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CAROLINA AFONSO DE LIMA

INVESTIGAÇÃO DO MECANISMO DE AÇÃO ANTICÂNCER DO COMPOSTO "BRACHYDIN E" EM LINHAGENS TUMORAIS DE MAMA

Bragança Paulista
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Tese apresentada ao programa de Pós-Graduação *Stricto Sensu* em Ciências da Saúde da Universidade São Francisco, como requisito para obtenção do Título de Doutor em Ciências da Saúde

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“**Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer”**

Mahatma Gandhi

RESUMO

Uma forma de tratamento do câncer é através da quimioterapia, majoritariamente oriunda de produtos naturais, a exemplo dos pertencentes ao grupo dos taxanos (palictaxel e docetaxel), da podofilotoxina (etoposideo e teniposideo) e dos alcaloides vinca (vimblastina e vinorelbina), no entanto possuem amplos efeitos adversos ao paciente. Portanto, ainda há a necessidade da busca por novas moléculas para o desenvolvimento de fármacos antitumorais, que possuam menos efeitos adversos. Neste âmbito, a pesquisa envolvendo produtos naturais continua ganhando espaço no tratamento desta doença, principalmente porque seus representantes podem apresentar propriedades anticancerígenas que interferem no desenvolvimento desta doença, modulando variados mecanismos, como proliferação celular, apoptose e angiogênese. Dentre os compostos naturais, há um grupo de metabólitos especiais, os flavonoides, compostos polifenólicos que possuem um notável interesse científico e terapêutico. Uma espécie vegetal abundante em flavonoides e vem sendo bastante estudada pelo nosso grupo de pesquisa é a *Fridericia platyphylla* (Cham.) L.G.Lohmann (SINONÍMIA BOTÂNICA: *Arrabidaea brahypoda*). Os compostos isolados desta espécie tem se diferenciado por se enquadrarem no subgrupo de biflavonoides complexos pela sua organização molecular e em literatura ainda existem poucos relatos de sua atividade anticancerígena. Considerando a necessidade da busca por novas biomoléculas com capacidade antitumoral, o presente trabalho teve como objetivos investigar a potencial atividade anticâncer de biflavonoides e avaliar a atividade antitumoral *in vitro* do biflavonoide Brachydin E isolado das raízes da espécie vegetal *Fridericia platyphylla* em linhagens tumorais de mama, bem como traçar seu mecanismo de ação. Como resultados, Brachydin E apresentou valor de GI_{50} =7.5 µM para as linhagens de mama MCF7 e MDA-MB-231, demonstrando expressiva atividade antiproliferativa e foi capaz de reduzir em 32,6% a viabilidade das células tumorais arranjadas em modelo tridimensional (3D) para a linhagem MDA-MB-231. Também foi capaz de reduzir o número de colônias de células MCF7 em 70% e em 62,5% para a linhagem MDA-MB-231. Foi possível observar que o composto Brachydin E induziu ambas as linhagens em estudo à morte celular regulada (MCF7 aumentou o número de células marcadas em estágio de morte celular inicial, enquanto que na MDA-MB-231 o aumento de células em estágio de morte celular tardia foi maior), foi capaz de clivar proteína PARP que é a responsável pela reparo do DNA e reduzir a expressão de c-myc que é responsável pela proliferação e replicação de células tumorais de mama . Este composto também induziu parada do ciclo celular em fase S e interferiu em proteínas responsáveis pela sua regulação como p21 e CDK4, além de ativar as caspases-8 e -9 (juntamente a -3 e -7), que são as responsáveis por desencadear morte por apoptose pelas vias extrínseca e intrínseca, respectivamente. Desta forma, conclui-se que o composto Brachydin E possui promissora atividade anticâncer.

Palavras-chave: Neoplasias da mama. Biflavonoides. Apoptose. Ciclo celular. Cultura 3D.

ABSTRACT

One way of treating cancer is through chemotherapy, mostly from natural products, such as those belonging to the group of taxanes (palictaxel and docetaxel), podophyllotoxin (etoposide and teniposide) and vinca alkaloids (vinblastine and vinorelbine), however have broad adverse effects on the patient. Therefore, there is still a need to search for new molecules for the development of antitumor drugs, which have fewer adverse effects. In this context, research involving natural products continues to gain ground in the treatment of this disease, mainly because its representatives may have anti-cancer properties that interfere in the development of this disease, modulating various mechanisms, such as cellular cells, apoptosis and angiogenesis. Among natural compounds, there is a group of special metabolites, flavonoids, polyphenolic compounds that have notable scientific and therapeutic interest. A plant species that is abundant in flavonoids and has been extensively studied by our research group is Fridericia platyphylla (Cham.) L.G.Lohmann (BOTANICAL SYNONYMY: Arrabidaea brahypoda). The compounds isolated from this species have been differentiated because they fall into the subgroup of complex biflavonoids due to their molecular organization and in the literature there are still few reports of their anticancer activity. Considering the need to search for new biomolecules with antitumor capacity, the present work aimed to investigate the potential anticancer activity of biflavonoids and evaluate the in vitro antitumor activity of the biflavonoid Brachydin E isolated from the roots of the plant species Fridericia platyphylla in breast tumor lines as well as trace its mechanism of action. As a result, Brachydin E presented a GI50 value =7.5 µM for the MCF7 and MDA-MB-231 breast lines, demonstrating significant antiproliferative activity and was able to reduce the observation of burning tumor cells in a three-dimensional model by 32.6%. (3D) for the MDA-MB-231 strain. It was also able to reduce the number of MCF7 cell colonies by 70% and by 62.5% for the MDA-MB-231 line. It was possible to observe that the compound Brachydin E induced both cell lines under study to regulated cell death (MCF7 increased the number of cells marked in the initial cell death stage, while in MDA-MB-231 the increase in cells in the cell death stage later was higher), was able to cleave PARP protein which is responsible for DNA repair and reduce the expression of c-myc which is responsible for dermatology and replication of breast tumor cells. This compound also induced cell cycle arrest in S phase and interfered with proteins by regulating them such as p21 and CDK4, in addition to activating caspases-8 and -9 (along with -3 and -7), which are responsible by triggering death by apoptosis through the extrinsic and intrinsic pathways, respectively. Therefore, it is concluded that the compound Brachydin E has promising anti-cancer activity. ***Key words:*** *Breast neoplasms. Biflavonoids. Apoptosis. Cell Cycle. 3D Culture.*

LISTA DE ABREVIATURAS E SIGLAS

ADME	*Absorption Distribution Metabolism Excretion*
BCL-2	*Gene or Protein Bcl-2*
Capes	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CDK	Cyclin-Dependent Kinases
c-MYC	Oncogene c-MYC
COX-2	Cicloxigenase-2
DHT	Dihidrotestosterona
DNA	Ácido desoxirribonucleico
ER	Receptor de Estrôgeno
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FAS	*Fas Cell Surface Death Receptor*
FDA	*Food and Drug Administration*
GI	Sistema Gastrointestinal
GR	Receptor de Glicocorticóide
Globocan	Observatório Global do Câncer
HER-2	Fator do Crescimento Humano Epidérmico Receptor-2
IgG	Imunoglobulina G
INCA	Instituto Nacional do Câncer
MDR	*Multidrug resistance* – fenômeno de resistência a múltiplos fármacos
MRP1	*Multidrug resistance protein 1*
NCI	Instituto Nacional do Câncer
PARP	(Poli (ADP-ribose) polimerase)

PDL-1	*Programmed death-ligand 1*
P - gp	*P- glycoprotein*
PLA - 2	*Phospholipase A 2*
PR	Receptor de Progesterona
P21	Protein p21
RNA	Ácido ribonucleico
SNC	Sistema Nervoso Central
TNFR	*TNF receptor*
TPSA	*Topological polar surface area*
TRAIL-R	*Death Receptor Trail*

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1. INTRODUÇÃO

1.1 CÂNCER

Segundo o Observatório Global do Câncer (Globocan) é estimado que mais de 6,2 milhões de pessoas sejam diagnosticadas com esta doença até o ano de 2040. Enquanto que no Brasil, segundo Instituto Nacional do Câncer (INCA) são estimados 704 mil novos casos da doença para cada triênio de 2023 a 2025. Segundo a última estimativa de incidência de câncer no Brasil publicada pelo INCA (2023) (FIGURA 1), à exceção do câncer de pele não melanoma, os tipos de câncer mais incidentes no país serão de mama feminina (30,1%) e próstata (30,0%).

Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2023 por sexo, exceto pele não melanoma*

Localização Primária	Casos	%
Próstata	71.730	30,0%
Cólon e reto	21.970	9,2%
Traqueia, brônquio e pulmão	18.020	7,5%
Estômago	13.340	5,6%
Cavidade oral	10.900	4,6%
Esôfago	8.200	3,4%
Bexiga	7.870	3,3%
Laringe	6.570	2,7%
Linfoma não Hodgkin	6.420	2,7%
Fígado	6.390	2,7%

Localização Primária	Casos	%
Mama feminina	73.610	30,1%
Cólon e reto	23.660	9,7%
Colo do útero	17.010	7,0%
Traqueia, brônquio e pulmão	14.540	6,0%
Glândula tireoide	14.160	5,8%
Estômago	8.140	3,3%
Corpo do útero	7.840	3,2%
Ovário	7.310	3,0%
Pâncreas	5.690	2,3%
Linfoma não Hodgkin	5.620	2,3%

*Números arredondados para múltiplos de 10.

FIGURA 1: Distribuição proporcional dos dez tipos de câncer mais incidentes no Brasil estimados para o ano de 2023. Fonte: Instituto Nacional do Câncer, 2023

O câncer de próstata é o tipo mais comum entre os homens e estima-se que para o ano de 2040 ocorra um aumento no número de novos casos, passando de 1,4 milhões em 2020 para 2,9 milhões no ano de 2040, número bastante alarmante para a saúde pública (JAMES et al., 2024).

O diagnóstico de câncer de próstata pode ser realizado através de biópsia, através do PSA (teste de antígeno prostático específico), toque retal e ressonância magnética. Os fatores de risco para esse tipo tumoral incluem o histórico familiar, a etnia do indivíduo, idade, obesidade e também outros fatores ambientais (SEKHOACHA et al., 2022). Algumas pesquisas mostram a relação entre a incidência deste câncer e alguns hábitos alimentares, mostrando que a nutrição pode possuir um papel preventivo. Estudos epidemiológicos evidenciam que homens que praticam uma alimentação baseada em alto consumo de gordura animal e com baixo consumo de

frutas, verduras, cereais integrais, possuem uma alta propensão para o câncer de próstata (VOS et al., 2015).

Para a realização de um tratamento adequado e com maior probabilidade de sucesso, o ponto principal é identificar qual a fase do câncer que o paciente encontra-se, como por exemplo, em sua fase inicial o câncer de próstata é hormônio dependente, multiplicando-se a partir da ação de alguns hormônios, como a testosterona e dihidrostestoterona no receptor andrógeno, desencadeando assim a multiplicação celular; nesse caso, a terapia mais comum é a prostatectomia radical ou parcial, seguida de hormonioterapia, pela deprivação do andrógeno, crioterapia, radioterapia externa e braquiterapia e a quimioterapia convencional (NCI, 2024).

A hormonioterapia, também conhecida como terapia hormonal, é realizada através da privação de andrógeno, que possui como objetivo principal diminuir os níveis do hormônio masculino no paciente, impedindo dessa forma, o estímulo ao crescimento das células tumorais de próstata. Os principais andrógenos produzidos pelo homem são a testosterona e dihidrotestosterona (DHT), a sua grande maioria é produzida pelos testículos, mas também há uma pequena produção nas glândulas suprarrenais (SANTANA et al., 2015).

Outra forma de tratamento incluída mais recentemente para o câncer de próstata foi a crioterapia, que consiste em utilizar baixas temperaturas no tecido afetado, por meio do nitrogênio. O mecanismo baseia-se na destruição das células tumorais através do dano físico, causado por esses ciclos de congelamento e descongelamento, criando cristais de gelo que comprometem a integridade da membrana celular e alteram componentes celulares, como as proteínas e enzinas, levando como consequência a morte celular. Esse tratamento é comumento utilizado em tumores localizados na próstata de baixa a médio risco de disseminação, também utilizado como tratamento das recidivas do câncer de próstata, após radioterapia e cirurgia (AMARAL et al., 2022).

A radioterapia externa é também uma das técnicas utilizadas para o tratamento do câncer de próstata, onde ocorre a destruição das células cancerígenas, afetando o mínimo possível dos tecidos saudáveis, através de irradiação. Outra forma de radioterapia é denominada de braquiterapia, em que são inseridos implantes pequenos radioativos no interior da próstata, e cuja radiação vai sendo liberada de forma gradual, causando o mínimo dano aos órgãos próximos. Embora ainda apresentem efeitos adversos, esses tratamentos podem ser utilizados em conjunto

com a quimioterapia, de forma a obter sucesso no tratamento dos pacientes, realizando assim uma terapia combinada e indvidualizada (BRUM et al., 2019).

O quimioterápico convencional mais utilizado na clínica para o tratamento do câncer de próstata é o docetaxel (FIGURA 2A), um análogo semi-sintético do paclitaxel (FIGURA 2B), produzido a partir da casca do teixo europeu *Taxus baccata*. Ele se torna diferente do paclitaxel em sua estrutura química em duas posições, o que o torna mais solúvel em água; entretanto, o docetaxel apresenta alguns graves problemas de toxicidade como, mielosupressão e neuropatia periférica, e ainda possui algumas reações de hipersensibilidade aguda e o fenômeno de resistência (SANTOS, 2013).

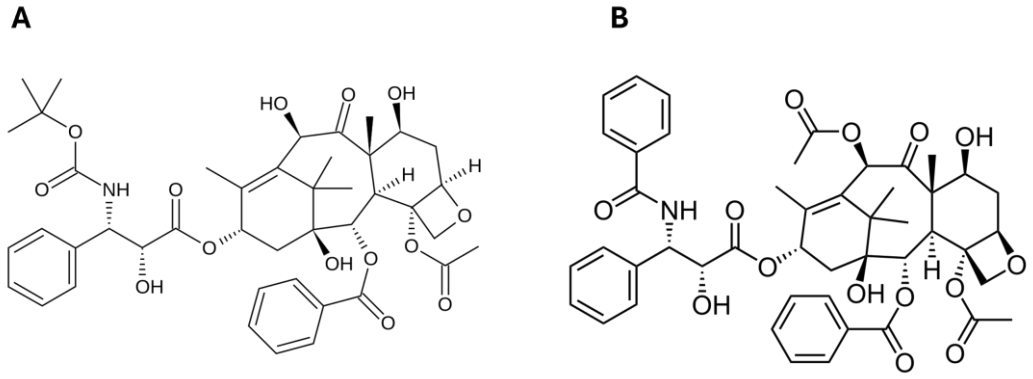

FIGURA 2: **A:** Estrutura molecular do quimioterápico Docetaxel. **B:** Estrutura molecular do quimioterápico Paclitaxel.

Outros três quimioterápicos também utilizados na clínica são a mitoxantrona, estramustina e enzalutamida. A mitoxantrona é derivada da família das antracenedionas que são quimicamente análogas as antraciclinas. Este quimioterápico se intercala ao DNA da célula inibindo sua replicação e impedindo a síntese de RNA por interagir com a enzima topoisomerase II que é responsável pela quebra das cadeias do DNA (ALVES; GUIMARÃES, 2012).

A estramustina é um composto híbrido sintético, uma combinação de estradiol e a normustina, uma mostarda nitrogenada. O estradiol possui como função principal direcionar o fármaco híbrido, apresenta atividade anti- gonadotrófica, reduzindo os níveis de testosterona do paciente, efeito similar ao que acontece após castração cirúrgica. Já a mostarda provoca uma inibição na formação de microtúbulos na metáfase e uma diminuição de microtúbulos na intérfase

(DIAS, 2017). A estramustina apresenta efeitos adversos como indução de leucemia e formação de coágulos sanguíneos, respectivamente (PETRYLAK et al., 2004).

Já a enzalutamida é classificada como antagonista do receptor de andrógeno, inibindo sua translocação para o núcleo e consequente ligação com a molécula de DNA assim inibe a síntese de fatores essenciais para a multiplicação celular (MADEIRA, 2017). Esse quimioterápico prolonga a sobrevida global de pacientes com câncer de próstata metastático, porém apresenta efeitos adversos como fadiga e hipertensão (BEER et al., 2014; HUSSAIN et al., 2018).

