseases, low selectivity for neoplastic cells, relevant side effects and tumor resistance to chemotherapy are often observed, situations that motivate the development of new drugs with antineoplastic action. Phenolic compounds have been considered potential candidates for new antineoplastic agents based on studies that suggest cytotoxic, cytostatic and antimetastatic action for these substances. However, several studies in the literature on the potential antineoplastic effect of phenolic compounds present relevant inconsistencies and their possible mechanism of action are not adequately elucidated. The present study evaluated possible effects of the treatment of human malignant cells derived from breast and prostate cancers with caffeic (CA), cinnamic (CIA) and ferulic (FEA) acids, phenolic compounds obtained from different natural sources, such as the propolis produced by bees. Human cell lines with different levels of biological aggressiveness were used, evaluated in terms of cell viability, migration rates and in vitro invasion. Cells were incubated with phenolic compounds at final concentrations of 25 - 800µM for 24, 48 and 72h. Cell viability was estimated by the MTT assay and cell migration and invasion rates were evaluated in Boyden chamber assays. There was a consistent reduction in cell viability in all lines exposed to CA (dose and time dependent), but no effects were observed with CIA and FEA under the same conditions. IC50 values for CA treatment (72h) were: 177 ± 15.3 µM for MCF-7 cells and 260 ± 45.8 µM for MDA-MB-231 (both derived from breast adenocarcinomas), and 90 ± 19.4 µM for LNCaP and 59.29 ± 5.3 µM for PC3 (derived from prostate adenocarcinomas). At non-cytotoxic concentrations, CA treatment significantly reduced the migration of MCF-7 ($p<0.05$) and MDA-MB-231 ($p<0.0001$) cells; in addition, the combination of CA and CIA at non-cytotoxic concentrations significantly reduced the inhibition of PC3 cell migration ($p<0.05$) and the invasion of MCF-7 cells ($p<0.05$), MDA-MB-231 ($p<0.001$), LNCaP ($p<0.001$) and PC3 ($p<0.0001$). These results indicate that the use of CA, alone or combined with CIA, may be promising for new anticancer protocols aimed at breast and prostate cancers.

Keywords: cancer treatment, breast cancer, prostate cancer, phenolic compounds, caffeic acid, cinnamic acid, ferulic acid, pre-clinical study.

Capítulo 1: Revisão de literatura

Introdução

Produtos naturais em medicina

O uso medicinal de produtos naturais para o alívio e cura das doenças é uma prática humana ancestral, perpetuada como tradição popular. Civilizações antigas se valiam do consumo e/ou aplicação de diversos extratos de plantas, produtos derivados de animais e alguns microrganismos para seus cuidados de saúde, sedimentando as bases da medicina e da farmacologia modernas. Com o passar do tempo, diversas propriedades desses produtos foram descobertas, demonstrando seus efeitos curativos ou mesmo revelando propriedades tóxicas ou mortais, dependendo da quantidade do produto administrado (DIAS; URBAN; ROESSNER, 2012a; HARVEY, 2008).

Os produtos naturais são um recurso valioso para o desenvolvimento de fármacos que auxiliam no tratamento de diferentes doenças (ATANASOV et al., 2021), servindo de matéria-prima para medicamentos com efeitos analgésico, antibiótico, antifúngico, antiviral e antineoplásicos (DIAS; URBAN; ROESSNER, 2012a; HARVEY; EDRADA-EBEL; QUINN, 2015; NEWMAN; CRAGG; SNADER, 2000). Nesse último caso, é importante ressaltar que os produtos naturais são os principais componentes de pelo menos 75% dos agentes antineoplásicos mais comumente utilizados (DEMAIN; VAISHNAV, 2011; MANN, 2002).

Compostos fenólicos figuram dentre os produtos naturais com maior potencial farmacêutico. Trata-se de um grupo de substâncias bioativas reconhecidas por sua ação antioxidante, além de uma ampla variedade de efeitos biológicos, incluindo efeitos anti-inflamatórios, anti-hipertensivo e antitrombóticos. Ademais, são também relatados resultados satisfatórios no controle da diabetes, efeitos imunomoduladores e antitumorais (GÓMEZ-MAQUEO; ESCOBEDO-AVELLANEDA; WELTI-CHANES, 2020a; KUMAR; GOEL, 2019). Os efeitos antitumorais decorrem de inibição de diversos fenômenos da patogênese dos cânceres, incluindo a proliferação e sobrevida das células neoplásicas, a regulação da população de células tronco cancerígenas, a modulação de características do microambiente tumoral, inibição da angiogênese e efeitos no remodelamento da matriz extracelular (ANANTHARAJU et al., 2016; CIANCIOSI et al., 2020; LS et al., 2016; MILEO; MICCADEI, 2016; STANIFORTH et al., 2012; SUN; HEILMANN; KÖNIG, 2015). Em termos gerais, admite-se que polifenóis são substâncias

promissoras para o desenvolvimento de fármacos, incluindo novos medicamentos para o tratamento de cânceres (ANNA MASEK; CHRZESCIJANSKA, EWA; LATOS, MALGORZATA, 2016; DAVIDSON; TOUGER-DECKER, 2009; LEE et al., 2006).

Cânceres: generalidades

Câncer é um termo genérico que remete a doenças neoplásicas malignas, que em comum apresentam acúmulo de células que não respondem aos estímulos fisiológicos de diferenciação celular e homeostase tecidual. Essas células tendem a invadir e destruir o tecido onde surgem e – a depender do nível de sua agressividade biológica – podem se disseminar para órgãos distantes e gerar doença sistêmica ("World Health Organization - Cancer - key facts", 2016).

Denomina-se *carcinogênese* o processo biológico de formação dos cânceres, sendo tipicamente dividido em três etapas sucessivas, conhecidas como iniciação, promoção e progressão. Na etapa de iniciação, células normais de um tecido sofrem alterações irreversíveis em seu genoma (mutações), quer seja por condições hereditárias específicas, induzidas por exposição a agentes físicos ou químicos de natureza genotóxica, ou ainda por mutações de surgimento espontâneo (e.g., por infidelidade de replicação do DNA) (DEVI, 2004). Na etapa de promoção ocorre à expansão clonal das células iniciadas e o acúmulo de novas alterações genéticas e epigenéticas que levam ao surgimento de células com evidências de fenótipo maligno, denominadas de *células transformadas*. Por fim, com o contínuo acúmulo de alterações moleculares genéticas e epigenéticas, na fase de progressão tipicamente são observadas as manifestações clinicas da doença oncológica, incluindo o crescimento tumoral, invasão e disseminação neoplásica por metástase, por exemplo (YOKOTA, 2000).

Dadas as elevadas taxas de morbidade e mortalidade associadas, essas doenças constituem importante problema de saúde pública em todo mundo. Segundo dados da Agência Internacional de pesquisa sobre Câncer (*International Agency for Research on Cancer* - IARC), agência especializada em câncer da Organização Mundial da Saúde (*World Healt Organitation* - WHO), apenas em 2020 foram registrados 19.3 milhões de novos casos de cânceres e 10 milhões de óbitos associados a essas doenças. Ainda, estima-se que para o ano de 2040, as cifras de incidência e mortalidade aumentem significativamente, alcançando cerca de 28,4 milhões de novos casos e 16,3 milhões de mortes ao ano, respectivamente (SUNG et al., 2021).

Com base em dados do Observatório Global do Câncer (*Global Cancer Observatory* - GCO), são descritos como cânceres de maior incidência aqueles que ocorrem em mama (2,3 milhões), pulmão (2,2 milhões), colón/reto (1,9 milhões) e próstata (1,4 milhões). Os de maior prevalência entre as mulheres são os de mama e os colorretais, com 7,8 e 2,4 milhões de casos registrados respectivamente. Para os homens, são mais prevalentes os de pulmão e próstata, com 1,5 e aproximadamente 5 milhões de casos, respectivamente (FERLAY J et al., 2020; SUNG et al., 2021; WORLD HEALTH ORGANIZATION: REGIONAL OFFICE FOR EUROPE, 2020).

Segundo o ministério da saúde a través do Instituto Nacional de Câncer José Alencar Gomes da Silva, estima que no Brasil, entre os anos 2020 e 2022 serão registrados aproximadamente 625 mil novos casos por ano, com incidência de 66 mil novos casos de câncer de mama e o mesmo número de casos de câncer próstata, com distribuição homogênea da incidência calculada em cada região geográfica analisada ("Instituto Nacional de Câncer - INCA", [s.d.]).

Os cânceres de mama são um grupo heterogêneo de doenças neoplásicas, diferindo tanto em características clínicas e histológicas como no prognóstico e na sua resposta aos tratamentos. Representam aproximadamente 33,7% de todos os cânceres diagnosticados na população feminina, responsáveis por pelo menos 685 mil óbitos anuais em todo mundo (FERLAY J et al., 2020; SUNG et al., 2021; WORLD HEALTH ORGANIZATION: REGIONAL OFFICE FOR EUROPE, 2020). A etiologia dos cânceres de mama é multifatorial e inclui fatores endócrinos, reprodutivos, consumo de bebidas alcoólicas, uso de terapia hormonal (contraceptivos e reposição hormonal pós-menopausa), exposição à radiação ionizante, hábitos alimentícios (e.g., dieta hipercalórica), sedentarismo e histórico familiar de câncer de mama (BARNES et al., 2011; HOWELL et al., 2014).

Os cânceres de próstata, por sua vez, são o segundo tipo de câncer mais diagnosticado em homens, com cerca de 1,4 milhões de novos casos anuais. Figuram dentre os cinco cânceres mais letais, responsáveis por cerca de 400 mil óbitos em 2020 (FERLAY J et al., 2020; SUNG et al., 2021). A probabilidade do desenvolvimento do adenocarcinoma prostático está diretamente relacionado à idade dos indivíduos, aumentando significativamente a partir dos 65 anos de idade (CUZICK et al., 2014). Fatores genéticos são preponderantes na etiopatogenia do adenocarcinoma prostático, posto que o risco de desenvolvimento da doença aumenta em até 11 vezes em indivíduos com pai ou irmão que manifestaram esse câncer antes dos 40 anos (CUZICK et al., 2014;

STEWART; WILD, 2014). A etnia também é um fator modificador de risco relevante: em indivíduos afrodescendentes, o risco de desenvolvimento da doença é 1,6 vezes superior ao de caucasianos, principalmente por mutações herdadas, identificadas em 8q24 e 17q21(CUZICK et al., 2014; HENDERSON et al., 2012).

Como de praxe, o prognóstico de pacientes com câncer de mama ou de próstata é influenciado principalmente pelo estágio da doença ao diagnóstico, sendo mais favorável quando a doença é detectada nas fases iniciais (DEVI, 2004; TALMADGE; FIDLER, 2010). Por outro lado, o prognóstico passa a ser desfavorável nos casos de doença avançada, com disseminação sistêmica de células neoplásicas. Estima-se que 90% dos casos de doença com metástases não respondem satisfatoriamente às terapias convencionais e causam o óbito do paciente (HANAHAN; WEINBERG, 2011a; MEHLEN; PUISIEUX, 2006).

O uso de produtos naturais ou derivados tem se popularizado como possível recurso complementar as diversas estratégias terapêuticas para o tratamento da doenças oncológicas, razão pela qual tem recebido mais atenção dos pesquisadores nas últimas décadas (DIAS; URBAN; ROESSNER, 2012b; MORRIS et al., 2000; TAUTZ et al., 2012; WODE et al., 2019). Entre 1981 e 2014, 136 agentes farmacológicos comumente empregadas no tratamento contra o câncer foram aprovados, dos quais aproximadamente 83% são derivados de produtos naturais e 17% eram moléculas sintéticas (AMARAL; SEVERINO; CARVALHO, 2019; NEWMAN; CRAGG, 2016). Ademais, dados experimentais acumulados na literatura sugerem que outros medicamentos derivados de produtos naturais podem surgir, eventualmente a partir de compostos fenólicos, o que será abordado em maiores detalhes a seguir.

Compostos fenólicos

Compostos fenólicos são fitoquímicos amplamente distribuídos na natureza, constituintes de uma ampla variedade de vegetais. Quimicamente são definidos como polifenóis, pois sua estrutura varia entre uma molécula simples de um anel aromático unido a um ou mais grupos hidroxílicos, até polímeros complexos de alto peso molecular (CHEYNIER, 2005b; HARRIS et al., 2007). São metabólitos secundários essenciais para o crescimento e reprodução das plantas, originados da atividade de vias como a da pentose-fosfato, chiquimato e a fenilpropanóide. Cerca de 8.000 compostos fenólicos são conhecidos, agrupados em quatro categorias principais: 1) ácidos fenólicos; 2) cumarinas; 3) flavonoides; e 4) taninos. A estrutura dessas substâncias é diversa, o

que se relaciona a diferentes atividades biológicas. Por outro lado, em geral se verifica capacidade antioxidante como ação biológica principal dos polifenóis (BALASUNDRAM; SUNDRAM; SAMMAN, 2006a; TSAO, 2010b).

Os ácidos fenólicos estão distribuídos nos grupos dos ácidos hidroxibenzoicos e dos ácidos hidroxicinâmicos. Ambos os grupos são encontrados *in natura* principalmente em frutas, vegetais, grãos e sementes, quer seja como substâncias livres ou conjugadas. Do ácido hidroxicinâmico são derivados os ácidos p-cumarínico, ferúlico, cafeico e sinápico, que são os fitoquímicos mais comuns e aos que são geralmente atribuídos os diversos efeitos biológicos descritos para esse grupo de substâncias (CHEYNIER, 2005b; GÓMEZ-MAQUEO; ESCOBEDO-AVELLANEDA; WELTI-CHANES, 2020a; HARRIS et al., 2007; KUMAR; GOEL, 2019; MILEO; MICCADEI, 2016).

Em particular, os ácidos fenólicos são um importante grupo de antioxidantes. Dada suas propriedades de alta solubilidade (tanto em lipídios como em água), são compostos bioativos promissores para o desenvolvimento de novos fármacos que beneficiem à saúde, no entanto, devem ser amplamente estudados para determinar seu potencial farmacológico, assim como os afeitos tóxicos no organismo, promovidos pelo consumo dos ácidos fenólicos. Ações antimicrobianas e anti-inflamatórias são descritas para esses compostos, além de efeitos cardioprotetores e propriedades anticancerígenas (KIOKIAS; PROESTOS; OREOPOULOU, 2020). A seguir, serão sumarizadas as informações relevantes dos efeitos biológicos atribuídos especificamente a três compostos fenólicos, os ácidos cafeico, cinâmico e ferúlico.

Ácido cafeico

O ácido cafeico (ACA), ou 3,4-dihidroxicinamico, é o composto fenólico mais amplamente distribuído em tecidos vegetais. Sua biossíntese ocorre como metabolito secundário da atividade das vias chiquimato e do ácido mevalônico. O ACA participa ativamente dos mecanismos de defesa contra insetos, fungos e bactérias, contribuindo também na proteção contra a radiação ultravioleta B (SILVA; OLIVEIRA; BORGES, 2014). Suas propriedades biológicas nos organismos é principalmente atribuída a uma potente ação antioxidante (GENARO-MATTOS et al., 2015; SILVA; OLIVEIRA; BORGES, 2014; SON; LEWIS, 2002) por diversos mecanismos, como a captura e eliminação direta de radicais livres, inibição específica de enzimas que induzem a

formação dessas espécies reativas, bem como as capacidades de quelar íons metálicos e a de inibir a peroxidação lipídica (SUGIURA et al., 1989).

O ACA tem sido estudado pela sua ampla variedade de efeitos biológicos, sendo proposto como substância promissora para o desenvolvimento de novos medicamentos (WANG et al., 2020). Nesse sentido, dados experimentais subsidiam seu uso para o controle de infecções induzidas por vírus, bactérias e fungos, por exemplo (KHAN et al., 2021). Ainda, foram demonstrados efeitos anti-inflamatórios e imunomoduladores do ACA por inibir a ativação de fatores de transcrição NF-kB de forma especifica (NATARAJAN et al., 1996; XIAO et al., 2018; ZIELIŃSKA et al., 2021) e pela sua capacidade de induzir aumento significativos nas interleucinas-2 e -4 em roedores (PARK et al., 2004).

Uma possível atividade antineoplásica desse composto fenólico têm surgido com base em resultados de experimentos com modelos de células cultivadas *in vitro* (OZTURK et al., 2012). Alguns estudos sugerem que o ACA pode reduzir significativamente a viabilidade de células malignas, controlar a proliferação celular e inibir a formação de metástases de cânceres mediante modulação e alteração do ciclo celular e indução de apoptose, bem como reduzir taxas de migração e invasão de células neoplásicas (AKYOL et al., 2013; OZTURK et al., 2012; PELINSON et al., 2019). Dados que dão suporte às propriedades anticancerígenas do ACA indicam que a ação antiproliferativa pode estar associada a regulação direta da fosforilação do gene *TP53* (HGNC:11998) (REZAEI-SERESHT et al., 2019), e/ou por causar disfunção mitocondrial das células neoplásicas (JAGANATHAN, 2012; JIN et al., 2008; RAJENDRA PRASAD et al., 2011). Digno de nota, o ACA aparenta ter atividade antiproliferativa bastante segura e específica, pois as propriedades citotóxicas parecem ser seletivas para células neoplásicas (HERNANDES et al., 2020; SARI; SÜMER; CELEP EYÜPOĞLU, 2020).

Adicionalmente, é descrito que o ACA pode exercer um efeito antimetastático, pois reduz os níveis de expressão das metaloproteinases da matriz (*Matrix Metaloproteinases* - MMPs) nas células neoplásicas, especificamente a MMP-2 e MMP-9(HWANG et al., 2006; LI et al., 2017; PENG et al., 2012), proteínas que quando expressas, tem a função de degradar a matriz extracelular para favorecer a migração e invasão das células neoplásicas, auxiliando com isso o desenvolvimento das metástases (SHAY; LYNCH; FINGLETON, 2015).

Ácido cinâmico.

O ácido cinâmico (ACI), ou ácido 3-fenil-2-propenóico, é um polifenol sintetizado nas plantas na forma de ácido graxo aromático. Compõe o grupo das auxinas – hormônios vegetais responsáveis pela regulação do crescimento e a diferenciação celular – e como o ACA também cumpre funções de proteção contra microrganismos (BUXTON et al., 2020; STEENACKERS et al., 2019). O ACI é encontrado naturalmente numa ampla variedade de alimentos, incluindo o café, a canela, mel e seus derivados, azeite de oliva, frutos cítricos, cereais e maçãs (NACZK; SHAHIDI, 2006). Possui uma estrutura química nas formas *trans*- e *cis*- e serve como precursor dos ácidos fenólicos, principalmente o ácido cafeico e o ácido ferúlico (PEI et al., 2016; RUWIZHI; ADERIBIGBE, 2020).

São diversas as propriedades biológicas atribuídas ao ACI e seus derivados, sendo que as aparentes atividades antimicrobiana, anti-inflamatória, imunomoduladora o tornam um composto bioativo promissor para o desenvolvimento de novos fármacos, perspectiva reforçada por suas propriedades benéficas sobre doenças crônicas como a diabetes, hipertensão e neoplasias malignas (RUWIZHI; ADERIBIGBE, 2020).