O câncer de mama pode ser dividido em subgrupos biológicos e clinicamente significativos, de acordo com o grau histológico. Grau histológico é uma avaliação do tumor quanto à sua atividade proliferativa, através da porcentagem de Ki67, que é um marcador imuno-histoquímico que está localizado no núcleo. Sua imunorreatividade nuclear para Ki67 é capaz de indicar em que fase as células se encontram no ciclo celular e fornecer uma estimativa da fração de crescimento em amostras tumorais, refletindo sua agressividade, para assim auxiliar na determinação da terapia (WEIGELT; GEYER; REIS-FILHO, 2010; KREIPE; HARBECK; CHRISTGEN, 2022).

Existem ainda outros fatores que são apontados como importantes para a identificação do subgrupo tumoral e posterior tratamento, como a expressão dos receptores de estrógeno (RE) e receptores de progesterona (RP) e a superexpressão ou a amplificação do receptor-2 do fator do crescimento humano epidérmico (HER-2+), como demonstrado na TABELA 1 (SALLES et al., 2009; HAMMOND; HAYES; DOWSETT, 2009).

Segundo o INCA (2024) cirurgia, quimioterapia e radioterapia são tratamentos convencionais utilizados para o tratamento do câncer no geral, sendo também escolhas para o tratamento do câncer de mama e, além destes, hormonioterapia e imunoterapia também podem ser escolhas terapêuticas, a depender do tipo do câncer de mama e em qual estágio se encontra.

O câncer de mama do subgrupo Luminal A é o mais frequente, quase metade do total de casos de câncer de mama corresponde a esse fenótipo, é frequentemente positivo para o RE, está associado a maior sobrevida, melhor prognóstico e menores taxas de recorrência. Entretanto, a heterogeneidade clínica e molecular está presente nos tumores deste subgrupo, em que as diferenças nas alterações genômicas são associadas à resistência à terapia endócrina ou hormonioterapia (GÁRCIA-CORTÉS; HERNÁNDEZ-LEMUS; ESPINAL-ENRÍQUEZ, 2021).

Em comparação com o subgrupo Luminal A, o Luminal B apresenta maior taxa de proliferação, menor expressão do RP e está associado com uma maior incidência de mestástases à distância, devido ao risco aumentado da recidiva do câncer. O tratamento sistêmico para pacientes com câncer de mama pertencentes ao subgrupo Luminal B inclui quimioterapia e hormonioterapia (DIECI et al., 2022).

O subgrupo HER2+ é um subtipo da família de receptores de membrana celular de tirosina quinase (juntamente com HER1, HER3 e HER4). O HER2 é um proto-oncogene, capaz de codificar o receptor de crescimento epidérmico através da atividade da tirosina quinase. No câncer de mama a sua amplificação está ligada diretamente com a superexpressão da proteína HER2 e com um mau prognóstico, alta taxa de recorrência e mortalidade. Umas das terapias utilizadas para o tratamento desse subgrupo é a quimioterápica associada à imunoterapia ou ainda à terapia-alvo (BRENDIN; WALSHE; DENDULURI, 2020; AHN et al., 2019).

O último subgrupo pertencente ao câncer de mama é o triplo negativo, que recebe este nome por não possuir expressão de RE, RP e nem de HER2, sendo o subgrupo com maior taxa de recidiva e mortalidade, para o qual hormonioterapias e terapias alvo a Her2 não são úteis, embora algumas opções de tratamento podem ser tomadas, como a quimioterapia isolada ou em conjunto com imunoterapia (HOWARD; OLOPADE, 2021).

TABELA 1 . Classificação dos subgrupos tumorais para o câncer de mama

Subgrupos	***Prevalência***	***Ki67***	***Receptor Hormonal***	***HER2***
Luminal A	50 a 60%	Menor que 14%	+	-
Luminal B	10 a 20%	Maior ou igual a 14%	+	-
HER2+	15 a 20%	-	Independente de receptor	Superexpressão
Triplo Negativo	20%	-	-	-

Fonte: adaptado de LAZARETTI; ASSIS; MACHADO (2014, pág. 144-145).

Na quimioterapia, destaca-se a doxorrubicina, que é uma droga antraciclina, extraída de *Streptomyces peucetius var. Césio* e que age na célula intercalando o DNA e interrompendo a sua replicação mediada pela topoisomerase II ou gerando radicais livres e danos à membrana celular, ao DNA e proteínas, mas que pode acarretar alguns efeitos adversos como dores, náuses e vômitos,

diarreia, além de apresentar cardiotoxicidade. Derivados do taxol (paclitaxel e docetaxel) também são utilizados para pacientes triplo-negativo, Luminal A e B e Her2-. Estes quimioterápicos agem pelo mecanismo de estabilizarem microtúbulos e inibirem a despolimerização dos mesmos, causando a morte celular, porém apresentam como efeitos adversos, mielodepressão, alopecia, neuropatia periférica, distúrbios gastrointestinais e reações de hipersensibilidade. Outros efeitos adversos pelo uso da quimioterápia incluem sangramento e corrimento vaginal; erupções cutâneas; fadiga; anemia; eventos cerebrovasculares isquêmicos e eventos tromboembólicos (SHAPIRO; RECHT, 2001; CAGEL et al., 2016; SIDNEY et al., 2019).

Na hormonioterapia, são utilizados moduladores seletivos para o receptor de estrógeno, que bloqueiam a proliferação celular e controlam a recidiva da doença, como o tamoxifeno e o fulvestranto, porém estes medicamentos causam alguns efeitos adversos no útero como formação de pólipos, hiperplasia e carcinoma endometrial. Podem ser utilizados também inibidores de aromatase, que diminuem os níveis séricos de estrogênio a partir da inibição da enzima aromatase, reponsável pela conversão periférica de androgênio em estrogênio, privando assim as células malignas desse fator de crescimento, como o anastrozole, mas que possui alguns efeitos adversos como mutação genética, evento adverso cutâneo localizado, síndrome da vasoconstrição cerebral reversível, vasculite leucocitoclástica, alucinações, distúrbios gastrointestinais, náusea, vômitos, diarreia, cefaléia, sonolência, confusão mental e paralisia motora (LEAL, 2010; PAULA et al., 2021).

A imunoterapia consiste em uma forma de tratamento medicamentoso que estimula as células do sistema imunológico a reconhecer células tumorais. Os principais imunoterápicos utilizados na clínica são anticorpos monoclonais, alguns conhecidos como terapia-alvo, pela especificidade de tratamento a determinado subtipo tumoral, a exemplo do trastuzumabe, que é um anticorpo monoclonal recombinante humanizado, do tipo IgG e que possui alta afinidade de ligação com o receptor HER2, sendo indicado para o tratamento de pacientes com câncer de mama metastático que apresentam tumores com superexpressão do HER2 (MORROW; ZAMBRANA; ESTEVA, 2009) e do atezolizumabe, anticorpo monoclonal que se liga ao PDL-1, uma molécula reguladora que controla a atividade do sistema imunológico, sendo bastante estudado para combater o subtipo tumoral triplo negativo. Esta terapia não causa náuseas e perda de cabelo, como as quimioterapias convencionais, mas podem gerar inflamações severas no intestino, causando diarreia.

1.2 MECANISMOS DE REGULAÇÃO DO CÂNCER

A maioria dos quimioterápicos age impedindo a progressão do ciclo celular (SUN et al., 2021). A divisão celular é regulada por vários mecanismos de controle pertencentes ao ciclo celular, para garantir a produção de duas células idênticas geneticamente. O ciclo celular possui pontos de vetificação, que funcionam como mecanismos de checagem do DNA, que conseguem impedir a propagação e acúmulo de erros genéticos durante a divisão celular (Figura 3A). Em células cancerígenas os pontos de verificação de danos ao DNA são geralmente comprometidos, fazendo com que a divisão celular continue apesar do acúmulo de erros genéticos (MATTHEWS; BERTOLI; BRUIN, 2022).

O ciclo celular é controlado por várias ciclinas e ciclinas dependentes quinases (CDKs). O câncer de mama, como muitos outros cânceres, envolve o aumento da proliferação celular, que resulta da interrupção na regulação do ciclo celular pela expressão desregulada e ativação de CDKs. A desregulação da CDK durante o câncer de mama leva à proliferação descontrolada de células cancerígenas, mantendo assim a progressão do câncer e outros fatores (SOFI et al., 2022).

Existem diversos genes e proteínas que estão diretamente ligados com a progressão ou parada da proliferação das células tumorais. Uma dessas proteína, a p21, age como inibidor da CDK, e é capaz de desempenhar um papel importante no controle da progressão do ciclo celular, promovendo uma interrupção da síntese de DNA e levando a uma parada do ciclo celular na fase S, o que pode culminar em morte celular (SHAMLOO; USLUER., 2019).

A apoptose é um dos principais mecanismos de morte celular programada, crítico para o desenvolvimento, sobrevivência e funcionamento de organismos multicelulares. Como a apoptose é fundamental para que se mantenha a homeostase nas células normais, defeitos em seu funcionamento favorecem a proliferação das células cancerígenas, causando até mesmo a resistência à terapia padrão (SINGH; LIM, 2022). Consequentemente, a desregulação da apoptose está comumente associada a uma ampla gama de doenças, desde câncer até distúrbios degenerativos. Existem duas vias bem definidas para a apoptose - a mitocondrial, também conhecida como via intrínseca, induzida por estresse ou regulada por BCL-2, e a via induzida pelo receptor de morte, também conhecida como via extrínseca (Figura 3 B).

Várias proteínas participam do processo de morte celular programada, incluindo caspases

(proteases específicas do cisteinil aspartato), seus adaptadores/ativadores, os membros da família de proteínas BCL-2 que constituem os reguladores críticos de apoptose, PARP (poli (ADP-ribose) polimerase) que é responsável pelo reparo do DNA, porém quando clivado essa sua função é inativada e ocorre um aumento na taxa de apoptose (KALONI et al., 2022).

A proteína c-myc, está ligada diretamente no desenvolvimento e progressão do câncer de mama, portanto a inibição ou redução de c-myc pode levar à diminuição da capacidade das células de formar colônias, indicando uma redução na capacidade de crescimento e replicação celular (WANG et al., 2004; MAWSON et al., 2005).

Envolvida na apoptose existem as caspases, sendo a caspase-8 uma protease específica da cisteína-aspartato que desencadeia a via apoptótica extrínseca em resposta à ativação de receptores de morte de superfície celular como FAS, TRAIL-R e TNF-R. Já a via apoptótica intrínseca é regulada após uma série de estímulos à proteína Caspase-9, como estresse intracelular e danos ao DNA. Ambas as caspases, -8 e -9, culminam na ativação das caspases efetoras-3 e -7. Estratégias terapêuticas continuam sendo desenvolvidas para atingir esses reguladores da apoptose para o tratamento do câncer (MANDAL et al., 2020; MOONEY et al., 2002).

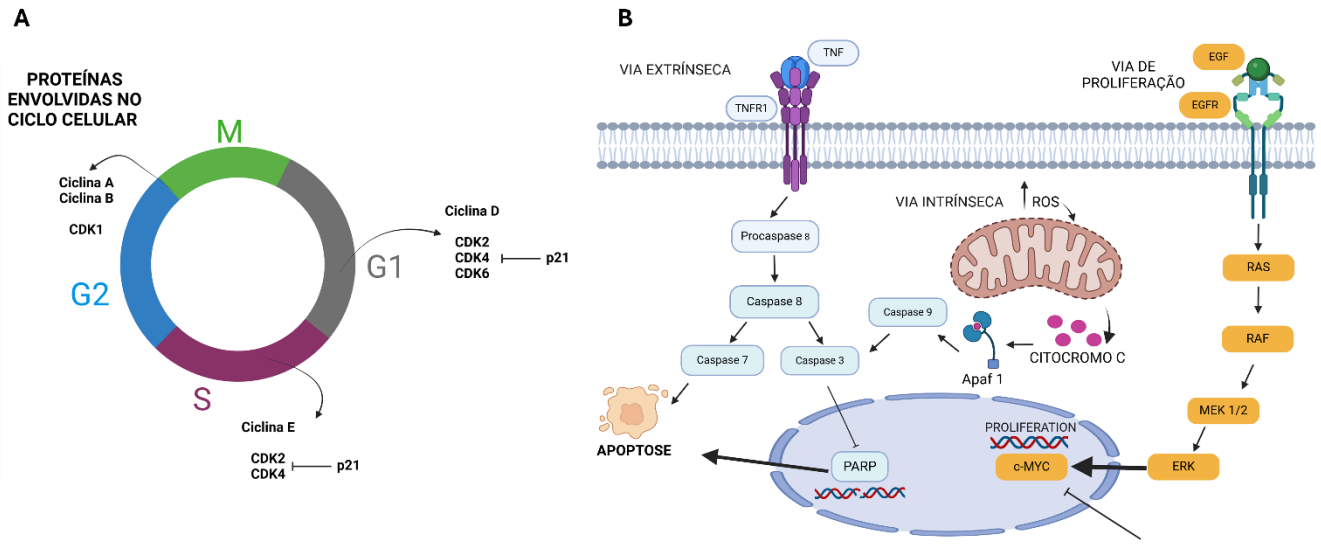

FIGURA 3. **A**: proteínas envolvidas na regulação do ciclo celular. **B**: Vias extrínseca e intrínseca de morte celular por apoptose e via de proliferação celular. Fonte: Do Autor. Criado através de biorender.com.

1.3 COMPOSTOS NATURAIS E CÂNCER

Estima-se que 64,9% dos quimioterápicos utilizados na clínica para o tratamento de câncer sejam de origem vegetal ou sintetizados a partir da observação da estrutura molecular de produtos naturais (NEWMAN; CRAGG, 2020).

Vários exemplos de antineoplásicos obtidos de plantas e amplamente utilizados terapeuticamente incluem vimblastina (Velban ®), vincristina (Oncovin ®) e seus análogos vindesina (Eldisine ®) e vinorelbina (Navelbine ®); paclitaxel (Taxol ®) e os análogos docetaxel (Taxotere ®) e cabazitaxel (Jevtana ®); podofilotoxina e análogos etoposídeo (Etopophos ®), teniposídeo (Vumon ®); e camptotecina e análogos topotecano (Hycamtin ®), irinotecano (Camptosar ®) e belotecano (Camptobell ®). As bactérias do solo também são uma fonte notável de drogas anticâncer, que podem ser claramente ilustradas por compostos como as antraciclinas doxorrubicina (Doxil®; Adriamycin®), daunorrubicina (Cerubidine®) e epirrubicina (Ellence®); a bleomicina glicopéptido (Blenoxane®); e o peptídeo não ribossômico dactinomicina (Cosmegen®) (JIMENEZ et al., 2018).

Dentre os compostos naturais tem-se os flavonoides, um grupo amplo de metabólitos especiais formado por compostos polifenólicos (FANG et al., 2010) que se destacam pelas várias atividades benéficas que apresentam, incluindo atividades antibacteriana, antiviral, anti-inflamatória, antioxidante, antialérgica, hepatoprotetora, vasodilatadora e antitrombótica (TIEMY et al., 2013). Segundo Ponte e colaboradores (2021), estudos clínicos estão sendo desenvolvidos incluindo flavonoides na terapia oncológica, como terapia auxiliar ao tratamento ou como forma de prevenção de vários tipos de câncer, com resultados positivos (PONTE et al., 2021).

Existe uma subclasse de flavonóides, conhecida como biflavonóides, tem despertado interesse científico. São considerados biflavonoides aqueles formados por dois monoflavonóides que ocorrem de maneira natural. São classificados em três grupos: C-C (FIGURA 4A), C-fragmento linear-C (FIGURA 4B) ou biflavonóides complexos (FIGURA 4C), dependendo se o ligante entre os dois resíduos contém um átomo. A maioria dos membros desta subclasse consiste em dímeros de flavona-flavona e flavanona- flavanona, com os dímeros de chalconas e isoflavonas ocorrendo mais raramente (MERCADER et al., 2013; HE et al., 2021).