Acerca dos efeitos antineoplásicos, foi relatado que o ACI exerce ação antiproliferativa *in vitro* sobre células de carcinoma de cólon Caco-2 (RRID:CVCL_0025), inibindo sua síntese de DNA (EKMEKCIOGLU; FEYERTAG; MARKTL, 1998a), além de atuar como agente pró-apoptótico em células neoplásicas de glioblastoma A172 (RRID:CVCL_0131) e U251 (RRID:CVCL_0021) (LIU et al., 1995b), de melanoma HT-144 (RRID:CVCL_0318) (DE OLIVEIRA NIERO; MACHADO-SANTELLI, 2013), de adenocarcinomas de pulmão A549 (RRID:CVCL_0023) e de próstata, linhagens PC3(M) (RRID:CVCL_9555), DU145 (RRID:CVCL_0105) e LNCaP (RRID:CVCL_0395) (DE OLIVEIRA NIERO; MACHADO-SANTELLI, 2013; LIU et al., 1995b). O ACI aparenta ter uma atividade segura para o organismo e específica para células neoplásicas, pois demostra baixa toxicidade em células não-neoplásicas, sendo necessário o aumento das doses em até 20 vezes para gerar a mesma toxicidade (DE OLIVEIRA NIERO; MACHADO-SANTELLI, 2013; LIU et al., 1995b). Em modelos murinos o ACI apresenta baixa toxicidade, com dose letal 50 (LD_{50}) corresponde a aproximadamente 2850mg/kg de peso (PEI et al., 2016).

Ácido ferúlico.

O ácido ferúlico (AFE), também denominado ácido 4-hidroxi-3-metoxicinâmico ou ácido coniférico, é um importante derivado do ACI. Encontra-se em abundância no trigo, aveia e arroz, sendo também observado em diversos outros vegetais, incluindo a beterraba, alcachofra e algumas plantas forrageiras, encontrado ligado a polímeros das paredes das células dessas espécies (BARBEROUSSE et al., 2008; BEZERRA et al., 2017). Como outros compostos fenólicos, o AFE possui ação antioxidante, que é atribuída à sua capacidade de neutralizar o peroxido de hidrogênio, o ânion superóxido e o radical hidroxila (SOARES, 2002; ZDUŃSKA et al., 2018). Outra importante ação atribuída ao AFE é a de absorção da radiação UV, com consequente redução dos danos associados a sua exposição (GRAF, 1992).

Algumas evidências experimentais indicam capacidade do AFE de bloquear vias de sinalização intracelular relevantes na carcinogênese, incluindo a via FGFR1/PI3K/AKT, importante reguladora da angiogêneses tumoral (YANG; JIANG; LU, 2015a). Um outro estudo determinou que células neoplásicas de bexiga T24 (RRID:CVCL_0554) apresentaram maiores taxas de apoptose após tratamento com AFE, consequência da inibição da proteína bcl-2 e a indução de bax, (proteínas anti e pró-apoptóticas respectivamente) (PENG et al., 2013). Há ainda dados indicando que células neoplásicas tratadas com AFE sofrem parada do ciclo celular na fase G0/G1, além de uma redução dos níveis de expressão do transcrito para MMP-9 (GAO et al., 2018). Ademais, alguns autores propõem que o AFE inibe a formação de metástases de células neoplásicas de glândula mamária pela regulação de transições epitelial-mesenquimal (*Epithelial-Mesenquimal Transitions* - EMTs) (ZHANG et al., 2016), fenômeno da biologia dos cânceres que favorece a disseminação de células neoplásicas epiteliais e o desenvolvimento de metástases de carcinomas (HANAHAN; WEINBERG, 2011b).

Com base em dados experimentais indicando efeitos inibitórios em fenômenos relevantes da biologia dos cânceres, sugere-se que compostos fenólicos como o ACA, ACI e AFE são promissores para o desenvolvimento de novos fármacos de ação antineoplásica. A reconhecida atividade antioxidante, por exemplo, pode ter papel em diferentes etapas da carcinogênese, prevenindo a formação e acúmulo de espécies reativas de oxigênio e seus consequentes efeitos genotóxicos, que propicia a instabilidade genética crucial para o desenvolvimento dos cânceres(GRAF, 1992; KIOKIAS; PROESTOS; OREOPOULOU, 2020; YIN et al., 2019). Há

também relatos de que esses compostos podem bloquear o ciclo celular, induzir apoptose e a de inibir a migração e invasão de células neoplásicas, o que lhes pode conferir ação moduladora ou mesmo inibitória de fenômenos da progressão tumoral (DE OLIVEIRA NIERO; MACHADO-SANTELLI, 2013; GAO et al., 2018; HUNKE et al., 2018; PELINSON et al., 2019; QI et al., 2016). Por outro lado, diversos estudos publicados sobre esses compostos carecem de rigor experimental(FAEZEH; REZA; MARYAM, 2016; KABAŁA-DZIK et al., 2017, 2018; SERAFIM et al., 2011), o que suscita dúvidas sobre a fidelidade dos resultados. De modo a gerar informações relevantes para melhor entendimento do potencial antineoplásico do ACA, ACI e AFE, o presente estudo buscou avaliar a influência desses compostos fenólicos na viabilidade celular e eventos biológicos de migração e invasão celular avaliados *in vitro* empregando células humanas derivadas de adenocarcinomas de mama e próstata, empregados em tratamentos com agente único ou em combinação.

Objetivos

Objetivo geral

Este estudo visa verificar se os componentes fenólicos ACA, ACI e AFE podem alterar a viabilidade celular e propriedades *in vitro* relevantes para o potencial metastático de células humanas neoplásicas derivadas de cânceres prevalentes.

Objetivos específicos

Baseado em modelos de células malignas derivadas de adenocarcinomas de mama (linhagens MCF-7 e MDA-MB-231) e de próstata (LNCaP e PC3), este estudo avaliou os efeitos do tratamento isolado ou combinado com ACA, ACI e AFE em relação a:

1. Citotoxicidade;
2. Possíveis efeitos sinérgicos da combinação dos compostos avaliados;
3. Efeitos sobre a capacidade de migração e invasão in vitro das células malignas;

Delineamento experimental

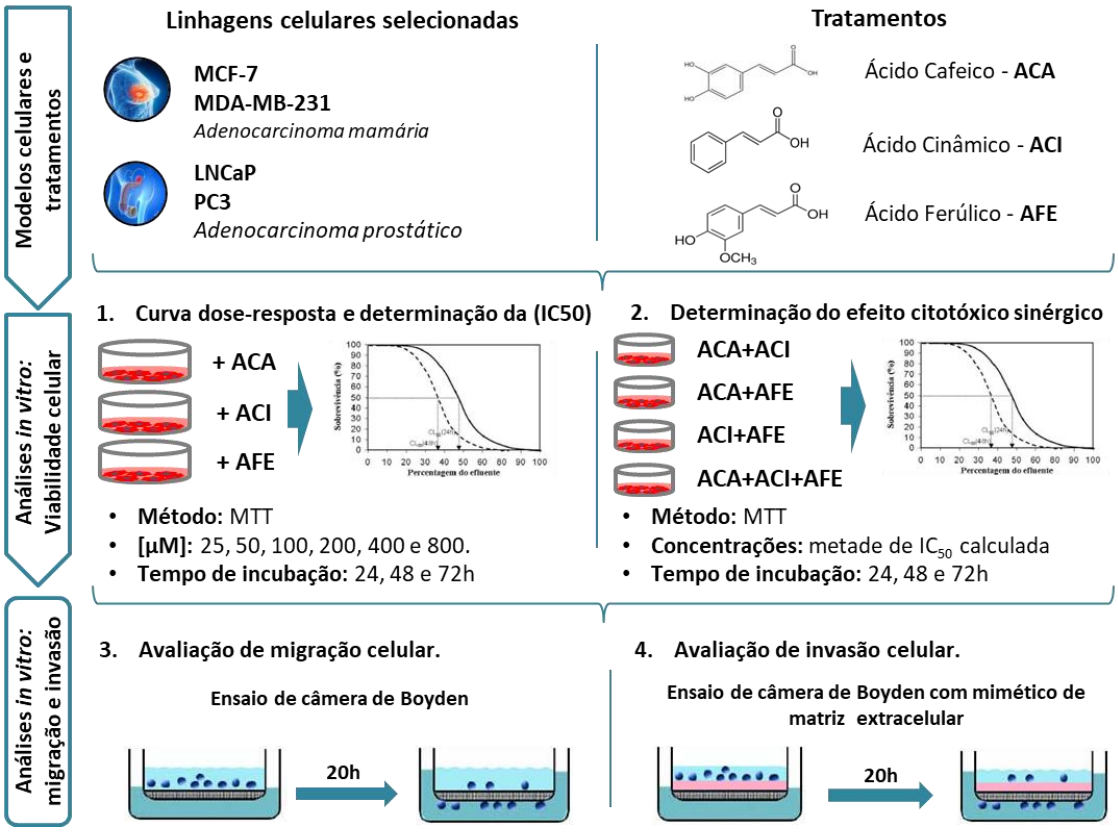

FIGURA 1. Delineamento experimental do estudo.

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Capítulo 2 – Manuscrito*

* O manuscrito a seguir será apresentado conforme as recomendações do "International Committee of Medical Journal Editors – ICJME" (normas de Vancouver).