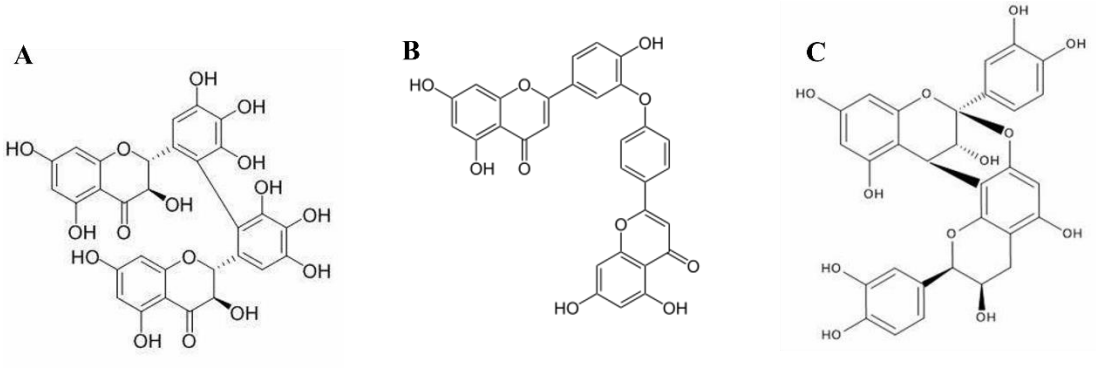

FIGURA 4: **A:** Estrutura molecular representativa de biflavonoide com ligação C-C. B: Estrutura molecular representativa de biflavonoide com ligação C-fragmento linear – C. C: Estrutura molecular representativa de biflavonoide com estrutura complexa. Fonte: He et al., 2021 (pág 4, 18, 20).

Na literatura, os flavonoides diméricos são descritos por suas propriedades farmacológicas, incluindo atividades antibiótica, analgésica, anti-inflamatória, antioxidante e inibidora de fosfolipase A2 (PLA2) e de ciclo-oxigenase-2 (COX-2) (KIM et al., 2008; GONTIJO; DOS SANTOS; VIEGAS JR., 2016). Estudos indicam que estes compostos também estão envolvidos no processo de reversão da resistência a múltiplas drogas (MDR), atuando como mediadores da superexpressão de proteínas de resistência, como MRP1 e glicoproteína-P (P-gp) em células tumorais multiresistentes. A MDR é um dos principais obstáculos na terapia antibeoplásica, sendo muitas vezes optado por combinação de drogas para contornar essa resistência tumoral (CHAN et al., 2012; DURY et al., 2017; LIMA et al., 2020).

Apesar da introdução de novos fármacos no arsenal terapêutico do câncer, vários tumores ainda não dispõem de tratamento adequado. As fontes naturais ainda estão disponíveis em abundância e oferecem as melhores possibilidades de encontrar substâncias de interesse terapêutico.

1.4 FAMÍLIA BIGNONIACEAE, GÊNERO *FRIDERICIA* E ESPÉCIE *Fridericia platyphylla* (Cham.) L.G.Lohmann (SINONÍMIA BOTÂNICA: *Arrabidaea brahypoda*)

A família Bignoniaceae é composta por 120 gêneros e 650 espécies, sendo o Brasil considerado o centro da diversidade de Bignoniáceas, pois, no país, ocorrem 60 gêneros e cerca de 338 espécies, que são distribuídas desde os cerrados até as florestas úmidas (ORTOLANI et al.,

2008; CHEN et al., 2022).Várias espécies são conhecidas pelas propriedades medicinais, sendo amplamente utilizadas na medicina popular (LOHMANN, 2018).

O gênero *Fridericia* (família Bignoniaceae), é encontrado principalmente em regiões tropicais, suas atividades são atribuídas aos componentes químicos dentre estes as lignanas, flavonoides, triterpenos, xantonas entre outros. Incluem 60 espécies do México à Argentina e sul do Brasil. Plantas que pertencem a esse gênero *Arrabidaea* foram reclassificadas e as espécies realocadas nos gêneros: *Cuspidaria, Fridericia, Tanaecium* e *Xylophragma* (ROZATTO, 2012; DA ROCHA et al., 2017; HENRIQUE et al., 2024).

Espécies do gênero *Fridericia* têm sido utilizadas na medicina tradicional para fins adstringentes, antioxidantes, antiinflamatórios, antimicrobianos, antitumorais e cicatrizantes (DA ROCHA et al., 2015), bem como para tratar doenças da pele, infecções por fungos, hemorragia pós-parto, malária, diabetes e pneumonia. Muitas literaturas relatam que as espécies contêm classes diferentes de constituintes ativos como feniletanóides, fenólicos, lignanos, flavonóides, cumarinas e xantonas. Além disso, estudos demonstraram uma ampla gama de atividades biológicas como citotóxica (em linhagens tumorais de colorretal, rim e melanoma), antidiabética, indução do sono, gastroproteção, antiobesidade e inseticida (MAHMOUD et al., 2019).

Dentre suas espécies, destacam-se 2 comumente utilizadas, *Fridericia chica* e *brachypoda.* A espécie *Fridericia chica*, popularmente conhecida como crajiru, é uma videira americana tropical nativa empregada na medicina popular para curar feridas, inflamações e cólicas gastrointestinais (SERVAT-MEDINA et al., 2015). Várias propriedades são atribuídas a esta espécie, como capacidade antioxidante e anti-ulcerogênica, melhora na formação de colágeno durante a cicatrização, efeitos anti- inflamatórios, atividade contra câncer bucal e uterino e leucemia (PAULA et al., 2013). Também possuem atividades antifúngica e tripanocida (COSTA et al., 2008). As folhas de *Fridericia chica* são empregadas no tratamento de cólica intestinal, diarréia com sangramento, anemia, inflamação uterina, psoríase, úlceras e piodermites. Estudos mostraram que a *A. chica* possui algumas atividades, como antihipentensiva, vaso-relaxante, antihepatotóxica, antioxidante, antitumoral (com seletividade para as linhagens tumorais MCF7 - mama e NCI-H460- pulmão, antifúngica e anti-inflamatória (BEHRENS; TELLIS; CHAGAS, 2012).

Fridericia platyphylla (Cham.) L.G.Lohmann é uma planta nativa do Brasil, amplamente distribuída em diferentes biomas, conhecida como “cervejinha do campo”, “cipó-una”, e popularmente usada para tratar pedras nos rins e artrite. Da Rocha et al. (2014) a partir de frações do extrato de raízes de *F. platyphylla*, identificaram e caracterizaram novos compostos, classificados como flavonoides diméricos incomuns, denominados Brachydin A, Brachydin B, Brachydin C, Brachydin D, Brachydin E, Brachydin F, Brachydin G, Brachydin H, Brachydin I e Brachydin J (SERPELONI et al., 2020). Dados da literatura apontam para suas atividades anti-inflamatória, anti-*trypanosoma cruzi* e antinociceptiva (DA ROCHA et al., 2011, 2014) e mais recentemente antiproliferativa e antimetastática (NUNES et al., 2017; RIBEIRO et al., 2022).

Dois destes biflavonoides braquidinas, denominados Brachydin E e Brachydin F, foram avaliados anteriormente pelo nosso grupo (LIMA et al., 2022) quanto a sua atividade antiproliferativa em um painel de linhagem de diversos tecidos tumorais (glioblastoma pulmão, próstata e colorretal), em que essas moléculas demonstraram ser mais seletivas para as linhagens tumorais de adenocarcinoma de próstata (PC-3). Brachydin E e F reduziram significativamente a viabilidade celular, a proliferação e o potencial clonogênico de células PC-3, induzindo-as ao processo de morte celular regulada.

Estudos *in silico* e de docagem molecular indicaram receptores nucleares como alvos para Brachydins E e F e apontaram sua interação no bolsão do ligante do receptor de glicocorticóide (GR). O direcionamento da via GR tem sido descrito como uma estratégia terapêutica importante para o câncer de próstata, pois sugere-se que ele participe do processo de resistência deste tipo tumoral. Este resultado sugere que as braquidinas poderiam interagir com o GR, o que, por sua vez, leva à supressão do tumor, culminando na ativação da apoptose.

Embora Brachydin E e F se diferenciem entre si apenas no anel aromático C (pela presença de um grupo metoxila em Brachydin F, enquanto que Brachydin E não ocorre substituição, mantendo com Hidrogênio no anel aromático C) – Figura 5 A, resultados obtidos *in vitro* apontaram que Brachydin E possui atividade antiproliferativa mais potente (LIMA et al., 2022).

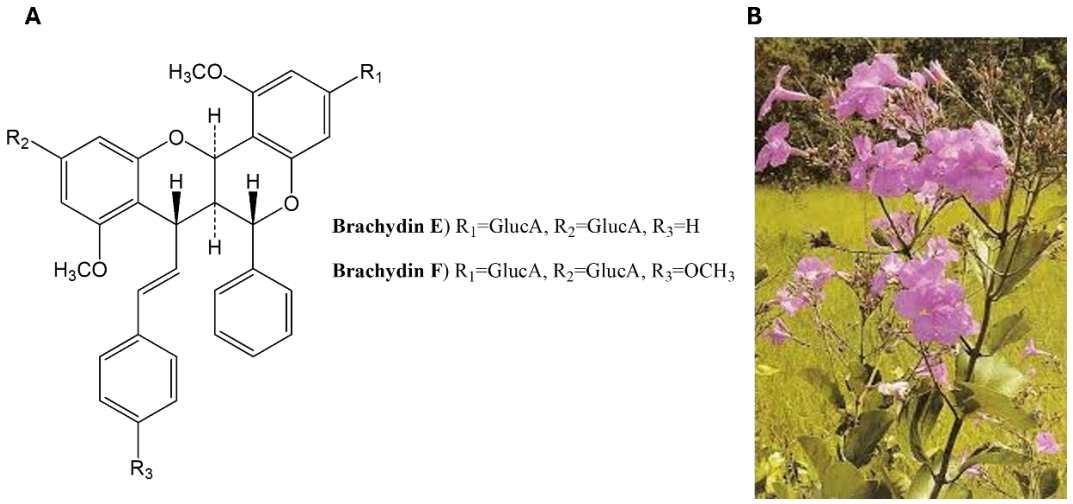

FIGURA 5 . A: Estrutura molecular dos biflavonoides Brachydin E e F. **B:** *Arrabidaea brachypoda (DC.) Bureau* (sinonímia botânica: *Friderichia platyphylla*). Fonte: LIMA et al (2022, pág. 4) DA ROCHA et al. (2014, pág. 1345).

Para verificar suas semelhanças e diferenças, foi realizado um estudo *in silico* de absorção, distribuição, metabolismo e excreção (ADME), que é uma ferramenta importante para o design racional de medicamentos. Embora o estudo ADME tenha previsto baixa absorção GI para Brachydins E e F (sugerindo baixa biodisponibilidade oral), nenhuma das moléculas tem potencial para permear a barreira hematoencefálica, o que pode indicar que não causam efeitos colaterais no sistema nervoso central (SNC). Além disso, o estudo ADME também sugeriu que Brachydin E e F não são capazes de inibir nenhuma enzima do metabolismo, sugerindo um menor risco de interação medicamentos.

Brachydin E e F apresentam características físico-químicas semelhantes, com exceção de dois parâmetros: Brachydin E possui maior LogP e menor TPSA do que Brachydin F. Esses achados indicam uma melhor permeabilidade através da membrana plasmática de E em comparação com F, que pode explicar suas diferentes respostas *in vitro*.

Tendo em vista a elevada incidência do câncer de próstata e mama e a busca incessante pela descoberta de novas moléculas que possuam atividade antitumoral mais eficaz e com efeitos adversos menores ao paciente; e considerando que os biflavonoides braquidinas tem se mostrado moléculas promissoras nesse âmbito (LIMA et al., 2022), surgiu-se a necessidade de dar continuidade na investigação da atividade anticâncer do composto Brachydin E, desta vez, em linhagens tumorais de mama.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a atividade antitumoral *in vitro* de Brachydin E isolado das raízes da espécie vegetal *Fridericia platyphylla* (Cham.) L.G.Lohmann em linhagens tumorais de mama.

2.2 OBJETIVOS ESPECÍFICOS

- Revisar e descrever os biflavonoides relatados em literatura que tem potencial ação anticâncer;

- Analisar o potencial antiproliferativo do composto Brachydin E em modelos bidimensional (2D) e tridimensional (3D) de cultivo de células tumorais de mama;

- Investigar seu mecanismo de ação no processo de morte celular e regulação do ciclo celular.

3. CAPÍTULO 1 – Biflavonoids: preliminary reports regarding their contributions to prostate and breast anticancer therapy **(artigo publicado)**

Tendo em vista o recente interesse que o grupo dos biflavonoides tem despertado na comunidade científica acerca de suas propriedades farmacológicas e considerando a lacuna científica que ainda existe na divulgação da sua atividade anticâncer, o presente estudo foi conduzido com o objetivo de destacar os biflavonoides de ocorrência natural que já foram avaliados *in vitro*, *in vivo* e *in silico* quanto à sua potencial ação citotóxica sobre os dois tipos de câncer mais incidentes na população, próstata e mama. Foram utilizadas para a busca dos artigos as plataformas PubMed e Scielo, com as palavras-chave: *"dimers of flavonoids", "flavonoid dimers", "biflavonoids", "dimeric flavonoids"*, isolados ou em conjunto com *"prostate cancer"* ou *"breast cancer"*.

Foram encontrados 38 artigos que relataram a atividade citotóxica e antiproliferativa de 33 biflavonoides em linhagens tumorais de próstata e mama. Os dados foram compilados em tabelas, contendo o subtipo de biflavonoide, a depender da ligação estabelecida entre os flavonoides (C-C, C-linear-C ou complex) e os tipos de monômeros que foram unidos, sendo classificados como: AA (flavana–flavana), BB (flavona–flavona), EE (isoflavona–isoflavona), EG (isoflavona–chalcona), e GG (chalcona–chalcona).

Os testes realizados *in silico*, *in vitro* e *in vivo* demonstraram o potencial de atividade citotóxica dos biflavonóides contra células tumorais de próstata e mama. Os biflavonóides foram capazes de interferir na migração e replicação de células cancerígenas e seu mecanismo de ação está relacionado às vias de morte celular, principalmente apoptose, necrose e ferroptose. Estes compostos diminuíram o potencial da membrana mitocondrial e aumentaram significativamente os níveis intracelulares de espécies reativas de oxigênio (ROS). Além disso, eles regularam significativamente a expressão de p21, Bax e caspase-3 clivada, enquanto regulam negativamente os níveis de Bcl-2 e caspase-3, indicando que seu mecanismo de ação de morte celular é através da via Bcl-2/Bax/caspase-3 clivada e parada do ciclo celular.

Os biflavonóides aqui relacionados mostraram atividade anticancerígena promissora e são considerados potenciais candidatos a medicamentos para tratamento de câncer de próstata e mama.

O artigo foi aceito para publicação na revista *Pharmaceuticals* (qualis Capes – A1).

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Review

Biflavonoids: Preliminary Reports on Their Role in Prostateand Breast Cancer Therapy

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Abstract: Dimeric flavonoids, also called biflavonoids, are bioactive compounds that exhibit various activities described in the literature, including antibacterial, antifungal, antiviral, anti-inflammatory, analgesic, antioxidant, vasorelaxant, and anticancer properties. This work focuses on the anticancer action of naturally occurring dimeric flavonoids against prostate and breast cancer, as well as on the mechanisms of action involved in their activity and presents the most current information on this subject in the literature. In the present review, we summarize the latest findings on the antiproliferative activity of 33 dimeric flavonoid-based compounds selected from recently published studies. The tests conducted were in silico and in vitro and demonstrated the cytotoxic activity potential of biflavonoids against prostate and breast tumor cells. Biflavonoids were capable of interfering with the migration and replication of cancer cells and their mechanism of action is related to cell death pathways, especially apoptosis, necrosis, and ferroptosis. These compounds decreased mitochondrial membrane potential and significantly increased intracellular levels of reactive oxygen species (ROS). Additionally, they significantly upregulated the expression of p21, Bax, and cleaved caspase-3, while downregulating Bcl-2 and caspase-3 levels, indicating their cell death mechanism of action is through the Bcl-2/Bax/cleaved caspase-3 pathway and cell cycle arrest. The biflavonoids here related have shown promising anticancer activity and are considered potential drug candidates for prostate and breast cancer treatment.

Keywords: biflavonoids; breast and prostate cancer; anticancer; natural product

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1. Introduction

According to the latest estimate published by the World Health Organization (WHO), in 2020, there were 1.4 million cases of prostate cancer worldwide, resulting in 397 thousand deaths from this disease and 19.3 million cases of breast cancer, resulting in 10 million deaths [1].

The incidence and mortality rates of prostate cancer worldwide are associated with increasing age, with the average age at the time of cancer diagnosis being around 66 years [2]. Breast cancer can be categorized into biologically and clinically significant subgroups based on histological grade. The histological grade, an assessment of the tumor's proliferative activity (percentage of Ki67), reflects its aggressiveness and aids in determining the appropriate therapy [3].

Cancer treatment may involve surgery, chemotherapy, radiotherapy, and often requires a combination of modalities [4]. Despite advances in chemotherapy, many patients either do not opt for this intervention or must discontinue treatment, primarily due to side effects or the development of resistance to multiple drugs. This underscores the necessity and importance of researching new molecules that are more effective and have fewer adverse effects [5].