Caffeic acid inhibits the growth of malignant cells derived from human breast and prostate adenocarcinomas and synergistically inhibits cell invasion in vitro when combined with cinnamic acid

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Abstract

Breast and prostate adenocarcinomas are prevalent human cancers worldwide. Although potentially curable, adverse effects and tumor resistance associated with conventional chemotherapeutic protocols require the development of new drugs and therapeutic regimens. Phenolic compounds are debated as promising agents for the treatment of malignant tumors due to putative cytotoxic, cytostatic, and even antimetastatic properties. Nonetheless, the available evidence is controversial, notably because of poor study design and overstated conclusions of studies based on cell culture models. Therefore, in this pre-clinical study, we assessed the effects of three phenolic compounds – caffeic acid (CA), cinnamic acid (CIA), and ferulic acid (FA) on cells derived from the human adenocarcinomas of breast (MCF-7 and MDA-MB-231) and prostate (LNCaP and PC3). The cultivated cells were treated with the phenolic compounds (25 to 800µM) for 24, 48, and 72h and the rates of cell viability, cell migration, and cell invasion in vitro were assessed. We found a consistent reduction in cell viability (dose and time-dependent) for all cell lines exposed to CA, whilst the treatment with CIA and FA did not show significant changes under the same experimental conditions. The following IC50 values for CA treatment (72h) were obtained: MCF-7 = 177 ± 15.3 µM; MDA-MB-231 = 260 ± 45.8 µM; LNCaP: 90 ± 19.4 µM; and PC3: 59.29 ± 5.3 µM. At non-cytotoxic concentrations, the treatment with CA reduced the migration of MCF-7 ($p<0.05$) and MDA-MB-231 ($p<0.0001$) cells. Also, CIA boosted the CA adverse effect on in vitro migration of PC3 cells ($p<0.05$), as well as the invasiveness of cell lines evaluated. Taken together, these findings suggest that the treatment of breast and prostate

adenocarcinoma human cells with CA, alone or in association with CIA, has a promising potential for the development of new antineoplastic agents.

Keywords: cancer treatment, breast cancer, prostate cancer, phenolic compounds, caffeic acid, cinnamic acid, ferulic acid, pre-clinical study.

Introduction

Globally, breast and prostate cancers are the two most common cancers in women and men. Breast cancers are a heterogeneous group of neoplastic diseases, differing in clinical and histological features, as well as in prognosis and response to treatments (1). They represent approximately 33.7% of all cancers diagnosed in the female population, responsible for at least 685,000 annual deaths worldwide. On the other hand, prostate adenocarcinoma is the second most diagnosed cancer in men, with about 1.4 million new cases annually. They are among the five deadliest cancers, responsible for about 400,000 deaths in 2020 (2). For both breast and prostate cancer, chemotherapy is the traditional treatment, but the drugs commonly used do not show optimal selectivity and activity against cancer cells. Consequently, treatment-associated adverse effects and tumor resistance observed after conventional chemotherapy protocols prompt the search for new antineoplastic drugs to treat these diseases (3).

Historically, natural products have proven to be an important source of compounds for medical drugs, including those with therapeutic potential against cancers (NEWMAN; CRAGG, 2020; RODRIGUES et al., 2016). In this regard, phenolic compounds are among the natural products with high pharmaceutical value. These compounds are a group of bioactive substances with known antioxidant action, in addition to a wide variety of biological effects. Published studies investigate the biological activity of phenolic compounds from a variety of natural sources, such as plants(DE CAMARGO et al., 2018; KISIRIKO et al., 2021; MIKLAVČIČ VIŠNJEVEC; SCHWARZKOPF, 2020) and fruits(BUSATTO et al., 2019; GÓMEZ-LÓPEZ et al., 2021; GÓMEZ-MAQUEO; ESCOBEDO-AVELLANEDA; WELTI-CHANES, 2020b), as well as bee propolis (CINEGAGLIA et al., 2013; DEVEQUI-NUNES et al., 2018; SALATINO; SALATINO; NEGRI, 2021; SFORCIN, 2016) – a resinous product made of plant exudates, beeswax, and salivary secretions, among other substances (MARCUCCI et al., 2001; OKIŃCZYC et al., 2021;

SFORCIN, 2016). Overall, phenolic compounds are phytochemicals of great interest due to their potential benefits to human health. The consumption of phenolic compounds in the diet has shown an inverse association with the prevalence of chronic diseases such as diabetes, cardiovascular diseases, and even neoplasms.(CIANCIOSI et al., 2018; DI LORENZO et al., 2021; ROBBINS, 2003; TUNGMUNNITHUM et al., 2018).

More than 8,000 phenolic compounds are currently described, grouped into four main categories: 1) phenolic acids; 2) coumarins; 3) flavonoids, and 4) tannins. They are defined as polyphenols, and their chemical structure varies from a single molecule of an aromatic ring linked to one or more hydroxyl groups to complex polymers of high molecular weight (BALASUNDRAM; SUNDRAM; SAMMAN, 2006b; CHEYNIER, 2005a; TSAO, 2010a; TUNGMUNNITHUM et al., 2018). Phenolic acids are distributed in the group's hydroxybenzoic acids and the hydroxycinnamic acids; in both groups, they are commonly found in fruits, vegetables, grains, and seeds, either as free or conjugated substances (VALANCIENE et al., 2020). Among the phenolic acids, caffeic (CA), cinnamic (ACI), and ferulic (FA) acids will be investigated here. CA (3,4-hydroxycinnamic acid) is a derivative of hydroxycinnamic acid and it is the most widely distributed phenolic compound in plants, fruits, and vegetables; ACI (3-phenyl-2-propenoic acid) is a polyphenol synthesized in plants as an aromatic fatty acid (BUXTON et al., 2020; STEENACKERS et al., 2019), and FA (4-hydroxy-3-methoxycinnamic acid) is an important derivative of ACI (BARBEROUSSE et al., 2008; BEZERRA et al., 2017).

CA, ACI, and FA have been investigated previously regarding their possible antineoplastic effects. For instance, CA's biological properties were essentially attributed to its potent antioxidant action (SILVA; OLIVEIRA; BORGES, 2014; SON; LEWIS, 2002). CA was also considered a promising antineoplastic agent due to reported activities decreasing the viability of neoplastic cells, inducing apoptosis, by modulating and altering the cell cycle, and reducing the rates of migration and invasion of neoplastic cells. (AKYOL et al., 2013; OZTURK et al., 2012; PELINSON et al., 2019). ACI was reported to exert antiproliferative activity on colon cancer cells *in vitro*, inhibiting their DNA synthesis.(EKMEKCIOGLU; FEYERTAG; MARKTL, 1998b); it was also described as a pro-apoptotic agent for cells derived from glioblastoma (A172 and U25)(LIU et al., 1995a) of melanoma (HT-144)(NIERO; MACHADO-SANTELLI, 2013) lung (A549) and prostate adenocarcinomas (PC3, DU145, and LNCaP) (LIU et al., 1995a; NIERO; MACHADO-

SANTELLI, 2013). Also, experimental evidence suggests that FA blocks intracellular signaling relevant to carcinogenesis, including the FGFR1/PI3K/AKT pathway, which is an important regulator of tumor angiogenesis (YANG; JIANG; LU, 2015b).

Despite some exciting data on phenolic acids as potential antineoplastic agents, the available literature is usually controversial due to the low replicability of the experimental findings. This can be attributed to flaws in the study design and overstated conclusions that do not take into account the limitations of cell line models. A better understanding of the potential use of phenolic acids for cancer treatment requires more pre-clinical studies with careful control of the experimental design to circumvent the most common issues identified in a variety of published studies. Based on this premise, in this study, we evaluated whether the phenolic acids CA, ACI, and AFE, alone or in combination, can affect selected in vitro properties relevant to the aggressiveness of human malignant cells derived from breast and prostate adenocarcinomas.

Material and Methods

Chemicals

The compounds caffeic acid (CA; CAS# 331-39-5), trans-cinnamic acid (CIA; CAS# 140-10-3), and ferulic acid (FA; CAS# 537-98-4), as well as the reagent 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; CAS: 298-93-1), were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO, USA).

Cell lines and cell culture conditions.

The cell line MCF-7 (RRID: CVCL_0031) is derived from a metastatic (pleural effusion) hormone-responsive human breast adenocarcinoma (ductal); MDA-MB-231 (RRID: CVCL_0062) is a triple-negative breast cancer (TNBC) cell line derived from the metastatic site (pleural effusion); LNCaP (RRID: CVCL_0395) is an androgen-sensitive human prostate adenocarcinoma cell line derived from lymph node metastasis; and PC3 (RRID: CVCL_0035) is an androgen-independent prostate adenocarcinoma cell line derived from bone metastasis.

MCF-7 and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), whereas LNCaP and PC3 were maintained in the RPMI-1640 medium. The culture medium was always supplemented with 10% fetal bovine serum (FBS), as well as 0,4% gentamicin

(MCF-7 and MDA-MB-231) or 1% penicillin/streptomycin plus 0,1% amphotericin B (LNCaP and PC3) for microbiological control. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and regularly evaluated to assess cell growth and viability using a conventional trypan-blue dye exclusion assay. All the cell lines were authenticated before experiments in our lab by short tandem repeats (STRs) analysis using the GenePrint 10 system (Promega, Madison, WI, USA), according to the manufacturer's instructions. The cell cultures were also confirmed to be free of *mycoplasma* contamination using a PCR-based protocol (UEMORI et al., 1992).

Preparation of experimental solutions

The phenolic compounds CA, CIA, and FA were dissolved in DMSO at a concentration of 1M/mL to prepare the stock solution, which was aliquoted and stored at -20°C. The working solution was prepared just before each experiment to minimize the degradation of phenolic compounds, by diluting the stock solution in a cold (~8°C) culture medium without FBS. The final concentration of DMSO in the cell culture medium was kept always below 0,1% (v/v).

Cell viability assay

The MTT assay was performed to assess the cytotoxic activity of the selected phenolic acids. Briefly, all cells were seeded at a density of 2x103 cells per well in flat-bottomed 96-wells cell culture plates filled with complete culture medium and incubated for 24h. Subsequently, the complete medium was removed, and the cells were incubated for another 24h with a serum-free medium for cell-cycle synchronization by starvation. The starving medium was then removed, and the cells were treated with different concentrations (0 - 800 µM) of the phenolic compounds CA, CIA, and FA in a complete medium (200µL) for the dose-response studies and the cells were incubated as usual for 24, 48, and 72h. The negative control was prepared with a culture medium only, whereas the vehicle control (absence of phenolic compounds) was prepared with a culture medium containing only DMSO. By the end of the treatment, the MTT solution (5mg/mL; 10µL) was added to each well and the cells were incubated for another 4h, following the removal of the supernatants and addition of DMSO (200µL) to dissolve the formazan crystals. The number of viable cells was estimated by measuring the 570nm absorbance using a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The cell viability estimations for each concentration of the phenolic acids were determined relative to untreated cells (exposed to DMSO only). The half maximal inhibitory concentration (IC50) values for each cell line were

subsequently calculated based on dose-response curves after 24, 48, and 72h of treatment with CA, CIA, or FA. In all experiments, the obtained data were normalized to be presented as the percentage of the values obtained for the negative controls (cells not treated). The results were obtained from three independent experiments with three technical replicates each.

Assessment of putative synergistic activities

Possible synergistic effects due to the combined treatment of more than one of the investigated phenolic acids (CA+CIA, CA+FA, CIA+FA, and CA+CIA+FA) were investigated using non-cytotoxic doses of each compound. Accordingly, breast and prostate adenocarcinoma cells were treated using half of the IC50 calculated for each cell line treated for 24, 48, and 72h and evaluated regarding the cell viability using MTT assay, as well as the migration and invasion rates in vitro using Boyden chamber assays, as described in the following section.

Assessment of cell migration and invasion in vitro

The potential of migration and invasion in vitro by MCF-7, MDA-MB-231, LNCaP, and PC3 cells exposed to CA, CIA, and FA (alone or combined, as stated in the previous section) was assessed using Boyden chambers with 8µm pores ThinCert® inserts (Greiner Bio-One, Nürtingen, Germany) coupled to wells in a 24-well plate. Initially, 2x104 cells were seeded into 12-well cell culture plates and incubated for 24h for adherence and cell cycle synchronization (serum starvation). Following, the cells were preincubated in a complete medium (3mL final volume) for 72h with the compounds alone or in combination, using half of their IC50 (estimated for the specific cell line under evaluation). The negative control (no treatment) and vehicle control (DMSO only, <0,1%) were used as usual. After the preincubation, the cells were resuspended in serum-free medium and seeded (5x104 cells) into the transwell inserts (upper chamber), previously prepared with serum-free media with the phenolic compounds alone (CA, CIA, FA), and the selected combinations (CA+CIA, CA+FA, CIA+FA, and CA+CIA+FA) to a final volume of 150µL. The lower well chamber was prepared with fresh medium supplemented with 20% FBS, used as a chemo-attractant.

For the in vitro invasion; the inserts were coated with 100µL/cm2 of GeltrexTM LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Thermo Fisher Scientific, Waltham, MA, USA), in the case of assay prostate cancer cells, or 100µL/cm2 of GeltrexTM diluted 1:4 in DPBS, for breast cancer cells. In both cases, after incubation for 20h, the cells that did not migrate or

invade were carefully removed from the inner face of the inserts using a cotton swab. The inserts were then fixed (100% ethanol for 20 min), stained (1% crystal violet solution for 20 min), and washed with tap water to remove the excess dye. Migrating or invading cells were identified via microscopy using the inverted phase-contrast microscope Nikon TS100F (Nikon Inc., Minato, Japan). Two observation fields (200x magnification) were photographed for each insert in each of the experimental conditions evaluated. The stained cells were counted manually using the image processing software ImageJ (40,41).

Statistical analysis

All experiments were carried out in triplicate and the results were expressed as the mean ± standard deviation (SD). The normality of the distribution was assessed with the Shapiro-Wilk test. Differences between groups were analyzed by comparing the means using a one-way analysis of variance test (ANOVA), followed by Dunnett's test, using the software Prism v8 (Graphpad Software Inc, San Diego, CA, USA). Differences with $p<0.05$ were considered statistically significant.

Results

Caffeic acid inhibited the cell viability of breast and prostate cancer cells.

We examined the influence of phenolic compounds CA, CIA, and FA on cell viability and proliferation in vitro of malignant cells derived from the human breast (MCF-7, MDA-MB-231) and prostate (LNCaP and PC3) cancers using the MTT assay. As shown in figure 1, the cells exposed to CA showed a reduction in the number of viable cells in culture over time, notably in concentrations above 100µM of CA, overall. On the other hand, CIA and FA did not affect reducing the number of viable cells in culture under the same experimental conditions, even at concentrations of CIA or FA alone as high as 800µM.

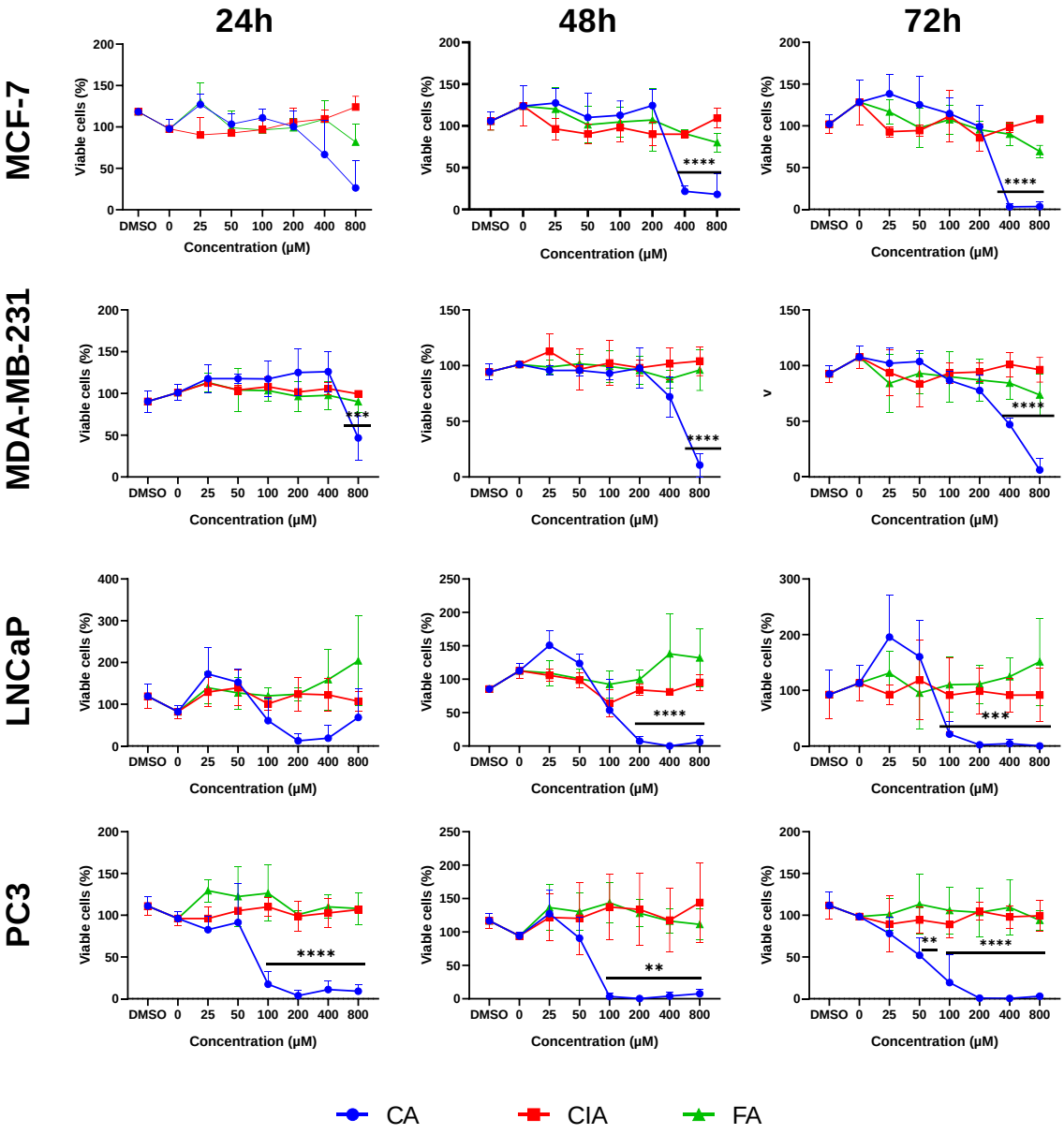

FIGURE 1. Effect of the phenolic compounds on the cell viability of malignant cells. Cells derived from the human breast (MFC-7 and MDA-MB-231) and prostate (LNCaP and PC3) cancers were treated with caffeic acid (CA), cinnamic acid (CIA), and ferulic acid (FA) for 24, 48, and 72h, at concentrations ranging from 25μM to 800μM. Cell viability was detected by MTT assay. The results are shown as a percentage (%) of the values obtained for the negative control (untreated cells). Cells exposed to CA showed a significant reduction in cell viability rate over time. The observed differences were analyzed with the one-way ANOVA, followed by the Tukey test. (* P<0.05. ** P<0.01. *** P<0.001. ****P<0.0001).

Under the same experimental conditions, the concentration required to reduce by 50% the viability of LNCaP and PC3 prostate cancer cells was lower by a magnitude of 4-5 times compared to breast cancer cells MCF-7 and MDA-MB -231. The estimated IC50 values for CA at 24h, 48h, and 72h of treatment are indicated in Table 1. Neither CIA nor FA affected the viability of all cell lines tested at the phenolic acid concentrations and time points evaluated.

Time points	Caffeic Acid IC_{50} (µM) for the indicated cell lines			
	MCF-7	**MDA-MB-231**	**LNCaP**	**PC3**
24h	330 ± 92,9	440 ± 46,2	76 ± 13,2	88,53 ± 20,9
48h	230 ± 43,6	330 ± 10	80 ± 16,1	53,72 ± 1,6
72h	177 ± 15,3	260 ± 45,8	90 ± 19,4	59,29 ± 5,3

CA, CIA, and FA do not show synergistic inhibition on cell viability for breast and prostate cancer cells.

Pharmacological synergism can be defined as the intensification of the effect obtained by the association of two or more substances, being significantly greater than the effects obtained with each substance alone (42) We examined whether the combination of phenolic acids reduces the cell viability of cancer cell lines MCF-7, MDA-MB-231, LNCaP and PC3 in 24, 48 and 72 hours of exposure. The treatments were CA+CIA, CA+FA, CIA+FA, and CA+ CIA +FA, using half of the IC50 (IC50/2) value of CA for each cell line estimated at the 72h of treatment (Table 1), combined at an equipotent molar ratio of CIA and FA. As shown in figure 2, no synergistic effects were observed for any of the phenolic acid combinations in the selected cell line models of human breast and prostate adenocarcinomas.