Natural products have long been used in the treatment of various diseases and are a crucial source for drug discovery research. One third of the chemotherapeutic drugs

used in clinical cancer treatment originate from natural products or their derivatives [6]. Indeed, most molecules in clinical research, especially those targeting cancer and microbial infections, have been developed from natural products [7].

The natural products, particularly phytochemicals, have been the focus of extensive study. This is largely because they possess anticancer activities capable of interfering with the initiation, development, and progression of cancer by modulating several mechanisms, including cell proliferation, differentiation, apoptosis, angiogenesis, and metastasis [8]. These molecules generally have a higher molecular mass, a greater number of sp3 carbonand oxygen atoms, fewer nitrogen and halogen atoms, more hydrogen-bond acceptors and donors, a lower calculated octanol–water partition coefficient indicating higher hydrophilicity, and significantly greater molecular rigidity compared to synthetic compounds. These characteristics may prove beneficial in targeting protein–protein interactions in the drug discovery process [9].

Among natural compounds, flavonoids stand out as a broad group of secondary metabolites formed by polyphenolic compounds [10]. They exhibit diverse activities, including antibacterial, antiviral, anti-inflammatory, antioxidant, antiallergic, hepatoprotec- tive, vasodilator, and antithrombotic effects. Additionally, they have the ability to inhibit cell proliferation, tumor growth, and carcinogenesis [11,12].

A new subclass of flavonoids, known as biflavonoids, has awakened scientific inter- est. Biflavonoids, composed of two monoflavonoid residues, occur naturally in angiosperms, bryophytes, ferns, and gymnosperms. They can be classified into three groups: C-C, C-linear fragment-C, or complex biflavonoids, depending on whether the linker between the two residues contains an atom. Since the linker can be established between two arbitrary rings from different residues, the C-C type encompasses various subtypes, as does the C-linear fragment-C type. [13]. Most members of this subclass consist of flavone–flavone and flavanone–flavanone dimers, with the dimers of chalcones and isoflavones occurring more rarely [14]. Biflavonoids have demonstrated a variety of biological activities, including anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, analgesic, antioxidant, vasorelaxant, anticlotting [15], antibiotic, phospholipase A2 (PLA2), and cyclooxygenase-2 (COX-2) inhibition [16,17]. They are also involved in reversing multidrug resistance (MDR), and acting as mediators in the overexpression of resistance proteins such as MRP1 and P-glycoprotein (P-gp) in multidrug-resistant tumor cells [18].

Given the importance of natural products as sources of new medicines, and consider-ing that prostate and breast cancer are the second most common cancer among the male and female population, respectively, and both present incidence rates that continue to increase every four years, there is a critical need to seek and investigate scientific data on the anticancer properties of biflavonoids on these specific types of cancer.

2. Results

Twenty-eight articles related to the anticancer properties of dimeric flavonoids against prostate (Table 1) and breast (Table 2) cancer cells were found.

Table 1. List of biflavonoids that have exhibited cytotoxic effects on prostate cancer cells.

Biflavonoids	Subtype	Monomer Type	Cell Lines	Assays	*IC50/#EC50/@TGI	Authors
Oxitrodiflavanone A (**1**)	C-C	BB	PC-3	MTT (cell viability)	6.64 µM (**1**)	[19]
Cupressuflavone (**2**)	C-C	AA	PC-3	MTT (cell viability)	19.9 µM (**2**)	[20]
Propolone B (**3**) Propolone A (**4**)	C-linear fragment-C	EE	PC-3	MTT (cell viability)	19.1 µM (**3**) 21.9 µM (**4**)	[21]
Neochamaejasmin A (**5**)	C-C	AA	LNCaP	MTT (cell viability) Western blot analysis Cell cycle analysis	12.5 µg/mL (**5**)	[22]
Ginkgetin (**6**)	C-C	BB	PC-3	MTT (cell viability) Western blot analysis Cell cycle analysis	15–30 µM (**6**)	[23]

Table 1. *Cont.*

Biflavonoids	Subtype	Monomer Type	Cell Lines	Assays	*IC50/#EC50/@TGI	Authors
Ginkgetin (**6**)	C-C	BB	DU-145	MTT (cell viability) Western blot analysis Cell cycle analysis Mouse xenograft in vivo	5 µM (**6**)	[24]
Brachydin E (**7**) Brachydin F (**8**)	Complex	GG	PC-3	MTT (cell viability), Molecular docking, Wound healing assay, Clonogenic assay, Phosphatidylserine (PS) Externalization assay, In silico pharmacodynamics	6.9 µM (**7**) 37.1 µM (**8**)	[25]
Brachydin A (**9**) Brachydin B (**10**) Brachydin C (**11**)	Complex	GG	PC-3	MTT (cell viability) Neutral red assay, LDH activity release assay, Cell death assay, Comet assay, Western blot analysis	23.41 µM (**9**) 4.28 µM (**10**) 4.44 µM (**11**)	[26]
Brachydin A (**9**)	Complex	GG	DU145	Cytotoxicity assay, Tumor spheroids, Clonogenicity, Cell migration, Cell death assay, Protein Expression	60.0–100.0 µM (**9**)	[27]
Brachydin B (**10**)	Complex	GG	DU145	MTT (cell viability), Clonogenicity, Cell death assay, LDH, Cell migration	7.45 µM (**10**)	[28]
Brachydin C (**11**)	Complex	GG	DU145	Cytotoxicity assay, Cell migration, Clonogenicity, Protein expression	47.31 µM (**11**)	[29]
DCMF containing Brachydin A (**9**) Brachydin B (**10**) Brachydin C (**11**)	Complex	GG	DU145	Sulforhodamine B (cell viability), Comet (genotoxicity), Clonogenicity (reproductive capacity) and Wound healing (cell migration) assays, and Atomic force microscopy (AFM) for ultrastructural cell membrane alterations	2.51 µg/mL (**9**–**11**)	[30]

*IC50—Half-maximal inhibitory concentration. #EC50—Half-maximal effective concentration. @TGI—Totalgrowth inhibition concentration.

Table 2. List of biflavonoids that presented cytotoxic effects on breast cancer cells.

Biflavonoids	Subtype	Monomer Type	Cell Lines	Assays	*IC50/#EC50/@TGI	Authors
DCMF containing Brachydin A (**9**) Brachydin B (**10**) Brachydin C (**11**)	Complex	GG	MCF7	Sulforhodamine B (cell viability), Comet (genotoxicity), Clonogenicity (reproductive capacity), Wound healing (cell migration) assays, and Atomic force microscopy (AFM) for ultrastructural cell membrane alterations	2.77 µg/mL (**9**–**11**)	[30]
Robustaflavone (**12**)	C-C	BB	MCF7	MTT (cell viability), Detection of apoptotic cells, RNA extraction and sequencing, ROS, Molecular docking, Western blot	11.89 µM (**12**)	[31]
(2R,2″R″)-7-O-methyl-2,3,2″,3″-tetrahydrorobustaflavone (**13**)	C-C	AA	MCF7	MTT (cell viability)	5.4 µM (**13**)	[32]

Table 2. *Cont.*

Biflavonoids	Subtype	Monomer Type	Cell Lines	Assays	*IC50/#EC50/@TGI	Authors
Calodenin B (**14**) Lophirone A (**15**)	C-C (**14**) Complex (**15**)	AA (**14**) EG (**15**)	MCF7	Cytotoxicity assay	219.3 µM (**14**) 19.2 µM (**15**)	[33]
7-O-methyl-2,3,2″,3″-tetrahydro-3′,3‴-biapigenin (**16**) 4′-O-methylrobustaflavone (**17**)	C-C	BB	MCF7	MTT (cell viability)	41.44 µM (**16**) 16.68 µM (**17**)	[34]
4′,7,7″-tri-O-methylcupressuflavone (**18**) 4‴,7,7″-tri-O-methylagathisflavone (**19**)	C-C	BB	MCF7	MTT (cell viability)	91.74 µg/mL (**18**) 314.44 µg/mL (**19**)	[35]
Amentoflavone (**20**) Cupressuflavone (**2**)	C-C	AA	MDA-MB-231	MTT (cell viability)	16.1 µM (**20**) 12.7 µM (**2**)	[20]
Psocorylin R (**21**) Psocorylin S (**22**) Psocorylin U (**23**) Psocorylin V (**24**) Psocorylin W (**25**) Psocorylin Y (**26**)	C-C or C-linear fragment-C	GG	MCF7	Cytotoxicity assay and Apoptosis assay	7.35 µM (**21**) 17.40 µM (**22**) 10.01µM (**23**) 21.98 µM (**24**) 8.42 µM (**25**) 9.01 µM (**26**)	[36]
Neocryptomerin (**27**) Hinokiflavone (**28**)	C-linear fragment	BB	MCF7	MTT (cell viability)	30.09 µg/mL (**27**) 39.32 µg/mL (**28**)	[37]
Hinokiflavone (**28**)	C-linear fragment	BB	MDA-MB-231	MTT (cell viability), Clonogenicity (reproductive capacity), Western blot, cell migration, In vivo tumor model in mice	40 µM (**28**)	[38]
Isoginkgetin (**29**)	C-C	BB	MDA-MB-231	Cytotoxicity assay Western blot	20 µM (**29**)	[39]
Chamaejasmin (**30**)	C-C	AA	MDA-MB-231	MTT (cell viability), Western blot, Cell cycle analysis	5.11 µM (**30**)	[40]
7,7″-di-O-methylchamaejasmin (**31**)	C-C	AA	MDA-MB-231	Cytotoxicity assay, Western blot, Apoptosis assay	7.76 µM (**31**)	[41]
Podocarpusflavone-A (**32**) II-4″,I-7-Dimethoxyamentoflavone (**33**)	C-C	BB	MCF7	MTT (cell viability), Cell cycle Topoisomerase I assay	16.24 µg/mL (**32**) 15.17 µg/mL (**33**)	[42]
Amentoflavone (**20**)	C-C	AA	MCF7	MTT (cell viability), Cell cycle Western blot Cometa assay	150 µM (**20**)	[43]
Ginkgetin (**6**)	C-C	BB	MCF7	MTT (cell viability), Cell cycle Western blot Apoptosis assay ER-α expression	10 µM (**6**)	[44]

*IC50—Half-maximal inhibitory concentration. #EC50—Half-maximal effective concentration. @TGI—Totalgrowth inhibition concentration.

All of these studies employed in vitro and in silico tests, with the MTT assay being the most commonly selected method to evaluate the cell viability of both tumor types. Tests involving the ability of these compounds in inhibiting the migration and clonogenicity were also executed. Furthermore, genes and proteins associated with apoptosis pathways were investigated. The prostate cancer cell lines PC-3, DU145, LNCaP, and non-tumoral PNT2 and the breast cancer cell lines MCF7 and MDA-MB-123 were used in these tests. Thirty- three dimeric flavonoids, numbered 1 to 33 (Figure 1), were identified and their anticancer activities were reported below, in accordance with their findings. The biflavonoids were cat- egorized by subtype (C-C, C-linear fragments-C, and complex biflavonoids) and monomer type, including: AA (flavan–flavan), BB (flavone–flavone), EE (isoflavone–isoflavone), EG (isoflavone–chalcone), and GG (chalcone–chalcone) [13].

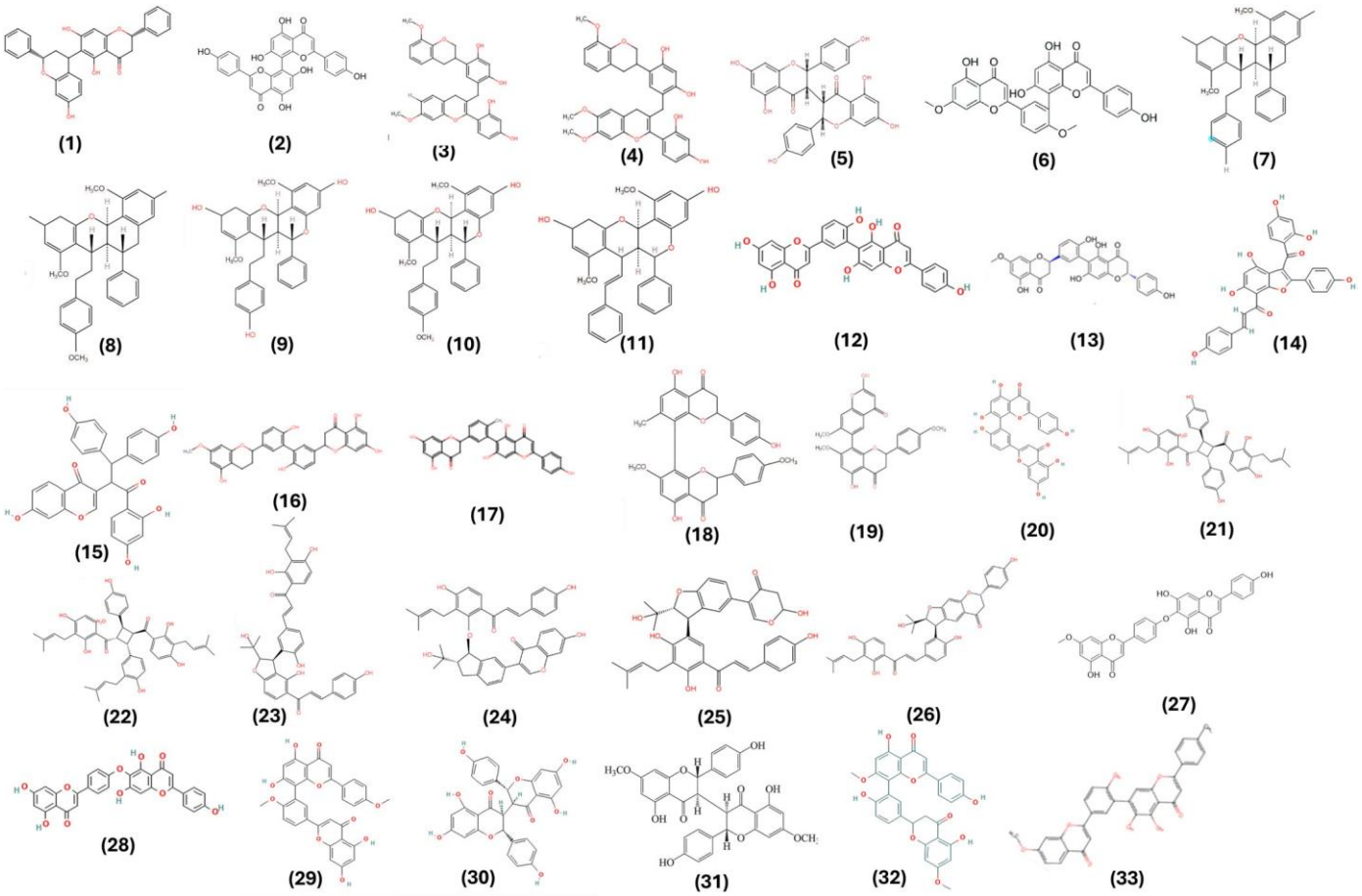

Figure 1. Molecular structure of biflavonoids **1**–**33**.

Oxitrodiflavanone A (**1**) is a biflavonoid isolated from *Oxytropis chiliophylla*. Its cytotoxic activity against prostate tumor cells (PC-3) was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. The IC50 value obtained was 6.64 µM, indicating that the biflavonoid exhibits potential cyto- toxic activity against the PC-3 cell line [19].

The biflavonoid Cupressoflavone (**2**) was isolated from ground leaves of *Juniperus phoenicea* L. This compound has been previously tested and demonstrated anticancer, antioxidant, antimicrobial, antinociceptive, and anti-inflammatory activities. Its cytotoxic effect was evaluated against the prostate cancer cell line PC-3 and the non-tumor prostate cell line PNT2 using the MTT assay. Cupressoflavone showed high cytotoxic selectivity for prostate cancer cells (PC-3) with an IC50 value of 19.9 µM, while showing no cytotoxicity against the normal prostate cell line (PNT2). This selectivity for prostate tumor cells is noteworthy. The result is particularly significant when compared to etoposide, a commonly used anticancer drug, which had an IC50 value of 61 µM—three times higher than that of Cupressoflavone [14,21].

Red propolis is derived from plant sources and contains various compounds that can be isolated, including two dimeric flavonoids named Propolone B (**3**) and Propolone A (**4**). These compounds demonstrated antiproliferative effects in the prostate cancer cell line (PC-3) using the MTT assay, with Propolone B showing a TGI (total growth inhibition) of 19.1 µM and Propolone A showing a TGI of 21.9 µM. This highlights their potential antiproliferative effects in 2D in vitro cultures [21].