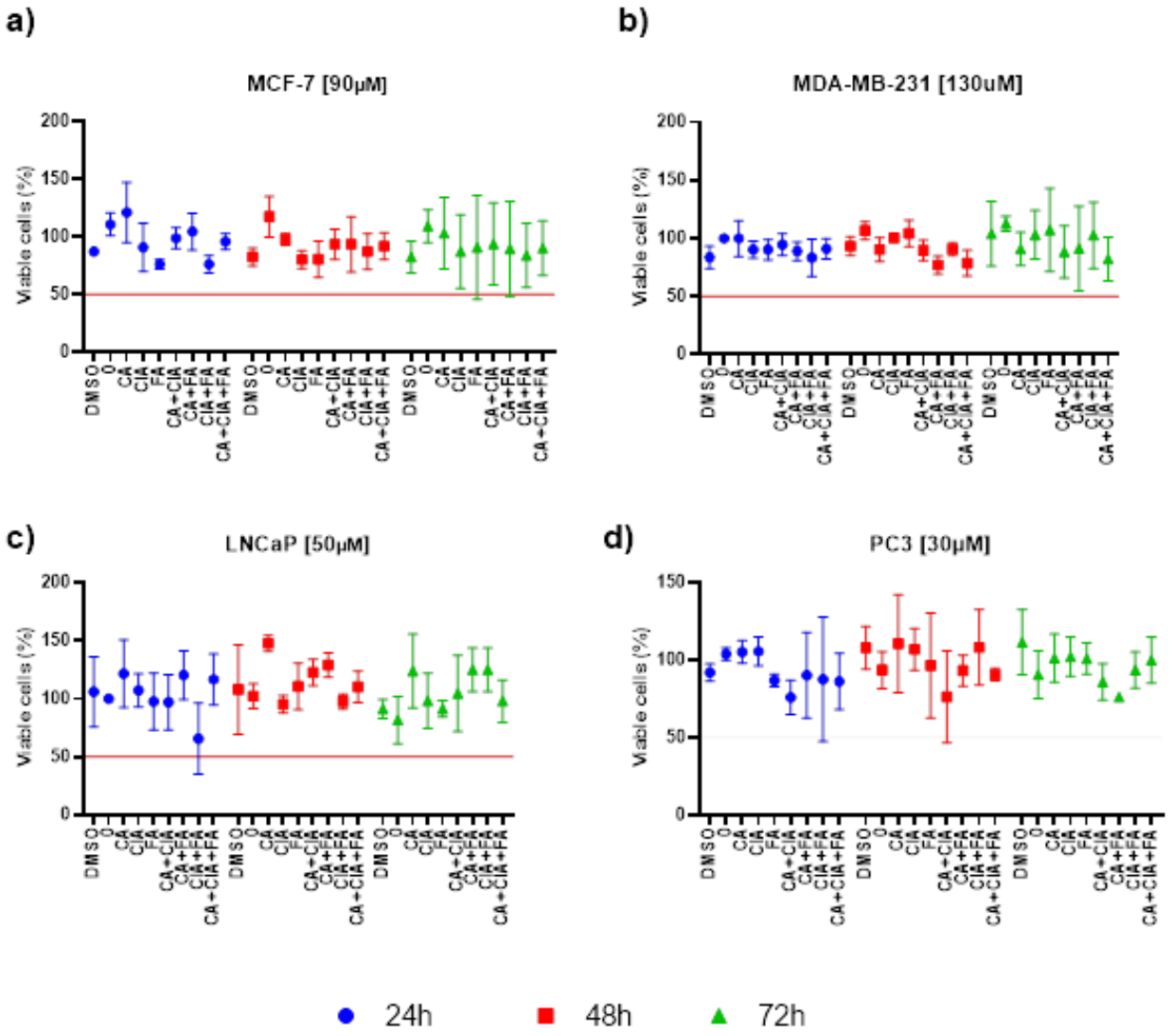

FIGURE 2. Effects of the combination of phenolic acids on the viability of malignant cells. Cells derived from the human breast (MFC-7 and MDA-MB-231) and prostate (LNCaP and PC3) were incubated for 24, 48, and 72h using half of the IC50 (IC50/2) value of CA estimated for each cell line, combined CIA and/or FA at equipotent molar ratios. Cell viability was detected by MTT assay. there are no differences in the cell number for all the cell lines assayed: a) MCF-7; b) MDA-MB-231; c) LNCaP, and d) PC3. caffeic acid (CA), cinnamic acid (CIA), and ferulic acid (FA), as assessed by the MTT assay

The combined exposure to CA and CIA has a greater effect on the migration and invasion in vitro of breast and prostate adenocarcinoma cells.

In this study, cell migration and invasion in vitro were assessed using Boyden chambers, in a Transwell-based assay. Briefly, 2x104 cells/well were seeded in 12-well culture plates and pre-incubated for 72h with CA at half of its respective IC50 (IC50/2) for each cell line, in combination with CIA and/or FA at equipotent molar ratios and kept under usual cell culture conditions.

To investigate the possible effects of phenolic compounds and their combinations on the invasive behavior of the breast and prostate adenocarcinoma cells under evaluation, we selected the treatments in which any effect in reducing the migration rates of cells was observed (Figures 3a, 3c, 4a, and 4c).

Overall, CA significantly reduced the migration of MCF-7 cells when used at 90µM ($p<0.05$), as well as in combinations with CIA ($P < 0.05$) and FA ($P < 0.001$) (Figure 3a). Also, the association of CA+CIA significantly inhibited cell invasion in vitro ($P < 0.05$) (Figure 3b). Similarly, breast adenocarcinoma cells MDA-MB-231 treated with non-cytotoxic concentrations of the phenolic acids for 72h showed a reduction of cell migration (Figure 3c) and invasion rates when exposed to CA alone (130µM), CA in association with CIA, or all the phenolic acids combined (CA+CIA+FA) (Figure 3).

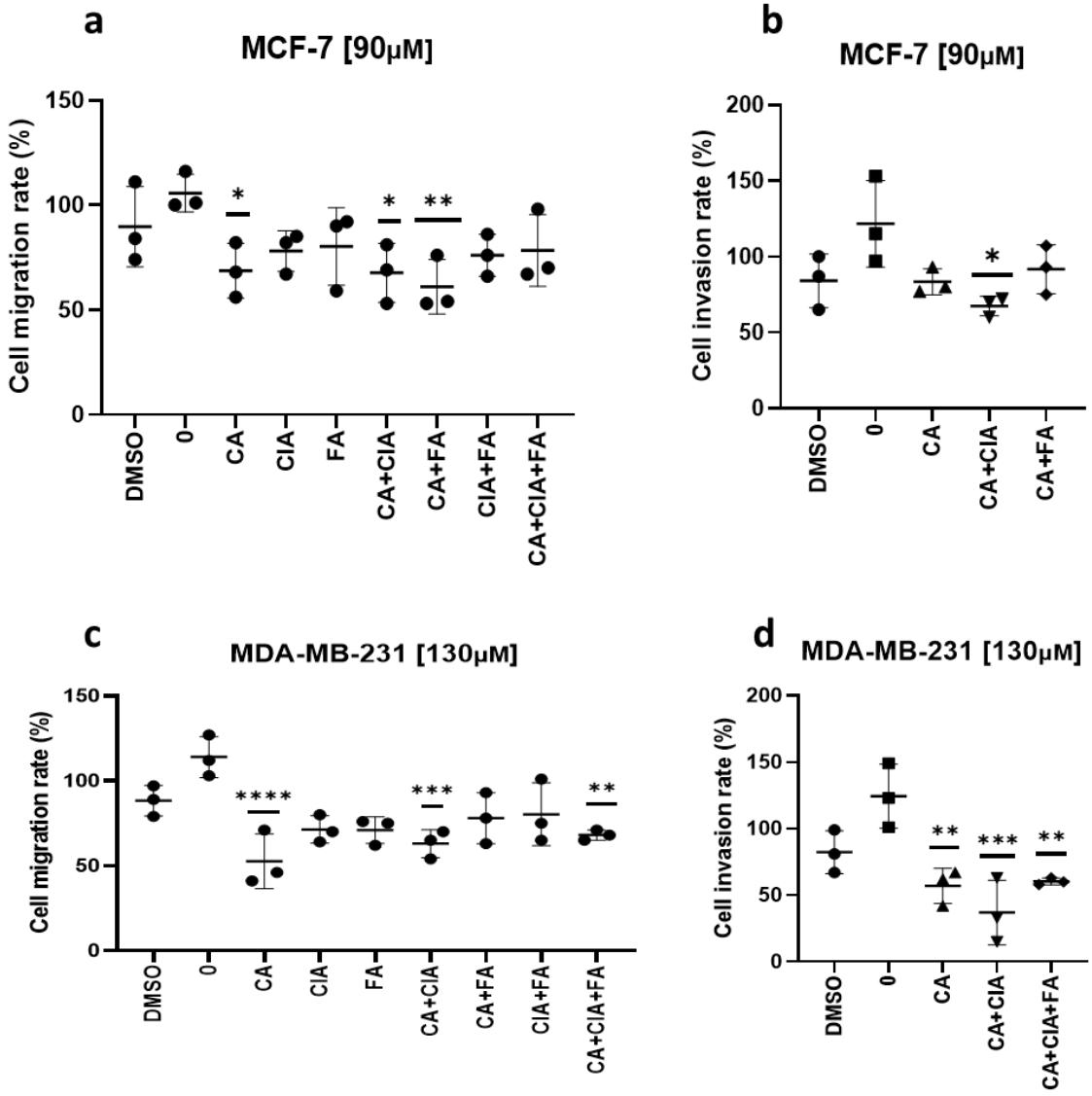

FIGURE 3. The association of phenolic acids decreased the migration and invasion of MCF-7 and MDA-MB-231 breast cancer cell lines. Cells were treated with phenolic acids caffeic acid (CA), cinnamic acid (CIA), and ferulic acids (FA) alone and their combination CA+CIA, CA+FA, CIA+FA, and CA+CIA+FA at a non-cytotoxic concentration for 72h. Transwell assay was used to determine the migration and Invasion rates. CA induced significant inhibition of the migration of MCF-7 (a) and MDA-MB-231 (c) breast cancer cells, in addition, when using the CA+CIA association, it shows synergism, potentiating the inhibitory effect and significantly reducing the invasion of both cell lines (b, d). The differences observed were analyzed with the one-way ANOVA test, followed by Dunnet's test. (* $P<0.05$. ** $P<0.01$. *** $P<0.001$. **** $P<0.0001$).

The combination of phenolic acids also inhibited the migration and invasion of prostate adenocarcinoma cells (Figure 4). Although CA alone did not significantly affect the migration of LNCaP (50µM) and PC3 (30µM) cells, the association of CA+CIA significantly inhibited cell invasion in both cell lines. Therefore, the results described above suggest that the phenolic acids CA and CIA exert an inhibitory activity on the migration and invasion of breast and prostate cancer cells, in a synergistic and time-dependent manner.

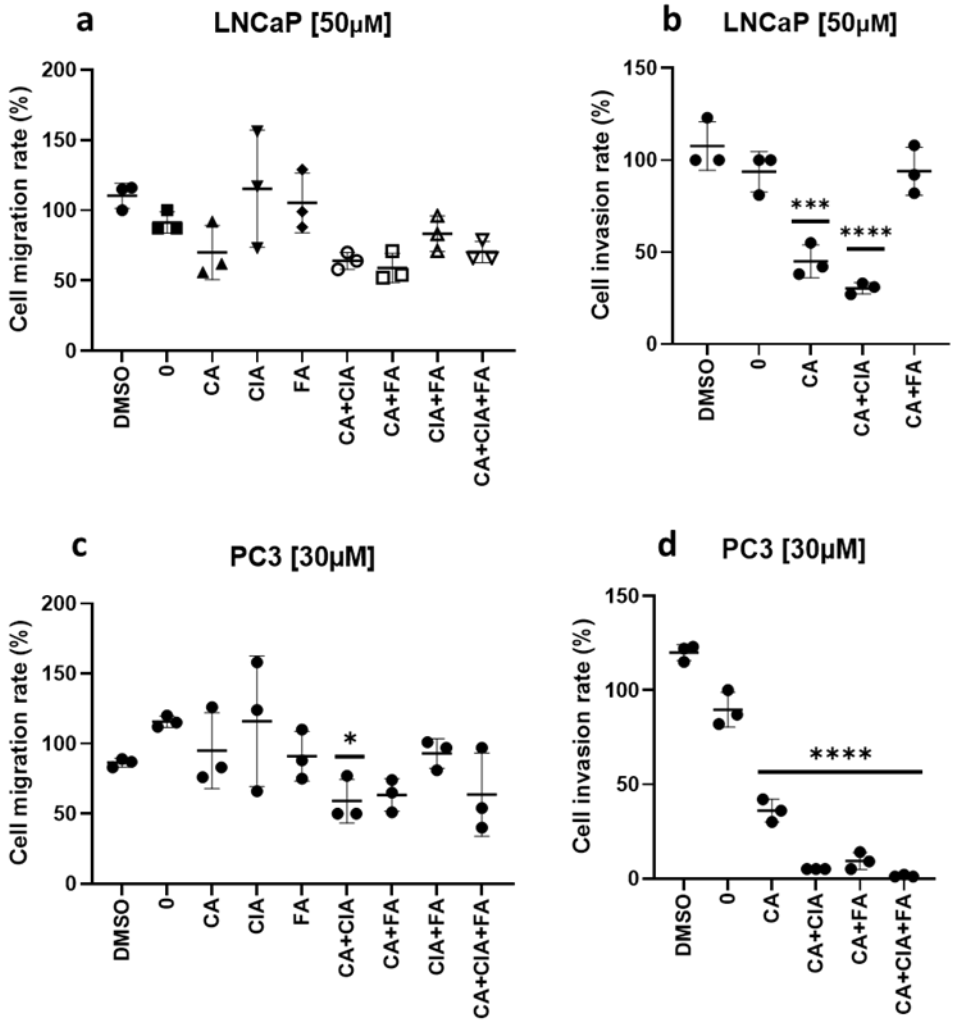

FIGURE 4. The association of phenolic acids decreased the migration and invasion of LNCaP and PC3 prostate cancer cell lines. Cells were treated with phenolic acids caffeic acid (CA), cinnamic acid (CIA), and ferulic acid (FA) alone and their combinations CA+CIA, CA+FA, CIA+FA, and CA+CIA+FA at a non-cytotoxic concentration for 72h. Transwell assay was used to determine the

migration and Invasion rates. the results suggest that CA promotes a non-significant reduction in cell migration of both neoplastic prostate cell lines (a, c), but when using the association of phenolic compounds CA+CIA, the inhibitory effect of migration becomes significant in the PC3 cell line (c) and promotes significant inhibition of cell invasion and both strains tested (b, d). The differences observed were analyzed with the one-way ANOVA test, followed by Dunnett's test. (* P<0.05. ** P<0.01. *** P<0.001. ****P<0.0001).

Discussion

Natural products are being evaluated as possible sources of new pharmaceutical agents over the years and they are among the main components of at least 75% of the most common anticancer drugs. (43,44). Phenolic compounds deserve attention for their high anticipated therapeutic potential for a variety of pathological conditions. Even though they are well known for their antioxidant properties (45–47), these compounds have a broader array of biological and pharmacological properties, including anti-inflammatory and antimicrobial effects (48–52), and even antineoplastic activities. (11,24,25,33,53–56).

In this study, we assessed the influence of the phenolic compounds CA, CIA, and FA in the properties in vitro of cell lines derived from the human breast (MCF-7 and MDA-MB-231) and prostate (LNCaP, and PC3) adenocarcinomas. Besides being popular experimental models for prevalent human cancers, these cell lines are derived from metastatic lesions, and presumably have a higher degree of intrinsic aggressiveness compared to cells derived from primary tumors. This biological feature is relevant to investigate the possible effects of compounds on cellular capabilities relevant to cancer progression, such as cell migration and invasion.

In this study, we first evaluated the viability rates for cells exposed to each compound (0-800µM) in 24, 48, and 72h – experiments intended to estimate the IC50 for each compound and cell line under investigation. Only cells treated with CA showed were adversely affected, in a dose and time-dependent way. Subsequently, we asked whether the combination of two or all the phenolic compounds at non-cytotoxic doses could modify the behavior of the treated cells regarding cell viability, migration, or invasion in vitro, in comparison with the observed results for the treatment using each of the compounds alone. For this, we choose to use half of the IC50 value of the CA calculated for each cell line and adjusted the concentrations of other phenolic compounds (CIA and FA) equipotentiality. Even though none of the combinations assayed (CA+CIA; CA+FA;

CIA+FA, and CA+CIA+FA) had an impact on the viability rates of any of the breast or prostate adenocarcinoma cell lines (even after 72h of exposure), the migration and invasion potential of cells previously incubated for 72h with the phenolic compounds were affected. Worth to note, that cells previously exposed (72h) to CA+CIA consistently showed a significant reduction in their migration and invasion capabilities compared to the cells exposed to these compounds alone or other combinations of the phenolic compounds under evaluation (Figure 4).

Despite the substantial number of reports showing data on the putative effects of phenolic compounds on human neoplastic cells, many of these published studies have weaknesses in their experimental design or unclear procedures that may compromise the confidence in their results. These caveats may culminate in unreliable data, overstated conclusions, and non-reproducible research. Even lacking or incomplete descriptions of very basic experimental methods may raise questions about the fidelity of the results reported. For instance, the preparation of stock and working solutions of phenolic compounds for use with cell lines in vitro may be challenging due to a lack of consensus in the literature regarding the best protocol to be used. After several tests (data not shown), DMSO 100% was identified as the best vehicle for the cell culture experiments with all the three phenolic compounds assayed in this study, allowing us to prepare 1M stock solutions without oversaturation or the need for further measures to achieve complete dissolution of the compounds, such as adding water, as suggested in some published studies (57).

Cellular effects in terms of cytotoxicity, cell proliferation, and the rates of migration and invasion of malignant cells (both common proxies for the metastatic potential of cancers in vivo) are experimental parameters commonly evaluated for phenolic compounds aiming to identify possible antineoplastic activities (58,59). The results obtained here show that the treatment of the breast and adenocarcinoma cell lines to CA causes a significant reduction in the number of viable cells over time, in a dose-dependent manner. Some explanations for this effect include the CA-mediated regulation of TP53 phosphorylation (60), and/or mitochondrial dysfunction of neoplastic cells (61–63).