A biflavonoid isolated from the roots of traditional Chinese medicine, *Stellera chamae- jasme* L., named Neochamaejasmin A (**5**), was analyzed in a prostate cancer cell line (LNCaP). The IC50 value obtained was 12.5 µg/mL. Treatment with low concentrations

≤6.25 µg/mL) of compound 5 inhibited the expression of cell cycle regulatory proteins such as cyclin D and the cyclin-dependent kinase inhibitor p21, leading to cell cycle arrest in the G1 phase. Additionally, this biflavonoid altered mitochondrial membrane potential and induced cellular apoptosis in LNCaP cells through the Fas-caspase8-caspase3 pathway [22].Ginkgetin (**6**) is a natural biflavonoid isolated from the leaves of *Ginkgo biloba* L. This molecule exhibits cytotoxic effects on prostate cancer cell lines PC-3 and DU-145, with IC50 values of 15 µM and 5 µM, respectively. It induces cell cycle arrest in the sub-G1 phase and activates apoptosis through caspase-3 and PARP cleavage [23,24].

Furthermore, Ginkgetin inhibits tumor growth as demonstrated in a mouse xenograft model using DU-145 cells, highlighting its potential as a potent anticancer agent suitable for clinical use. Ongoing studies by the research group aim to further elucidate the compound's mechanism of action [24].

Brachydins (Br) are biflavonoids extracted from the roots of *Fridericia platyphylla* (Cham.) L.G. Lohmann. They have recently been extensively studied by several research groups, and the results concerning their effects on prostate and breast cancer cells are presented here. Brachydin E (**7**) and Brachydin F (**8**) significantly reduced the cell viability of the prostate cancer cell line PC3, with IC50 values of 6.9 µM and 37.1 µM, respectively. In addition, wound healing and clonogenicity assays were conducted. Both biflavonoids effectively inhibited cell repopulation, resulting in only 20% and 13.2% closure of the wound area after 48 h of treatment, respectively. Furthermore, they reduced the number of PC-3 cell colonies by more than 60%.

The cell death assay assessed phosphatidylserine externalization in PC3 cells using Annexin-V labeling. Brachydin E (**7**) and Brachydin F (**8**) induced an increase in Annexin V-labeled cells, indicating their ability to induce regulated cell death in prostate tumor cells. Additionally, an in silico study demonstrated that Brachydins E and F target nu- clear receptors, specifically molecularly coupling with the glucocorticoid receptor (GR). Glucocorticoid hormones (GC) are known to exert an antiproliferative effect on various cells through the GR, which acts as a transcription factor. Thus, the direct binding of these compounds to the glucocorticoid receptor contributes to their antiproliferative function in cancer cells [25].

The Brachydins (Br) A (**9**), B (**10**), and C (**11**) were evaluated for their cytotoxicity against the prostate cancer cell line (PC-3) using the MTT assay, yielding IC50 values of 23.41 µM (**9**), 4.28 µM (**10**), and 4.44 µM (**11**). They were further assessed by the neutralred cytotoxicity assay, revealing that compounds **9**, **10**, and **11** initiated cytotoxic effects at concentrations of 15.36 µM, 6 µM, and 3.84 µM, respectively. At these concentrations, these biflavonoids induced cell death in the PC-3 cell line, as confirmed by the LDH activity release assay. The cell death mechanisms of Brachydins were investigated, showing that BrA and BrC induce necrosis in cells, while BrB can induce both necrosis and apoptosis in prostate cancer cells. Protein analysis via Western blot revealed overexpression of the p21 protein, leading to cell cycle arrest in PC-3 cells treated with BrB or BrC, but not BrA. The p27 protein, another cell cycle regulator, remained unchanged with the treatments. Expression of pAKT was reduced in cells treated with BrA and BrB, indicating disruption of cell survival processes. Additionally, cleaved PARP expression was elevated across all treatments, suggesting cell death via apoptosis. These findings underscore the potent antiproliferative effects of these biflavonoids, although further studies are needed to fully elucidate their mechanisms of action [26].

The antiproliferative and antimetastatic activity of Brachydin A (**9**) was evaluated in a three-dimensional (3D) culture of DU145 prostate cancer cells. Brachydin A exhibited cytotoxic effects at concentrations ranging from 60 to 100 µM, leading to alterations in spheroid morphology and volume, as well as suppression of cell migration and tumor invasiveness. In addition, Brachydin A caused a reduction in mitochondrial membrane potential, resulting in increased markers of apoptosis and necrosis, including activation of cleaved PARP and p-γ-H2AX. It also decreased levels of anti-apoptotic/pro-apoptotic markers such as BCL-2, BAD, and RIP3K, as well as cell survival markers p-AKT1 and

p-44/42 MAPK. There was an elevation in the protein levels of effector caspases (CASP3, CASP7, and CASP8) and a positive regulation of inflammation markers (NF-kB and TNF-α). These findings highlight Brachydin A as a potential candidate for preclinical studies against metastatic prostate cancer due to its multifaceted effects on cell viability, apoptosis, necrosis, and inflammation [27].

Brachydin B (**10**) was evaluated in both two-dimensional (2D) and three-dimensional (3D) cultures of the prostate cancer metastatic cell line DU145. It was found to induce cytotoxic effects within 24 h at a concentration of 7.45 μM in 2D culture, whereas in 3D culture, cytotoxic effects were observed at concentrations above 50 μM within 48 h. Brachydin B reduced clonogenicity in 2D culture and decreased the area/volume of 3D spheroids. It also demonstrated the ability to inhibit cell migration and invasion in both 2D and 3D assays, highlighting its potential as a potent anticancer agent in vitro. Further in vivo studies are planned to confirm its candidacy for therapy against metastatic prostate cancer [28].

Brachydin C (**11**) was also investigated in prostate cancer cells (DU145) using both 2D and 3D culture models. The IC50 values after 24 h of treatment were determined to be 47.31 μM (2D) and 229.8 μM (3D). Brachydin C impaired both horizontal (wound healing) and vertical (transwell assay) cell migration and invasion in 2D culture. Additionally, Brachydin C modulated the expression of several genes including BIRC5, TNF-α, CASP3, NKX3.1, MMP9, MMP11, CDH1, and ITGAM. In Western blot analysis, it downregulated proteins such as CASP7, BAX, and TNF-α. Overall, Brachydin C induced cell death and affected epithelial–mesenchymal transition processes [29].

The cytotoxic effect of a dichloromethane fraction (DCMF) containing the three brachydins (**9**–**11**) was evaluated on the prostate cancer cell line DU145 and the non-tumoral prostate cell line PNT2. The IC50 value for the prostate cancer cells was found to be half the concentration observed in the non-tumoral cell line, indicating the selectivity of DCMF towards cancer cells. These compounds together significantly inhibited colony formation and reduced migration of prostate tumor cells. Atomic force microscopy (AFM) was employed to examine changes in the cell membrane, revealing an increase in the number of membrane holes and roughness, particularly in tumor cells, in a concentration-dependent manner. These findings corroborate the observed effects of cytotoxicity, inhibition of clonogenicity, and suppression of migration in cells treated with DCMF [30].

The dichloromethane fraction (DCMF) containing Brachydin A (**9**), B (**10**), and C (**11**) was also analyzed for breast cancer, through the MCF7 lineage. It was observed that DCMF reduces cell viability, with IC50 values of 2.77 μg/mL for the strain under study. At higher concentrations, they were able to significantly inhibit the migration of cell lines and altered the membrane structures of tumor cells without causing toxic effects to normal cells [30].

The cytotoxic effect of the biflavonoid Robustaflavone (**12**), isolated from a species of *Selaginella* called *S. trichoclada*, on MCF7 breast cancer cells, was evaluated through MTT assay and the IC50 found was 11.89 μM. Subsequent transcriptome analysis identi- fied VDAC2 (voltage-dependent anion selective channel protein 2) as a potential target of compound **12**, a key regulator in the ferroptosis cell death pathway. These findings suggest that compound **12** may induce MCF7 cell death through non-apoptotic pathways, characterized by diminished or absent mitochondrial cristae, a hallmark of ferroptosis due to the accumulation of reactive oxygen species, making it an interesting study, given that the vast majority of dimeric flavonoids do not act through cell death by ferroptosis [31].

The antiproliferative activity of a new biflavonoid called (2R,2′R′)-7-O-methyl-2,3,2″,3″-tetrahydrorobustaflavone (**13**), isolated from *Aster tataricus*, whose structure was confirmed through spectroscopic and circular dichroism analysis, was evaluated against seven human cancer cell lines: A549 (lung), NCI-H1975 (lung), PC3 (prostate), DU145 (prostate), HepG2 (liver), LoVo (colon) and MCF-7 (breast) using the MTT assay. The compound under study showed cytotoxicity (IC50 = 5.4 μM) exclusively against MCF-7 cells [32].

Two biflavonoids isolated from the same plant species *Brackenridgea zanguebarica*, Calodenin B (**14**) and Lophirona A (**15**), were analyzed for their cytotoxic activity against MCF7 breast tumor cells, and their EC50 values were 19.2 μM for Lophirone A and 219.3 μM

for Calodenin B, indicating that Lophirone A is cytotoxic to MCF7 breast cancer cells, while Calodenin B is not effective [33] and this may be attributed to their chemical structure.

Other biflavonoids with cytotoxic activities were discovered in *Selaginella doeder-leinii*, highlighting their potential as plant-derived anticancer agents, called 7″-O-methylrobustaflavone (**16**)and 4‘-O-methylrobustaflavone (**17**). The cytotoxic activity of these compounds was testedagainst four human tumor cell lines: A549 (lung), MCF7 (breast), SMMC7721 (liver) and LoVo (colon) using the MTT assay. The results revealed that 7″-O-methylrobustaflavone (**16**) exhibited selective modest activity for MCF7 cells with an IC50 value of 15.09 μM, and 4′-O-methylrobustaflavone (**17**) showed moderate inhibitory effects in three tumor cell lines (MCF7, SMMC-7721, and LoVo) with IC50 values ranging from 16.68 to 33.47 μM [34].

The compounds 4′,7,7″-tri-O-methylcuppressuflavone (**18**) and 4″,7,7″-tri-O-methylagathisflavone (**19**) were initially isolated from the leaves of the Indonesian *A. hun-steinii* and later from other Araucaria plants. An MTT assay on MCF7 breast cancer cells revealed IC50 values of 91.74 μg/mL for compound **18** and 314.44 μg/mL for compound **19**, showing that compound **18** can be active against MCF7 cancer cells, while compound **19** is not, although bothpresented IC50 values higher than the positive control epirubicin HCl (IC50 0.52 μg/mL) [35]. The cytotoxicity of the flavonoids Amentoflavone (**20**) and Cupressoflavone (**2**), de- rived from the active fractions (F3 and F4) of the methanolic extract of *J. phoenicea* leaves, was investigated by Groshi (2019). Among all human cancer cell lines evaluated, MCF7 and MDA-MB-231 were most sensitive to compounds **20** and **2**, with IC50 values of 25 μM for MCF7 and 12.7 μM for MDA-MB-231, indicating specific cytotoxicity. In relation to breast cancer cell lines, their mechanisms will be studied for better analysis, as they are two different types of breast tumors and with different receptors [20].

Nine new biflavonoids were isolated from the fruits of *Psoralea corylifolia*. In MTT assays, compounds **21**, **23**, **25,** and **26** demonstrated cytotoxicity with IC50 values of 7.35, 10.01, 8.42, and 9.01 μM, respectively, while compounds **22** and **24** showed mild cytotoxic activities, with IC50 values of 17.40 and 21.98 μM, respectively. Compounds **21** and **26** were notable for their antiproliferative activity against breast cancer cells, as they led to a decrease in mitochondrial membrane potential, significantly increased intracellular reactive oxygen species (ROS) levels, and induced apoptosis in MCF7 cells. Specifically, compound **26** exhibited an apoptosis ratio of 6.17 ± 0.39% at low concentration (4 μM) with a significant decrease in mitochondrial membrane potential, and compound **21** showed an apoptosis ratio of 24.58 ± 1.68% at 8 μM. Furthermore, they upregulated the expression of Bax and cleaved caspase-3, while downregulated the levels of Bcl-2 and caspase-3, indicating their action through the Bcl-2/Bax/cleaved caspase-3 pathway. These biflavonoids have demonstrated promising anticancer activity and are considered potential drug candidates for the treatment of breast cancer [36].

The biflavonoids cryptomerin (**27**) and hinokiflavone (**28**) were isolated from *Selaginella tamariscina* (Beauv.). Initial trials revealed IC50 values of 30.09 μg/mL for cryptomerin (**27**) and 39.32 μg/mL for hinokiflavone (**28**) against the breast cancer cell line MCF7. These findings indicate cytotoxic effects of these biflavonoids against breast cancer cells. Further studies are required to fully explore and characterize their potential anticancer properties [37].

Hinokiflavone (**28**) is a biflavonoid isolated from *Selaginella p*. Beauv, *Juniperus phoenicea*,and *Rhus succedanea*. Known for its various biological activities such as anti-HIV-1, reverse transcriptase inhibition, anti-sialic acid influenza enzyme, and antioxidant properties, its anticancer activity against MDA-MB-231 breast tumor cells has been thoroughly investigated both in vitro and in vivo. In vitro studies demonstrated that hinokiflavone induced apoptosis in MDA-MB- 231 cells at a concentration of 40 μM. Additionally, it exhibited significant anti-migration and anti-invasion effects in a dose-dependent manner. In vivo evaluation using a mouse xenograftmodel of MDA-MB-231 tumors showed that hinokiflavone treatment over 21 days significantly inhibited tumor growth at doses of 20 mg/kg and 40 mg/kg. Tumor weight reduction was observed in a dose-dependent manner, and immunohistochemical staining revealed fewer Ki67-positive and MMP-2-positive cells in tumors treated with hinokiflavone compared to the

control group. These findings underscore hinokiflavone's ability to suppress the growth of human breast cancer cells in vivo, supporting the results obtained from in vitro studies [38].

Isoginkgetin (**29**), a biflavonoid extracted from the leaves of *M. glyptostroboides* (Dawn redwood, family: Taxodiaceae), has demonstrated potent antitumorigenic properties. In studies conducted on MDA-MB-231 breast cancer cells, Isoginkgetin was found to reduce the production of matrix metalloproteinase MMP-9, Akt, and PI3K. These proteins play crucial roles in cancer progression, particularly in invasion processes. Given that Isoginkgetin effectively decreases these proteins implicated in invasion, it emerges as a promising candidate for therapeutic intervention against tumor invasion of MDA-MB-231 cells [39].

Chamaejasmin (**30**), a new biflavonoid derived from *Stellera chamaejasme* L., exhibited an IC50 value of 4.72 μM against MDA-MB-231 breast cancer cells. It induced cell cycle arrest in the G2/M phase by inhibiting cyclins Cdk2 and cdc2, and activating WAF1/p21 and KIP1/p27. Additionally, Chamaejasmin induced apoptosis by activating Bax and inhibiting Bcl-2. These findings highlight Chamaejasmin as a potent anticancer agent that acts on multiple cell death pathways, inhibiting the growth of MDA-MB-231 cells [40].

7,7″-di-O-methylchamaejasmin (**31**), isolated from the Kenyan medicinal plant *Ormocarpum kirkii*, demonstrated an IC50 value of 7.76 μM against MDA-MB-231 breast cancer cells. It induced apoptosis by altering mitochondrial membrane potential and increasing reactive oxygen species [41].

Two biflavonoids, Podocarpusflavone-A (**32**) and II-4″,I-7-Dimethoxyamentoflavone (**33**), were isolated from the dry twigs of *Podocarpus nakaii* Hayata (Podocarpaceae). Their cytotoxic activity was evaluated in the breast cancer cell line MCF7, revealing ED50 values of 16.24 μg/mL and 15.17 μg/mL, respectively. Mechanistic studies indicated that both biflavonoids induce cell cycle arrest at the S phase in MCF7 cells, which was associated with alterations in Topoisomerase I enzyme activity [42].

Ginkgetin (**6**), previously mentioned, was also evaluated in MCF7 breast cancer cells, where it exhibited an IC50 value of 10 μM. It increased the number of cells labeled with annexin V and PI, indicating cell death by apoptosis. Ginkgetin also inhibited the estrogen receptor signaling pathway by downregulating ER-α expression. This explains the lack of promising results in the MDA-MB-231 cell line, which lacks estrogen receptors [44].

The biflavonoid amentoflavone (**20**), previously mentioned, was also assessed in MCF7 breast cancer cells, revealing an IC50 of 150 μM. It induced cell cycle arrest in the G1 phase and promoted chromatin condensation and apoptosis. The comet assay demonstrated that amentoflavone induced DNA damage. Protein analysis indicated a decrease in BCL2 levels and an upregulation of BAX in MCF7 cells, underscoring its significance for clinical research [43].

3. Discussion

In recent decades, special attention has been given to dimeric flavonoids, which are prevalent in various species of the plant kingdom. Although well reported for their rich pharmacological properties in the literature, there remains a scarcity of articles on their anticancer activity [16].

The results regarding the anticancer potential of the dimeric flavonoids discussed here align with findings in the literature on the anticancer activity of this subclass of flavonoids in other cancer types, such as glioblastoma, lung, and neuronal cell lines. Most of the trials described herein are preliminary, indicating that research in this area is still in its early stages and merits further attention from the scientific community.

This review presented the results of 33 dimeric flavonoids on the antiproliferative activity against prostate and breast cancer. Generally, the tests carried out with these compounds were in vitro, with the majority demonstrating cytotoxicity through the MTT assay. The lowest IC50 values found for prostate and breast cancer cells were obtained with brachydins derived from the genus *Fridericia platyphylla* (Cham.) L.G. Lohmann. These molecules are classified as monomer type G-G, resulting from the union of two chalcones. The presence of chalcones in the structure of Brachydins contributes significant pharmacological potential, as this group of compounds exhibits diverse properties, including

antioxidant, cytotoxic, anticancer, antimicrobial, antiprotozoal, antiulcer, antihistamine, and anti-inflammatory activities. Various pharmacologically active compounds have been developed based on the chalcone skeleton [25].

Biflavonoids were capable of interfering with the migration and replication of cancer cells,on the cell cycle progression and their mechanism of action is related to cell death pathways, specially apoptosis, necrosis and ferroptosis. Apoptosis is a disordered cellular process and also one of the most studied regulated deaths by scientists. In cancer, this process occurs in a minimal way, allowing cells to survive for longer and to multiply progressively, allowing the transformation of cancer cells, tumor metastasis and developing resistance to multiple drugs, therefore compounds that can induce apoptosis in tumor cells play an important role in the treatment of cancer and are a target of many treatment strategies [45]. The term necrosis refers to a series of events, such as a gain in cell volume, swelling of organelles, rupture of the plasma membrane with consequent loss of intracellular content. In the same way as apoptosis, it is considered a regulated cell death, and also a type of cell death for the treatment of cancer, as it causes the total extravasation of the contents of that cancer cell treated [46]. Ferroptosis is a form of regulated cell death specific to iron overload, accumulation of lipid reactive oxygen species (ROS) and lipid peroxidation. Evidence demonstrates that this type of cell death is directly linked to cancer suppression [47].

Therapy that targets cancer also acts by interrupting the functions of proteins that play essential roles during cancer progression. Biflavonoids were shown to be positive in inhibiting proteins that induce tumor growth (oncoproteins), as well as activating proteins directly related to regulated cell death (tumor suppressor proteins). We can also highlight that dimeric flavonoids have selectivity for tumor cells and presented, in vitro, less toxicity than other chemotherapeutic drugs still used clinically. Only two articles have investigated the in vivo anticancer effects of the bioflavonoids ginkgetin (**6**) and hinokiflavone (**28**). Pre-clinical tests involving toxicity still need to be conducted [48].

The most studied cell lines were PC-3 (prostate) and MCF7 (breast), likely because these tumor types are more responsive to existing treatments and are commonly used inin vitro research. Additionally, some research groups explored the effects of biflavonoids on the MDA-MB-231 breast cancer cell line, derived from a triple-negative strain. The findings related to compounds **2**, **20**, and **28–31** are particularly noteworthy since triple-negative breast cancer lacks estrogen and progesterone receptors and has insufficient HER2 protein, making it ineligible for hormone therapy or HER2-targeted medications, thus limiting treatment options compared to other types of invasive medications for breast cancer.

The research on biflavonoid anticancer properties is still emerging in the literature, indicated by the limited number of articles published over a period of six years. This scarcity can be attributed to biflavonoids because it is only recently that they have gained significant scientific interest as potent bioactive compounds. We strongly encourage further research into the anticancer properties of biflavonoids, as they presented promising results and are candidates for new anticancer agents.

Cancer is considered a global problem, due to its high rate of occurrence and mortality, and unfortunately the treatments that exist present many adverse effects. Considering that prostate and breast cancer are the most recurrent in the population, the option of new treatments is necessary, as a way to have fewer adverse effects and a high rate of selectivity for tumor cells. In this sense, biflavonoids have high selectivity for tumor cells and low adverse effects, so studies need to move on to the next phases of development to better explore the mechanism of action by which they act on these certain types of cancer and obtain new compounds for the treatment of prostate and breast cancer. We strongly encourage further in vivo experiments with biflavonoids.

4. Materials and Methods

This study was carried out through a literature review, using the PubMed and Sci-ELO platforms to search for articles published in recent years, with the keywords “dimers of flavonoids”, “flavonoid dimers”, “biflavonoids”, “dimeric flavonoids” isolated or together

with "prostate cancer" or "breast cancer". The articles selected for this review were those categorized as studies on naturally occurring flavonoid dimers, with their structures explicitly stated. Only original research articles were considered.

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4. CAPÍTULO 2 - Manuscrito em elaboração

Neste artigo foi analisada a potencial atividade anticâncer do biflavonoide BrachydinE frente a dois subgrupos de células tumorais de mama, o grupo Luminal B e o triplo negativo (MCF7 e MDA-MB-231, respectivamente). Objetivou-se traçar o mecanismo de ação deste composto no ciclo e na morte celular.

Para tanto, Brachydin E foi submetido a ensaios bi e tridimensionais de atividade antiproliferativa e anticlonogênica, que revelaram a potente ação citostática do composto. Proteínas envolvidas na regulação do ciclo celular, na ativação da via de apoptose e na inativação da via de proliferação celular foram investigadas pelas técnicas de western blotting e citometria de fluxo.

Brachydin E induz a ativação de caspases iniciadoras, tanto da via extrínseca quanto da via intrínseca da apoptose, que culminam na ativação de caspases efetoras deste processo de morte celular. A clivagem de PARP induzida por esta molécula leva à fragmentação de DNA que também culmina em apoptose e a inativação de c-myc pelo Brachydin E impede a proliferação celular.

No que diz respeito à regulação do ciclo celular, foi observado que Brachydin E leva à parada das células na fase de síntese do DNA devido à ativação na expressão de CDK4 e p21, proteínas envolvidas nos pontos de checagem da transição entre as fases G1 e S.

Estes resultados destacam a promissora atividade anticâncer do biflavonoide Brachydin E e reforçam a necessidade de se continuar a investigar suas propriedades *in vitro* e *in vivo*.

MDPI

Article

Breast anticancer properties of Brachydin E, an unusual dimeric flavonoid.

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Abstract:.The number of cancer cases is increasing every year, and although treatments keep pace with developments, chemotherapy drugs that are derived from plant origin continue to be the vast majority as the basis for the treatment of various types of cancer. Fridericia platyphylla is a shrub found in the Brazilian cerrado that presents anti-inflammatory, analgesic and cytotoxic properties, although it has few reports of anticancer activity. From this species many dimeric flavonoids were isolated, which are flavone-flavone, flavanone-flavanone, in addition to the more rare occurrence of chalcone and isoflavone dimers. The aim of this study was to investigate the anticancer potential of the dimeric flavonoid Brachydin E, in two breast tumor cell lines, MCF7 (Luminal A) and MDA-MB-231 (triple negative). The compound Brachydin E showed antiproliferative, replicative and antitumorigenic activity for both lineages, led to cell death by apoptosis and necroptosis (MCF7 and MDA-MB-231), respectively, and induced cell cycle arrest in S phase. Proteins involved in the process of cell death through apoptosis, arrest in the cell cycle, were also explored. The preliminary results show that Brachydin E is a very promising compound for its anticancer effects.

Keywords: natural product; breast cancer; cell cycle arrest; cell death.

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1. Introduction

Cancer is among the leading causes of death in the world and, according to the World Health Organization (WHO), 21.4 million new cases and 13.2 million deaths caused by cancer are expected by 2030 due to population growth and aging. There are more than 2.3 million cases of breast cancer occurring each year, making it the most common cancer among adults. In 95% of countries, breast cancer is the first or second cause of female cancer death [1].

Diagnosis and treatment of breast cancer are increasingly gaining advances, but it is still necessary to search for new treatments that provide less side effects. Natural products

have been used for the treatment of various diseases and are becoming an important research area for drug discovery. [2,3].

It is estimated that 60% of chemotherapy drugs used in the clinic for cancer treatment come from natural products [4]. Observing the list of new medicines that have been approved in the last three decades by the Food and Drug Administration (FDA), it is possible to conclude that one third of medicines are based on natural products or derived from natural products. Most of the molecules involved in clinical research, specifically anticancer and antimicrobial agents, were developed from natural products [5].

In this context, flavonoids constitute a broad class of natural compounds of considerable scientific and therapeutic interest. They are a widely distributed secondary metabolites group consisting of polyphenolic compounds found in foods, used as cosmetics and possibly involved in the prevention of cancers, cardiovascular diseases and neurodegeneration [6-7]. In recent decades, special attention has been given to bioflavonoids or dimeric flavonoids, which consist of two flavonoids linked by either –C, C–C, –O, or a C–O–C bond. In the literature, dimeric flavonoids are extensively reported for their pharmacological properties, including antiproliferative, anti-inflammatory, antioxidant, inhibitory activity against phospholipase A2 (PLA2) and antiprotozoal activity [8].

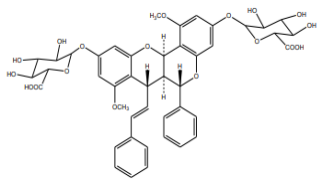
Among the plant species that present several dimeric flavonoids, *Fridericia platyphylla* (botanical synonym: *Arrabidaea brachypoda*) stands out. It is a plant native to the Brazilian cerrado, popularly known as "cervejinha do campo" or "tintureiro" and has bushes up to 70 cm tall [9]. The flavonoid Brachydin E (chalcone dimer) was isolated from the roots of this plant native to the Brazilian cerrado and there is only one article describing the anticancer property of this dimeric flavonoid in the literature, developed by our research group [10]. Therefore, the objective of this article is to highlight the antiproliferative activity of the compound Brachydin E in breast tumor lines, as well as elucidate its mechanism of action.

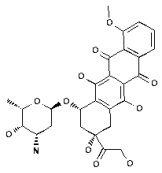
2. Results

2D and 3D Antiproliferative assays

The antiproliferative activity of compound Brachydin E was evaluated for breast cancer cell lines MCF7 and MDA-MB-231 and the GI50 value obtained was 7.5 μM, indicating that Brachydin E exerts a cytostatic effect on both lineages, as shown in TABLE 1. Comparing to chemotherapeutic agent doxorubicin, Brachydin E was less cytotoxic to breast cancer cells, but it is important to emphasize that it was also less cytotoxic to keratinocytes cell line HaCat.

TABLE 1. Antiproliferative activity of Brachydin E in cultured human breast tumor cells, expressed in GI50 (μM)

Structural formula	Samples	MCF7		MDA-MB-231	HaCat
	Brachydin E	7.50 0.39	±	7.50 ± 0.25	4.42 ± 0.46

Compound				
C		Doxorubicin Hydrochloride	1.20 ± 0.27	3.88 ± 1.22	2.19 ± 0.36

d Brachydin E was also effective in reducing the MDA-MB-231 cell viability by 32,6% in three-dimensional tumor model when compared to the DMSO negative control, as seen in FIGURE 1. 3D spheroids are composed of 3 zones: proliferative zone on the surface, normoxic zone in the middle and a hypoxic or anoxic zone in the core [11]. Brachydin E destroyed the external proliferative zone, modified the normoxic middle zone and affected the cell arrangement on hypoxic zone (Figure 1).

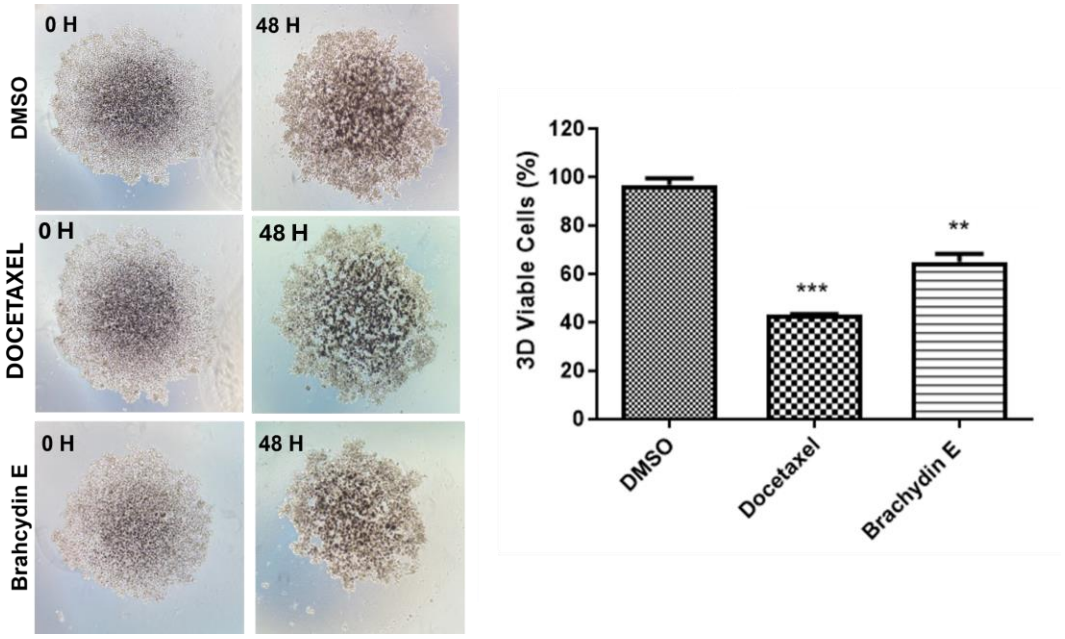


FIGURE 1. MDA-MB-231 cell viability (%) after 48 hours of treatment with DMSO (negative control), docetaxel 3,8 μM (positive control) and 7,5 μM Brachydin E. **p<0.01 ,***p<0.001 (two-way ANOVA: Bonferroni).

Brachydin E was able to reduce the number of colonies by 70% for MCF-7 (Figure 2A and B) and by 62.5% for MDA-MB-231 cancer cell lines (Figure 2C and D), corroborating to antiproliferative activity observed through MTT assay.

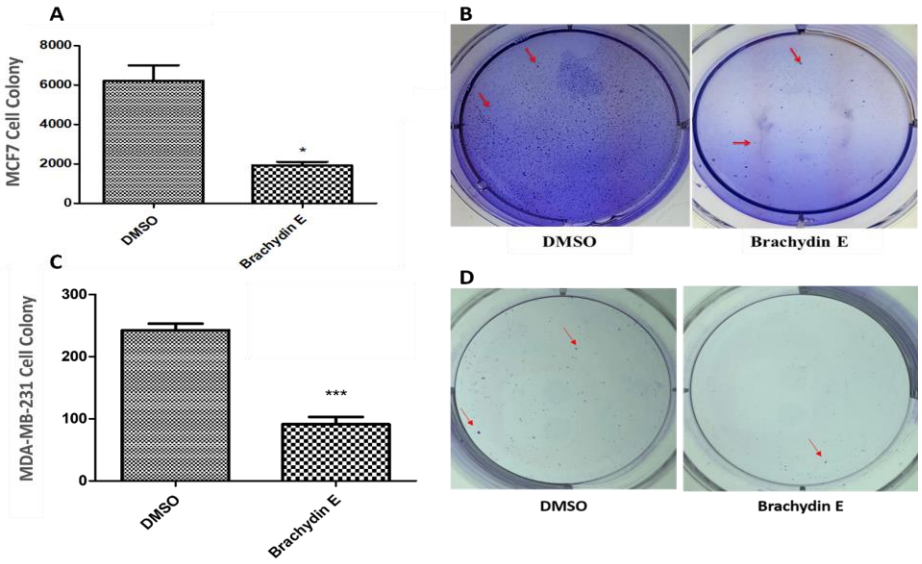


FIGURE 2. MCF7 (A and B) and MDA-MB-231 (C and D) colonies formed after treatment with DMSO at a concentration >0.01% and Brachydin E at a concentration of 7,5 μM /10 days. * p<0.05; *** p<0.001 (T-Test). Arrows show colonies containing between 50-100 cells each.