Some reports suggest that CA does not have antiproliferative activity. For instance, Serafim and colleagues reported that the CA at 25, 50 e 75µM had no cytotoxic effect on the MCF-7 and MDA-MB-231 cell lines, even after 7 days of treatment (64). The differences observed considering these results and what we found in this study can be understood as a consequence of distinct

experimental designs, notably regarding the different concentrations of the compound and the duration of cell treatment. The in vitro effects attributed to CA, for instance, are highly dependent on the experimental conditions and cell models used. Accordingly, we found that the prostate adenocarcinoma cell lines LNCaP and PC3 are more sensitive to CA effects compared to the breast adenocarcinoma cells MCF-7 and MDA-MB-231, which showed higher IC50 values. This stresses that the number, variety, and intrinsic properties of cell lines are key to properly assess possible antineoplastic effects of phenolic compounds in pre-clinical studies, being a key element in proper experimental design. Some published studies also fail to address this issue, investigating the effects of phenolic compounds with only one cancer cell line or two from different histogenesis(53,54,54,60,64–67). Also, most of the published studies do not explicit that the cell lines used were authenticated before the experiments. This is critical to certify the validity of results and conclusions in pre-clinical studies, notably considering the cell identity crises that emerged, which put into check the quality of a vast amount of published scientific data. (68–70)

Another experimental issue frequently not addressed when evaluating the antineoplastic effects of compounds is the control for the cell proliferation bias when using MTT-based assays to assess the cytotoxic effect and to estimate IC50s. Serum starvation before the cell treatment is a popular and cost-effective procedure to minimize this bias; it essentially reduces the cell activity to basal levels, inducing cells to stay in a quiescent state at the G0/G1 phase of the cell cycle. This simple procedure reduces analytical interference and provides more reproducible experimental conditions (71,72) Nonetheless, many studies do not indicate this or any alternative procedure (treatment with mitomycin, for instance) in their experimental protocol (65,73–77). Therefore, we cannot rule out that their results could be adversely impacted and are not reliable for a proper assessment of the antineoplastic effects of the phenolic compounds evaluated.

The combination of polyphenols with similar or complementary antineoplastic effects might allow for enhanced synergistic interactions between them, thus expanding different antineoplastic mechanisms simultaneously and in a coordinated way (78–80). In this study, we associated CA, CIA, and FA using non-cytotoxic concentrations to assess the effects on the cell viability/proliferation of breast and prostate cancer cell lines treated for 24, 48, and 72h. Overall, most combinations did not demonstrate a synergistic effect in any of the cell lines tested, but we found that the association of phenolic acids can potentiate the inhibitory effect promoted by CA

observed in migration and invasion assays. Our results revealed that CA when used as a single treatment can significantly inhibit migration rates in both breast cancer cell lines (MCF-7: $p<0.05$; MDA-MB-231: $p<0.0001$) and when associated with CIA, this combination potentiates the anti-migratory effect of CA by significantly inhibiting the migration of the PC3 prostate cancer cell line ($p<0.05$) and significantly inhibiting the invasiveness of all cell lines (MCF-7: $p<0.05$; MDA-MB-231: $p<0.001$; LNCaP: $p<0.001$ and PC3: $p<0.0001$). It is important to note that the effects described in our study are a consequence of the interaction of several factors, including 1) the biological activity exerted by each phenolic compound; 2) the potentiation of the effects by the controlled association of phenolic acids (pharmacological synergism), and 3) longer exposure to the compounds.

In conclusion, the results reported here indicate that some phenolic compounds are promising sources for the development of new drugs against prevalent cancers. For instance, CA showed a significant reduction in cell viability when used as a single agent to treat either breast or prostate adenocarcinoma cells, in a dose and time-dependent manner. Furthermore, a pharmacological synergism may be obtained by combining some phenolic compounds, as demonstrated here with CA+CIA at non-cytotoxic concentrations, which significantly inhibits both cell migration and invasion of the malignant cells. Our study also stresses the need for rigorous study design and careful description of the experimental procedures, as pre-clinical studies with relevant issues that compromise the confidence in their results and the validity of their conclusions are found frequently in the literature.

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Conflict of Interest

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

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Data Availability Statement

The data supporting the findings reported in this study is available in the main manuscript and its supplementary materials. Upon publication, all material regarding this study will be publicly available on the Open Science Framework (OSF) repository, at https://osf.io/xh7b4/ (DOI: 10.17605/osf.io/xh7b4)

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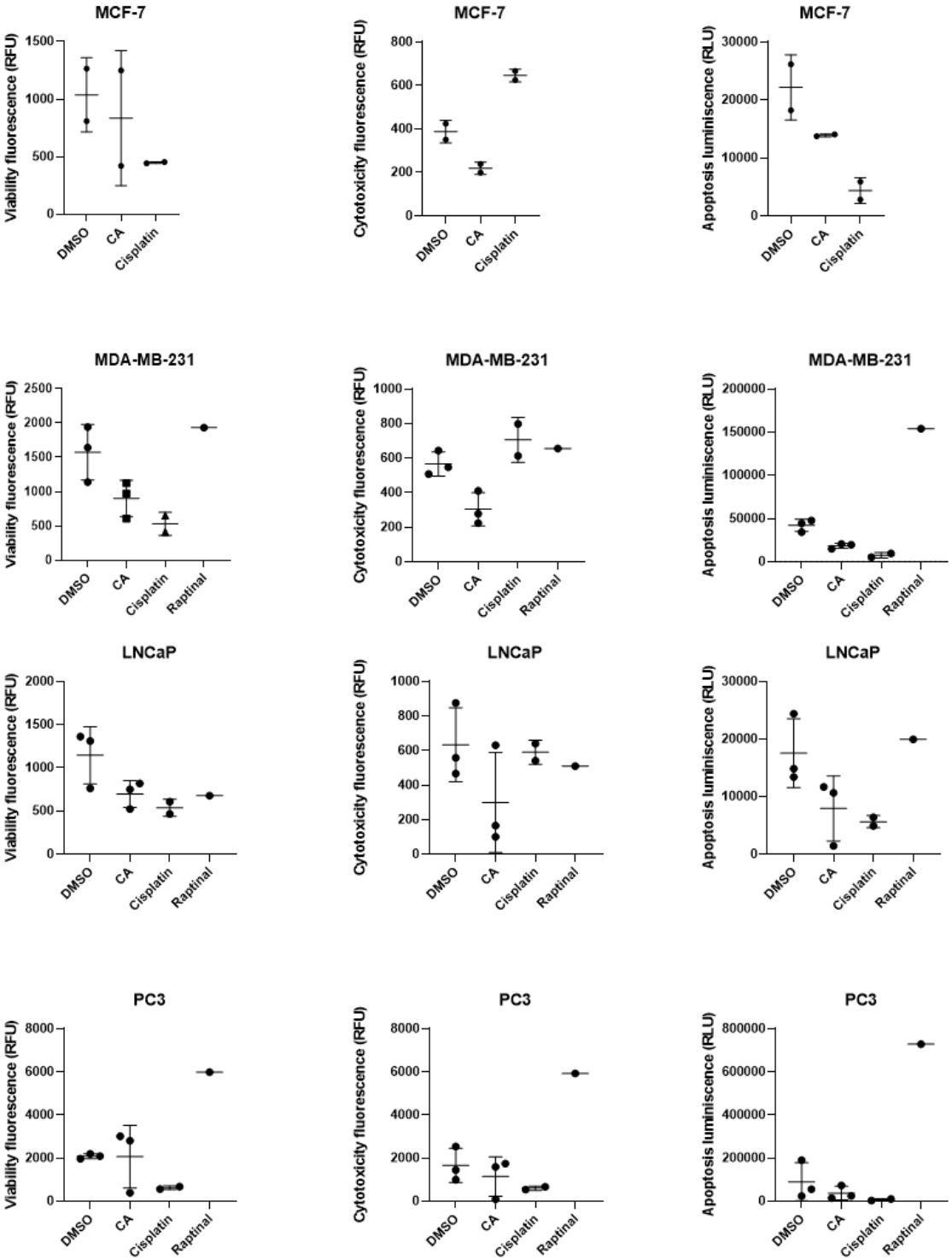
Capítulo 3 – Considerações finais

As seguintes conclusões se aplicam com base nos resultados obtidos, consideradas as condições experimentais utilizadas:

1. O ácido cafeico reduz significativamente a viabilidade das células neoplásicas humanas de mama (MCF-7, MDA-MB-231) e de próstata (PC3, LNCaP), de maneira dose-tempo dependente.
2. A combinação dos ácidos fenólicos utilizados neste estudo não revelou efeito citotóxico sinérgico nas linhagens celulares testadas.
3. A associação entre compostos fenólicos específicos pode revelar sinergismo farmacológico, conforme notado pela associação entre o ACA e o ACI, que incrementou os efeitos inibitórios verificados na capacidade de migração e invasão de células neoplásicas de mama e próstata.
4. Tendo em vista a necessidade de desenvolvimento de novos medicamentos com propriedades antineoplásicas, consideramos importante insistir na necessidade do rigor metodológico durante os ensaios pré-clínicos, com o objetivo de garantir a confiabilidade dos resultados e a reprodutibilidade dos estudos. Por tanto, este estudo oferece uma base metodológica sólida para avaliar a influência de biocompostos sobre modelos celulares de doenças oncológicas.

Capítulo 4 – Apêndices

APÊNDICE A - Resultados da análise de saúde celular utilizando o kit comercial ApoTox-GloTM- Triplex Assay **(**Promega®, Madison, USA). Devido a inconsistências dos controles, não foi possível considerar os resultados obtidos.



APÊNDICE B - Quadro com os recursos de pesquisa

"Ação de compostos fenólicos nas propriedades in vitro de células malignas humanas derivadas de adenocarcinomas de mama e de próstata"

Brayan Sayed Lopez Castañeda

Reagent or Resource	Source//Manufacturer	Identifier / Reference
Assay kits		
ApoTox-GloTM	Promega	#G6320
Cell lines		
PC3		CVCL_0035
LNCaP		CVCL_0395
MCF-7		CVCL_0031
MDA-MB-231		CVCL_0062
Chemicals, peptides, recombinant proteins		
ácido caféico	MilliporeSigma	CAS# 331-39-5
ácido trans-cinâmico	MilliporeSigma	CAS# 140-10-3
ácido ferúlico	MilliporeSigma	CAS# 537-98-4
MTT	Sigma-Aldrich	CAS# 298-93-1
ThinCert	Greiner	CLS3399
Geltrex	Gibco	A1413201
Software		
ImageJ2	OpenSource (https://imagej.net/)	RUEDEN C. T. et al. BMC Bioinformatics 18:529, 2017. DOI: 10/gdsmf9 [PMID: 29187165].

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