Cell Cycle Regulation Assays

The cell cycle assay was performed for the MCF7 line in order to investigate the action of Brachydin E on the progression of cell division. As observed, there was an increase in the number of cells in the S phase treated with positive control etoposide at 10 μM (from 9.3% to 26.7%) and Brachydin E at 12 μM (from 9.3% to 21.5%), with a consequent reduction of cells in the G1 phase, from 87.5% to 71.8% for etoposide and 87.5% to 75.8% Brachydin E, as shown in FIGURE 3A.

The analysis of proteins involved in the progression and control of the cell cycle, CDK4 and p21, revealed that Brachydin E was able to increase the expression of p21 with a consequent decrease in the expression of CDK4, showing that it is involved in cell cycle arrest in the S phase, corroborating the results found through cytometry, as shown in FIGURE 3B.

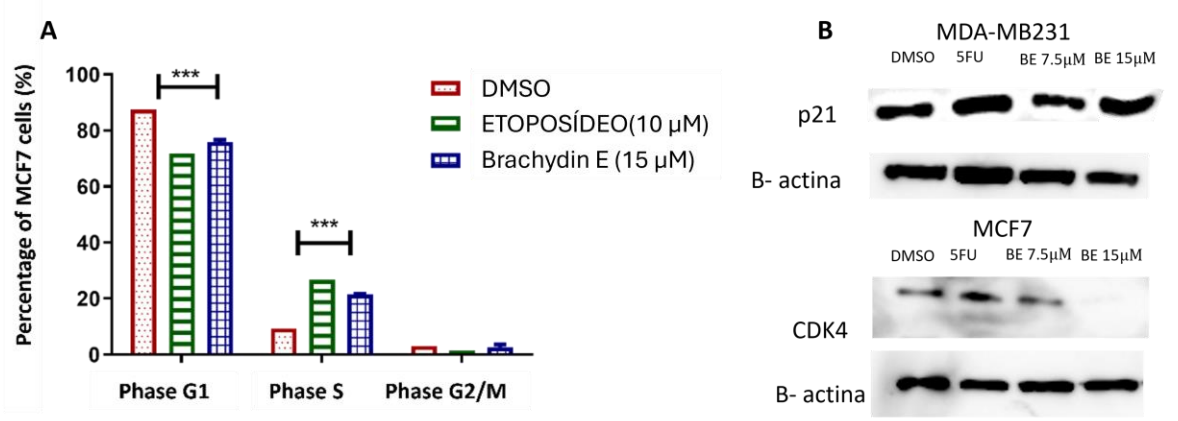


FIGURE 3. A. Percentage of MCF7 cells (%) in the different phases of the cell cycle: G0/G1, S and G2/M. Treatment: Brachydin E (12μM) and Etoposide (10μM). ***p<0.01 (two-way ANOVA: Bonferroni); B. Analysis of p21 and CDK4 proteins with DMSO, 5FU, Brachydin E (7.5 μM and 15 μM).

Cell death mechanism assays

To better characterize the effect of the compound, assays to investigate its mechanism of action were conducted using the flow cytometry technique.

The Annexin V-PE/7AAD data (Figure 4 A-C) were very expressive for both breast cancer cell lines and revealed that there was an increase in cells labeled only with annexin (from 3.5±0.7 to 29.7± 0.1%), with a consequent reduction in viable cells (from 79.7±2.1 to 41.4±6.6%) for MCF7 cells. Differently, for the MDA-MB-231 lineage, Brachydin E increased cells double-labeled with Annexin V-PE/7AAD (from 0% to 65.9%), with a consequent drastic reduction of viable cells (from 99.3% to 13.6%).

Brachydin E induced an increase in cells double labeled for caspase 8, 3 and 7, from 5.64% to 13.01% (FIGURE 5 A, B and C) and also for caspase 9, 3 and 7, from 4.10 % to 13.78% (FIGURE 5 D, E and C) in MDA-MB-231 cells. This molecule was also able to interfere with some proteins involved with the progression, replication of cancer cells such as activation of cleaved PARP and decreased c-myc expression (FIGURE 5 F).

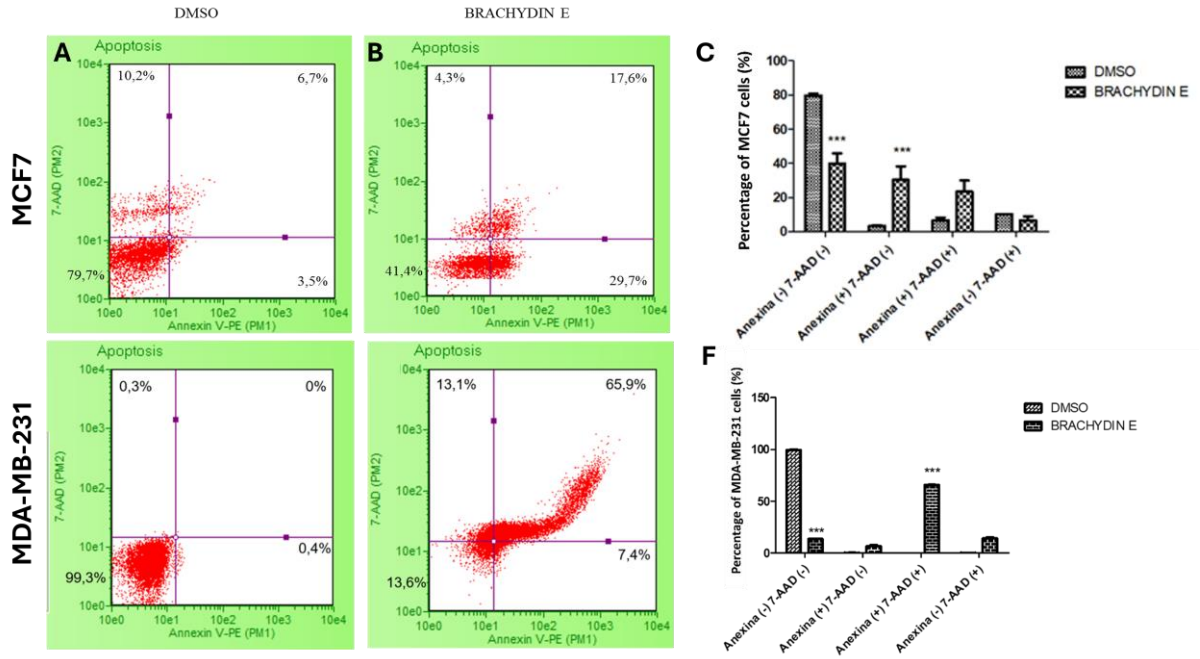


FIGURE 4. Percentage of MCF7 (A-C) and MDA-MB-231 (D-F) cells at different cell death stages: Viable: lower left quadrant; initial process of apoptosis: right lower quadrant; late process of programmed cell death: upper right quadrant; non-viable: upper left quadrant. Treatment: DMSO (A and D) at a concentration of 0.01% and Brachydin E (B and E) at a concentration of 7,5 μM/30h for MCF7 and /24h for MDA-MB-231. ***p<0.001 (two-way ANOVA: Bonferroni).

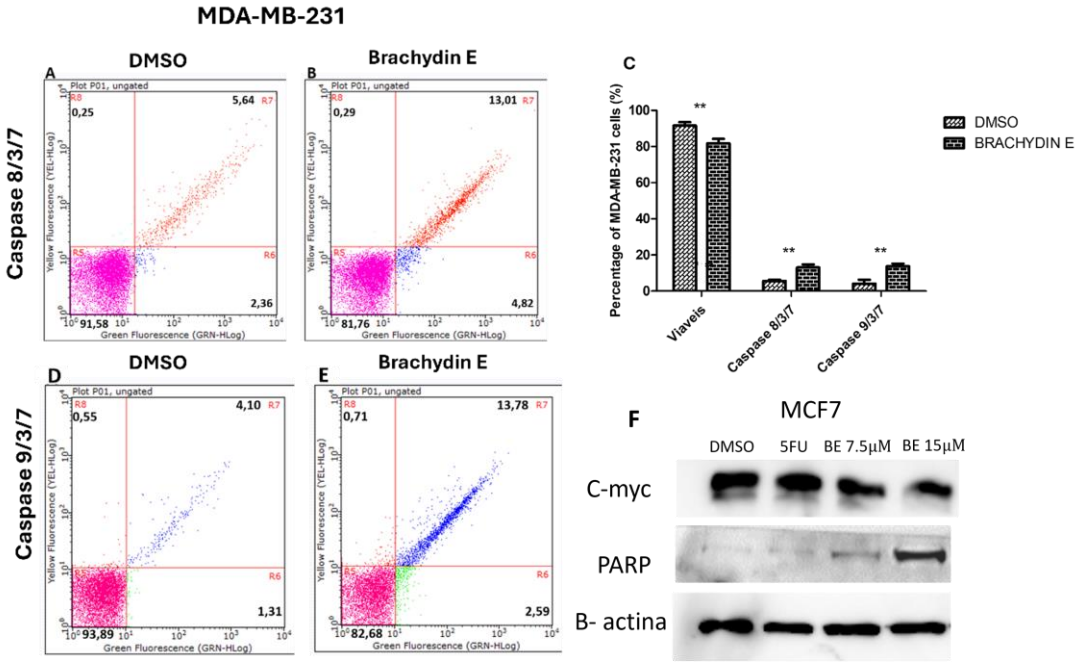


FIGURE 5. Percentage of MDA-MB-231 cells marked with caspases after 12 hours of treatment with 0.01% DMSO (A and D) and 7,5 µM of Brachydin E (B and E). C: caspases 8, 9, 3 and 7. A and B - Lower left quadrant: (-) Caspase-8 (-) Caspase-3/7; lower right quadrant (+) Caspase-8 (-) Caspase-3/7; upper right quadrant (+) Caspase-8 (+) Caspase-3/7; upper left quadrant (-) Caspase-8 (+) Caspase-3/7. D and E - Lower left quadrant: (-) Caspase-9 (-) Caspase-3/7; lower right quadrant (+) Caspase-9 (-) Caspase-3/7; upper right quadrant (+) Caspase-9 (+) Caspase-3/7; upper left quadrant (-) Caspase-9 (+) Caspase-3/7. ***p<0.001 (two-way ANOVA: Bonferroni). F. Analysis of PARP and c-myc proteins with DMSO, 5FU, Brachydin E (7.5 µM and 15 µM).

3. Discussion

Despite advances in chemotherapy treatments, many cancer patients do not use this type of intervention or need to interrupt treatment, mainly due to the side effects that the treatment causes and the development of resistance to multiple drugs, indicating the need for and importance of researching new molecules with more effective anticancer activity and with fewer adverse effects [12]. Natural products have been used to treat various diseases, becoming an important source of research for drug discovery. These products, especially phytochemicals, have been extensively studied, mainly due to the fact that they exhibit anticancer activities, as they are capable of intervening in the initiation, development and progression of cancer through the modulation of several mechanisms, including cell proliferation, differentiation, apoptosis, angiogenesis and metastasis [3].

With the advancement and improvement of genetic techniques for the production of plant secondary metabolites, combinatorial syntheses and high-throughput screening, it is expected that new compounds from natural sources, including medicinal plants, will be identified and developed as chemopreventive and anticancer drugs with more efficacy and safety, with fewer side effects. Therefore, the search for new bioactive molecules of plant origin always offers great opportunities to investigate new chemical classes of anticancer agents, as well as new mechanisms of action with high relevant potential [13].

Studies developed in recent years have proven that flavonoids are capable of inhibiting cell proliferation, tumor growth and carcinogenesis. It has been widely reported that flavonoids interfere with the initiation, promotion and progression of cancer, by modulating different enzymes and receptors responsible for cell proliferation, differentiation, apoptosis, inflammation, angiogenesis, metastasis and reversal of resistance to multiple drugs [7].

Biflavonoids are bioactive natural products with two-unit bonding styles and have diverse pharmacological effects of antitumor, antioxidant, anti-inflammatory and antivirus properties [14]. Biflavonoids can be developed as excellent anticancer agents to combat sensitive and resistant breast cancer cell lines [15].

Some biological activities have been reported for dimeric flavonoids called brachydins isolated from *Arrabidaea brachypoda* specie. Brachydin B and Brachydin C significantly reduced the percentage of peritoneal macrophages infected with stationary phase promastigotes, as well as the number of *Leishmania amazonensis* amastigotes [16]. Recently Da Rocha and collaborators reported the antiulcerogenic activity of the hydroalcoholic extract from the roots of *Arrabidaea brachypoda*, from which several other brachydins were isolated and identified, which may be responsible for this activity [17]. Our previous results showed that the crude hydroethanolic extract did not present cytotoxic activity, but its subfraction presented lower IC50 values for glioblastoma (U-251) and prostate adenocarcinoma (PC-3) cell lines. Brachydins E and F significantly reduced cell viability, proliferation, and clonogenic potential of PC-3, inducing them to the process of regulated cell death. In silico studies have indicated nuclear receptors as targets for Brachydins E and F, and molecular docking has pointed out their binding into glucocorticoid receptor (GR) ligand pocket [10].

According to our results, Brachydin E presented antiproliferative potential, observed in two-dimensional and three-dimensional cell culture. This 3D conformation is better than 2D monolayer culture because 2D culture does not mimic the natural cellular environment. Cells are, for the most part, deprived of cell-cell and cell-extracellular matrix interactions, therefore, the three-dimensional model ends up mimicking cancer in a more realistic way [18].

Another characteristic of tumor cells is their ability to replicate, and the clonogenic assay performed is commonly used to investigate how compounds behave in cellular tumorigenesis. The results demonstrated the ability of the compound Brachydin E to inhibit the proliferation of human breast tumor cells. This result was also confirmed by western blot technique,by the reduction of the c-myc protein expression, which is directly linked to the development and progression of breast cancer. Overexpression of c-Myc has been observed in many breast cancer patients and is associated with a poor prognosis and high levels of c-Myc contribute to the growth of breast tumor cells, therefore, its reduction can be considered one of the promising strategies against these breast tumor cells [19-20].

Although cancer exhibits heterogeneous characteristics, all malignant tumors have the property of growing beyond normal. The clonal expansion of an altered cell depends on a failure of proliferative capacity and an inability to activate cell death mechanisms. Some evidences show that resistance to cell death mechanisms is a hallmark of most malignant tumors [21]. The observation that Brachydin E treatment decreased c-Myc expression in breast tumor cells is consistent with the understanding that c-Myc inhibition can lead to inhibition of tumor growth and cell replication, leading to a decrease in in the ability of cells to form colonies, indicating a reduction in the capacity for cell growth and replication. Therefore, the results observed for Brachydin E provide additional evidence that inhibition of c-Myc may be an effective strategy in the treatment of breast cancer [19-20].

Flow cytometry allows monitoring the distribution of cells in different phases of the cycle in response to certain stimuli or the action of drugs. It is also possible to visualize cells

with an abnormal pattern in DNA content [27]. Therefore, this analysis helps to detect disturbances in certain phases of the cycle, that is, changes in the DNA content of dividing neoplastic cells, which generate mutations and changes in the methylation of cell cycle regulatory genes; These situations are commonly caused by genetic instability present in the process of malignant transformation and tumor progression [27].

To analyze the DNA content, specific fluorescent compounds are used, such as propidium iodide, which is intercalated in the helical structure of DNA [28-31]; The intensity of the fluorescence resulting from the binding of the fluorochrome to DNA is directly proportional to the amount of genetic material present in the cell nucleus, thus demonstrating different phases of the cell cycle [28]. The results obtained show that the compound Brachydin E led to an increase in the number of cells in the S phase, indicating cell cycle arrest in the same way as the chemotherapy drug etoposide, which causes cycle arrest in S or G2/M, inhibiting topoisomerase II [9;32 -33].

Two proteins involved in cell cycle regulation were analysed. Firstly, p21 is considered a tumor suppressor and regulator of the transition from the G1 to S phase of the cell cycle, and Brachydin E led to an increase in the expression of p21 in breast tumor cell lines, as observed through cytometry analysis. [22]. The second protein analyzed CDK4 is responsible for driving the progression of the cell cycle from the G1 phase to the S phase, reducing the proliferation of cancer cells. In some studies, CDK4 inhibitors induced cytostatic and apoptotic effects in tumor cells and its reduction in expression may occur through the activation of p21. Brachydin E reduced CDK4 expression, most likely due to the consequent increase in p21 expression, as previously described for hesperetin, a known flavonoid [22].

Cell death can be defined as a process of damage to the cell's vital functions. Regulated cell death is based on a complex of molecular actions that can be modified by pharmacological and genetic factors. Furthermore, it can be triggered in different ways, through mechanisms that seek the balance of the organism without any external interference and through activation generated by intracellular or extracellular stimuli that result in a major disturbance in the cell and need to deal with stress or restore cellular homeostasis [23].

Apoptosis mechanisms involve a cascade of energy-dependent molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway, which converge on the same terminal or execution pathway. There are some proteins participating on the cell death signalization. Caspase 8 is a member of the extrinsic apoptosis pathway, initiated by perturbations of the extracellular microenvironment detected by plasma membrane receptors in which it propagates mainly to Caspase-3 [23]. This execution pathway initiated by the cleavage of caspase-3 results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins and formation of apoptotic bodies. Another biochemical characteristic is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent phagocytic cells, which thus allows the characterization of apoptosis through the externalization of phosphatidylserine from the plasma membrane [23].

Caspase-9 is a member of the intrinsic pathway and plays a central role in the mitochondrial apoptotic pathway. The intrinsic or mitochondrial pathway is initiated by the release of cytochrome from mitochondria in response to a variety of cellular stresses. Cytochrome-c interacts with apoptotic protease activating factor 1 (Apaf-1), procaspase-9, and deoxyadenosine triphosphate (dATP) to form a multiprotein complex called the apoptosome. Once bound to the apoptosome, caspase-9 is active, which subsequently triggers a cascade of executioners caspases, such as 3, 6 and 7. Some works have shown that caspase-9 is the direct target for regulatory phosphorylation by multiple protein kinases

activated in response to extracellular growth/survival factors, osmotic stress or during mitosis, which is linked to tumorigenesis and responses to thermotherapy [24].

In order to discover the possible mechanism of action of the compound Brachydin E, breast cancer cells were subjected to the flow cytometry technique for analysis phosphatidylserine exposure and caspase activation.

Brachydin induced different stages of the cell death process depending on the strain evaluated. In the triple negative subgroup, there was an increase in the number of cells in the late phase of cell death, as phosphatidylserine was externalized, but the plasma membrane was also ruptured, denoted by ANNEXIN/PE and 7AAD labeling, respectively, making it the subgroup most difficult tumor to treat, due to the lack of hormone receptors, Her2+ and KI67. In the luminal subgroup, Brachydin E increased the number of cells in the early phase of cell death without 7-AAD labeling. Brachydin E activated initiator caspases 8 and 9, as well as executors 3 and 7, demonstrating that both extrinsic and intrinsic apoptosis pathways can be regulated by this molecule.

Another result that confirms the action of Brachydin E in inducing cell apoptosis is through cleavage of the PARP protein. The PARP protein is responsible for DNA repair, however, when cleaved, its function is inactivated and there is an increase in the rate of apoptosis, confirming the tests carried out with Brachydin E [26]. Previously reported, Brachydins A, B, and C demonstrated potential anticancer activity in prostate and breast tumor cell lines. Brachydin B and C exerted cytotoxic effects, reduced clonogenicity and cell migration/invasion and stimulated cell death by apoptosis, as observed, the compound under study already has some similar characteristics within its family, causing cell death by apotosis and exerting cytotoxic effects in tumor lineages [35-36].

As demonstrated the mechanism of action of Brachydin E, a scheme was created, demonstrating the form of mechanism of action of this compound, through FIGURE 6, Brachydin E induces the activation of initiated caspases, both from the extrinsic and intrinsic pathways of apoptosis, which culminates in the activation of effector caspases in this cell death process. The cleavage of PARP caused by this molecule leads to DNA fragmentation which consequently triggers apoptosis and through the inactivation of c-myc by Brachydin E, cell survival is prevented.

Regarding cell cycle regulation, it was observed that Brachydin E leads to cell cycle arrest in the DNA synthesis phase due to the activation of p21 expression and inhibition of CDK4, proteins involved in the checkpoints of the transition between the G1 and G1 phases. S. These results highlight the promising anticancer activity of the biflavonoid Brachydin E in tumor lines, with positive results and reinforce the need to continue investigating its in vitro and in vivo properties.

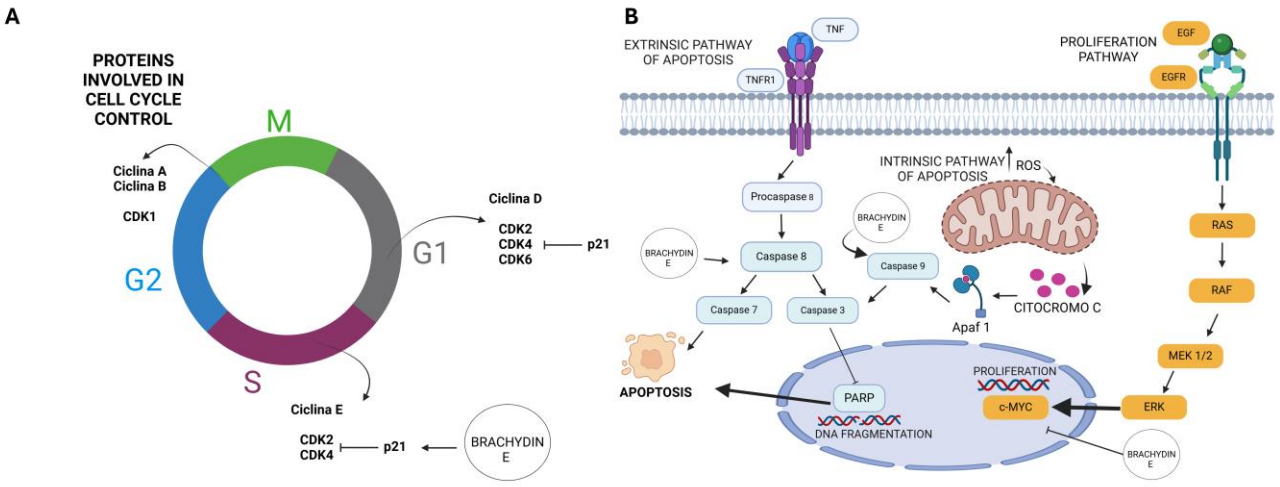


FIGURE 6. A: proteins involved in regulating the cell cycle. B: Extrinsic and intrinsic pathways of cell death by apoptosis and cell proliferation pathway. Source: From the Author. Created via biorender.com.

Brachydin E significantly reduced the proliferative potential of breast tumor lines, both in the two-dimensional and three-dimensional cell culture models and presented a significant anti-replicative effect. The mechanism of action studied suggests that Brachydin E is capable of inducing cell death regulated by caspases and inducing cell cycle arrest in S phase, with the activation or inactivation of proteins regulating cell death by apoptosis, as well as those involved in the stages of regulation of the cell cycle.

4. Materials and Methods

Cell culture and sample

Human tumor cell lines (MCF7 (HTB-22) and MDA-MB-231 (CRM-HTB-26)) and non-tumor/keratinocyte cell line (HaCat (PCS-200-011)) were obtained from National Cancer Institute at Frederick MA-USA. Stock cultures were grown in RPMI 1640 medium supplemented with 5% FBS and 100 U/mL penicillin, 100 lg/mL streptomycin at 37 ºC with 5% CO2. Brachydin E was obtained as previously described by our research group [10].

Antiproliferative assay

For this assay, the colorimetric method 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide, MTT (Sigma) was used to indirectly evaluate cell viability by the mitochondrial enzymatic activity of living cells [38]. The cell suspensions were prepared in RPMI-1640 (Gibco) medium containing 5% FBS (Nutricell) and 1% PS (Nutricell). One hundred μL of cell suspension containing 5000 cells were inoculated per well into 96-well plates and incubated for 24 hours at 37 °C in a 5% CO2 atmosphere and humidity. After 24 hours, the samples were diluted in DMSO and added to the cells at concentrations of 1.8; 3.6; 7.2; 14.5; 29; 58 and 116 μM (100 μL/well) in triplicate and then incubated for 48 hours at 37 °C in an atmosphere with 5% CO2 and humidity. As a positive control, the chemotherapy drug doxorubicin hydrochloride, doxo (Eurofarma) was used at concentrations of 0.3; 0.5; 1.1; 2.2; 4.3; 8.6; 17.3 μM (100 μL/well) in triplicate. The final concentration of DMSO (less than 1%) did not affect cell viability [39–40]. Cells seeded on the control plate (T0) were stained with MTT salt (Sigma®) to determine the number of viable cells present at the time of sample addition. After 48 hours of treatment, the treated cells were then stained with MTT and read spectrophotometrically at 570 nm using a microplate reader (Epoch BIOTEK®). The absorbance data was analyzed and compiled into graphs representing the percentage of viable cells with the sample concentration. GI50 values (50% growth inhibition) were calculated, which refers to the concentration of samples necessary to reduce cell viability by 50%.

Cell viability assay in three-dimensional (3D) cell culture

Cells were trypsinized and counted at the density required for each well of 96 compartments to receive $5x10^3$ cells. Next, Nanoparticles (GREINER®) were added to the cells, after which the cells were kept in an oven at 37ºC in an atmosphere of 5% CO2 and a humid environment. The nanoparticles consist of an aqueous solution of poly-L-lysine containing iron oxide nanoparticles (Fe2O3) and Au nanoparticles (gold) [41]. The cells with the nanoparticles are kept in an oven for a period of 48 hours, with the spheroid magnet below the plate. After this time, the magnetic magnet is removed, time zero photography is performed and treatment with DMSO (negative control), Docetaxel (positive control) and

Brachydin E. The concentration of Docetaxel was used in accordance with the literature and described by Seneme et al. al., 2022. After 48 hours of treatment, taking photographs at each treatment time (24 and 48 hours), the spheroids were mechanically dissociated with a tip and MTT - 3-(4,5-Dimethylthiazol-2- il)2,5-Diphenyl Tetrazolium Bromide - for analysis of cell viability by absorbance, according to the manufacturer's recommendations and the reading was done on a Glomax Microplate reader at a wave length of 640 nm (Promega).

Colony Formation Assay (Clonogenic Assay)

Colony formation or clonogenic assay is a quantitative in vitro technique to examine the ability of a single cell to grow into a large colony through clonal expansion. It was performed as described by Silva et al. [42]. For this assay, 5,000 MCF7 cells were seeded in agar medium (Kasvi) in 6-well plates to prevent these cells from adhering to the well; for the MDA-MB-231 line, 5,000 cells were seeded per well, directly in the plate, without half agar. Treatments with DMSO and Brachydin E diluted in serum-free culture medium were performed every three days. After 21 days in culture, colonies were fixed by the gentle addition of 0.005% formaldehyde (Scientific Exodus) and stained with crystal violet (Nuclear). The wells were photographed and the colonies were counted using the ImageJ software and statistics were performed.

Investigation of the mechanism of action by flow cytometry

Studies on the mechanism of action of the Brachydin E compound were carried out on the Guava Easy Cyte 5HT Benchtop Flow Cytometer, Millipore®, Billerica, MA, USA. The commissions for the compounds chosen for evaluation were based on the previous antiproliferative assay, considering the GI50 values. 10,000 events were acquired from each sample.

Phosphatidylserine (PS) externalization assay

This test is based on labeling with Annexin V-PE (phycoerythrin) and 7-amino-actinomycin D (7-aminoactinomycin D - 7-AAD), representing the externalization of phosphatidylserine (typically characteristic of regulated cell death) and loss of cell membrane integrity (typical of advanced cell death), respectively. Double negative cells are considered viable. MCF7 and MDA-MB-231 cells were inoculated in 6-well plates at a density ranging from $1x10^5$ to $1x10^6$ cells/mL in RPMI medium + 5% FBS and 1% PS and incubated at 37 °C in an atmosphere of 5% CO2 and humidity. After 24 hours of treatment with DMSO and Brachydin E, cells were trypsinized and 100 µL of the suspension was transferred to a round-bottom 96-well plate and mixed with 100 µL of Guava Nexin Reagent (Merck/Millipore) for 20 minutes in the dark. The plate was then analyzed on a GuavaEasyCyte 5HT benchtop flow cytometer (Merck/Millipore).

Cell Cycle Analysis Assay

This test is based on the quantification of cells at different stages of the cell cycle by labeling DNA with propidium iodide (PI), a fluorescent DNA intercalator. This analysis is only permitted after membrane permeabilization with 70% ethanol at 4 ºC [41]. MCF7 and MDA-MB-231 cells were seeded into 6-well plates at a density ranging from $1x10^5$ to $1x10^6$ cells/mL in RPMI medium + 5% FBS and 1% PS and incubated at 37 °C in a 5 % CO2 and humidity. FBS was removed from the medium for a period of 24 hours for starvation, aiming to synchronize cells in the G0 phase, the beginning of the cell cycle. As a positive control, the chemotherapy drug etoposide was used, at a concentration of 10 µM, which is

known to cause cycle arrest in S or G2/M [31-33], treatment for 24 hours with DMSO (<0.001%), etoposide and Brachydin E (7,5 μM), MCF7 cells were trypsinized, the cell pellet was resuspended in 200 μL of Guava Cell cycle reagent (Merck/Millipore: 4500-0220), being incubated for 30 min protected from light and then analyzed on a GuavaEasyCyte 5HT benchtop flow cytometer (Merck/Millipore).

Detection of active caspases

The Guava® Multicaspase Detection Kit (4500-0101) uses a caspase inhibitor conjugated to a fluorochrome called sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK). This inhibitor is cell permeable and non-cytotoxic. Once inside the cell, this inhibitor covalently binds to caspases that have been activated by the apoptotic pathway [44]. Cells with positive staining for SR-VAD-FMK correspond to cells in the apoptosis stage. Included in this kit is also the dye 7-AAD, which is impermeable to viable cells, but permeable to cells in the final stage of apoptosis. The MDA-MB-231 breast tumor line was inoculated at a density of 1x105 cells/mL in RPMI/5% SFB medium plus 1% penicillin/streptomycin. After 24h of incubation Brachydin E was added. As a negative control, only cells and RPMI culture medium containing 0.25% DMSO, the compound diluent, were used. After 12h of treatment, Guava Caspase Reagent Working Solution was added to an eppendorf tube. Incubation carried out for 60 min. in a dark environment, at room temperature. After incubation, 1x Apoptosis Wash Buffer was added to the sample, the cell pellet was resuspended in 100 μL Caspase 7AAD Reagent Working Solution, and the samples were incubated room temperature in the dark.

Western Blotting

Cells were collected and placed in lysis buffer and protease inhibitor cocktail (after 24-hour treatment). The protein concentration in the cell lysate was determined by the Bradford method. Protein samples were filtered and separated by SDS-PAGE (10% and 15% acrylamide gel) and transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Little Chalfont, UK) using a TransBlot Turbo Transfer (Bio-Rad Laboratories , Hercules, CA, 414 USA). Membranes were blocked using 5% skim milk in Tris-buffered saline with 415 Tween 20 for 1 h at room temperature. Then, the membranes were incubated overnight with primary antibody (CDK4, p21, c-myc, PARP, β-actin) and then incubated with secondary antibody at room temperature. β-Actin expression was used as an internal loading control. Immune detection was performed with ECL Western Blotting detection reagents on automatic ImageQuant mini 419 LAS4000 (GE Healthcare Life Science). Densitometric quantification was performed using 420 ImageJ software (NIH, Bethesda, MD, USA) [45]

Statistical Analysis

Results were expressed as mean ± standard deviation (SD) of three independent experiments conducted in duplicate. Statistical analyses were performed with the GraphPad Prism 5 software. Test T and ANOVA followed by Tukey, or Bonferroni post-hoc test was used, and p-values less than 0.05 were considered significant. For the cytotoxic activity assay, the linear regression of the curves was obtained using the mean growth percentage and calculated with Origin software (OriginLab).

Author Contributions: Conceptualization, C.A.d.L. and G.B.L.; methodology, C.A.d.L., Y.E.M.F., C.D.P.R., J.R.d.N., J.M.S.., G.R.S., M.C.F.P., R.J.d.S.O., C.Q.d.R.; data curation, G.B.L.; writing—original draft preparation, C.A.d.L., G.B.L.; writing—review and editing, C.A.d.L., G.B.L.; funding acquisition, C.Q.d.R., G.B.L.

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Conflicts of Interest: The authors declare no conflict of interest.

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5. CONCLUSÃO

O biflavonoide Brachydin E foi capaz de inibir o crescimento das células tumorais de mama (MCF7 e MDA-MB-231) em modelo bi e tridimensional de cultivo celular, possuindo ação antireplicativa e migratória. Pelos resultados, sugere-se que seu mecanismo de ação está relacionado à morte celular por apoptose, através da ativação da cascata de caspase e clivagem de PARP; e também à interrupção do ciclo celular na fase S, através da ativação de p21 e inibição de CDK4 e c-myc. Considera-se que Brachydin E é um importante composto para o estudo do tratamento do câncer e sugere-se a continuação da investigação de seu mecanismo de ação.

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